Mechanical stress to bone plays a critical role in maintaining bone mass and strength. However, the molecular mechanism of mechanical stress-induced bone formation is not fully understood. In the present study, we demonstrate that FosB and its spliced variant ΔFosB, which is known to increase bone mass by stimulating bone formation in vivo, is rapidly induced by mechanical loading in mouse hind limb bone in vivo and by fluid shear stress (FSS) in mouse calvarial osteoblasts in vitro both at the mRNA and protein levels. FSS induction of FosB/ΔFosB gene expression was dependent on gadolinium-sensitive Ca²⁺ influx and subsequent activation of ERK1/2. Analysis of the mouse FosB/ΔFosB gene upstream regulatory region with luciferase reporter gene assays revealed that the FosB/ΔFosB induction by FSS occurred at the transcriptional level and was conferred by a short fragment from −603 to −327. DNA precipitation assays and DNA decoy experiments indicated that ERK-dependent activation of CREB binding to a CRE/AP-1 like element (designated “CRE2”) at the position of −413 largely contributed to the transcriptional effects of FSS. These results suggest that ΔFosB participates in mechanical stress-induced intracellular signaling cascades that activate the osteogenic program in osteoblasts.

Mechanical stress to bone plays a critical role in maintaining bone mass and strength. Reduced mechanical loading because of long-term bed rest or immobilization, or microgravity conditions in space, has been shown to cause significant bone loss and mineral changes. Evidence from human and animal studies has indicated that impaired bone formation significantly contributes to such unloading associated osteoporosis (1–3). Therefore, it is an important task for us to elucidate the mechanism by which mechanical loading induces bone formation to understand the pathophysiology and to establish an effective treatment of osteoporosis caused by reduced mechanical loading.

Bone cells have the ability to sense mechanical loading to activate multiple intracellular signaling pathways and transcription of various genes. Although the molecular device and the cellular repertoire in bone responsible for sensing such mechanical stimuli in vivo has not been precisely determined, it has been proposed that mechanotransduction in bone is mediated by changes in extracellular fluid flow caused by dynamic loading, which engenders fluid shear stress (FSS) to bone cells, particularly osteocytes that are a terminally differentiated form of osteoblasts embedded in calcified bone (4, 5). Consistently, FSS has been shown to elicit a divergent array of intracellular signaling pathways including intracellular calcium rise, activation of enzymes such as protein kinase B/Akt, mitogen-activated protein kinase family, and activation of transcription factors such as AP-1 (activator protein-1) and CREB (cyclic AMP response element-binding protein) in cells of the osteoblast lineage (6–8). Downstream of such signaling events is induction of various gene expression including extracellular matrix proteins such as type I collagen and osteopontin, and growth factors such as insulin-like growth factor-I (7). However, the molecular mechanisms as well as relevance to bone formation of such gene induction by mechanical stress in bone cells are largely unknown.

One of the earliest transcriptional events caused by mechanical loading in bone cells is induction of c-Fos (9), a proto-oncogene that belongs to the AP-1 transcription factor family consisting of three Fos and four Jun family members. As a prototype of “immediate early genes,” c-Fos expression is known to be rapidly and transiently induced by various stimuli including serum and growth factors (10–12). Induction of c-Fos expression by mechanical force has also been demonstrated in non-bone cells such as cardiac, muscle, and endothelial cells (13). Because mechanical loading induces several AP-1-responsive genes presumably involved in bone formation, c-Fos has been assumed to play a role in induction of such AP-1 target genes, thereby contributing to mechanical stress-induced bone formation. However, ubiquitous overexpression of c-Fos in transgenic mice resulted in development of osteosarcoma without evidence for increased bone formation (14). Therefore, although c-Fos may stimulate proliferation and/or survival of the osteoblast lineage cells, it is not able to induce bone formation when overexpressed alone in bone cells.

Another “immediate early gene” type member of the Fos family is FosB. Recently, transgenic mice overexpressing...
ΔFosB (15–17), a short splicing isoform of FosB gene, have been reported to exhibit a progressive increase in bone mass because of enhanced bone formation (18). Expression of ΔFosB was observed within the osteoblast lineage and regulated in a differentiation-associated manner, and the effects of ΔFosB overexpression on osteoblasts appeared to be cell-autonomous and reversible (19, 20). These results indicate that expression of ΔFosB alone is sufficient to induce bone formation, and further imply that ΔFosB may be involved in signaling pathways induced by osteogenic stimuli such as mechanical loading.

In the present study, we investigated a potential role of ΔFosB in mechanical stress-induced bone formation. The results indicated that FosB/ΔFosB gene expression in bone cells was induced by mechanical stress both in vitro and in vivo. The induction occurred in a manner dependent on gadolinium (Gd³⁺)-sensitive Ca²⁺ influx and ERK (extracellular signal-regulated kinase) but independent of prostaglandin production, and involved a transcriptional mechanism with a major contribution of CREB. Our results suggest that ΔFosB acts as a downstream effector of mechanical loading and participates in mechanical stress-induced osteogenic signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials—** A selective cyclooxygenase (COX)-2 inhibitor, JTE-522 (21, 22), was a kind gift from Japan Tobacco Inc. (Osaka, Japan). Indomethacin, nifedipine (L-type Ca²⁺ channel blocker), BAFTA-AM (intracellular Ca²⁺ chelator), AS3817 (calcium ionophore), dibutylde (dibutyryl cAMP), U0126 (ERK inhibitor), and SB203580 (p38 kinase inhibitor) were purchased from Calbiochem. pAP1-Luc, pCRE-Luc, and pSRE-Luc plasmids, which are cis-reporter plasmids containing tandem consensus AP-1-binding sites, CRE (cAMP-response element), and c-Fos-derived SRE (serum response element), respectively, were from Invitrogen. All chemicals for dual-luciferase reporter assays were from Promega (Madison, WI). Antibody against FosKAP63 was from Santa Cruz Biotechnology (San Diego, CA). Polyconal antibodies against ERK1/2 and activated (phospho) ERK1/2 were from Promega, and anti-CREB and phospho-CREB antibodies were from New England Biolabs (Beverly, MA). All DNA modifying enzymes used in this study were from New England Biolabs Inc. and the other reagents were from Sigma unless otherwise indicated.

**Experimental Animals—** For in vitro experiments, 7–9-week-old ICR male mice were purchased from Charles River Japan (Tokyo, Japan), and were acclimatized for 1 week. The body weight of the mice ranged from 20 to 25 g. All mice were allowed free access to food and water, housed in stainless cages in an air-conditioned environment (temperature: 24–25 °C, humidity: 50–55%) that was illuminated from 8:00 a.m. to 8:00 p.m. The experimental protocols in the current study have been performed according to the guideline principles in the Care and Use of Animals.

**Cell Culture—** Primary osteoblasts were prepared by calvariae of newborn ICR mice by sequential digestion with 0.1% (w/v) type IA collagenase and 0.2% (w/v) dispase as previously described (25). The osteoblasts were cultured in α-MEM supplemented with 10% fetal bovine serum (fetal bovine serum) (Sigma), penicillin/streptomycin (Invitrogen) for 48 h. Before induction experiments, 1 × 10⁵/ml osteoblasts were plated on culture dishes, grown to 70–80% confluence, and serum-deprived in α-MEM with 1% fetal bovine serum for 24 h. For mechanical loading in vitro, osteoblasts were exposed to FSS by placing 6-well culture dishes on a horizontal shaking apparatus fixed inside the culture incubator. All the experiments in the present study were performed at 100–120 rpm. The shear stress force in our system was estimated to be slightly less than that produced by 200 rpm with a cone viscometer, which was ~2 Pascal at the edge (26), and thus theoretically stayed within the physiological range.

**Adenovirus Infection—** A recombinant adeno vector for constitutively active MEK1 (MEKCA) was a kind gift from Dr. Sakae Tanaka (University of Tokyo). Primary osteoblasts at passage two were infected with MEKCA adenoviruses at a multiplicity of infection varying from 0 to 100. At 48 h after infection, cells were harvested and examined for expression of FosB/ΔFosB and other proteins by Western blot analysis.

**RNA Analysis—** Total RNA was extracted from tibia and femur of the mice or cultured primary osteoblasts using TRIzol reagents (Invitrogen) and random primers (Promega Corp., Madison, WI). One of a 20-μL RT reaction was used for PCR analysis. Primer sets used for amplification of the mouse GAPDH gene were designed as follows: sense, 5′-aagaggcgattgaggtgctgg-3′; and antisense, 5′-ttgacaggggtcgaaacgcggaggg-3′ for mouse FosB, which amplifies both the short spliced ΔFosB isoform and the long FosB transcripts; and sense, 5′-tggctcaccatttgaggaaggg-3′, and antisense, 5′-ttggtgactcaggttggggctgg-3′, for mouse GAPDH gene. The PCR cycle numbers were 28 for FosB/FosB and 22 for GAPDH, which were determined so that quantitative information was not lost. Amplified products were separated on 1.5–2.0% agarose gels and stained with ethidium bromide for visualization. For the RNase protection assay, a fragment of mouse FosB cDNA (nucleotides 1480–2051) was subcloned into pBlueScript SKII(+) (Stratagene, La Jolla, CA). The resultant plasmid was linearized and purified with Gel Extraction Kit (Qiagen Inc., Valencia, CA) and in vitro transcribed, then probe using the MAXIscript In Vitro Transcription Kit (Ambion Inc., Austin, TX). A control template for mouse β-actin was purchased from Ambion. RNase protection assays were performed using the RPAII Kit (Ambion) as previously described (27). Briefly, RNA samples were incubated with [α-32P]UTP-labeled cRNA probes at 42 °C for 18 h and digested with an RNase A/T1 mixture. Digestion RNA was precipitated, separated on a 6% polyacrylamide gel, transferred to 3MM Whatman filter paper, dried, and autoradiographed.

**Protein Analysis—** Preparation of nuclear extracts was described previously (25). Briefly, 1–2 × 10⁶ cells were collected, washed twice with ice-cold phosphate-buffered saline, incubated in ice-cold hypotonic buffer (10 mM HEPES-KOH, pH 7.2, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermine, 0.15 mM spermidine, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Sigma), and 10 mM Na₂MoO₄) for 10 min, vortexed for 10 s, and centrifuged at 15,000 × g at 4 °C for 60 s. The pellet was resuspended in NLB buffer (20 mM HEPES-KOH, pH 7.2, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Sigma), and 10 mM Na₂MoO₄) on ice for 15 min, and centrifuged at 4 °C at 15,000 × g for 10 min. The supernatant was saved as nuclear extracts. Total cell lysates were prepared using 1× Cell Lysis Buffer (Cell Signaling Technology Inc., Beverly, MA) according to the manufacturer’s instructions. The nuclear extracts and total cell lysates were stored at −80 °C until use. For Western blot analysis, 30 μg of protein was separated on a 5–20% gradient SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp.). The resultant blots were reacted with antibodies against FosB and other proteins by Western blot analysis.

**Cloning of the Mouse FosB Gene Promoter—** Fragments of mouse FosB gene (GenBank™ number AF095624) upstream regulatory region from nucleotides −1000 to +307 (1.0K), −603 to +307 (0.6K), and −327 to +307 (0.3K) (with the transcription start site being numbered +1) were cloned from a mouse genomic library (Toyobo Co., Ltd., Osaka, Japan) by PCR with the following primer sets: sense, 5′-aagggacctgtagctctgcggctgaggaac-3′, and antisense, 5′-acaaggtcttcatgctgcttggaggaac-3′, for 1.0K; sense, 5′-ttaaatggccagaggtgaacgcaggtgctggaggaac-3′, and antisense, acaaggtcttagtctgcggctgaggaac-3′, for 0.6K; and sense, 5′-acaaggtcttcatgctgcttggaggaac-3′, and antisense, 5′-acaaggtcttcatgctgcttggaggaac-3′, for 0.3K. PCR products were purified, digested with KpnI/HindIII (1.0K and 0.3K) or Smal/HindIII (0.6K), and subcloned into a luciferase reporter plasmid pGL3-basic (Promega, Madison, WI), which lacked eukaryotic promoter and enhancer sequences. The luciferase vector containing the longest 1.0K promoter, a series of mutant plasmids were generated by site-directed mutagenesis using the QuickChange™ Site-directed Mutagenesis Kit (STRATAGENE) with mutagenic primers including 5′-attatatTCG-3′ for SRE mutation (g547c and g548c) and 5′-tgCACA-3′ for CRE2 mutation (c556g, c557c, and t558a). All the constructs were verified by sequencing.

**Dual Luciferase Assay—** Osteoblasts were seeded in 6-well culture
plates at 50% confluence and cultured in α-MEM supplemented with 10% fetal bovine serum. After 18–24 h, the cells were co-transfected with 1 µg of chimeric luciferase reporter plasmids and 0.025 µg of pRL-TK Renilla luciferase plasmids (Promega) using GenePorter 2 Transfection Reagents (Gene Therapy Systems, Inc., San Diego, CA) in Opti-MEM supplemented with 1% fetal bovine serum. At 6 h after transfection, the medium was replaced by 2 ml of Opti-MEM supplemented with 1% fetal bovine serum, and the cells were incubated for an additional 24 h. The transfected cells were then treated with various reagents and/or exposed to FSS by placing the culture plates on a shaking apparatus at 100 to 120 rpm. For dual luciferase assays, the cells were washed twice with PBS and lysed with 100 µl of passive cell lysis buffer (Promega). Luciferase activities were measured with luminometer (ATTO, Tokyo, Japan) by mixing 50 µl of luciferase substrate solution (Promega) with 10 µl of cell lysates. Transcriptional activity was normalized for Renilla luciferase activity or protein concentrations.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay was performed as previously described (25). Radiolabeled double-stranded oligonucleotide probes were prepared by annealing complementary oligonucleotides end-labeled with [α-32P]dATP and DNA polymerase I fragment (Klenow) (New England Biolabs). The labeled probes were purified by Sephadex G-25 columns (Quick Spin Columns, Roche Molecular Biochemicals), diluted with distilled water, and 3 × 10⁴ cpmp was incubated with nuclear extracts (10 µg) and 4 µg of poly(dI-dC)poly(dI-dC) in a total volume of 20 µl of 1/2 × NLB buffer at 4 °C for 30 min. For competition analysis, a hundred times molar excess of cold oligonucleotide probes were added to the electrophoretic mobility shift assay reaction. Resolution was accomplished by electrophoresing 15 µl of the reaction mixture on 4.5 to 5.0% polyacrylamide gels using 0.25 TBE buffer (22.3 mM Tris-HCl, 22.3 mM boric acid, and 0.25 mM EDTA, pH 8.0). Gels were then transferred onto 3MM filter paper and dried, and protein-DNA complexes were visualized by autoradiography.

**DNA Precipitation Assay**—Biotinylated double-stranded oligonucleotide probes containing three tandem repeats of SRE/CRE2 (28, 29) were synthesized to form a double-stranded DNA oligomer with the both ends hinged by a stretch of seven Ts. This structure has been demonstrated to confer resistance to endogenous nucleases (29). As controls, we also synthesized mutant CDODNs including: Scramble, 5′-ataaggactgctgcctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt...
were introduced into osteoblasts by GenePorter 2 Transfection Reagents according to the manufacturer's instruction.

RESULTS

In Vivo Mechanical Forces and In Vitro FSS to Osteoblasts Induce FosB/ΔFosB Gene Expression at Both the mRNA and Protein Levels—

We first investigated whether or not expression of the FosB/ΔFosB gene in bone is induced by mechanical loading in vivo. Seven to 9-week-old ICR male mice were tail suspended for 4 days to reduce the background expression, and then mechanically reloaded in rotating cages. As shown in Fig. 1A, expression of (long) FosB and ΔFosB mRNA in tibiae and femurs was undetectable in tail-suspended mice, but was induced as early as 30 min after mechanical reloading and reached the maximum within 2 h. In the in vitro experiments shown in Fig. 1B, primary osteoblasts derived from newborn mouse calvariae were subjected to FSS on a shaking apparatus. As a result, we observed that FosB/ΔFosB mRNA was induced by FSS in primary osteoblasts, recapitulating the in vivo induction in reloaded mice. These results are consistent with the assumption that the in vivo mechanical forces caused FSS to bone cells of the osteoblast lineage, leading to increased expression of FosB/ΔFosB. To confirm that the induction of FosB/ΔFosB mRNA by FSS leads to an increase in the amount of their protein products, nuclear extracts were obtained from cells that had been exposed to FSS and were analyzed for FosB/ΔFosB protein expression by Western blotting. The results indicated that FosB (50 kDa) and ΔFosB (32 kDa) proteins were both induced either by mechanical loading in vivo or FSS in vitro in a time-dependent manner (Fig. 1, C and D). These findings were similarly observed in other osteoblastic cell lines such as a murine calvarial cell line, MC3T3-E1, and a murine bone marrow stromal cell line, ST-2 (data not shown). Therefore, induction of FosB/ΔFosB gene expression by mechanical stress occurs at both the mRNA and protein levels in cells of the osteoblast lineage.

Induction of FosB/ΔFosB mRNA Is Independent of Prostaglandin Production—Previous reports suggest that prostaglandins are important mediators of mechanical stress-induced bone formation. Mechanical stress has been shown to cause activation of a constitutive type of prostaglandin G/H synthase (COX-1) and transcriptional up-regulation of an inducible isoform (COX-2) (8, 30), and both events would lead to increased production of prostaglandins, especially the E series that have been shown to induce c-Fos (31). To determine whether FSS-induced FosB/ΔFosB expression is mediated by prostaglandins, tail-suspended mice were subcutaneously injected with indo-

![Fig. 3. FSS-induced FosB/ΔFosB induction is dependent on ERK. A, primary calvarial osteoblasts were pretreated with 10 μM U0126, 10 μM SB203580, or a vehicle for 30 min. Cells were then exposed to FSS for 30 min and analyzed for FosB/ΔFosB and GAPDH mRNA expression by RT-PCR. Control is a negative control without FSS. B, to evaluate ERK activation by FSS, cells were exposed to FSS for the indicated times, lysed, and examined by Western blot analysis using antibodies against phosphorylated (pERK1/2) and total ERK1/2. C, primary osteoblasts were infected with an adenovirus expressing constitutively active MEK1 (MEKCA) (44) at increasing multiplicity of infection for 48 h, and analyzed for FosB/ΔFosB, MEK1, and β-actin expression by Western blotting.](#)

![Fig. 4. FSS-induced FosB/ΔFosB induction was dependent on Ca²⁺ influx through a Gd³⁺-sensitive cation channel. Mouse calvarial osteoblasts were pretreated with vehicles, 10 μM nifedipine, 10 μM gadolinium chloride (Gd³⁺), 2.5 mM EGTA, or 50 μM BAPTA-AM for 30 min and then exposed to FSS for 30 min. Levels of FosB/ΔFosB and GAPDH mRNA expression and ERK phosphorylation were determined by RT-PCR (A) and Western blot analysis, respectively (B).](#)
methylene chloride, a general COX inhibitor (both COX-1 and COX-2), prior to reloading, and FosB/ΔFosB mRNA induction in tibiae and femurs was analyzed by RT-PCR. As shown in Fig. 2A, FosB/ΔFosB mRNA induction was not blocked by indomethacin. Similarly, pretreatment with either indomethacin or a selective COX-2 inhibitor, JTE-522, had no major effects on FSS-induced FosB/ΔFosB expression in osteoblasts in vitro (Fig. 2B). These results indicated that mechanical stress-induced FosB/ΔFosB induction was independent of pros-taglandin production both in vivo and in vitro.

FSS-induced FosB/ΔFosB mRNA Expression Is Dependent on ERK—We then attempted to delineate the intracellular signaling pathways leading to FosB/ΔFosB induction. We first investigated involvement of the mitogen-activated protein kinase family members including ERK, p38 kinase, and c-Jun N-terminal kinase in response to FSS. As shown in Fig. 2A, FosB/ΔFosB mRNA induction was completely blocked by extracellular (EGTA) and intracellular (BAPTA-AM) Ca²⁺ chelators, and Gd³⁺, suggesting involvement of a Gd³⁺-sensitive Ca²⁺ channel. Neither voltage-dependent Ca²⁺ channel inhibitors, nifedipine (Fig. 4A) nor verapamil (data not shown), affected the FosB/ΔFosB mRNA induction. We examined the same set of inhibitors for the effects on FSS-induced ERK activation, and obtained virtually the same results (Fig. 4B). We also confirmed that Ca²⁺ ionophores such as A23187 and ionomycin induced both ERK activation and FosB/ΔFosB induction.
mRNA expression in osteoblasts (data not shown). Taken together, these results suggested that FSS induced FosB mRNA expression in a manner dependent on Ca\(^{2+}\)-influx through a Gd\(^{3+}\)-sensitive cation channel and the subsequent ERK activation.

Induction of FosB mRNA Expression by FSS Occurs at the Transcriptional Level—To determine whether induction of FosB mRNA expression by FSS occurred at the transcriptional level, we cloned and analyzed the mouse FosB gene promoter (34). A genomic fragment (1,000 to 307) containing a TATA box and a 1.0-kb long upstream regulatory region of the FosB gene was obtained by genomic polymerase chain reaction and subcloned into a luciferase reporter vector PGL3-basic. The resultant vector pGL3–1.0K was transfected into primary osteoblasts, and the promoter activities were measured in the absence or presence of FSS in vitro.

As shown in Fig. 5A, FSS stimulated FosB gene promoter activity in a time-dependent manner, with a peak being 4-fold induction at 6 h. Consistent with the results of mRNA expression studies shown in Figs. 3 and 4, the induction was almost completely abrogated by an ERK inhibitor U0126, Gd\(^{3+}\), or an intracellular Ca\(^{2+}\) chelator BAPTA-AM (Fig. 5B). Thus, FSS induced FosB mRNA expression in a manner dependent on a Gd\(^{3+}\)-sensitive cation channel and ERK.

Cyclic AMP Response Element-like Sequences (CRE2) Contribute to FSS-induced FosB Gene Transcription—We then attempted to determine DNA elements mediating FSS-induced FosB/ΔFosB gene transcription. For this purpose, we first generated two deletion constructs, PGL-0.6K containing a promoter fragment from 603 to 307 and PGL-0.3K containing a fragment from 327 to 307, and tested FSS effects on promoter activities of these two deletion constructs together with the original PGL-1.0K containing a region between 1,000 and 327. As shown in Fig. 5C, the response was retained in PGL-0.6K but significant induction by FSS was lost in PGL-0.3K, indicating that the transcriptional response to FSS was conferred by a region between 603 and 327.

It has been suggested that FosB and c-Fos genes have evolved from a common ancestor gene by gene duplication (34). Accordingly, these genes have a similar genomic organization and are subject to a similar mode of transcriptional regulation as an immediate early gene, and most of the critical DNA elements in the c-Fos gene promoter are also conserved in the upstream regulatory region of the FosB gene (11, 12). We found that such key elements were present in the FosB/ΔFosB promoter region between 607 and 327 that we have identified to confer the shear stress response: an upstream CRE or AP-1 like sequences from 479 to 470 that we designated CRE1, and SRE from 428 to 419 with an immediately downstream CRE or AP-1 from 413 to 407 (we designated SRE/CRE2) (Fig. 5C). We therefore examined whether each of these elements, whose counterparts in the c-Fos gene have been shown to play an important role in its transcriptional regulation, were able to respond to FSS. When an oligonucleotide corresponding...
Mechanoinduction of ΔFosB in Bone

In the present study, we have demonstrated that FSS to osteoblasts induces ΔFosB gene expression via a Ca^{2+}- and ERK-dependent manner.}

**Fig. 7.** FSS causes phosphorylation of CREB, which binds to the CRE2 sequences, in a Ca^{2+}- and ERK-dependent manner. Primary osteoblasts were subjected to FSS for the indicated times. Total cell lysates (A–C) or nuclear extracts (D–E) were prepared and analyzed by Western blot using antibodies against phosphorylated or total CREB (A–C) or analyzed for DNA binding by DNA precipitation followed by immunoblotting with phospho-CREB antibody. A, effect of FSS was compared with that of treatment with 100 nM A23187. FSS was applied in the presence of vehicle alone, 25 mM EGTA, or 50 μM BAPTA-AM. C, FSS was applied in the presence of vehicle alone or 10 μM U0126. D, nuclear extracts from cells subjected to FSS were analyzed for binding of phosphorylated CREB to CRE1 or SRE/CRE2 oligonucleotide probes by DNA precipitation assay. E, DNA precipitation assay for phospho-CREB binding to SRE/CRE2 were performed in the presence of free competitors including intact (Wt), SRE-mutated (Sm1), or CRE-mutated (Cm) SRE/CRE2 oligonucleotides.

**Binding of Activated CREB to the CRE2 Element Is Critical to Transcriptional Induction of ΔFosB Gene by FSS.**

Our results have demonstrated that SRE/CRE2 sequences alone can confer an FSS response to a comparable extent to the full-length promoter and further suggest that the factor binding to the CRE2 site is critical to the transcriptional induction. We therefore examined whether CREB is activated and binds to this element in response to FSS. The results indicated that FSS, as well as a calcium ionophore, A23187, did induce CREB phosphorylation in osteoblasts (Fig. 7A), which occurred in a manner dependent on Ca^{2+} and ERK (Fig. 7, B and C). Moreover, DNA precipitation assays revealed that phosphorylated CREB induced by FSS did bind to the SRE/CRE2 sequences, but not to the CRE1 site (Fig. 7D). We also confirmed that binding of activated CREB to the SRE/CRE2 site occurred in a manner dependent on the CRE2 sequences by competition analysis (Fig. 7E). Taken together, these results suggest that FSS induces ΔFosB gene expression by activating CREB in a Ca^{2+}- and ERK-dependent manner, which then interacts with CRE2 to promote gene transcription.

Although our results demonstrate a role of CREB activation and subsequent binding to the CRE2 site, its relative importance in the context of full-length promoter remained obscure. Therefore, we applied an oligonucleotide decoy strategy to determine contribution of the SRE/CRE2 sequences to the transcriptional induction by FSS. We utilized circular dumbbell decoy oligonucleotides (CDODN) to inhibit binding of transcription factors to the SRE/CRE2 sequences. CDODN have been demonstrated to show efficient cellular uptake and increased stability (28, 29). We first confirmed by DNA precipitation assay that SRE/CRE2 CDODN actually bound activated CREB, whereas scrambled CDODN did not (Fig. 8A). Introduction of SRE/CRE2 CDODN into osteoblasts caused a dose-dependent inhibition of transcriptional induction by FSS. The maximal dose (10 μg/ml) caused nearly complete inhibition, indicating a major contribution of the transcription factors binding to the SRE/CRE2 site (Fig. 8B). Consistent with a critical role for CREB binding to the CRE2 site, we further demonstrated by mutant CDODN that the ability of SRE/CRE2 CDODN to inhibit FSS-induced ΔFosB gene transcription was dependent on the CRE2 sequences (Fig. 8C). An SRE-mutated SRE/CRE2 CDODN were still able to block the FSS response, whereas a CRE-mutated CDODN lost the ability to block ΔFosB gene transcription. Moreover, we generated mutant promoter constructs in which either or both of the SRE and CRE2 sites were disrupted by site-directed mutagenesis and demonstrated that disruption of the CRE2 site in the context of the full-length promoter resulted in a significant reduction in the FSS response, whereas disruption of the SRE site showed a minimal effect (Fig. 8D). We therefore conclude that the CRE2 sequences at −407 in the upstream regulatory region of the ΔFosB gene make a major contribution to the transcriptional response to FSS in osteoblasts.

**DISCUSSION**

Our results demonstrate that FSS to osteoblasts induces ΔFosB gene expression via a Ca^{2+}- and ERK-dependent manner. Primary osteoblasts were subjected to FSS for the indicated times. Total cell lysates (A–C) or nuclear extracts (D–E) were prepared and analyzed by Western blot using antibodies against phosphorylated or total CREB (A–C) or analyzed for DNA binding by DNA precipitation followed by immunoblotting with phospho-CREB antibody. A, effect of FSS was compared with that of treatment with 100 nM A23187. FSS was applied in the presence of vehicle alone, 25 mM EGTA, or 50 μM BAPTA-AM. C, FSS was applied in the presence of vehicle alone or 10 μM U0126. D, nuclear extracts from cells subjected to FSS were analyzed for binding of phosphorylated CREB to CRE1 or SRE/CRE2 oligonucleotide probes by DNA precipitation assay. E, DNA precipitation assay for phospho-CREB binding to SRE/CRE2 were performed in the presence of free competitors including intact (Wt), SRE-mutated (Sm1), or CRE-mutated (Cm) SRE/CRE2 oligonucleotides.

**Fig. 7.** FSS causes phosphorylation of CREB, which binds to the CRE2 sequences, in a Ca^{2+}- and ERK-dependent manner. Primary osteoblasts were subjected to FSS for the indicated times. Total cell lysates (A–C) or nuclear extracts (D–E) were prepared and analyzed by Western blot using antibodies against phosphorylated or total CREB (A–C) or analyzed for DNA binding by DNA precipitation followed by immunoblotting with phospho-CREB antibody. A, effect of FSS was compared with that of treatment with 100 nM A23187. FSS was applied in the presence of vehicle alone, 25 mM EGTA, or 50 μM BAPTA-AM. C, FSS was applied in the presence of vehicle alone or 10 μM U0126. D, nuclear extracts from cells subjected to FSS were analyzed for binding of phosphorylated CREB to CRE1 or SRE/CRE2 oligonucleotide probes by DNA precipitation assay. E, DNA precipitation assay for phospho-CREB binding to SRE/CRE2 were performed in the presence of free competitors including intact (Wt), SRE-mutated (Sm1), or CRE-mutated (Cm) SRE/CRE2 oligonucleotides.

**Binding of Activated CREB to the CRE2 Element Is Critical to Transcriptional Induction of ΔFosB Gene by FSS.** Our results have demonstrated that SRE/CRE2 sequences alone can confer an FSS response to a comparable extent to the full-length promoter and further suggest that the factor binding to the CRE2 site is critical to the transcriptional induction. We therefore examined whether CREB is activated and binds to this element in response to FSS. The results indicated that FSS, as well as a calcium ionophore, A23187, did induce CREB phosphorylation in osteoblasts (Fig. 7A), which occurred in a manner dependent on Ca^{2+} and ERK (Fig. 7, B and C). Moreover, DNA precipitation assays revealed that phosphorylated CREB induced by FSS did bind to the SRE/CRE2 sequences, but not the CRE1 site (Fig. 7D). We also confirmed that binding of activated CREB to the SRE/CRE2 site occurred in a manner dependent on the CRE2 sequences by competition analysis (Fig. 7E). Taken together, these results suggest that FSS induces ΔFosB gene expression by activating CREB in a Ca^{2+}- and ERK-dependent manner, which then interacts with CRE2 to promote gene transcription.

Although our results demonstrate a role of CREB activation and subsequent binding to the CRE2 site, its relative importance in the context of full-length promoter remained obscure. Therefore, we applied an oligonucleotide decoy strategy to determine contribution of the SRE/CRE2 sequences to the transcriptional induction by FSS. We utilized circular dumbbell decoy oligonucleotides (CDODN) to inhibit binding of transcription factors to the SRE/CRE2 sequences. CDODN have been demonstrated to show efficient cellular uptake and increased stability (28, 29). We first confirmed by DNA precipitation assay that SRE/CRE2 CDODN actually bound activated CREB, whereas scrambled CDODN did not (Fig. 8A). Introduction of SRE/CRE2 CDODN into osteoblasts caused a dose-dependent inhibition of transcriptional induction by FSS. The maximal dose (10 μg/ml) caused nearly complete inhibition, indicating a major contribution of the transcription factors binding to the SRE/CRE2 site (Fig. 8B). Consistent with a critical role for CREB binding to the CRE2 site, we further demonstrated by mutant CDODN that the ability of SRE/CRE2 CDODN to inhibit FSS-induced ΔFosB gene transcription was dependent on the CRE2 sequences (Fig. 8C). An SRE-mutated SRE/CRE2 CDODN were still able to block the FSS response, whereas a CRE-mutated CDODN lost the ability to block ΔFosB gene transcription. Moreover, we generated mutant promoter constructs in which either or both of the SRE and CRE2 sites were disrupted by site-directed mutagenesis and demonstrated that disruption of the CRE2 site in the context of the full-length promoter resulted in a significant reduction in the FSS response, whereas disruption of the SRE site showed a minimal effect (Fig. 8D). We therefore conclude that the CRE2 sequences at −407 in the upstream regulatory region of the ΔFosB gene make a major contribution to the transcriptional response to FSS in osteoblasts.
ERK/CREB signaling pathway at the transcriptional level in vitro. FSS-activated CREB stimulated FosB/ΔFosB gene transcription through interaction with the CRE2 element, which appeared largely responsible for the FSS effect as demonstrated by DNA decoy experiments. Consistent with the direct transcriptional effect, induction of FosB/ΔFosB gene expression by mechanical forces was independent of prostaglandins known to induce c-Fos. We also confirmed that mechanical loading to bone caused accumulation of the ΔFosB protein in vivo. Because transgenic overexpression of ΔFosB has been shown to stimulate bone formation and thereby cause osteosclerosis in mice (18), increased expression of ΔFosB should contribute to mechanical stress-induced bone formation (Fig. 9).

ΔFosB is a C-terminal truncated FosB gene product generated by alternative splicing (15–17). Although physiological roles for ΔFosB are not fully understood, it has been proposed that ΔFosB is a molecular mediator of long-term neural and behavioral plasticity (35–37). In contrast to other Fos family members that are transiently induced in brain by various acute stimuli including electrical stimulation, stress, and psychotropic drugs, ΔFosB has been demonstrated to accumulate in specific regions of the brain after chronic administration of drugs of abuse and compulsive running (37). Moreover, in vivo overexpression of ΔFosB in brain resulted in augmented locomotor responses to cocaine administration and enhanced rewarding effects of cocaine and morphine, suggesting a role for ΔFosB in drug addiction (35). Directly relevant to our current study is the extremely stable nature of the ΔFosB protein (37). Based on the analogy of mechanical effect on bone to drug addiction in the sense that both are intermittent and repetitive in nature and are dependent on its magnitude and frequency (8), we propose that ΔFosB may also act as a molecular mediator of the mechanical loading effect on bone, which accumulates an intermittently loaded mechanical stress and further enhances mechanosensitivity. Validity of such a hypothesis is currently being tested in our laboratory.

A signaling pathway that involves Ca²⁺, ERK, and CREB has already been described in other cell lineages (38–40). As a mechanism of ERK-dependent CREB activation, p90 ribosomal S6 protein kinase 1, a downstream effector of ERK, has been suggested to directly phosphorylate and activate CREB in human airway epithelial cells (41). And, although roles for CREB in bone formation remain to be established, a recent report has demonstrated that CREB is involved in transcriptional induction of cyclooxygenase-2 gene expression by FSS in a mouse osteoblastic cell line, acting in concert with C/EBP and AP-1 (30). Therefore, it is likely that the ERK/CREB pathway, which induces multiple transcriptional targets, constitutes a significant part of divergent intracellular signaling events induced by mechanical loading that leads to enhanced bone formation.

Initial characterization of the FosB/ΔFosB gene promoter (34) has revealed that the upstream regulatory region of the FosB/ΔFosB gene shares most critical transcription factor binding sites with that of c-Fos gene (11, 12): CRE and immediate downstream CRE/AP-1-like elements, which we designated CRE2 in the current paper, intervened by 6 nucleotides. Our results indicate that shear stress response elements in the FosB/ΔFosB promoter are present in a short region between −607 and −327 containing the above-mentioned important sequences. This region considerably overlaps with recently reported mechanoresponsive regions in the c-Fos promoter (42). Because induction of FosB/ΔFosB gene transcription was de-
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References

1. Morey, E. R., and Baylink, D. J. (1978) Science 204, 1138–1141
2. Schneider, V., Ogawara, V., LeBlanc, A., Rakmonov, A., Taggart, L., Bakulin, A., Hunton, C., Gregorier, A., and Varonlin, L. (1995) Acta Osteontol. 36, 463–466
3. Inoue, M., Tanaka, H., Moriwake, T., Oku, M., Sekiguchi, C., and Seino, Y. (2000) Bone (N.Y.) 26, 281–288
4. Baur, D. B., Nohring, A. G., and Turner, C. H. (2002) Bone (N.Y.) 30, 781–786
5. Knothe-Tate, M. L. (2003) J. Biomech. 36, 1409–1424
6. Mikuni-Takagaki, Y. (1999) J. Bone Miner. Res. 14, 57–60
7. Nomura, S., and Takano-Yamamoto, T. (2000) Matrix Biol. 19, 91–96
8. Ehrlich, P. J., and Lanyon, L. E. (2002) Osteoporos. Int. 13, 688–700
9. Lean, J. M., Mackay, A. G., Chow, J. W., and Chambers, T. J. (1996) Am. J. Physiol. 270, E937–E945
10. Kato, M., Liu, Z., and Zandi, E. (1997) Cell. Signal. 12, 435–445
11. Chinnadurai, A. E., Schenck, K., Wang, Z. Q., and Wagner, E. F. (1993) J. Cell Biol. 122, 685–701
12. Munder, D., Lucibello, F. C., Schuermann, M., and Muller, R. (1991) Genes Dev. 5, 1212–1223
13. Ven, J., Wisdom, H. M., Tratnir, I., and Verma, I. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5077–5081
14. Nakabeppu, Y., and Nathans, D. (1991) Cell 64, 751–759
15. Sabatkos, G., Sims, N. A., Chen, J., Anki, K., Kelz, M. B., Andling, M., Bouali, Y., Mukhopadhyay, K., Ford, K., Nestler, E. J., and Baron, R. (2000) Nat. Med. 6, 985–990
16. Krefting, M., Chiuasrili, R., Sims, N. A., Wu, M., Sabatkos, G., Gorre, H. W., and Baron, R. (2002) Endocrinology 143, 4304–4309
17. Sims, N. A., Sabatkos, G., Chen, J. S., Kelz, M. B., Nestler, E. J., and Baron, R. (2002) Bone (N.Y.) 30, 32–39
18. Matsushita, M., Masaki, M., Yagi, Y., Tanaka, T., and Wakiiri, K. (1997) Inflamm. Res. 46, 461–467
19. Ono, K., Akatsu, T., Murakami, T., Kitamura, R., Yamamoto, M., Shinomiya, N., Rokutanda, M., Sasaki, T., Amizuka, N., Ozawa, H., Nagata, N., and Kugari, N. (2000) J. Bone Miner. Res. 15, 774–781
20. Sakata, T., Sakai, A., Tsukurumi, H., Okimoto, N., Okazaki, I., Ikeda, S., Norimura, T., and Nakamura, T. (1999) J. Bone Miner. Res. 14, 1596–1604
21. Matsumoto, T., Nakayama, K., Kodama, Y., Fuse, H., Nakamura, T., and Nakamoto, S. (1999) Bone (N.Y.) 25, 985–992
22. Inoue, D., Santiago, P., Horne, W. C., and Baron, R. (1997) J. Biol. Chem. 272, 25386–25393
23. Sakai, K., Mohitai, M., Shida, J., Harayama, K., Benvenuti, S., Brandt, M. L., Kukita, T., and lwamoto, Y. (1999) J. Bone Miner. Res. 14, 2089–2098
24. Inoue, D., Reit, M., Loom, L., Krasznear, K., Wessakum, G., Muong, Y. M., Baron, R., and Blohel, C. P. (1999) J. Biol. Chem. 274, 4180–4187
25. Ahn, J. D., Kim, C. H., Mages, K., Kim, Y. H., Kim, H. J., Park, K. K., Hang, S. P., Park, K. G., Lee, I. K., and Chang, Y. C. (2003) Biochem. Biophys. Res. Commun. 310, 1048–1053
26. Lee, I. K., Ahn, J. D., Kim, H. S., Park, J. Y., and Lee, K. U. (2003) Curr. Drug Targets 4, 619–623
27. Ogawa, A., Arakawa, T., Kaneda, T., Takuma, T., Sato, T., Kano, K., Kumeogawa, M., and Hakeda, Y. (2001) J. Biol. Chem. 276, 7048–7054
28. Weinreb, M., Rutledge, S. J., and Rodan, G. A. (1997) Bone (N.Y.) 20, 247–353
29. Takahashi, M., Ishida, T., Tsuchi, T., Okuma, M., and Watanabe, J. (1999) Blood 93, 281–286
30. Lanza, P. S., Dorrman, K., Neguchi, T., Mattei, M. G., and Brave, R. (1992) Nucleic Acids Res. 20, 340–353
31. Kelz, M. B., Chen, J., Carlezen, W. A., Jr., Wieder, K., Gilden, L., Beckman, A. M., Steffen, C., Zhang, Y. J., Marotti, L., Self, D. W., Tacket, T., Baraauanusa, G., Surmeier, D. J., Neve, R. L., Duman, R. S., Picciotto, M. R., and Nestler, E. J. (1999) Nature 401, 272–276
32. Nestler, E. J., Kelz, M. B., and Chen, J. (1999) Brain Res. 855, 10–17
33. Nestler, E. J., Barret, M., and Self, D. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11042–11046
34. Okubo, N., Mitsuda, N., Tamatami, M., Yamaguchi, A., Lee, D. Y., Ogihara, T., Tite, M. P., and Tokuyama, M. (2003) J. Biol. Chem. 278, 3046–3053
35. Zanazzl, P., Saulillo, M., Felicierle, A., Avendino, E. V., Gallo, V., and Schinelli, S. (2001) J. Biol. Chem. 276, 11487–11495
36. Nashat, A. H., and Langer, K. (2003) Mol. Cell. Biol. 23, 4783–4786
37. Song, S. K., Seong, J. K., Chung, H. C., Lee, W. J., Kim, C. H., Cho, K. N., Kang, C. D., Koo, J. S., and Yoon, J. H. (2003) J. Biol. Chem. 278, 34890–34896
38. Peake, M. A., and El Haj, A. J. (2003) FEBS Lett. 537, 117–120
39. Sadoshima, J., and Izzono, Y. (1993) Circ. Res. 73, 424–438
40. Miyazaki, T., Katagiri, H., Kanaegi, Y., Takayanagi, H., Sawada, Y., Yamamoto, A., Pando, M. P., Asano, T., Verma, I. M., Oda, H., Nakamura, K., and Tanaka, S. (2000) J. Cell Biol. 148, 333–342

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