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Developmental Profiles of Phosphorylated and Unphosphorylated CREBs in Murine Calvarial MC3T3-E1 Cells[1]

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The cAMP-responsive element (CRE) binding protein/activating transcription factor (CREB/ATF) family plays a major role in the expression of skeletal-specific genes and skeletal tissue development. We analyzed the changes of the amount, degree of phosphorylation and binding activity of the CREB/ATF family in the course of development of the murine calvarial osteoblastic cell line MC3T3-E1 as an *in vitro* model system of bone formation. The amount of CREB in the whole-cell extract detectable by Western blot analysis was high through all stages of development and maximal in the proliferation stage. The degree of phosphorylation estimated with anti-phosphorylated CREB antibody changed greatly and reached high levels in the proliferation stage and early mineralization stage. The ratio of phosphorylated CREB to total CREB in the CREB-CRE complex was also examined by gel shift assay. Although the binding to the consensus/CRE probe reached almost equally high levels in the proliferation stage and early mineralization stage, the relative level of phosphorylated CREB in the CREB-CRE complex was different in these two stages. In the early mineralization stage, most CREB bound to consensus/CRE was phosphorylated, while both phosphorylated and unphosphorylated CREB were bound to consensus/CRE in the proliferation stage. ATF-1 was also detected as a minor component bound to the consensus/CRE probe. The alteration of the binding of CREB to consensus/CRE over the course of osteoblast development supports the hypothesis that CREB may regulate the expression of genes defining the developmental sequence of MC3T3-E1 cells.

Key words: binding activity, cyclic AMP response element, CRE-binding protein, osteoblast development, phosphorylation.

The transcription factors of the leucine zipper family play major roles in the regulation of skeletal-specific gene expression and skeletal tissue development (*1-8*). These transcription factors fall primarily into two broad classes, the cAMP response element (CRE) binding protein/activating transcription factor (CREB/ATF) family and the AP-1 family. The CREB/ATF family includes a variety of CREB, cAMP response element modulator (CREM), and ATF classes of factors, and the AP-1 family includes *fos*, *jun*, and *fra* (*9-14*). The CREB/ATF family can be activated by phosphorylation catalyzed by cAMP-dependent protein kinase (PKA) to stimulate gene transcription (*15-18*). Both parathyroid hormone (PTH) and prostaglandin E_2 (PGE_2) are known to influence the growth and differentiation of osteoblasts (*19-22*), and the CREB/ATF family has been established as the main mediator of transcription activation induced by PTH (*23*) or PGE_2 (*24*). However, the role of CREB/ATF members in controlling osteoblast growth and differentiation is still largely unknown.

Transgenic mice that overexpress c-*fos* display bone abnormalities, including nonmalignant bone neoplasms and collagenase-producing bone tumors (*1, 25*). Similarly, the ablation of c-*fos* expression in transgenic animals leads to osteochondrodysplasia with deficiencies in bone remodelling and tooth eruption in these animals (*3*). In recent studies, the ablation of ATF-2 led to a defect in endochondral ossification, which has a histopathology similar to human hypochondroplasia (*8*). These data suggest that specific members of this large family of transcription factors have specific roles in the development of the skeletal tissue. Therefore, we hypothesized that the CREB/ATF family regulates genes which define the developmental sequence of skeletal tissues. To study the role of the CREB/ATF family in osteoblast development, we chose clonal murine calvarial osteoblastic cell line MC3T3-E1 (*26-28*).

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Abbreviations: ALP, alkaline phosphatase; AP-1, activating protein-1; ATF, activating transcription factor; BSP, bone sialoprotein; *bsp*, gene encoding bone sialoprotein; CRE, cyclic AMP response element; CREB, CRE-binding protein; c-*fos*, gene encoding c-Fos; IGF-I, insulin-like growth factor-I; PKA, cAMP-dependent protein kinase; PGE_2, prostaglandin E_2; PMSF, phenylmethylsulfonyl fluoride; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide.

MC3T3-E1 cells undergo three stages of development: proliferation, matrix maturation, and extracellular matrix mineralization. The proliferation stage is the initial phase of development (days 1-9 of culture after plating), in which MC3T3-E1 cells actively replicate, as evidenced by the high rates of DNA synthesis and progressive increase in cell numbers (*26*). A decrease in replication and expression of differentiated osteoblast functions characterize the next developmental stage, matrix maturation, which occurs approximately 10 days after plating. In this period, these osteoblasts produce alkaline phosphatase and process procollagens to collagens. The final phase of MC3T3-E1 development, the extracellular matrix mineralization stage, begins at about day 20 and is defined by matrix calcification associated with progressive increases in extracellular matrix accumulation (*26-28*).

In this work, we showed the time-dependent changes in the amount and degree of phosphorylation, and in the binding activity of CREB in the course of development of MC3T3-E1 cells. The ratio of phosphorylated CREB in the CREB-CRE complex changed dramatically during osteoblast development.

MATERIALS AND METHODS

Cell Culture—Osteoblastic, clonal MC3T3-E1 cells derived from C57BL/6 of newborn mouse calvaria (*26, 27*), were donated by Dr. H. Kodama (Ohu University) and Dr. Kuboki (Hokkaido University) (*28*). Cells were plated at a density of 5,000 cells/cm^2 into 100-mm-diameter plastic culture dishes, grown for periods ranging from 3 to 42 days in α-MEM containing 10% fetal bovine serum (Dainippon Pharmaceutical, Tokyo) and 60 μg/ml kanamycin sulfate, and maintained at 37°C in a fully-humidified atmosphere of 5% CO_2-95% air. The medium was changed every 2 days.

Alkaline Phosphatase Activity—After culture for the indicated number of days, MC3T3-E1 cells were washed 3 times with 250 mM sucrose and 1 mM $MgCl_2$, harvested and ultrasonicated in the same solution. Alkaline phosphatase (ALP) activity was measured as previously described (*29*) using a *p*-nitrophenyl phosphatase substrate.

Western Blotting—MC3T3-E1 cells cultured for various periods were washed three times with phosphate-buffered saline, then solubilized by the addition of hot SDS sample buffer solution [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol (DTT)] followed by ultrasonication. After further heating the cell lysate at 100°C for 5 min, it was centrifuged at 18,000$\times g$ for 10 min and the resultant supernatant was submitted to Western blotting. Equal amounts of extracted protein from each sample (10-20 μg) were electrophoresed on 10% SDS-polyacrylamide gel, then transferred electrophoretically to Immobilon-P (Millipore, Bedford, USA). Membranes were incubated with a rabbit anti-CREB polyclonal antibody and anti-phosphorylated CREB antibody with a PhosphoPlus CREB (Ser 133) Antibody Kit (New England Biolabs, USA). Blots were developed using enhanced chemiluminescence Western Detection System (New England Biolabs) and exposed to X-ray film. The anti-phosphorylated CREB antibody cross-reacts with the phosphorylated form of ATF-1 (according to the specifications provided by the manufacturer), and it detected ATF-1 of 38 kDa together with CREB protein in the control extract in the Antibody kit.

Nuclear Protein Extracts—Crude nuclear extracts were prepared by the method of Lee *et al.* (*30*) with minor modifications. Cells were harvested after plating for various periods and washed in phosphate-buffered saline. Pelleted cells were suspended in a hypotonic buffer (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT) with phosphatase inhibitors (0.5 mM sodium fluoride, 1 μM Microcystin LR), protease inhibitors [0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin], and 0.2% (v/v) Nonidet P-40, and incubated for 10 min on ice. The nuclei were centrifuged at 2,000$\times g$ for 1 min. The nuclear proteins were extracted by suspending the nuclei in the extraction buffer (10 mM HEPES, pH 7.6, 0.5 M NaCl, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 0.5 mM DTT) including the phosphatase inhibitors and protease inhibitors with additional vortexing and incubation for 30 min on ice. Following centrifugation at 18,000$\times g$ for 15 min at 4°C, the supernatants were collected and stored at −80°C. The concentration of protein in the extracts was estimated by the method of Bradford (*31*), using a Bio-Rad protein assay kit.

Gel Shift Assay—The nucleotide sequences of probes and competitors used in the gel shift assay are shown in Table I. Equal amounts of complementary oligonucleotides for each probe were mixed and annealed by heating to 70°C and cooling slowly to room temperature. Oligonucleotide probes were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Nuclear extracts (2-8 μg of protein) were preincubated in 20 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 mM NaCl, 1 mM spermidine, 5% glycerol), and 1 μg of poly(dI-dC)·poly(dI-dC) for 10 min at 4°C. Following the preincubation, the oligonucleotide probe was added, and the incubation was continued for 10 min. The reaction mixture was then applied to 5% polyacrylamide gel containing 5% glycerol. After electrophoresis at 150 V in a buffer containing 37 mM Tris-HCl (pH 8.5), 280 mM glycine, and 0.75 mM EDTA, the gel was dried and autoradiographed. For competitive binding

TABLE I. **Nucleotide sequence of probes and competitors.** All sequences (top strand) are written 5′ to 3′ and the CRE core motifs are underlined.

Oligo name	Sequence
C/CRE (CRE consensus oligonucleotide)	AGAGATTGCCTGACGTCAGAGAGCTAG
C/Oct-1 (Oct consensus oligonucleotide)	TGTCGAATGCAAATCACTAGAA
AP-1 (AP-1 consensus oligonucleotide)	CGCTTGATGAGTCAGCCGGAA
c-*fos*/CRE	TTCCGCCCAGTGACGTAGGAAGTCCAT
IGF-1/CRE	TTCAGAGCAGATAGAGCCTGCGCAATCGAAA
Osteocalcin/CRE	TTGGGTTTGACCTATTGCGCACATGACCCCCAATTAGTCCTGGCAGCA

studies on the consensus/CRE probe, competitor oligonucleotides were added at the preincubation step. For supershift assays, antibodies were added before the addition of the labeled oligonucleotide. A mouse monoclonal antibody to ATF-1 (G41-5.1) and a rabbit polyclonal antibody to ATF-2 (C-19) were purchased from Santa Cruz Biotechnology (USA).

RESULTS

Characterization of MC3T3-E1 Cells: Morphological Observations and ALP Activity Expression—The MC3T3-E1 cells showed rapid growth after plating and had fibroblast-like shapes until they attained the confluent state (Fig. 1A). When the cells attained a confluent state at about day 4 after plating, they changed from fibroblast-like to polygonal in shape. They continued to grow thereafter and formed multilayered structures (Fig. 1B). At about day 20 after plating, colonies with clusters of the characteristic spherical cells and small mineralized opaque nodules appeared (Fig. 1C). These colonies steadily increased in number and size. At about day 40, nodular structure caused an increase in the density (Fig. 1D).

Alkaline phosphatase activity has been used as an important indicator of bone formation activity. The enzyme activity is known to increase markedly at the initiation of mineralization of hard tissue (*32*). As shown in Fig. 2, alkaline phosphatase in the cell matrix fraction increased rapidly until day 20, then decreased slowly until day 30,

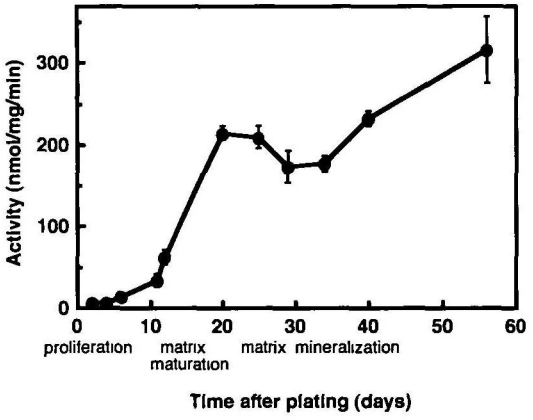

Fig. 2. **Changes of alkaline phosphatase activity of the MC3T3-E1 cells during osteoblast development.** At the time indicated, samples were collected and ALP activity was assayed as described in "MATERIALS AND METHODS." Stages of osteoblast development judged from morphological observation are noted under the horizontal axis, which represents the number of days after plating.

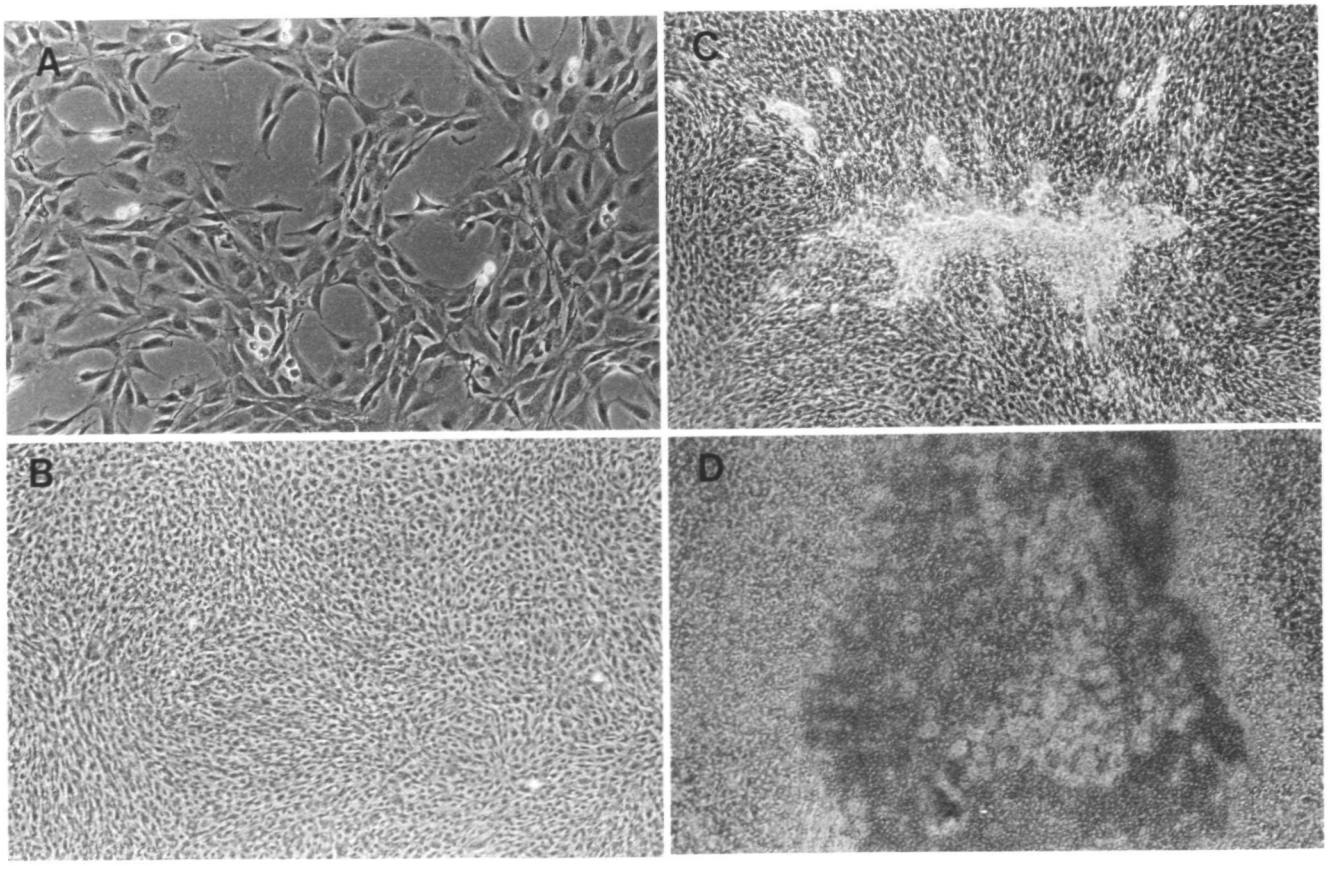

Fig. 1. **Phase-contrast microscopic appearance of MC3T3-E1 cells in the course of development.** The cells were grown as described in "MATERIALS AND METHODS." Fibroblast-like appearance of cells at day 3 after plating (magnification, ×100) (A). Multi-layered structures at day 14 after plating (×40) (B). The early mineralized stage of colonies with the characteristic spherical cells and small opaque nodules at about day 20 after plating (×40) (C). Mineralized opaque nodular structures at day 40 after initiation of culture (×40) (D).

after which it started increasing again.

MC3T3-E1 cells display a time-dependent and sequential expression of osteoblast characteristics analogous to *in vivo* bone formation. From morphological observation (Fig. 1), the MC3T3-E1 cells used here differentiated into osteoblasts and formed mineralized bone tissue in culture as has been reported previously (*28*).

Differential Change of Phosphorylated and Unphosphorylated CREB Protein Levels during Osteoblast Development—To determine the cellular levels of CREB and its phosphorylation during MC3T3-E1 development, immunoblotting analysis was performed using anti-CREB and anti-phosphorylated CREB antibodies. The former antibody recognizes both phosphorylated and unphosphorylated CREB, but the latter recognizes only the phosphorylated form of CREB at Ser 133. The whole-cell proteins of MC3T3-E1 in the proliferation, matrix maturation, and extracellular matrix mineralization stages were electrophoresed and subjected to Western blotting as described in "MATERIALS AND METHODS." As shown in Fig. 3, bands corresponding to CREB protein that reacted with both antibodies were observed at 47 kDa in all stages. The intensities of the band of 47 kDa at each stage were compared with a densitometer (Fig. 3C). The level of detectable CREB was maximal in the proliferation stage (day 3 after plating), while remaining significantly high in other stages. On the other hand, the level of phosphorylated CREB changed dramatically and was higher in the proliferation stage (day 3) and in the early mineralization stage (day 21) than in the other stages (Fig. 3B), but the ratio of the amount of phosphorylated CREB to the total amount of CREB reached its peak in the early mineralization stage (Fig. 3C). The anti-phosphorylated CREB antibody used here also cross-reacted with the phosphorylated form of ATF-1 (see "MATERIALS AND METHODS"). Detection of

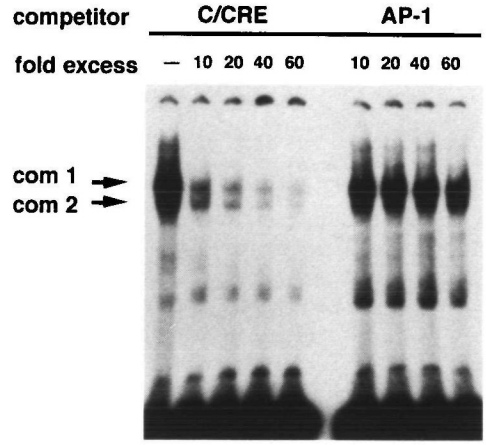

Fig. 4. **Competition analysis of CRE complexes.** Nuclear extracts prepared from MC3T3-E1 cells at 3 days after initiation of cultures were used in the competition gel shift assay in the presence of the consensus/CRE probe and increasing amounts (10 to 60-fold molar excess) of unlabeled consensus/CRE or AP-1 oligonucleotides. The specific complexes are labeled com 1, and com 2. —, no competitor oligo. The probes and competitors used are shown in Table I.

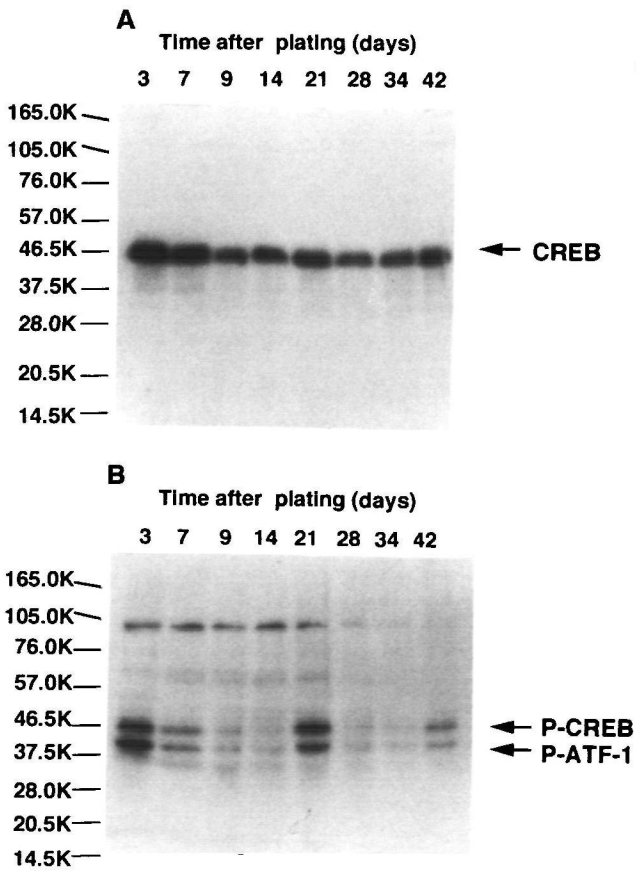

Fig. 3. **Developmental changes of the levels of phosphorylated and unphosphorylated CREB in MC3T3-E1 cells.** The whole-cell proteins were extracted from MC3T3-E1 cells at various stages of culture and subjected to Western blot analysis. Blots were probed with antibodies specific for CREB (A) and phosphorylated CREB (P-CREB) (B). Densitometric measurements of detectable CREB levels (○) and the ratio of the levels of phosphorylated CREB to CREB (●) shown in A and B are expressed as a percentage of the maximum levels (C).

another band of 38 kDa with the antibody may indicate that the phosphorylated form of ATF-1 was also contained in MC3T3-E1 cells.

Binding Activities to Consensus/CRE in MC3T3-E1 Cells during Osteoblast Development—To assess CRE binding activities in MC3T3-E1 cells, a gel shift assay was carried out using the consensus/CRE probe. Nuclear extracts were incubated with the probe containing the consensus CRE motif as described in "MATERIALS AND METHODS." The probe is known to have a high affinity for CREB/ATF family members (*33, 34*). As shown in Fig. 4, two major protein complexes bound to the consensus/CRE probe were detected (com 1, com 2). To verify further the specificity of these complexes, a competition assay was performed in the presence of unlabeled consensus/CRE or unlabeled AP-1 oligonucleotides (Fig. 4). The binding to the consensus/CRE probe was subjected to competition, in a dose-dependent manner, by the unlabeled CRE oligonucleotide but not by the AP-1 oligonucleotide.

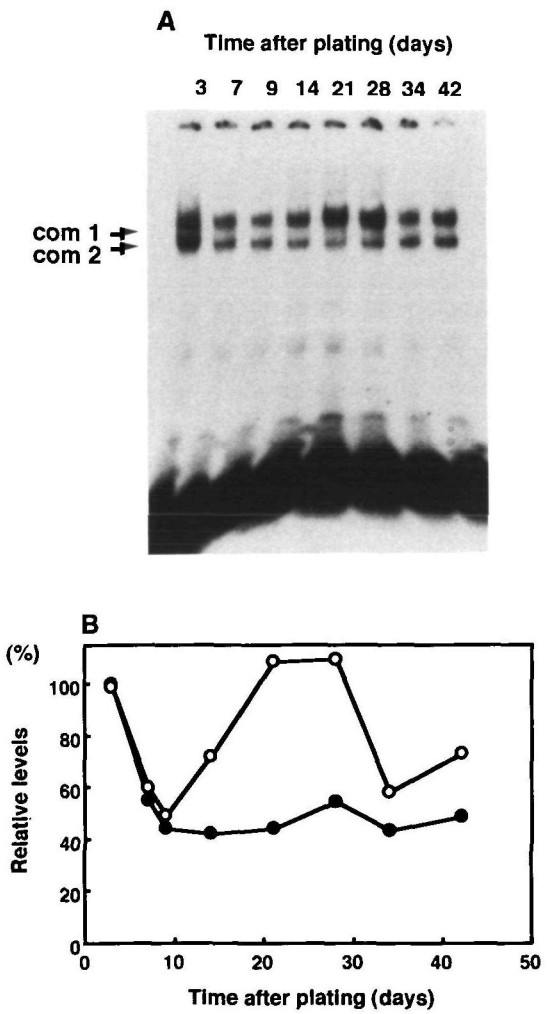

Fig. 5. **Changes of CREB bound to the CRE in MC3T3-E1 nuclear extract during osteoblast development.** Gel shift assay was performed using a labeled consensus/CRE probe and nuclear extracts (2.5 μg/lane) obtained from MC3T3-E1 cells after various periods of culture (A). Arrows indicate the specific DNA-protein complexes observed (com 1, com 2). Densitometric measurements of levels of binding to consensus/CRE in com 1 (○) and com 2 (●) shown in A are expressed as a percentage of day 3 expression levels (B).

To determine the binding activities to consensus/CRE during osteoblast development, we performed a gel shift assay with nuclear extracts obtained from cells after various periods of culture (Fig. 5A), and the intensity of the binding at each stage was compared with a densitometer (Fig. 5B). The upper band, com 1, was strongly formed in the proliferation stage (day 3) and in the early mineralization stage (days 21 to 28), whereas the lower band, com 2, reached its peak at the proliferation stage (day 3) and then decreased.

To assess whether the decrease of the CREB complexes at days 7 and 9 had been affected by the extraction process, we performed a gel shift assay using the consensus binding site for the Oct family (consensus/Oct-1). As shown in Fig. 6, the binding activity to the octamer motif reached its peak in the proliferation stage (days 3 to 9) and declined during the maturation and mineralization stages. Oct-1 is a *trans*-activator for the histon H2B gene (*35–38*). H2B is one of the osteoblast developmental markers, and the decrease of Oct-1 corresponds with the decrease of proliferation in MC3T3-E1 cells. The time-dependency of the Oct-1 binding activity was different from that of the binding activity to consensus/CRE with the same nuclear extracts. These results demonstrated that changes in the binding activity to CRE were selective and dependent on osteoblast development.

Identification of CREB/ATF Family Members Assembling on the Consensus/CRE—We examined whether CREB was present in the CRE complexes that were induced during the osteoblast development (Fig. 7). The gel shift assay was performed both with and without the anti-CREB antibody. The antibody abolished com 1 in a dose-dependent manner and a supershift complex appeared concomitantly (Fig. 7), indicating that proteins in com 1 reacted with the antibody. Intensity of the super shifted band also decreased as the antibody increased. One possible cause for this is that because polyclonal anti-CREB IgGs were used in this study, dimer and multi-CREB-antibody complexes

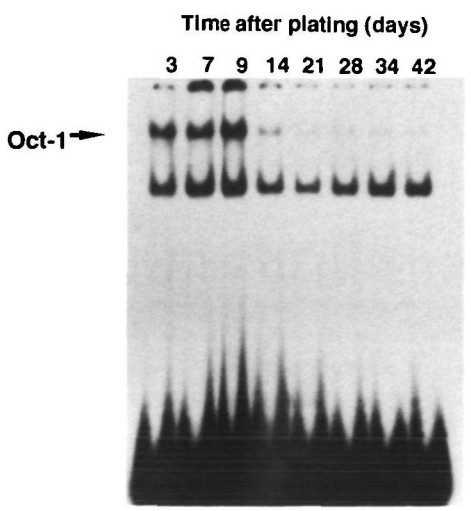

Fig. 6. **Changes of Oct-1 bound to consensus/Oct-1 in MC3T3-E1 nuclear extract during osteoblast development.** A gel shift assay was performed using the labeled consensus/Oct-1 probe and nuclear extracts (2.5 μg/lane) obtained from MC3T3-E1 cells after various periods of culture. The probes used are shown in Table I.

were formed which could not enter into the gel, or which, through their formation, interfered structurally with the DNA binding domain. Proteins in com 2 did not react with the antibody as indicated by the lack of change in intensity for com 2. Thus, we concluded that the CREB protein was involved in the upper complex, com 1, and not in the lower complex, com 2.

Because CREB is reported to heterodimerize with another CRE binding protein, ATF-1, an antibody specific to ATF-1 was also used in the gel shift assay (Fig. 8). Although the ATF-1 antibody forms a supershifted complex, it did not cause a significant decrease in the total amounts of the proteins in com 1 or com 2. Therefore, we could not determine whether ATF-1 was involved in com 1 or com 2. To identify ATF-2 in the CRE complexes, we also performed a supershift assay using anti-ATF-2 antibodies. No effect was observed on either com 1 or com 2, indicating that ATF-2 was not present in the nuclear complexes that were induced to bind the consensus/CRE motif (Fig. 8).

To determine the level of phosphorylation of CREB bound to consensus/CRE in each stage, we performed a supershift assay using the anti-CREB antibody and anti-phosphorylated CREB antibody. As shown in Fig. 9, proteins in com 1 reacted with these antibodies as indicated by the loss of com 1, and the results were analyzed with a densitometer. The value of com 1 with each antibody was subtracted from the value of com 1 with no antibody on each day and the difference was evaluated for total CREB or phosphorylated CREB bound to com 1. Then the ratio of phosphorylated CREB to total CREB was calculated on each day. The ratio of phosphorylated CREB to total CREB in com 1 in the proliferation stage (day 4, lanes 1, 4, and 7) was 0.63, that in the matrix maturation stage (day 14, lanes 2, 5, and 8) was 0.95, and that in the early mineralization stage (day 21, lanes 3, 6, and 9) was 0.96. The ratio in the proliferation stage was lower than in the mineralization stage, although the intensity of com 1 was the almost same in both stages.

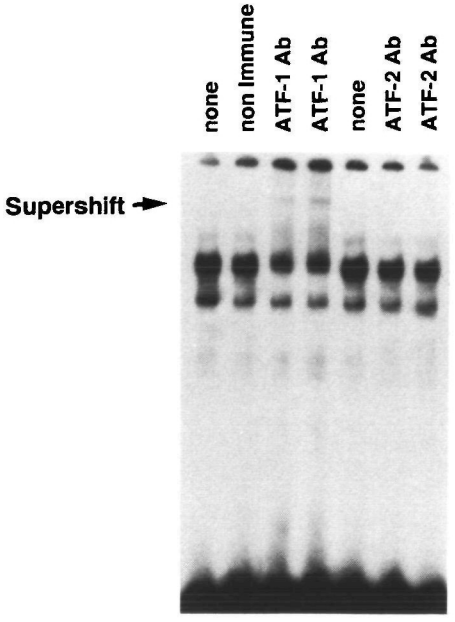

Fig. 8. **Identification of ATF-1 as one of the CRE binding proteins.** Nuclear extracts (8 μg/lane) from MC3T3-E1 cells at 3 days after initiation of plating were used in a gel shift assay with the consensus/CRE probe. The nuclear extracts were preincubated before proceeding to gel shift assays in the absence (lanes 1 and 5) or presence of ATF-1 Ab (1:20 and 1:10 dilution in lanes 3 and 4, respectively), ATF-2 Ab (1:20 and 1:10 dilution in lanes 6 and 7, respectively), or in the presence of non immune serum (1:10 dilutions in lane 2). Lanes are numbered from left to right.

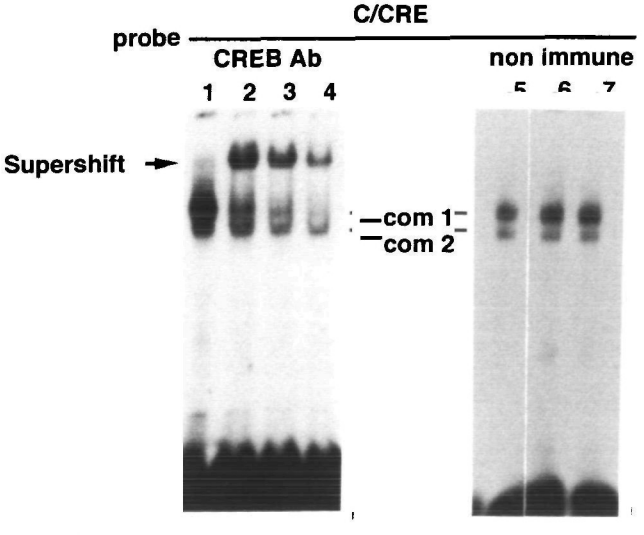

Fig. 7. **Identification of CREB as one of the CRE binding proteins.** Nuclear extracts (8 μg/lane) from MC3T3-E1 cells at 3 days after initiation of plating were used in a gel shift assay with the consensus/CRE probe. The nuclear extracts were preincubated before proceeding to gel shift assays in the absence (lanes 1 and 5) or presence of CREB Ab (1:40, 3:40, and 3:20 dilution in lanes 2, 3, and 4, respectively), or in the presence of non immune serum (3:40 and 3:20 dilutions in lanes 6 and 7, respectively).

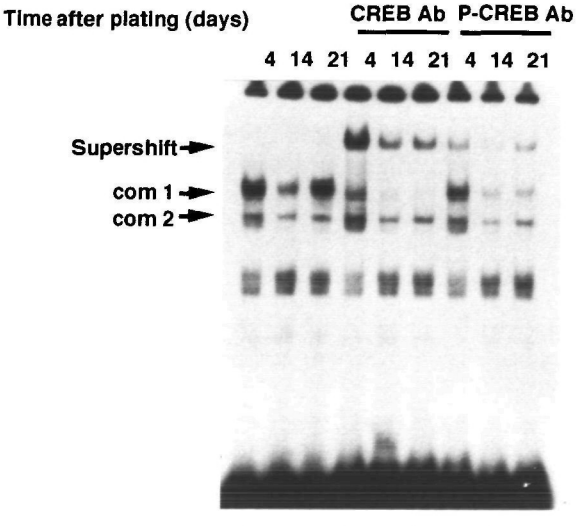

Fig. 9. **Changes of phosphorylated CREB complex during osteoblast development.** Nuclear extracts (2 μg/lane) were obtained from proliferation (day 4), matrix maturation (day 14), and extracellular matrix mineralization (day 21) stages of osteoblast development. The nuclear extracts were preincubated before proceeding to gel shift assays in the absence or in the presence of the anti-CREB antibody (CREB Ab), or presence of the anti-phosphorylated CREB (P-CREB) antibody (P-CREB Ab). The complexes are labeled com 1 and com 2, and the supershift complexes are indicated.

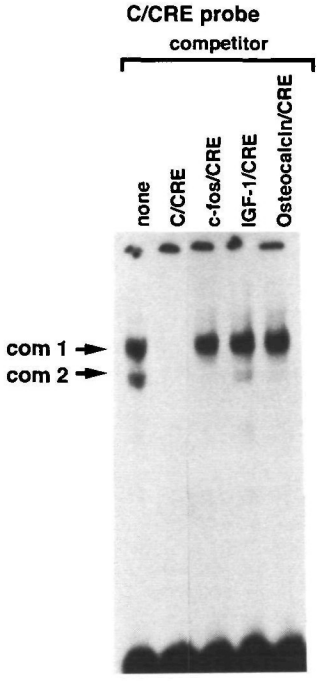

Fig. 10. **Effects of diverse CREs on CREB complexes in MC3T3-E1 cell nuclear extracts.** Nuclear extracts prepared from MC3T3-E1 cells at 3 days after initiation of cultures were used in the competition gel shift assay in the presence of the consensus/CRE probe and competitor oligonucleotides. All competitors are present in 60-fold molar excess. The probes used are shown in Table I.

Competition of CREB Complex in MC3T3-E1 Cells with c-fos, IGF-I or the Osteocalcin Promoter cAMP Response Region—Diverse nucleotide sequences have been identified as putative CREs for various genes. The mouse c-*fos* 5′ regulatory region has a CRE-like element (c-*fos*/CRE), which is required for PTH-mediated c-*fos* induction (*23*). CRE within the 5′-untranslated region of IGF-I exon 1 (IGF-I/CRE) is required for the hormonal activation of rat IGF-I gene transcription in osteoblasts (*24*). Rat osteocalcin promoter possesses a cAMP response region (osteocalcin/CRE), which contains two hexamer steroid hormone response motifs (*39*). We examined whether the CREB complex in MC3T3-E1 cells bound these diverse CREs. As shown in Fig. 10, the complex containing CREB bound to consensus CRE (com 1) was not subjected to competition by c-*fos*/CRE, IGF-I/CRE or osteocalcin/CRE, all of which possess the diverse CRE motifs (Table I). Another complex, com 2, was competed for binding by these CRE regions. Although the identity of the factor(s) in com 2 is unknown, common factors other than CREB may serve for regulation of these CRE regions.

DISCUSSION

In this work we analyzed the time-dependent changes in the amount, degree of phosphorylation, and binding activity of the CREB/ATF family in the course of development of MC3T3-E1 cells, which can serve as a model for the growth and differentiation of osteoblasts into bone tissue-like nodules. The purpose of this work has been to demonstrate that CREB plays an important role in regulating the transition of growing osteoblasts to differentiated osteoblasts.

We showed that the amount of CREB was high through all stages of development and maximal in the proliferation stage. Two peaks were observed in the phosphorylation level of CREB, in the proliferation stage and in the early mineralization stage, the latter level being the higher. This peak of the CREB phosphorylation level in the early mineralization stage suggested that CREB played a role in regulating the extracellular matrix mineralization in MC3T3-E1 cells. Several reports have revealed the importance of phosphorylation of CREB in cell growth. For instance, lymphocyte progression induced by IL-2 is associated with the phosphorylation of CREB (*40*). It has also been reported that CREB mediates the growth factor response in PC12 cells (*41*). These observations indicate that the maximum level of total CREB in the proliferation stage and its high level of phosphorylation in MC3T3-E1 may be related to osteoblast proliferation.

The strength of binding affinity of the diverse CRE elements for CREB, as well as the magnitude of phosphorylation-induced increase in this binding, depends on the sequence of each element (*42*). The group of CREs that contain the consensus CRE motif (TGACGTCA) binds to CREB with high affinity regardless of its phosphorylation state. The other group of CRE motifs, which contain only one CGTCA motif or do not have the motif, have a lower binding affinity for CREB: these CREs scarcely bind to unphosphorylated CREB but show a sharp increase in binding upon phosphorylation of CREB (*42*). We showed that the binding activity to the consensus/CRE reached almost equally high levels in the proliferation and early mineralization stages, while the relative levels of phosphorylated CREB to total CREB assembling on the consensus/CRE in the proliferation stage was lower than in the early mineralization stage as seen in Fig. 9. These results agree with the previous reports that the consensus CRE motif, which is a high-affinity site, should bind to both phosphorylated CREB and unphosphorylated CREB in amounts proportional to the ratio of the presence of phosphorylated CREB to unphosphorylated CREB in the nuclear extracts (*42*). Potential CREB/ATF binding sites are found in promoter regions of many genes involved in skeletal development (*43-45*). This predicts that in the proliferation stage, where the degree of the CREB phosphorylation is lower than in the early mineralization stage, unphosphorylated CREB should inhibit the binding of phosphorylated CREB to the consensus CRE motif. The consensus CRE motif may induce a lower level of activation of transcription in the proliferation stage than in the early mineralization stage. On the other hand, the low-affinity CRE motif may induce an similar level of activation of transcription in the proliferation stage and in the mineralization stage, because in the low affinity CRE site, unphosphorylated (transcriptionally inactive) CREB does not compete with phosphorylated CREB.

Changes in the binding activity to consensus/CRE during MC3T3-E1 development appeared to be similar to the changing levels of phosphorylation (Fig. 5). The low binding activity in the matrix maturation stage may be due to a change of the dimerizing partner, or the phosphorylation of CREB may directly influence binding activity to consensus/CRE in MC3T3-E1 cells.

Transcriptional activation by CREB serves as the final step in the signal transduction cascade initiated at cell surface receptors to activate adenyl cyclase, increase intracellular cAMP, and activate cAMP-dependent protein kinase (PKA). Phosphorylation of CREB by PKA is essential for its *trans*-activating functions (*17*). Results from our investigations showed that CREB in MC3T3-E1 cells was highly phosphorylated without any agents that elevate the intracellular cAMP concentration in the proliferation and early mineralization stages. One possible reason for this is that the synthesis of parathyroid hormone-related peptide (PTHrP) may serve as an autocrine or paracrine factor by which these cells maintain their cellular cAMP concentration and thus CREB is phosphorylated in MC3T3-E1 cells in certain stages. A previous report that PTH-(3-34), a PTH antagonist, inhibits basal *bsp* gene expression *via* decreasing activation of the CRE element in the *bsp* promoter region in primary cultures of embryonic osteoblasts (*45*) supports this hypothesis. Furthermore, PTH and forskolin, which elevate intracellular cAMP concentrations, increase transcription of osteocalcin and *bsp* gene (*45, 51*). Osteocalcin and BSP are both major noncollagenous proteins of bone that are restricted almost exclusively to bone, and their mRNAs peaked in the extracellular matrix mineralization stage. Given these reports, our results indicate that the increase of phosphorylated CREB in the early mineralization stage may contribute to the expression of these genes.

CREB has been reported to dimerize with ATF-1 (*46-48*) and the structurally related CRE-modulating protein (CREM) (*49, 50*). CREM was shown to cause repression of CRE-dependent transcription. ATF-1 was reported to be capable of antagonizing CREB-dependent activation of somatostatin promoter by limiting the amount of CREB that can form homodimers (*47*). By using specific antibodies in the gel mobility shift assay, we identified two known CREB/ATF transcription factors, CREB and ATF-1. ATF-1 is a 38 kDa protein that can bind to the CRE motif either as a homodimer or heterodimer with CREB. Because ATF-1 can suppress the promoter activity which is mediated by phosphorylated CREB (*47*), phosphorylated ATF-1 may contribute to selective control of the promoter activity during the osteoblast development. However, since ATF-1 was only weakly detected in the CRE complexes, it is a minor partner for CREB in MC3T3-E1 cells. Experiments are now in progress to determine if another member of the CREB/ATF family binds to the consensus/CRE probe.

The consensus/CRE, which contains an 8-bp palindrome with the sequence 5′-TGACGTCA-3′, is highly conserved in promoters of other genes regulated by cAMP (*15, 33*). Recently, however, many diverse nucleotide sequences have been identified as putative CREs for various genes in a variety of cellular contexts. We demonstrated in this study that the complex containing CREB bound to the consensus CRE was not subject to competition by c-*fos*/CRE, IGF-I/CRE, or osteocalcin /CRE, all of which possess the diverse CRE motif (Fig. 10). Except for c-*fos*/CRE, these results agree with those of previous reports (*24, 39*). However, another study reported that CREB in osteoblastic cell line UMR 106-01 binds to the c-*fos* promoter region CRE elements (*23*). Our finding that the CREB-consensus/CRE complex was not competed for by c-*fos*/CRE might be related to the fact that the expression of c-*fos* mRNA was not detectable in MC3T3-E1 cells without stimulation (*52*).

We demonstrated a significant difference in the expression of the CREB/ATF family over the course of MC3T3-E1 development. These findings provide a more effective basis for functionally defining the selective contribution of the CREB/ATF family to developmentally regulated signaling pathways, which control the expression of the genes involved in cell growth, differentiation, and mineralization of osteoblasts.

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