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Correlation between Induction of Expression of Biglycan and Mineralization by C-Type Natriuretic Peptide in Osteoblastic Cells[1]

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We reported previously that C-type natriuretic peptide (CNP) promotes the differentiation and mineralization of osteoblastic cells [*Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1311-C1318, 1996]. However, little information is available about the mechanism of action of CNP in differentiating osteoblastic cells. Using the technique known as differential display-polymerase chain reaction, we attempted to identify the mRNAs whose levels are regulated by CNP in mouse clonal preosteoblastic MC3T3-E1 cells. One species of mRNA whose level was increased by CNP was 99% homologous to the 3′-untranslated region of a mouse gene for biglycan (BGN), a small proteoglycan. BGN is known to be involved in bone formation by osteoblastic cells. Therefore, we investigated the relationship, during the formation of mineralized nodules, between CNP and BGN using calvarial osteoblast-like cells (ROB cells) from newborn rats, that are a good model for studies on bone formation *in vitro*. Northern blot analysis revealed that transcription of the mRNA for BGN was up-regulated by CNP in ROB cells on days 6 and 8, whereas no effect of CNP was observed on days 3 and 12. Brief treatment with 10^{-7} M CNP on days 3 through 9 exclusively enhanced the deposition of calcium, a result that suggests that CNP might regulate the expression of mineralization-related genes and, probably, the gene for BGN during a specific time period.

Key words: biglycan, C-type natriuretic peptide, differential display-polymerase chain reaction, mineralization, osteoblast.

The formation of bone occurs *via* a series of events that are regulated by various hormones and cytokines in chondrocytes, osteoblasts/osteocytes and osteoclasts. We proposed previously that specific vasoactive peptides, such as natriuretic peptides (*1, 2*), angiotensin II (*3*), and endothelin (*1, 4*), might regulate osteoblastic metabolism. The natriuretic peptide family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Osteoblastic cells express both CNP and the specific receptor for CNP, known as natriuretic peptide receptor-B (NPR-B), which is a membrane-bound guanylate cyclase (*1, 2*). Cyclic GMP produced in response to CNP promotes the differentiation and mineralization of osteoblastic cells (*1, 2, 5*). The purpose of the present study was to identify the genes induced by CNP in osteoblastic cells using the method known as differential display-polymerase chain reaction (DD-PCR), and to determine whether the identified genes are involved in osteoblastic differentiation and/or mineralization by monitoring their expression in cultured osteoblast-like cells derived from the calvariae of newborn rats (ROB cells).

Biglycan (BGN) belongs to a family of small proteoglycans that have dermatan or chondroitin sulfate side chains attached to their N-terminal regions (*6*). The cores of mature BGN proteins are highly conserved among mammalian species (*7*). As is the case for other members of this family, namely, decorin, fibromodulin, and lumican, BGN is characterized by several leucine-rich repeats. The promoters of the genes for mouse BGN (*7*) and human BGN (*8*) have been cloned, and the human promoter was found to be responsive to interleukin-6 and tumor necrosis factor-α (*8*). The level of the mRNA for BGN was also enhanced by a number of factors, such as insulin-like growth factors I and II (*9*). However, the regulatory factors and signal transduction pathways that lead ultimately to alterations in the level of expression of the gene for BGN are poorly defined. BGN binds to fibrillar proteins, such as collagen (*10*). BGN might also affect cell migration by modulating the interaction of cell surface receptors with their ligands in the matrix, and by influencing the availability and functions of growth factors (*11*). In addition, it has been suggested that BGN might play an important role in matrix formation and in the maturation of osteoblastic cells (*12, 13*). A recent study of BGN-deficient mice confirmed that BGN

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Abbreviations: ANP, atrial natriuretic peptide; BGN, biglycan; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; DD-PCR, differential display-polymerase chain reaction; NPR-B, natriuretic peptide receptor-B; ROB cells, osteoblast-like cells from newborn rat calvariae; TGF-β, transforming growth factor-β.

plays a regulatory role in bone development (*14*).

We found, in the present study, that CNP stimulated the expression of mRNA for BGN in osteoblastic cells at a specific time during their differentiation, and that their induction of BGN by CNP might be correlated with the mineralization of osteoblastic cells.

EXPERIMENTAL PROCEDURES

Materials—CNP was purchased from the Peptide Institute, Osaka. ^{32}P-labeled nucleotides were obtained from Amersham Pharmacia Biotech., Tokyo. Dulbecco's modified Eagle's medium (DMEM), α-modified minimum essential medium (α-MEM), a penicillin/streptomycin antibiotic mixture, and fetal bovine serum were obtained from Life Technologies, Grand Island, NY, USA.

Culture of MC3T3-E1 Cells—The mouse clonal preosteoblastic MC3T3-E1 cells were a generous gift from Dr. M. Kumegawa (Meikai University, Sakado). Cells were maintained in 55-cm^2 dishes in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin under a humidified atmosphere of 5% CO_2 in air at 37°C. After reaching confluence, the cells were detached by treatment with 0.05% trypsin. The cells were replated on 12-well plates at a density of 1×10^4 cells/cm^2, and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. During subculture, the medium was replaced every 4 days.

Isolation and Culture of ROB Cells—Cells were isolated enzymatically from the calvariae of newborn Wistar rats as described previously (*1*). The calvariae were cut into pieces and then subjected to six sequential 20-min digestions (yielding digests 1 through 6) with 3 ml of an enzyme mixture that comprised 1 mg/ml collagenase (150–250 units/mg; Wako Pure Chemical Industries, Osaka) and 0.5 mg/ml trypsin (Sigma, St. Louis, MO, USA). Cells from the pool of digests 4, 5, and 6 were plated on 75-cm^2 dishes, and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2 in air at 37°C. The medium was replaced by fresh medium after 24 h. After reaching 70% confluence, the cells were detached by treatment with 0.05% trypsin and the cells from three dishes (from the original pool of digests 4, 5, and 6) were combined. The cells that were combined were replated for experiments on 12-well plates (3.8 cm^2/well) or 55-cm^2 dishes at a density of 1×10^4 cells/cm^2, and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. During subculture, the medium was replaced every 3 days.

Differential Display-Polymerase Chain Reaction (DD-PCR) (15–17)—Total RNA was isolated from MC3T3-E1 cells that had been treated with 10^{-7} M CNP for 13 days by the acid guanidinium-phenol-chloroform method (*18*). Five micrograms of total RNA were reverse-transcribed with a T_{18} primer. DD-PCR was performed with a RAP-PCR kit (Stratagene, La Jolla, CA). For DD-PCR, an arbitrary primer was used. Its nucleotide sequence was 5′-AATCTAGAGCTCCCTCCA-3′. PCR was performed in reaction mixtures that comprised 10 mM Tris/HCl, pH 8.8, 50 mM KCl, 3 mM $MgCl_2$, 0.01% gelatin, 1 μM arbitrary primer, 200 μM dNTP, and 1 unit Taq DNA polymerase. The amplification reaction consisted of 1 cycle of 94°C for 1 min, 36°C for 5 min, and 72°C for 5 min, and then 40 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min. The reaction mixture was applied to a 5% (w/v) polyacrylamide gel and DNA was stained with ethidium bromide (Fig. 1A). Bands that showed differential expression were cut out and the DNA was eluted by boiling for 1 h in distilled water. After ethanol precipitation, the DNA fragments were reamplified by 40-cycle PCR. The DNA fragments obtained were subcloned into the cloning vector, pBluescript II SK$^-$ (Stratagene), and then the DNA fragments were sequenced by the dideoxynucleotide chain-termination method with a SequiTherm Long-Read Cycle Sequencing Kit-LC (LI-COR, Lincoln, NE, USA). The sequences were analyzed with GENETYX-MAC ver. 8.0 (Software Development, Tokyo).

Northern Blot Analysis—RNA was extracted from MC3T3-E1 cells and ROB cells. Twenty micrograms of total RNA were subjected to electrophoresis on a 1% agarose gel that contained 2.2 M formaldehyde, and the RNA bands were then transferred to a MagnaGraph nylon membrane (Micron Separations, Westborough, MA, USA). After the membrane had been baked, the RNA on the membrane was allowed to hybridize overnight with the cDNA for BGN (the 3′-noncoding region that was obtained by DD-PCR and the mouse coding region, 439–1010 bp) or for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 42°C in 50% formamide that contained 6×SSPE (1× SSPE is 0.15 M NaCl, 15 mM NaH_2PO_4, pH 7.0, 1 mM EDTA), 2×Denhardt's solution (0.1% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll), 0.5% SDS, and 100 μg/ml herring sperm DNA. Each cDNA probe was radiolabeled with a Ready-to-Go kit (Amersham Pharmacia Biotech., Tokyo). Each membrane was washed twice in 1×SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) that contained 0.1% SDS at room temperature for 5 min each and twice in 1×SSC that contained 0.1% SDS at 55°C for 1 h each, and then it was exposed to an imaging plate for 4 h. The plate was analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo).

von Kossa Staining—Osteoblastic cells on 12-well plates (3.8 cm^2/well) were fixed with 10% formaldehyde for 30 min and then washed three times with 10 mM Tris-HCl, pH 7.2. The fixed cells were incubated with 5% silver nitrate for 5 min in the sunlight, washed twice with H_2O, and then treated with 5% sodium thiosulfate (*1*). Mineralized nodules were assessed with respect to their number and the total area of nodules with an automated imaging system, which consisted of a BH microscope (Olympus, Tokyo), a camera (CCD/ICD-740; Olympus), and the Mac SCOPE program (Mitani, Fukui).

Quantitation of Calcium—The mineralized nodules from one well of a 12-well plate (3.8 cm^2/well) were washed twice with PBS, and then incubated overnight in 1 ml of 2 N HCl with gentle shaking. The calcium in the sample was quantitated by the *o*-cresolphthalein complexone method with Calcium C-Test Wako (Wako Pure Chemical Industries) (*1*).

RESULTS

Identification of CNP-Induced mRNAs in MC3T3-E1

Cells by DD-PCR—We proposed previously that CNP and its second messenger, cGMP, stimulate the differentiation of and bone formation by osteoblastic cells (*1, 2, 5*). To identify the CNP-inducible mRNAs in osteoblastic cells, we used the DD-PCR technique. mRNA was prepared from clonal preosteoblastic MC3T3-E1 cells that had been incubated with or without 10^{-7} M CNP. Two bands of PCR products, C1 and C2, whose levels were up-regulated by CNP, were detected, as shown in Fig. 1A. Nucleotide sequence analysis revealed that the C2 cDNAs encoded apolipoprotein D, a minor component of high-density lipoproteins (*19*), and eukaryotic initiation factor 4B, essential for the binding of mRNA to ribosomes (*20*). Fifteen of the 38 clones that were identified on the basis of the C1 band exhibited 99% homology to the 3′-untranslated region (348 bp) of the mouse gene for BGN (*7*), which is a small proteoglycan. Furthermore, we obtained, from the C1 band, four kinds of clones of which the sequences were unknown. BGN has been shown to be a protein that is related to the formation of bone. We confirmed by Northern blot analysis that expression of mRNA (2.9 kb) for BGN in MC3T3-E1 cells was up-regulated by 10^{-7} M CNP (Fig. 1B).

Temporal Regulation of the Expression of the Gene for BGN by CNP in Osteoblast-Like Cells (ROB Cells) from Newborn Rat Calvariae—We investigated the relationship between induction of the expression of the gene for BGN by CNP and the stimulation of mineralization by CNP in osteoblastic cells since BGN has been reported to be involved in osteoblastic bone formation (*12-14*). In this experiment, we used ROB cells from newborn rat calvariae instead of MC3T3-E1 cells because the former have been well characterized as a good model for studies on bone formation *in vitro* (*1*). ROB cells spontaneously form mineralized nodules with the characteristics of woven bone. We first examined if CNP induced the expression of mRNA for BGN in ROB cells. As shown in Fig. 2, 10^{-7} M CNP increased the level of mRNA for BGN in ROB cells to 150 and 180% of the control level after 48-h treatment that was started on days 6 and 8, respectively, while CNP at the indicated concentrations had no effect when the treatment was started on day 3 or 12. Thus, CNP increased the expression of BGN at a specific time. 8-Br-cGMP gave similar results (data not shown).

We next examined the effect of the pulsed administration of 10^{-7} M CNP on the deposition of calcium by ROB cells. The total number and area of mineralized nodules were determined after von Kossa staining with an automated imaging system (Fig. 3A). The presence of 10^{-7} M CNP from day 3 through 9 significantly enhanced the formation of mineralized nodules by ROB cells and the effect was the same as that of continuous treatment with CNP from day 0 to day 12 (Fig. 3, B and C). Figure 4A shows the accumulation of calcium in cells and the matrix layer when ROB cells were incubated with 10^{-7} M CNP for the indicated times. The exposure of ROB cells to CNP from day 3 through 9 was effective for stimulation of the deposition of calcium. The effect of CNP from day 6 through 9 on the deposition of

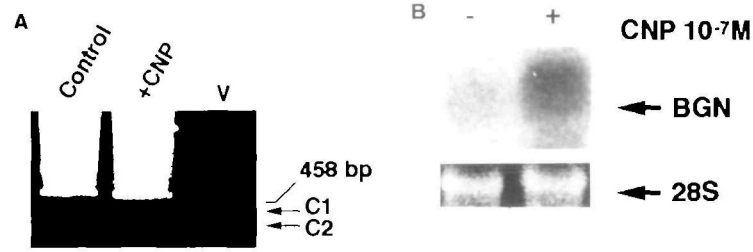


Fig. 1. **Detection of CNP-inducible mRNAs in MC3T3-E1 cells by differential display-PCR.** (A) Total RNA was isolated from osteoblastic cells after they had been treated with 10^{-7} M CNP (+CNP) for 13 days. Differential display-PCR was carried out as described under "MATERIALS AND METHODS." Two cDNA bands, designated as C1 and C2, increased in intensity in response to CNP (arrows). Size markers (DNA molecular weight marker V from Boehringer Mannheim, Mannheim, Germany) are denoted by V. (B) Confirmation of the up-regulation of BGN by CNP. MC3T3-E1 cells were grown in a 55-cm² dish for 6 days and then treated with 10^{-7} M CNP for 48 h before harvesting. Total RNA (20 μg) was isolated and subjected to Northern blot analysis. The blot was allowed to hybridize with ^{32}P-labeled cDNAs for BGN, as described under "MATERIALS AND METHODS." Data are representative of the results of three experiments that yielded similar results.

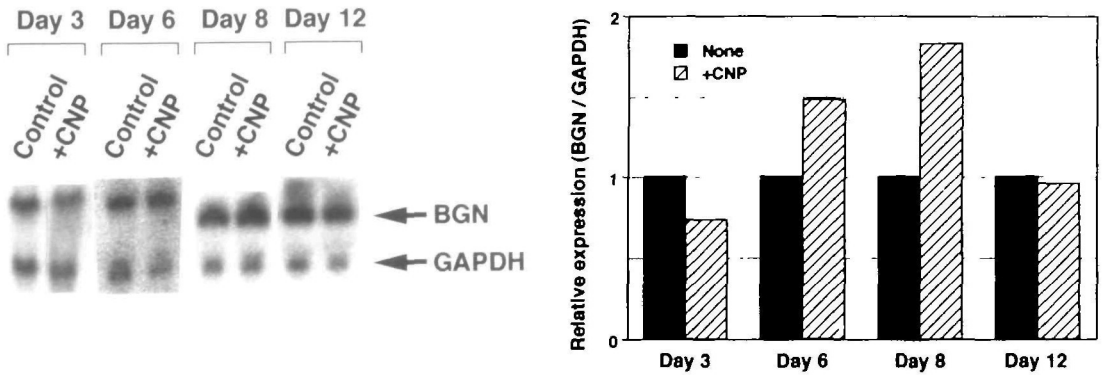


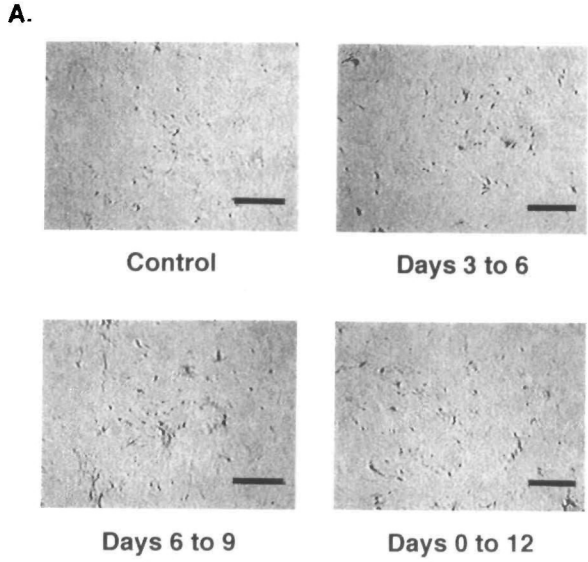
Fig. 2. **Stage-specific expression of mRNA for BGN in ROB cells in response to CNP.** ROB cells were grown in 55-cm² dishes for the indicated times and then subcultured with 10^{-7} M CNP for an additional 2 days before harvesting. Northern blot analysis was performed as described under "MATERIALS AND METHODS." The relative levels of expression of mRNA for BGN were calculated after normalization with reference to the respective levels of GAPDH mRNA. The ratio of BGN mRNA to GAPDH mRNA for the control culture was defined as 1. Data are representative of the results of two experiments that yielded similar results.

calcium was dose-dependent (Fig. 4B). These results indicate that CNP effectively promotes mineralization at a specific time during the differentiation of ROB cells. The period when CNP effectively stimulated deposition of calcium corresponded to the time when CNP increased the expression of the gene for BGN.

DISCUSSION

We proposed previously that cGMP, produced in response to CNP, accelerates the differentiation and mineralization of osteoblastic cells (*1, 2, 5*). However, little is known about the molecular mechanism of action and the signaling pathways of CNP in osteoblastic cells. We found here, using DD-PCR, that CNP induced the expression of the gene for BGN in osteoblastic cells. Our results suggest, furthermore, that CNP might stimulate bone formation by osteoblastic cells through temporal induction of the expression of the gene for BGN.

In bone, mRNA for BGN is expressed at high levels in osteoblastic cells on bone surfaces and in osteocytes (*21*). BGN seems likely to contribute to the maturation of both osteoblastic cells and the matrix (*10, 13*), as well as to settlement for subsequent mineralization (*13*). These conclusions were drawn from the results of experiments *in vitro*. They are also supported by the observations for mice that were deficient in the gene for BGN (*14*). Knockout mice were slightly smaller, on average, than their wild-type



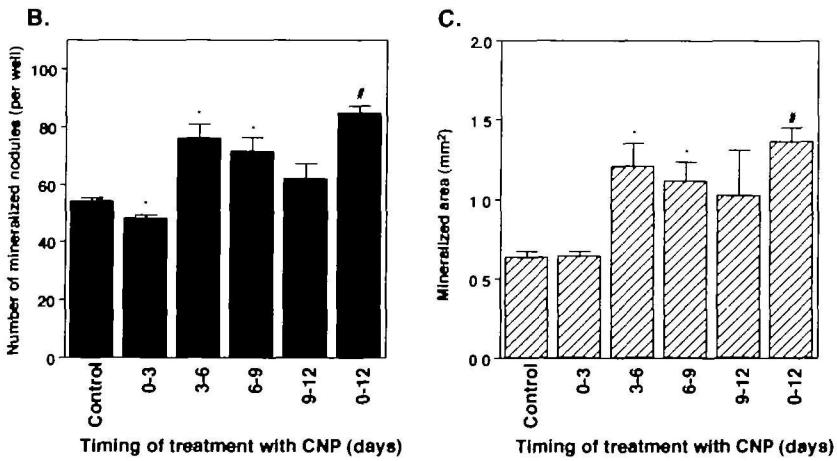


Fig. 3. **Temporal effects of CNP on the formation of mineralized nodules by ROB cells.** Cells in 12-well plates were cultured for 14 days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml L-ascorbic acid, with 10^{-7} M CNP included as indicated. Mineralized nodules were subjected to von Kossa staining as described under "MATERIALS AND METHODS" (A). All bars indicate 3 mm. The number (B) and area (C) of mineralized nodules were determined for four wells per sample with the Mac SCOPE program. Data are the means ± SE of the results for four wells and are representative of the results of four experiments that yielded similar results. $^{*}p<0.05$ *versus* control; $^{\sharp}p<0.01$ *versus* control.

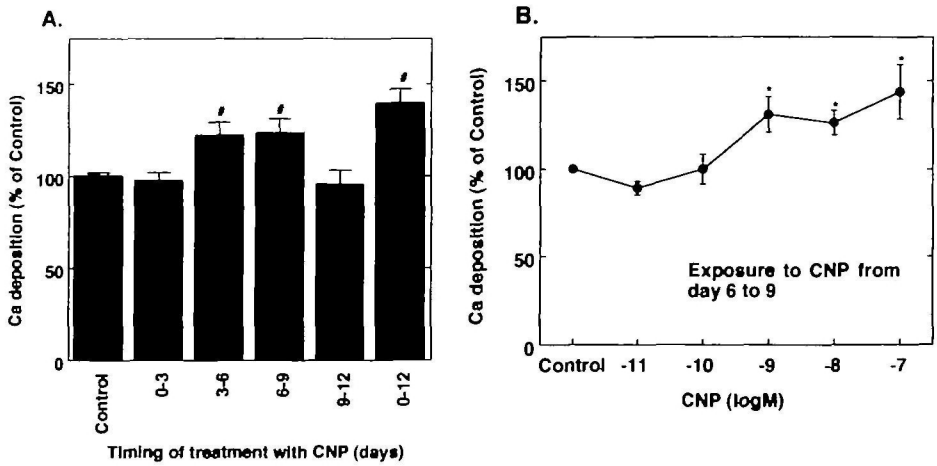


Fig. 4. **Timing of treatment with CNP on the deposition of calcium in ROB cells.** Cells in 12-well plates were cultured with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml L-ascorbic acid, and then treated with 10^{-7} M CNP continuously or during indicated periods (A). The exposure of ROB cells to CNP at various concentrations from day 6 through day 9 was performed on the stimulation of the deposition of calcium (B). On day 12, quantitative analysis of calcium ions derived from hydroxyapatite was performed by method described under "MATERIALS AND METHODS." Data are the means ± SE of the results for four wells and are representative of the results of three experiments that yielded similar results. $^{*}p<0.05$ *versus* control; $^{\sharp}p<0.01$ *versus* control.

littermates. Radiographic analysis of the long bones of 3-month-old mutant and wild-type animals revealed a lower bone density and reduced cortical thickness in the knockout mice. The gene for BGN is located on the X chromosome (*22*). Individuals with Turner's syndrome (monosomy X) have a decreased level of BGN, a short stature, and other skeletal deformities, that include early-onset osteoporosis (*23, 24*). In contrast, overexpression of BGN occurs in individuals with an abnormal number of X chromosomes. For example, individuals with Klinefelter's syndrome are excessively tall (*24*). Thus, BGN appears to play an important role in bone formation both *in vivo* and *in vitro*. In the present study, we showed that CNP stimulated the expression of mRNA for BGN in clonal preosteoblastic MC3T3-E1 cells and in ROB cells. The induction of mRNA for BGN by CNP was greater in MC3T3-E1 cells (Fig. 1) than in ROB cells (Fig. 2). The differences in the effect of CNP on the expression of mRNA for BGN might reflect differences in the cell culture system used: ROB cells comprise a variety of cells that include osteoblast-like cells at several stages of differentiation and other types of cells.

Several factors have been reported to regulate the synthesis of BGN in bone cells in culture. The promoter of the gene for BGN has a number of *cis*-acting elements, including SP-1, AP-1, AP-2, NF-1, and NF$\kappa\beta$ binding sites (*8*). The promoter of the gene for BGN in osteosarcoma cells is regulated by cAMP (*25*). TGF-β (*26*) and insulin-like growth factors (*9*) increase the level of mRNA for BGN in osteoblastic cells. In contrast, retinoic acid (*27*) and dexamethasone (*28*) have been reported to decrease the expression of BGN in bovine chondrocytes and in human osteoblasts and stromal cells from bone marrow, respectively. In the present study, we showed that CNP increased the level of mRNA for BGN in osteoblastic cells. Our data implicate the CNP/cGMP signal transduction pathway in the regulation of expression of the gene for BGN. CNP also stimulates the expression of mRNAs for TGF-β1 and TGF-β2 in MC3T3-E1 cells (our unpublished observations). CNP might increase the expression of the mRNA for BGN *via* the production of TGF-βs. Alternatively, cGMP produced by CNP might increase intracellular cAMP through inhibition of phosphodiesterase (type-III phosphodiesterase).

We reported previously that CNP increased the expression of mRNAs for osteoblastic differentiation marker proteins, such as ALPase and osteocalcin, in ROB cells (*1*). The expression of mRNA for ALPase increases before the onset of mineralization and reaches the maximum level almost coincident with the formation of mineralized nodules (*1, 13*). Osteocalcin mRNA expression occurs with the formation of mineralized nodules (*1*). The expression of mRNA for BGN shows a similar profile to that for type I-collagen or ALPase (*13*). CNP increased the expression of mRNA for ALPase at all times (data not shown). In contrast, the stimulation of the expression of mRNA for BGN by CNP was temporal (around day 8), as shown in Fig. 2. We showed previously that continuous culture of ROB cells with 10^{-7} M CNP for 14 days enhanced the formation of mineralized nodules and the deposition of calcium (*1*). In this study, the pulsed administration of CNP revealed that CNP was effective for the stimulation of mineralization on days 3 through 9 (Figs. 3 and 4). These results suggest that CNP might accelerate the formation of mineralized nodules by ROB cells at a specific time. Since BGN is known to be involved in acceleration of mineralization, it is possible that CNP might enhance the synthesis of matrix proteins and mineralization *via* the expression of BGN in osteoblastic cells. Using DD-PCR methods, we hope to identify additional CNP-controlled genes that are involved in the mineralization by osteoblastic cells.

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