Fibroblast Growth Factor 2 and Forskolin Regulate Mineralization-associated Genes in Osteoblast-like Cells and Breast Cancer Cells

（塩基性線維芽細胞成長因子とフォルスコリンによる骨芽細胞様細胞および乳ガン細胞における石灰化関連遺伝子の調節）

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Preface

This article is based on a main reference paper, “Forskolin Regulates Bone Sialoprotein Gene Expression in Human Breast Cancer Cells” in the International Journal of Oral-Medical Sciences, and a reference paper, “Fibroblast growth factor 2 and forskolin induce mineralization-associated genes in two kinds of osteoblast-like cells” in the Journal of Oral Science.

Abstract

BSP is a prominent mineral-associated protein in the extracellular matrix of bone that has been implicated in the metastatic activity of cancer cells. Fibroblast growth factor 2 (FGF2) and cyclic AMP (cAMP) play critical roles in controlling the differentiation of osteoblasts and mineralization of bone. We have previously reported that each of FGF2 and forskolin (FSK) alone increase transcription of the bone sialoprotein (BSP) gene, and that together (FGF/FSK) they upregulate BSP gene expression synergistically in rat osteoblast-like ROS 17/2.8 cells. However, other genes that are upregulated after stimulation by FGF2, FSK or FGF/FSK remain unclear. In the present study, we investigated candidate genes associated with mineralization after stimulation by FGF2, FSK and FGF/FSK in two kinds of osteoblast-like cells using DNA microarray, and how FSK regulates BSP gene transcription in human breast cancer MCF7 cells using reverse transcription PCR (RT-PCR), transient transfection assays and gel mobility shift analyses.

In ROS17/2.8 cells, FGF2 and FSK each increased the gene expression of c-FOS (7.2-fold and 10.7-fold, respectively). However, FGF/FSK did not induce c-FOS gene expression. FGF2 increased the expression of the dentin matrix protein 1 (DMP1, 129.8-fold) gene. In contrast, FGF/FSK increased the expression of the amphiregulin (AREG, 73-fold) gene maximally. In human osteoblast-like Saos2 cells, FGF2 increased the expression of the osteopontin (SPP1, 16.7-fold), interleukin-8 (IL8, 6.4-fold) and IL11 (4.8-fold) genes. FSK induced the expression of the IL6 (2.6-fold), IL11 (4.0-fold), chemokine ligand 13 (CXCL13, 2.8-fold) and bone morphogenetic protein 2 (BMP2, 2.5-fold) genes.

FSK (1 μM) increased BSP, Runx2 and Osterix mRNA levels in MCF7 cells at 12 h. From transient transfection analyses using various BSP promoter-luciferase constructs, a cAMP response element (CRE), a runt homeodomain protein 2 (Runx2) and a FGF2 response element (FRE) were identified as targets of transcriptional activation by FSK. Gel mobility shift analyses showed that FSK increased binding of CRE and FRE. These studies demonstrate that FSK stimulates BSP transcription in MCF7 cells by targeting the CRE and FRE in the BSP gene promoter, and suggest that FGF2 and FSK might be crucial regulators of mineralization and bone formation.

Introduction

Osteoblasts produce and secrete several kinds of growth factors that are also found in the extracellular matrix of bone, such as fibroblast growth factor 2 (FGF2),

transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF). These growth factors may regulate the initiation and development of bone formation and fracture callus. A particularly close relationship between FGF2 and bone formation has been reported (1, 2). Overexpression of the FGF2 gene in transgenic mice causes premature mineralization, flattening and shortening of long bones (3). Moreover, disruption of the FGF2 gene leads to a decrease of both bone mass and bone formation (4). Therefore, FGF2 could be crucial for the regulation of osteoblast proliferation and proper bone development. Forskolin (FSK) is a labdane diterpene produced by the Indian Coleus plant. FSK is commonly used to raise cyclic AMP (cAMP) levels in studies of cell physiology. FSK activates the enzyme adenylyl cyclase and increasing the intracellular levels of cAMP. Neurotransmitters and parathyroid hormone (PTH), which stimulate cAMP production and activation of protein kinase A (PKA) (5), can promote (6) or inhibit (7) growth and proliferation in a cell-specific manner. FSK and 8-Bromo-cAMP, which also elevate the intracellular level of cAMP, together with PTH, have been shown to increase the levels of both osteocalcin (OC) mRNA and protein (8).

OC and bone sialoprotein (BSP) are non-collagenous proteins expressed specifically in mineralized connective tissues (9). OC is expressed in bone matrix at the late stage of mineralization, whereas BSP is expressed in the early stage. The temporo-spatial deposition of BSP in the extracellular matrix of bone (10) and its ability to nucleate hydroxyapatite crystal formation (11) indicate a potential role of BSP in the initial mineralization of bone. Several studies have shown that FGF2 and FSK (cAMP) induce

expression of the OC gene (12,13), and that Msx2, Dlx5 and Runx2 are important transcription factors for OC gene expression (13-17). A low concentration of FSK induces differentiation of osteoprogenitor cells, possibly through a non-cAMP-dependent process, and intermittent elevation of intracellular cAMP has an inhibitory effect on bone formation (18). Moreover, FSK inhibits DNA synthesis, blocks protein kinase C (PKC)-stimulated tyrosine phosphorylation of p44MAPK (ERK1) and p42MAPK (ERK2), and also inhibits PKC-stimulated MAPK and Raf-1 activities in MC3T3-E1 osteoblast-like cells (19). Phorbol-12-myristate-13-acetate (PMA) activates the intermediate MKK step of the Raf-1/MKK/MAPK cascade in the presence of FSK (20). Inducible cAMP early repressor (ICER) expression in osteoblasts also requires activation of the cAMP-protein kinase A (PKA) signaling pathway (20).

Breast cancer cells are among the few human cancer cell types that exhibit the highest affinity for bone. The majority of patients with advanced breast cancer have evidence of skeletal metastases (21). Little is known about the molecular mechanisms responsible for breast cancer cells osteotropism, the phenomenon that defines the strong affinity of these cells for bone. A possible explanation could be provided by recent finding that breast cancer cells express high amounts of bone sialoprotein (BSP) (22). BSP is a highly phosphorylated and sulfated glycoprotein which is a major component of the mineralized bone and cementum matrix (23, 24). BSP mediates the attachment and activation of osteoclasts through the RGD motif (25, 26) and can facilitate attachment of normal bone or cancer cells to mineralized tissue surfaces (23-26). BSP and osteopontin

(OPN) can bind to factor H and protect cells from complement-mediated cell lysis, which may be important for cancer cell survival (27). BSP is expressed by mature osteoblasts, osteoclasts and hypertrophic chondrocytes (10, 28), and is also detectable in breast (21, 22), lung (29) and prostate cancers (30, 31), which could metastasized to bone, suggesting that BSP may play a role in the pathogenesis of bone metastases (32). The BSP gene promoters have been cloned and partially characterized (33-36). Rat BSP gene promoter have an inverted TATA box (-24 to -19) (37), an inverted CCAAT box (-50 to -46) which is required for basal transcription (38, 39). In addition, a cAMP response element (CRE; -75 to -68) (40-42), a fibroblast growth factor 2 (FGF2) response element (FRE; -92 to -85) (40, 43-45), a runt-related transcription factor 2 (Runx2) binding site (-84 to -79) (46), a pituitary-specific transcription factor-1 (Pit-1) motif (-111 to -105) (47), a homeodomain protein binding site (HOX; -199 to -192) (44, 48), a transforming growth factor-β (TGF-β) activation element (-499 to -485) (49) and a glucocorticoid response element (-931 to -906) overlapping an AP-1 site (-921 to -915) (50, 51) have also been characterized. We have previously reported FGF2 and cyclic AMP (cAMP) synergistically regulate BSP gene expression (46). The goal of the present study was to use microarray to investigate the candidate genes associated with mineralization after stimulation by FGF2, FSK and FGF/FSK in osteoblast-like cells and to elucidate the molecular mechanism of the FSK regulate BSP gene transcription in human breast cancer MCF7 cells.

Materials and Methods

Materials

Alpha minimal essential medium (α-MEM), fetal calf serum (FCS), Lipofectamine, penicillin, SuperScript one step RT-PCR with Platinum Taq, Penicillin-Streptomycin and TrypLE^{TM} Express were obtained from Invitrogen (Carlsbad, CA). Forskolin (FSK) was from Sigma-Aldrich Japan (Tokyo, Japan). Recombinant human FGF2 was from Genzyme, Techne (Minneapolis, MN). GeneChip (Human Genome U133A 2.0 Array and Rat Genome 230 2.0 Array) was purchased from Affymetrix (Santa Clara, CA). The pGL3-basic, pSVβ-galactosidase (β-Gal) control vector were purchased from Promega (Madison, WI). All chemicals used were of an analytical grade.

Cell culture

Human and rat osteoblast-like Saos2 and ROS17/2.8 cells (44, 46, 50), and human breast cancer cell line MCF7 (52) were cultured at 37 ^{o}C in 5% CO_2 air in α-MEM supplemented with 10% FCS. Cells were first grown to confluence in 60 mm tissue culture dishes in α-MEM medium containing 10% FCS, then Saos2 and ROS17/2.8 cells cultured in α–MEM without serum for 12 h, and stimulated by FGF2 (10 ng/ml), FSK (1 μM) or FGF2/FSK for 6 h. MCF7 cells were cultured in α-MEM without serum and incubated with FSK (1 μM) for time periods of 3-24 h. Total RNA was isolated from triplicate cultures and analyzed for the gene expressions. Nuclear proteins were extracted by the method of Dignam *et al.* (53) with the addition of extra proteinase

inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MaCl_2$, 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 aprotin, pH 7.9).

Microarray analysis

Three RNA samples were mixed together for gene expression profiling. Gene expression profiling was performed separately for each pooled RNA sample using a GeneChip (Human Genome U133A 2.0 Array, spotted 22,000 genes of human genes, and Rat Genome 230 2.0 Array, spotted 28,000 genes of rat genes). Hybridization and scanning of Gene Chip were done according to the Affymetrix manual. 20 μg of the fragmented biotinylated sample cRNA was hybridized to GeneChip, and washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA) using the EukGE-WS1 protocol by an Affymetrix GeneArray Scanner. The expressions of human and rat genes were monitored, and the data was imported into GeneSpring GX software (Agilent Technologies, Inc, Santa Clara, CA)

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (1 μg) was extracted with the guanidium thiocyanate described previously (49) and used as a template for cDNA synthesis. The cDNA was prepared by use of a SuperScript one step RT-PCR kit according to the supplier's protocol. Primers were synthesized on the basis of the reported human cDNA sequence for BSP, Runx2, Osterix

and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the primers used for PCR were as follow: human BSP forward, 5'-TGCTCAGCATTTTGGGAAT-3'; human BSP reverse, 5'-TGCATTGGCTCCAGTGACACT-3' (627 bp), human Runx2 forward, 5′-CAGTTCCCAAGCATTTCATCC -3′; human Runx2 reverse, 5′-TCAATATGGTCGCCAAACAG –3' (443 bp), human Osterix forward, 5′-GCAGCTAGAAGGGAGTGGTG -3′; human Osterix reverse, 5′-GCAGGCAGGTGAACTTCTTC -3′ (359 bp), human GAPDH forward, 5′-CCACCCA-TGGCAAATTCCATGGCA -3′; human GAPDH reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC -3′ (598 bp). Amplification was carried out for 30 (GAPDH) cycles and 35 (BSP, Runx2 and Osterix) cycles under saturation, each at 94°C, for 30 sec; 55°C, for 30 sec; 72°C, for 30 sec in a 50 μl reaction mixture. After amplification, 10 μl of each reaction mixture was analyzed by a 2% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining.

Transient transfection assays

Exponentially growing MCF7 cells were used for transfection assays. Twenty-four hours after plating, cells at 60–70% confluence were transfected using a Lipofectamine reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct (49, 50) and 2 μg of β-Gal vector as an internal control. After two days of post-transfection, cells were deprived of serum for 12 h, and 1 μM FSK was added 12 h prior to harvesting. The luciferase assay was performed according to the supplier's protocol (PicaGene;

Toyo Inki, Tokyo) using a Luminescence reader (Acuu FLEX Lumi 400; Aloka) to measure the luciferase activity.

Gel mobility shift assays

Confluent MCF7 cells in T-75 flasks incubated for 3, 6 and 12 h with 1 μM FSK in α-MEM without serum were used to prepare the nuclear extracts. Double-standard oligonucleotides encompassing the inverted CCAAT, CRE, FRE and Pit-1 in the rat BSP promoter were prepared. For the gel shift analysis, the double-standard 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 of radiolabeled double-standard oligonucleotide. Following incubation, the protein-DNA complexes were resolved by electrophoresis in 5% non-denaturing acrylamide gels (38:2 acrylamide/bis acrylamide) ran at 200 V at RT. After electrophoresis, the gels were dried and autoradiograms were prepared and analyzed using an image analyzer. Double-standard oligonucleotides encompassing the inverted CCAAT (-61 to -37, 5'-CCGTGACCGTG**ATTGG**CTGCTGAGA), CRE (-84 to -59, 5'-CCCACAGCC**TGACGTCG**CACCGGCCG), FRE (-98 to –79, 5'-TTTTCT**GGTGAGAA**CCCACA) and Pit-1 (-115 to -96, 5'-CGTG**TTGTAGT**TACGGATTT) in the rat BSP promoter were prepared.

Statistical analysis

Quadruplicate or triplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of the responses to the drugs. Significant differences between control and treatment groups were determined using the one-way ANOVA.

Results

Analyses of gene expression profile of osteoblast-like cells by DNA microarray

To study the regulation of human and rat genes by FGF2, FSK or FGF2/FSK, we performed DNA microarray analyses of total RNA extracted from Saos2 and ROS17/2.8 osteoblast-like cells. In human Saos2 cells, 418, 68 and 345 genes were upregulated more than two-fold by FGF2 (10 ng/ml), FSK (1 μM) and FGF/FSK in Saos2 cells (Fig. 1A, B, C). FGF2 increased synaptotagmin XII (SYT12) (60.2-fold), osteopontin (SPP1(OPN), 16.7-fold), colony stimulating factor 2 (CSF2, 15.6-fold), early growth response 1 (EGR1, 6.6-fold), interleukin 8 (IL8 6.4-fold), interleukin 11 (IL11, 4.8-fold), Wnt5B (WNT5A, 4.0-fold), histone deacetylase 4 (HDAC4, 3.5-fold), and bone morphogenetic protein 2 (BMP2, 3.3-fold), for mRNA levels (Fig. 1A). FSK stimulated sialophorin (LSN, 45.2-fold), synaptotagmin-like 3 (SYTL3, 6.1-fold), IL11 (4.0-fold), prostaglandin E synthase (PTGES, 2.9-fold), chemokine ligand 13 (CXCL13, 2.8-fold), IL6 (2.6-fold), and BMP2 (2.5-fold), for gene expressions (Fig.1B). FGF/FSK induced SYTL3 (33.9-fold), IL11 (13.6-fold), SPP1 (12.4-fold), CSF2 (10.3-fold), HDAC4 (7.5-fold), EGR1 (6.8-fold), IL8 (5.6-fold), BMP2 (4.4-fold), and WNT5A (4.1-fold),

for mRNA levels (Fig. 1C).

In rat ROS17/2.8 cells, 591, 127 and 390 genes were upregulated more than two-fold by FGF2 (10 ng/ml), FSK (1 μM) and FGF/FSK. (Fig. 2A, B, C). FGF2 stimulated dentin matrix protein 1 (DMP1, 129.8-fold), EGR1 (33.4-fold), c-FOS (7.2-fold), AXIN2 (4.6-fold), JUNB (4.1-fold), MYC (3.8-fold), and connective tissue growth factor (CTGF, 2.8-fold), for gene expressions (Fig.2A). FSK increased c-FOS (10.7-fold), EGR1 (5.6-fold), HDAC4 (3.6-fold), AXIN2 (2.8-fold), WNT4 (2.4-fold), CTGF(2.1-fold) and IL6 (2.1-fold) (Fig. 2B). Furthermore, FGF/FSK upregulated amphiregulin (AREG, 73.0-fold), EGR1 (22.6-fold), DMP1 (13.4-fold), PTGES (6.4-fold), CTGF (4.5-fold), cAMP response element modulator (CREM, 4.1-fold), JUNB (3.8-fold), MYC (3.8-fold), and AXIN2 (3.5-fold), for gene expressions (Fig. 2C).

Stimulation of BSP, Runx2 and Osterix mRNA levels in MCF7 Cells

To study the regulation of BSP expression by FSK in breast cancer cells, we performed RT-PCR using total RNA extracted from MCF7 cells. Since Runx2 and Osterix are considered master genes for osteogenesis, the effects of FSK on their expression were also analyzed by RT-PCR. Results of RT-PCR showed that FSK (1 μM) increased BSP mRNA level at 12 h and reached a maximum at 24 h. The Runx2 mRNA level was increased at 3 h, decreased at 6 h, and increased again at 12 and 24 h. The Osterix mRNA level increased at 6 h and reached maximal at 12 h (Fig. 3).

Transient transcription analysis

Transient transfection of chimeric constructs encompassing different regions of the rat BSP gene promoter that ligated to a luciferase reporter gene (pLUC3~6) were performed in MCF7 cells (Fig. 4). The results of the transfection assays indicated an increase in transcription after 12 h with constructs pLUC3 (-116~+60), pLUC4 (-425~+60), pLUC5 (-801~+60) and pLUC6 (-938~+60) following treatment with 1 μM of FSK (Fig. 2). Within the pLUC3 luciferase construct, an inverted TATA and CCAAT box, a CRE, a Runx2, a FRE and a Pit-1 motif are present (Fig. 5). To confirm the functional elements, we performed double mutation analyses. Two base pair mutation in CRE, Runx2 and FRE in pLUC3 abolished the FSK induced BSP promoter activities, whereas mutation in CCAAT had no effect (Fig. 6). The results suggested that CRE, Runx2 and FRE are functional response elements for FSK.

Gel mobility shift assays

To identify nuclear proteins which bind to CRE, Runx2 and FRE elements might be modulated by FSK, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent MCF7 cells that were either not treated (control) or treated with FSK (1 μM) for 3, 6 and 12 h. When the inverted CCAAT, CRE and FRE sequence were used as probes, the CCAAT-nuclear

factor-Y (NF-Y) protein complex did not change after FSK stimulation. NF-Y is a principal transcription factor that bound to the inverted CCAAT (39). In comparison, with nuclear extracts from confluent control cultures of MCF7 cells, CRE- and FRE-protein complexes were increased after stimulation by FSK (Fig. 7).

Discussion

In this study, we reported on candidate genes associated with mineralization induced by FGF2, FSK and FGF/FSK in two kinds of osteoblast-like cell lines (Fig. 1 and 2). These genes were classified into various fields depending on cell type, such as regulator of gene expressions (c-FOS, JUNB, MYC, WNT5 and HDAC4), extracellular matrix proteins (OC, OPN and DMP1), and inflammatory cytokines (IL6, IL8, IL1 and PTGES).

WNT5A and HDAC4 mRNA levels were increased by FGF2, FSK and FGF/FSK in human osteoblast-like Saos2 cells, respectively. While FGF2 induced WNT5B gene expression, FSK decreased WNT5B gene expression. WNT5A and WNT5B belong to the WNT family, and it is well known that the Wnt signaling pathway also plays a pivotal role in the regulation of bone mass (54). The Wnt signaling pathway is activated during postnatal bone regenerative events, such as ectopic endochondral bone formation and fracture repair. It has been reported that FGF signaling could control many aspects of osteoblasts differentiation through induction of SOX2 and regulation of the

Wnt-beta-catenin pathway (55). Ambrosetti et al. showed that ERK1/2 and Akt have distinct effects on FGF-induced osteoblasts proliferation and differentiation (56). The Wnt signaling pathway, which promotes osteoblast differentiation, also induces Akt phosphorylation. The cells expressing active Akt increase the levels of the stabilized beta-catenin and a central molecule in Wnt signaling (57). Takada et al. reported that histonelysine methyltransferase activated by non-canonical Wnt signaling suppresses PPAR-gamma transactivation, induces Runx2 expression, and promotes osteoblastogenesis (58). These results suggest a close relationship between FGF2, Wnt and osteoblastogenesis.

HDAC4 is one of the histone deacetylases (HDACs) (59), and is known to regulate osteoblast differentiation. HDAC4 or HDAC5 is required for TGF-beta-mediated inhibition of Runx2 function and is involved in osteoblasts differentiation (60). HDAC4 deacetylates Runx2, allowing the protein to undergo Smurf-mediated degradation. The inhibition of HDAC4 increases Runx2 acetylation and potentiates BMP-2-stimulated osteoblast differentiation and increases bone formation (61). PTH regulates HDAC4 in osteoblast-like cells through a PKA-dependent pathway, leading to removal of HDAC4 from the MMP13 gene promoter and enhancing gene transcription (62). These data suggest that HDAC4 could be related to the transcription factors induced by FGF and FSK in osteoblasts.

The WNT4 mRNA level was increased by FSK but not by FGF2. AXIN2 gene expression was induced FGF2, FSK and FGF/FSK. Also, the c-MYC mRNA level was

increased by FGF2 and FGF/FSK in ROS17/2.8 cells. AXIN2/conductin/Axil is a negative regulator of the canonical Wnt pathway that suppresses signal transduction by promoting degradation of beta-catenin. AXIN2 has a role in calvarial morphogenesis and craniosynostosis (63). The function of AXIN2 and beta-catenin have demonstrated that canonical signaling modulates most aspects of osteoblasts physiology including proliferation, differentiation, bone matrix formation/mineralization and apoptosis, as well as coupling to osteoclastogenesis and bone resorption.

Activator protein 1 (AP1) transcription factor was formed by c-FOS and c-JUN heterodimer. c-FOS is a key regulator of osteoblasts differentiation, whereas, c-JUN, JUNB and FRA1 are essential in embryonic and/or postnatal development (64). We have previously reported that FGF2 stimulates BSP gene transcription by targeting the FGF2 response element (FRE) and AP1/glucocorticoid response element (GRE) in rat BSP gene promoter (65). In this study, c-FOS was induced by FGF2 and FSK. Results of DNA microarray analyses showed that the JUNB mRNA levels were increased by FGF2 and FGF/FSK in ROS 17/2.8 cells (Fig. 2), whereas real-time PCR data indicated FGF2 and FGF/FSK could not induce JUNB gene expression, and FSK induced JUNB mRNA level maximally (data not shown). Further study to resolve the discrepancy is required. Osteoblast specific extracellular matrix proteins such as OC and BSP were strongly reduced in JUNB deficient mice (66).

Glucocorticoid increased mRNA levels of alkaline phosphatase (ALP), c-MYC, c-FOS and c-JUN mRNAs in human osteoblast-like cells (67). c-MYC induces

differentiation and apoptosis of human ES cells (68). In contrast, c-MYC has a positive role in mouse ES cells (69). Takahashi et al. demonstrated the generation of induced pluripotent stem cells (iPS cells) from adult human dermal fibroblasts using the four transcription factors of OCT3/4, SOX2, KLF4 and c-MYC (70). Thus, c-MYC has a close connection to cell differentiation and proliferation and c-MYC may have a role in osteoblasts differentiation and proliferation by FGF2 stimulation.

BSP has been characterized as a unique marker of osteogenic differentiation that can regulate the formation of mineral crystals (23, 24). Our studies have identified CRE, Runx2 and FRE elements in the proximal promoter of the BSP gene that mediates FSK-stimulated transcription of BSP gene in MCF7 human breast cancer cells.

From our previous research, we have observed that FSK and FGF2 regulated BSP expressions in DU145 prostate cancer cells and ROS17/2.8 osteoblast-like cells (31, 46). The presence of BSP in human breast cancers has been associated with an increased risk for subsequent bone metastases and poor survival rates (32, 71). The ability of BSP to bind to hydroxyapatite crystals and to mediate cell attachment through cell-surface integrins may be involved in the osteotropism of the metastatic cells (22-24). Various tumors express BSP (22, 29-32, 72), and FSK increased BSP and Runx2 mRNA and protein levels in prostate cancer cells (31). OC and BSP expression is coordinated and regulated through cAMP-dependent PKA signaling, which may define the molecular basis of the osteomimicry exhibited by prostate cancer cells (73).

RT-PCR results showed that FSK treatment of MCF7 breast cancer cells increased the

steady-state levels of BSP, Runx2 and Osterix mRNA (Fig. 3). From transient transfection assays, BSP promoter activities (pLUC3 (-116~+60), pLUC4 (-425~+60), pLUC5 (-801~+60), pLUC6 (-938~+60)) were increased by FSK (Fig. 4). Insertion of two base pair mutations in CRE, Runx2 or FRE sites in pLUC3 constructs abolished the FSK induced BSP promoter activities (Fig. 6). The results suggest that CRE, Runx2 and FRE are functional response elements for FSK. Results of gel shift assays showed that proteins from nuclear extracts that formed complexes with the CRE and FRE were increased by FSK (Fig. 7). Previously, a FRE (GGTGAGAA) was identified in the rat BSP proximal gene promoter that was juxtaposed to a putative Runx2 binding site (CCCACA) (Fig. 5) (46). FRE oligonucleotide which was used for the gel shift assay included both FRE and Runx2 sites. Therefore, FRE-protein complexes could contain FRE binding transcription factors and Runx2. Further study is necessary to investigate the FSK signal pathway in MCF7 human breast cancer cells and transcription factors that bind to CRE, Runx2 and FRE, and the functional interactions of these elements.

Breast cancer has become a serious health concern for women. It most commonly metastasizes to the lymph nodes and bone and causes significant morbidity in women with an advanced disease (21, 22, 32). Although there is little understanding of the molecular mechanisms responsible for bone metastasis in breast cancer, previous research found that noncollageous bone matrix proteins, such as OC, OPN and BSP, are expressed at high levels in advanced bone metastatic breast and prostate cancer cells. However, normal breasts and prostates do not express bone matrix proteins (30, 32).

These observations led us to focus specifically on the regulation of BSP expressions in breast cancer cells. BSP and OC expressions in prostate cancer cells are regulated by PKA signaling (73). Therefore, in this study, we used FSK, an activator of adenylate cyclase, which has been extensively used to stimulate the cAMP-PKA signaling pathway in order to elucidate how BSP gene transcriptions are regulated in breast cancer cells.

CREB1, ATF/CREB and AP1 family transcription factors can bind to CRE as homodimers or heterodimers (74). In addition, it was previously found that AP1 family transcription factors (c-Fos, c-Jun, JunD and Fra2) interacted with CRE1 and CRE2 elements in the human BSP gene promoter (31). Osteoblast-related transcription factors Runx2 and Msx2 mediate the expression of BSP in human metastatic breast cancer cells (75). We previously reported that FRE binding proteins in Saos2 osteoblast-like cells were Runx2, Dlx5 and Smad1, and that anti-Smad1 antibody co-precipitated Smad1 and Runx2 (44). It is possible that Runx2 may interact with CRE, because CRE is located adjacent to FRE in the proximal promoter of the rat BSP gene. CREB1 and AP1 transcription factors JunD and Fra2 regulated BSP gene expression in human breast cancer cells (76). Osterix is a C2H2-type zinc finger transcription factor from the SP gene family and a putative master regulator of bone cell differentiation. Osterix-deficient mice display an absence of bone due to arrested osteoblast differentiation (77). In this study, we showed that FSK induced Osterix mRNA levels in MCF7 human breast cancer cells (Fig. 3). The putative Osterix response element is a G/C-rich sequence; although, we could not identify the Osterix binding site yet. Further

study is necessary to elucidate the regulatory element in the rat and human BSP gene promoter

In conclusion, our study has suggested that FGF2, FSK and FGF/FSK induce many mineralization-associated genes. Therefore, the induced genes may interact with each other and regulate bone mineralization and formation. FSK-induced BSP transcription in human breast cancer cells was under the control of transcription factors interacted with CRE, Runx2 and FRE elements, and suggests that the cAMP-PKA signaling pathway inhibitor may have a potential to be a therapeutic agent for breast cancer.

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Fig. 1A **Saos2**

GenBank	Gene Name	Ratio FGF2/Cont
BE881590	ETV1	63.3
AK024280	**SYT12**	**60.2**
NM_001423	EMP1	35.7
AL572488	FLJ14490	29.3
BC003143	DUSP6	19.6
NM_002135	NR4A1	19.2
M83248	**SPP1 (OPN)**	**16.7**
M11734	**CSF2**	**15.6**
AB028021	FOXA2	14.3
AW193698	TGFBR3	14.3
AK026181	PHLDA1	14.2
NM_004454	ETV5	13.1
NM_000602	SERPINE1	12.0
BC015940	NT5E (CD73)	11.0
NM_003483	HMGA2	11.0
AW151660	RGS3	9.9
NM_004369	COL6A3	8.3
AW166825	S100A6	8.1
NM_00362	TIMP3	7.3
NM_002658	PLAU	7.0
AV733950	**EGR1**	**6.6**
AF043337	**IL8**	**6.4**
M27281	VEGF	5.5
U08839	PLAUR	5.2
M57765	**IL11**	**4.8**
M92934	CTGF	4.2
NM_030775	**WNT5B**	**4.0**
NM_006037	**HDAC4**	**3.5**
NM_001200	**BMP2**	**3.3**

Fig. 1B **Saos2**

GenBank	Gene Name	Ratio FSK/Cont
X52075	**LSN (CD43, GPL115)**	**45.2**
AI674404	**SYTL3**	**6.1**
AI799784	MGC45780	4.8
NM_018371	ChGn	4.7
M57765	**IL11**	**4.0**
NM_000693	ALDH1A3	3.9
D13891	ID2	3.8
AI422986	SIAT8D	3.8
AB018009	SLC7A5	3.5
AI084489	PITPNC1	3.5
BF246115	MT1F	3.3
NM_019058	DDIT4	2.9
NM_004878	**PTGES**	**2.9**
AI791187	FGFR1	2.8
NM_005985	SNAI1	2.8
NM_006419	**CXCL13**	**2.8**
NM_014746	RNF144	2.8
BF513244	DAAM2	2.7
AW118608	ATPAF2	2.7
NM_013281	FLRT3	2.7
NM_006169	NNMT	2.7
NM_005951	MT1H	2.6
NM_000600	**IL6**	**2.6**
R40917	PDE4D	2.5
NM_001200	**BMP2**	**2.5**
AI990526	AYP1	2.5
AA675892	TOB1	2.4
NM_001078	VCAM1	2.4
NM_005178	BCL3	2.3
NM_007348	ATF6	2.1

Fig. 1C **Saos2**

GenBank	Gene Name	Ratio FGF2+FSK/Cont
BE881590	ETV1	66.6
AL833750	**SYTL3**	**33.9**
AI631159	SLC2A3	26.2
NM_144613	COX6B2	21.3
NM_002135	NR4A1	18.7
NM_001423	EMP1	16.8
AI638433	PDE7B	16.0
AK026181	PHLDA1	14.8
BC015940	NT5E (CD73)	13.7
M57765	**IL11**	**13.6**
M83248	**SPP1 (OPN)**	**12.4**
AI674404	SYTL3	12.2
BC003143	DUSP6	12.0
AW193698	TGFBR3	11.9
M11734	**CSF2**	**10.3**
AW003173	STC1	10.0
AA748418	MCTP2	8.6
NM_002309	LIF	7.5
NM_006037	**HDAC4**	**7.5**
AV733950	**EGR1**	**6.8**
R40917	PDE4D	6.7
NM_000584	**IL8**	**5.6**
NM_002658	PLAU	5.2
NM_000600	IL6	4.9
M27281	VEGF	4.6
NM_001200	**BMP2**	**4.4**
AY029180	PLAUR	4.4
AI968085	**WNT5A**	**4.1**
NM_002166	ID2	3.9

Fig. 1 Representative genes increased by FGF2 (A), FSK (B) and FGF2 plus FSK (FGF/FSK) (C) in human osteoblast-like Saos2 cells. The indicated genes were upregulated more than two-fold compared with the control. Genes in bold print were described in the Results.

Fig. 2A ROS17/2.8

GenBank	Gene Name	Ratio FGF2/Cont
A106002	**DMP1**	**129.8**
NM_012551	**EGR1**	**33.4**
AI0059968	FLT1	25.0
AI231350	DUSP6	21.7
AI717725	MIST1	15.9
NM_017134	ARG1	13.2
NM_053714	ANK	12.8
AI602811	DUSP6	11.4
NM_032069	GRIP1	7.5
BF415939	**c-FOS**	**7.2**
NM_012620	SERPINE1	7.1
NM_012953	FOSL1	5.9
NM_012610	NGFR	4.9
BI299621	IGF2R	4.7
NM_024355	**AXIN2**	**4.6**
NM_022277	CASP8	4.2
NM_021836	**JUNB**	**4.1**
NM_012603	**MYC**	**3.8**
NM_022266	**CTGF**	**2.8**
BI275741	EMP1	2.6
NM_133578	CPG21	2.6
BM387324	GIG2	2.6
U67140	DAP4	2.6
M94288	NOPP1 40	2.6
NM_031668	MYBBP 1A	2.5
AI137869	ATP2B1	2.5
NM_130429	LEF1	2.5

Fig. 2B ROS17/2.8

GenBank	Gene Name	Ratio FSK/Cont
BF415939	**c-FOS**	**10.7**
AW533010	BHLHB2	6.8
NM_012551	**EGR1**	**5.6**
L08595	NR4A2	4.6
AF200684	SLC7A7	4.1
BF283073	PRSS8	3.8
BF419085	**HDAC4**	**3.6**
AB020480	SNF1 LK	3.5
NM_021770	OLIG1	3.3
NM_030851	BDKRB1	3.3
BF565001	PDE4D	3.1
AW527989	GRIP1	2.9
M22586	GLS	2.8
NM_024355	**AXIN2**	**2.8**
AW535310	ADAMTS5	2.6
AI603439	ULK1	2.6
NM_024134	DDIT3	2.5
NM_017334	CREM	2.5
AA875132	TPM1	2.5
NM_057125	PEX6	2.5
BI285710	VAD	2.4
NM_053402	**WNT4**	**2.4**
AA891661	AQP1	2.4
NM_022266	**CTGF**	**2.1**
NM_012589	**IL6**	**2.1**

Fig. 2C ROS17/2.8

GenBank	Gene Name	Ratio FGF2+FSK/Cont
NM_017123	**AREG**	**73.0**
NM_017134	ARG1	29.3
NM_012551	**EGR1**	**22.6**
AI231350	DUSP6	22.5
AI059968	FIT1	19.2
AI060002	**DMP1**	**13.4**
NM_053883	DUSP6	13.1
NM_012653	SLC9A2	13.0
NM_053714	ANK	10.9
NM_032069	GRIP1	10.6
AF007789	PLAUR	9.1
NM_133578	CPG21	7.3
NM_023981	CSF1	6.7
NM_030851	BDKRB1	6.5
AF280967	**PTGES**	**6.4**
X63434	PLAU	6.3
NM_012953	FOSL1	6.3
AW535553	CSF1	6.0
NM_031556	CAV	5.2
AA901341	SLC2A3	5.0
AA818382	DLC1	4.8
AI102530	NAB2	4.8
NM_022266	**CTGF**	**4.5**
NM_017334	**CREM**	**4.1**
NM_021836	**JUNB**	**3.8**
NM_012603	**MYC**	**3.6**
NM_012589	IL6	3.6
AF140346	SFRP4	3.6
NM_024355	**AXIN2**	**3.5**

Fig. 2 Representative genes induced by FGF2 (A), FSK (B) and FGF2 plus FSK (FGF/FSK) (C) in ROS17/2.8 cells. The indicated genes were upregulated more than two-fold compared with the control. Genes in bold print were described in the Results.

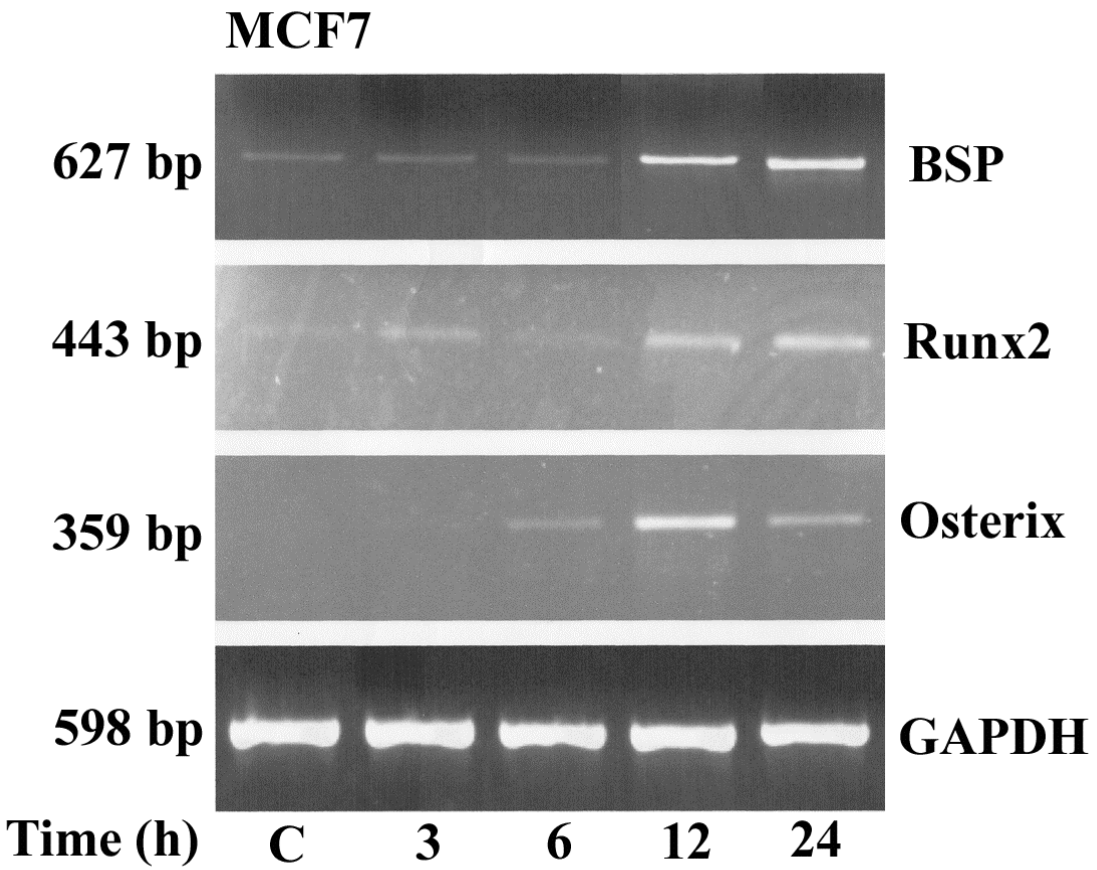

Fig. 3. Effects of FSK on BSP, Runx2 and Osterix mRNA levels in MCF7 cells. Results of time-course of RT-PCR for BSP, Runx2, Osterix and GAPDH mRNA levels in MCF7 cells after stimulation by FSK (1 μM) for 24 h. Total RNA was extracted, and the expression of BSP, Runx2, Osterix and GAPDH mRNA in these cells were analyzed by RT-PCR.

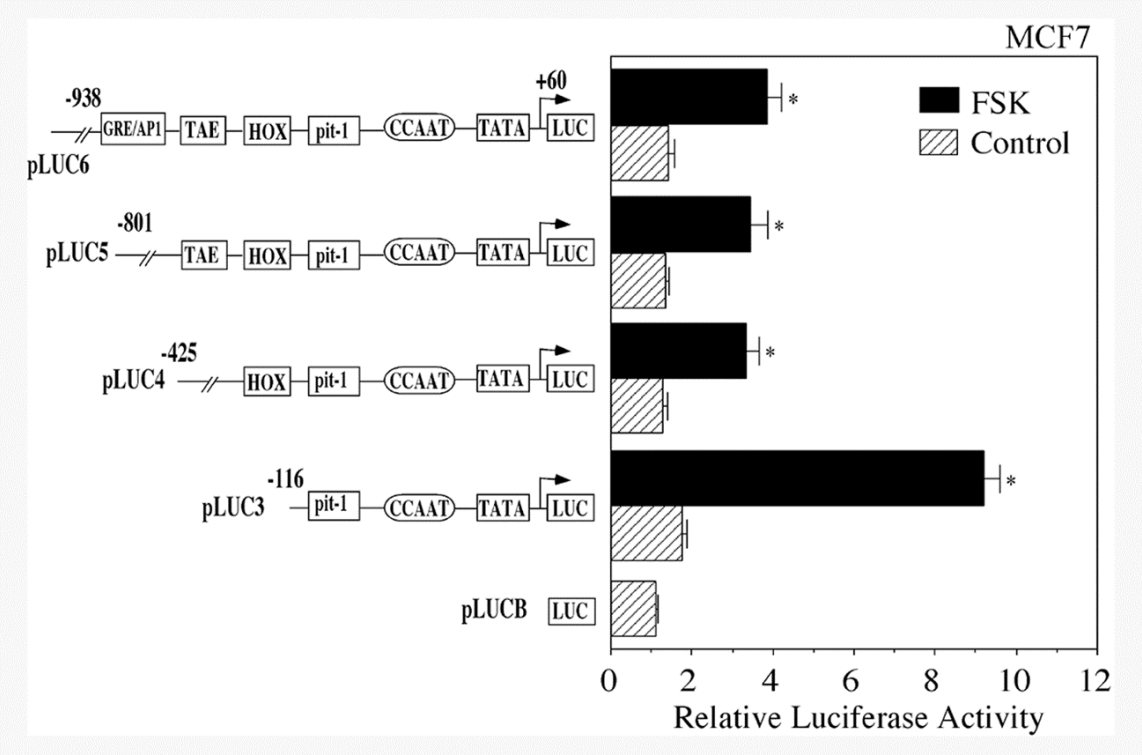

Fig. 4. FSK up-regulates BSP promoter activities. The transcriptional activities of pLUC3 (-116 to +60), pLUC4 (-425 to +60), pLUC5 (-801 to +60) and pLUC6 (-938 to +60) were increased after 12 h of treatment with 1 μM of FSK in MCF7 cells. The results of transcriptional activities obtained from three separate transfections with constructs, pLUC basic (pLUCB) and pLUC1 to pLUC6, have been combined and values expressed with standard errors. *($P<0.01$).

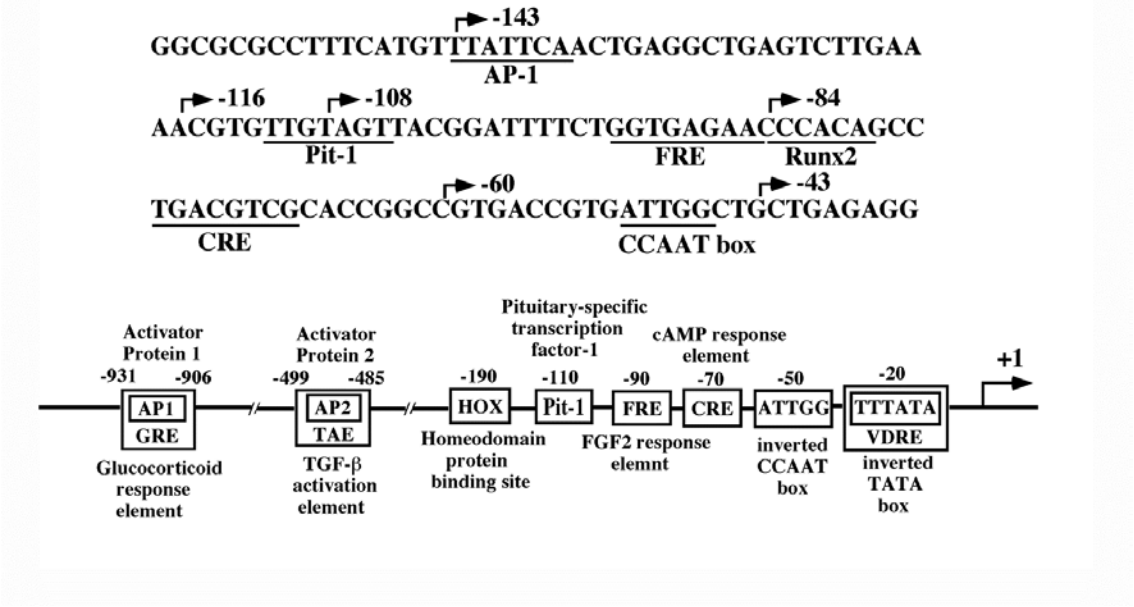

Fig. 5. Regulatory elements in the proximal promoter of rat BSP gene. **Upper:** The nucleotide sequence of the rat BSP gene proximal promoter is shown from -159 to -35. An inverted CCAAT box, CRE, Runx2, FRE, Pit-1 and AP-1 are present. **Lower:** The positions of inverted TATA and CCAAT boxes, vitamin D response element (VDRE) that overlaps the inverted TATA box, a CRE, a FRE, Pit-1, homeodomain protein-binding site (HOX), TAE (TGF-β activation element) overlaps AP-2, and GRE overlapping AP1 are shown.

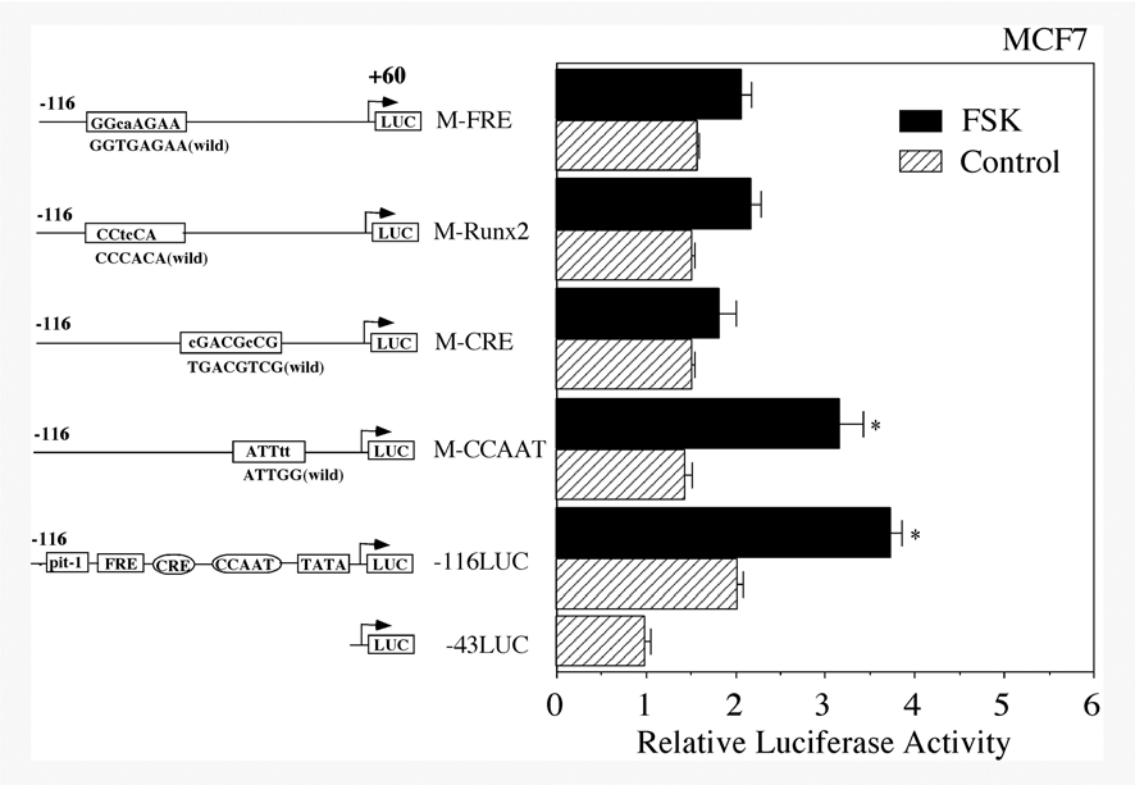

Fig. 6. Site-specific mutation analysis of luciferase activities. After introducing 2 bp mutations, transcriptional induction by FSK (1 μM) was partially inhibited in the M-CRE, M-Runx2 and M-FRE (pLUC3) constructs. The results of transcriptional activities obtained from four separate transfections with constructs were combined and the values expressed with standard errors. Significant differences from control: *($P<0.01$).

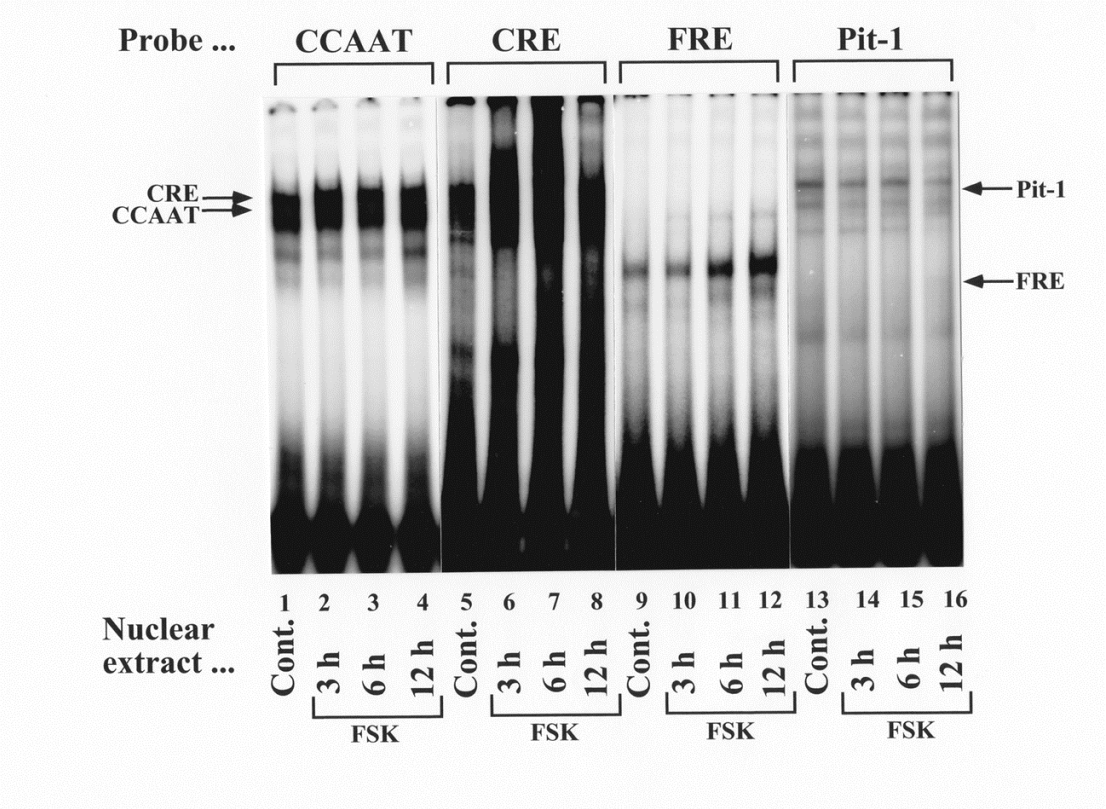

Fig. 7. Gel mobility shift assays using inverted CCAAT, CRE, FRE and Pit-1. After stimulation by 1 μM of FSK (3, 6 and 12 h), CRE-protein complexes increased at 3 h (lanes 5-8), and FRE-protein complexes increased at 6 h and reached a maximum at 12 h (lanes 9-12). CCAAT- and Pit-1-protein complexes did not change after stimulation by FSK (lanes 1-4, 13-16). DNA-protein complexes were separated on a 5% polyacrylamide gel in a low-ionic-strength Tris-borate buffer, dried under a vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.