Biochemical and Biophysical Research Communications **280,** 348–352 (2001)
doi:10.1006/bbrc.2000.4108, available online at http://www.idealibrary.com on IDEAL®

Phosphate Provides an Extracellular Signal That Drives Nuclear Export of Runx2/Cbfa1 in Bone Cells

Takashi Fujita,* Nobuo Izumo,* Ryo Fukuyama,* Tohru Meguro,* Hiromichi Nakamuta,* Takeyuki Kohno,† and Masao Koida*

**Department of Pharmacology and †Department of Environmental Health Sciences, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka, Japan*

Received November 20, 2000

Inorganic phosphate (Pi) supplement is generally used to accelerate mineralization of cultured bone cells but the mechanism of action is totally unknown. How the action is related with the transactivation of Runx2/Cbfa1, a master gene product of bone formation, was examined. Clonal bone cells (osteoblastic MC3T3-E1, chondrocytic ATDC5 and osteocytic MLO-Y4) on preculture in ascorbate-containing medium constantly expressed and accumulated Cbfa1 in the nuclei, and subsequent increase of Pi concentration to 3 or 10 mM was found to invariably induce nuclear export (not import) of Cbfa1 which was completed in a few hours. In addition, Pi was found to lower the expression of osteocalcin. Leptomycin B completely inhibited Pi-induced nuclear export, suggesting that CRM1/exportin 1 is involved in Pi-induced nuclear export. The result suggests that bone cells are equipped with a novel Pi sensing mechanism which is functionally linked to a nuclear export system of Cbfa1.

Key Words: **phosphate; Runx2/Cbfa1; nuclear export; CRM1/exportin; ossification.**

Bone formation in tissue culture is a relatively slow process and, when used for assays of drug effects, it requires a month long culture until detectable mineralization starts. In order to make such assays more practical, an appropriate "cocktail" of inorganic phosphate ion (Pi) or β-glycerophosphate as a Pi precursor, with ascorbate and/or a glucocorticoid, has been used as a starter of mineralization (1–4) (e.g., in a selected clone of MC3T3-E1 cells, a small increase of Pi from 1 to 3 mM was able to condense the pre-mineralization time from 2 weeks to 2 days. refer to 3). By far, however, no direct study has been reported to clarify why Pi is needed or how it accelerates the onset of mineralization. Phosphate is known to be actively transported into osteoblasts via sodium-dependent type III phosphate transporter (Glvr-1 and 2, etc.) (2, 5, 6), but little is known beyond the entry, namely about how the ion would be incorporated into extracellular matrices as the substrate and/or how it triggers a chain of reactions which induces matrix formation and mineralization, except a finding quite recently reported by Beck *et al.* (7) that Pi (4 to 10 mM) alone, even in the absence of ascorbate, induced osteopontin gene expression by MC3T3-E1 cells so far in a osteopontin-specific manner.

Runx2/Cbfa1 is a product of master gene of bone formation and its transactivation (nuclear import) is essential for osteoblastic and chondrocytic bone formation (8, 9). We addressed a possibility that Pi triggers the transactivation and/or potentiation of Cbfa1 as a basic mechanism to induce osteoblastic and chondrocytic ossification.

Using three different types of bone cells, osteoblastic MC3T3-E1, chondrocytic ATDC5 and osteocytic MLO-Y4 (10), we monitored the time course of Cbfa1 transactivation under the culture condition in which Pi condenses pre-mineralization time of two osteogenic cells, MC3T3-E1 and ATDC5. Such study provided, however, a totally unexpected result that Pi quickly evacuated Cbfa1 from the nuclei into the cytosol by a leptomycin-B (LMB)-sensitive nuclear export (not import) which completed within a few hours, thereafter keeping the cell nuclei free of Cbfa1, and days later accelerated mineralization occurred in spite of the nuclear absence of Cbfa1. This is the first observation of a nuclear export system of a transcription factor operating in mammalian bone cells and Pi as one of its operators. The biological significance of our finding is discussed because this system may be related with a well known adaptation mechanism of a yeast which also responds to Pi to start nuclear export of a transcription factor Pho4 (11).

Abbreviations used: AA, ascorbic acid; OCN, osteocalcin; LMB, leptomycin-B; ALP, alkaline phosphatase.

0006-291X/01 $35.00

MATERIALS AND METHODS

MLO-Y4 cells were a gift from Dr. Lynda Bonewald (Texas Health Science Center, Texas), anti-mouse Cbfa1 antiserum (9) from Dr. Gerard Karsenty of Baylor University (Houston, TX) and LMB from Dr. Minoru Yoshida (Tokyo University, Tokyo, Japan). MC3T3-E1 and ATDC5 cells were purchased from Riken Cell Bank (Ibaraki, Japan); monoclonal antibodies which recognize the central or the C-terminal portion of human osteocalcin (OCN) from Kyowa Medex Co. Ltd. (Tokyo, Japan); $Gla^{17,21,24}$-human osteocalcin from Peptide Inst. Inc. (Osaka, Japan).

Cell culture. MC3T3-E1 cells were precultured in 10% FCS/α-MEM for 8 days; ATDC5 cells were cultured to confluence in 5% DMEM and then transferred in 10% FCS/α-MEM; MLO-Y4 cells were cultured to confluence in 5% FCS and 5% FBS/α-MEM on collagen type-I coated plates. All the culture media contained ascorbic acid (AA: 50 μg/ml).

Measurements of protein content and mineralization. Protein concentration of Triton X-100 solubilized cells was determined using BCA protein assay kit (Pearce Chemicals, Rockford, IL, USA); alkaline phosphatase (ALP) activity colorimetracally using *p*-nitrophenylphosphate as a substrate; extracellular Ca deposits after decalcifying with 0.6 N HCl for 24 h by *o*-cresolphthalein complexone method (Calcium C-test Wako, Osaka, Japan).

Northern blot analysis. The analysis was carried out under high stringency. In brief, 20 μg as total RNA in cell extracts was separated by electrophoresis in 1.2% agarose-formaldehyde gel, transferred onto a nylon membrane filter (Hybond N^+, Amersham, UK) and hybridized with ^{32}P-labeled cDNA probes encoding the N-terminal of Cbfa1 gifted by Dr. Hideyuki Harada (Sumitomo Pharm. Co. LTD., Osaka, Japan). After the final washing, the membrane was exposed to a BAS imaging plate (Fuji Film, Tokyo, Japan), and the relative signal intensity was estimated.

Immunocytochemical localization of Cbfa1. Cells were fixed for 30 min in fresh 4% paraformaldehyde in PBS, rinsed with TBS-T (Tris-buffered saline-Tween 20), incubated with anti-Cbfa1 antibody (diluted 1:150) in 3% BSA (bovine serum albumin)/TBS-T, rinsed, and then treated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IG antibody (diluted 1:500) for examining by a MRC confocal laser scanning microscope (BIO-RAD Microscopy, Hemel Hempstead, UK).

Preparation of nuclear extract and gel retardation assay. Nuclear extracts were prepared and the assay conducted as originally described by Dignum *et al.* (12). Cells were homogenized using a Dounce homogenizer with a B-type pestle in 4 volumes of buffer A (10 mM HEPES-NaOH, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 μg/ml each of protease inhibitors: p-aminophenyl methansulfonyl fluoride, leupeptin, pepstatin A and aprotinin) and mixed with NP-40 to 0.6%. After 5 min standing on ice, the homogenates were centrifuged at 20,000*g* for 5 min. Supernatants were adjusted to buffer B by adding stock solution for buffer B, and stored as the cytosolic fractions at −80°C. All these procedures were performed at 4°C. Protein concentration was determined as described above. Double stranded oligonucleotides were individually labeled by [α-^{32}P]-ATP using krenow fragment and purified by Nick column. Each DNA binding reaction mixture contained poly (d[I-C]) (Amersham Pharmacia Biotech, UK), 3000–6000 cpm of labeled DNA fragments, 5 μg of nuclear extracts, and a buffer composed of 10 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, and 10% glycerol. For supershift assays, cellular extracts were preincubated with anti-mouse Cbfa1 antisera at 4°C for 30 min prior to the addition of labeled probe.

Immunoblotting of Cbfa1. Cells were solubilized in Tris-HCl buffer (pH 6.8) containing 3% sodium dodecyl sulfate (SDS) and 10% glycerol and after electrophoresis, proteins were transferred to polyvinylidene difluoride membrane for immunoblotting, using antibodies toward Cbfa1 and processed as described (13).

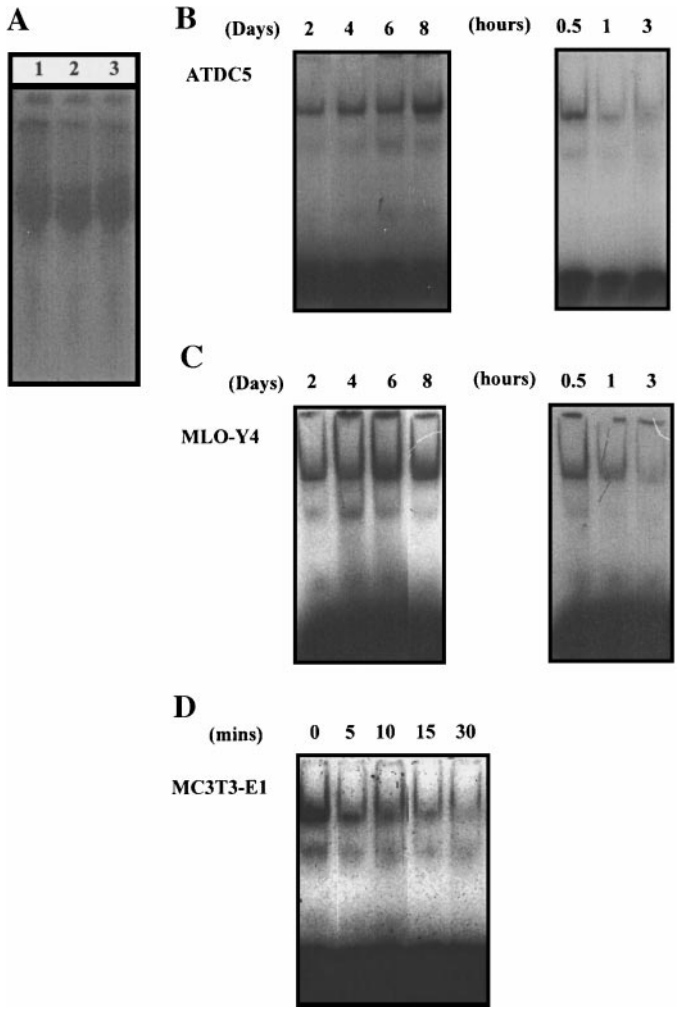

FIG. 1. Bone cells used consistently express Cbfa1 which gradually accumulates for nuclear OSE2 binding on daily basis and then Pi quickly evacuated the factor. (A) Universal expression of Cbfa1 by MC3T3-E1 cells after 8 days preculture and other cells after confluence in the presence of AA; lane 1, MC3T3-E1; lane 2, ATDC5; lane 3, MLO-Y4. (B and C) Nuclear accumulation of Obfa1 during 8 days preculture of ATDC5 and MLO-Y4 and 10 mM Pi-induced evacuation on the 9th day, respectively (data of MC3T3-E1 was not shown). D, time course of 3 mM Pi-induced evacuation in MC3T3-E1.

Enzyme immunoassay of OCN. Sampled culture medium or standard OCN was taken in 10 mM sodium phosphate buffer (pH 7.0), containing 0.4 M NaCl and 1.0 g/l BSA fraction V (Intergen Co., Purchase, NY). OCN in samples (0.15 ml) were assayed by two-site enzyme immunoassay using the two monoclonal antibodies.

Statistical analysis. Statistical analyses were performed using Student's *t* test. At $P < 0.05$, the difference was considered to be significant.

RESULTS

Figure 1 shows that three cell lines used herein universally expressed comparable levels of Cbfa1

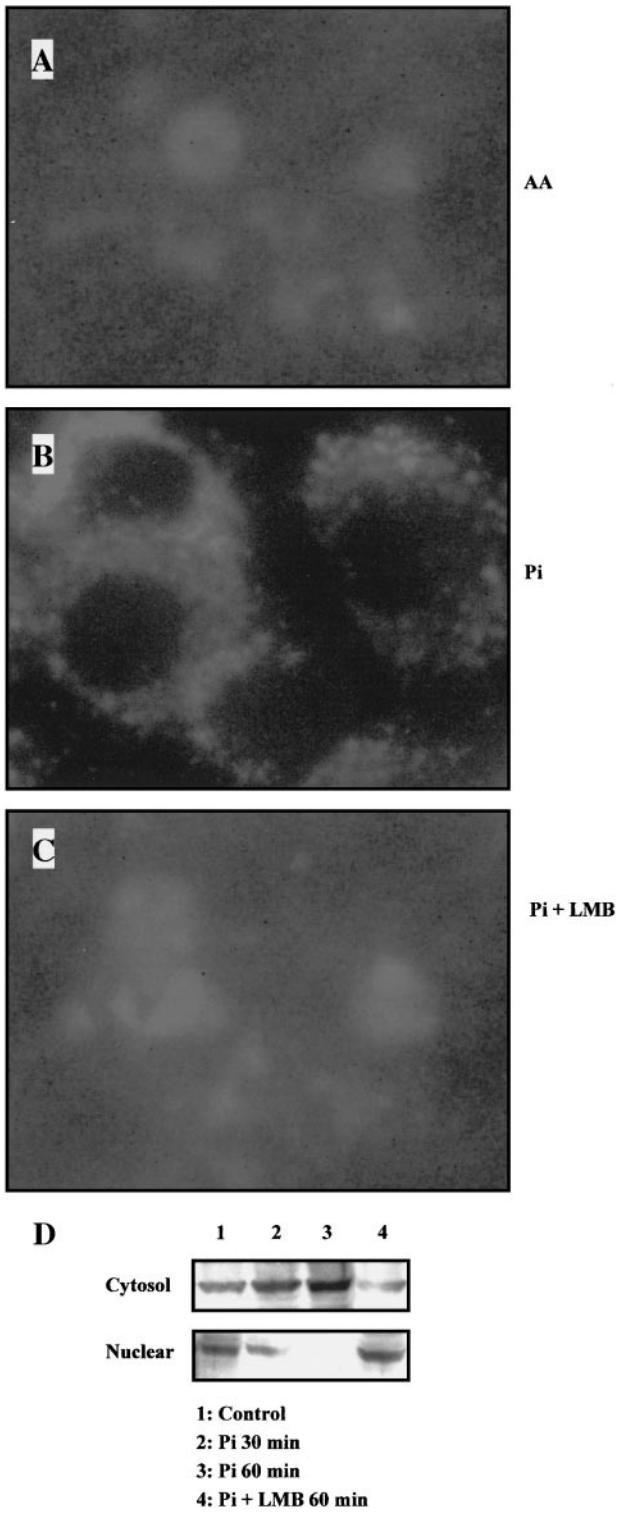

FIG. 2. Immunocytochemical visualization and immunoblot analysis of Pi-induced nuclear export of Cbfa1 via a LMB-sensitive way. (A) AA-induced nuclear accumulation of Cbfa1-immunofluorescence after 8 days culture of ATDC5. (B) 10 mM Pi-stimulated nuclear export. (C) Complete suppression of the export by LMB which was present from 60 min before Pi stimulus. Similar results were obtained with MC3T3-E1 and MLO-Y4 (data not shown). (D) Immunoblot analysis carried out in parallel with cytochemical study in A–C. Pi induced nuclear export of Cbfa1 which completed within 60 min, and LMB blocked nuclear export.

mRNA (Fig. 1A) after preculture 8 days for MC3T3-E1 cells or until confluence for other cells. The results of EMSA in Fig. 2B and C revealed that, in the presence of AA and 1 mM Pi, ATDC5 and MLO-Y4 cells constantly expressed and gradually accumulated Cbfa1 in the cell nuclei in a time-dependent manner during preculture which would possibly bind to the nuclear sites including OSE2 promoter of OCN. In the case of MLO-Y4, the accumulation was observed to occur even in the absence of AA.

Pi accelerates nodule formation by MC3T3-E1 and ATDC5 cells dose-dependently (data not shown) and, in our laboratory, 3 mM Pi for MC3T3-E1 and 10 mM for ATDC5 have been routinely employed for accelerating mineralization and ALP activity of these cell lines which became measurable within 2 and 4 days, respectively. MLO-Y4 cells did not mineralize under these conditions (the data not given). A series of experiments undertaken to chronologically relate the nuclear transfer of Cbfa1 with the initiation of mineralization led us to the finding that Pi increase induced quick evacuation of Cbfa1 from the cell nuclei which completed within a few hours (Figs. 1B and 1C) and within an hour in MC3T3-E1 cells (Fig. 1D), and days later accelerated mineralization occurred (data not shown).

Immunocytochemical studies of Cbfa1 in Figs. 2A to 2C provided a direct view of such evacuating action of Pi and also showed that the Pi action except on mineralization is completely suppressed by LMB, indicating that CRM1/exportin 1 is involved in the nuclear export of Cbfa1. OCN is a protein, the production of which is under the rigid control of nuclear Cbfa1 (9), and it is expected that Pi-induced evacuation of nuclear Cbfa1 would decrease the production. The result in Fig. 3 indicates that OCN production in fact decreased possibly due to Cbfa1 evacuation from the nuclei.

DISCUSSION

The results obtained herein shows that mammalian bone cells may be equipped with a novel Pi-sensing system which responds to extracellular Pi to induce the nuclear export (not import) of Cbfa1, a master gene product essential for osteoblastic and chondrocytic bone formation, as a quick response completing within a few hours. Though it was confirmed that thereafter the cell nuclei was kept free of Cbfa1, days later, Pi-induced mineralization followed as a delayed response in spite of the nuclear absence of Cbfa. It is possible

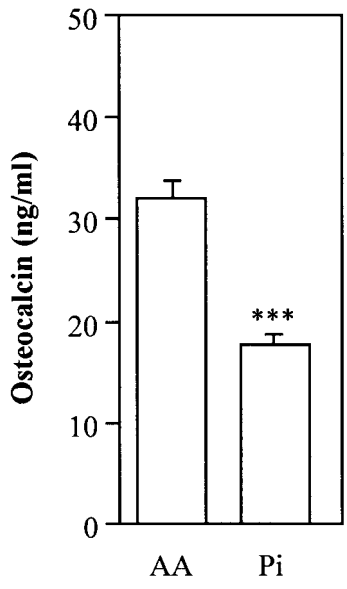

FIG. 3. Pi stimulus decreases OCN production. During 8 days of culture in α-MEM supplemented with 10% FCS and 50 μg/ml AA with 1 or 10 mM phosphate, the conditioned media in wells were collected daily for the last 3 days for EIA of OCN contents. Results are means ± SE of 6 wells obtained from 3 independent cultures. ***$P < 0.001$, significantly different from 1 mM phosphate samples.

that such quick and delayed responses are mutually related and mineralization which developed days later would have taken place in a Cbfa1-independent manner or have been effected by the cytosolic Cbfa1 itself. Recently, we have observed that PTH (1-34) can drive nuclear import of Cbfa1 in a related manner with its osteogenic action both *in vivo* and *in vitro*, indicating that the nuclear import is essential for bone formation and expression of a certain set of bone specific proteins such as bone sialoprotein, ALP and OCN (14 and unpublished observations). In order to unify these incompatible results, future more detailed studies appear to be needed.

Saccharomyces cerevisae, a yeast, is known to adjust its secretion pattern of acid phosphatase depending on phosphate availability. Under phosphate-rich condition, the transcription factor Pho4 is phosphorylated by a kinase for nuclear export, which turns off transcription of genes of phosphatase which turns on during phosphate starvation (15). The mechanism of its nuclear trafficking is now under intensive studies (refer to 16). Bone cells are also known to excrete mineralizing vesicles enriched with ALP (17) which appears to be essential for mineralization, and Pi effect observed herein may imply conservation of Pi-primed acid phosphatase secretion system of yeast as a novel Pi-primed excretion system of ALP-rich vesicles in mammalian bone cells.

Taken together, our results demonstrated that: (1) Pi in a high but physiological concentration range quickly stimulates nuclear export of Cbfa1 via a CRM1/exportin 1-related way, (2) the Pi stimulus then exerts accelerating effect on mineralization of osteoblastic MC3T3-E1 and chondrocytic ATDC5 (not on osteocytic MLO-Y4), with a delay of days by a signaling pathway in which at least nuclear Cbfa1 is not involved, (3) bone cells stimulated by Pi provide a good model to explore the basic mechanism underlying nuclear transport in mammalian bone cells, which may be a conserved form of a yeast export system.

REFERENCES

1. Ohgushi, H., Dohi, Y., Katsuda, T., Tamai, S., Tabata, S., and Suwa, Y. (1996) Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. *J. Biomed. Mater. Res.* **32,** 333–340.
2. Guicheux, J., Palmer, G., Shukunami, C., Hiraki, Y., Bonjour, J. P., and Caverzasio, J. (2000) A novel in vitro culture system for analysis of functional role of phosphate transport in endochondral ossification. *Bone* **27,** 69–74.
3. Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P. H., and Franceschi, R. T. (1999) Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. *J. Bone Miner. Res.* **14,** 893–903.
4. Fratzl-Zelman, N., Fratzl, P., Horandner, H., Grabner, B., Varga, F., Ellinger, A., and Klaushofer, K. (1998) Matrix mineralization in MC3T3-E1 cell cultures initiated by β-glycerophosphate pulse. *Bone* **23,** 511–520.
5. Palmer, G., Zhao, J., Bonjour, J., Hofstetter, W., and Caverzasio, J. (1999) In vivo expression of transcripts encoding the Glvr-1 phosphate transporter/retrovirus receptor during bone development. *Bone* **24,** 1–7.
6. Suzuki, A., Palmer, G., Bonjour, J. P., and Caverzasio, J. (2000) Stimulation of sodium-dependent phosphate transport and signaling mechanisms induced by basic fibroblast growth factor in MC3T3-E1 osteoblast-like cells. *J. Bone. Miner. Res.* **15,** 95–102.
7. Beck, Jr, G. R., Zerler, B., and Moran, E. (2000) Phosphate is a specific signal for induction of osteopontin gene expression. *Proc. Natl. Acad. Sci. USA* **97,** 8352–8357.
8. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S., and Kishimoto, T. (1997) Targeted disruption of Cbfa1 results in complete lack of bone formation owing to maturation arrest of osteoblast. *Cell* **89,** 755–764.
9. Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L., and Karsenty, G. (1997) Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. *Cell* **89,** 747–754.
10. Kato, Y., Windle, J. J., Koop, B. A., Mundy, G. R., and Bonewald, L. F. (1997) Establishment of an osteocyt-like cell line, MLO-Y4. *J. Bone. Miner. Res.* **12,** 2014–2023.
11. Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999) Leptomycin B inactivates CRM/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc. Natl. Acad. Sci. USA* **96,** 9112–9117.
12. Dignam, J. P., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11,** 1475–1489.
13. Takuma, K., Fujita, T., Kimura, Y., Tanabe, M., Yamamuro, A., Lee, E., Mori, K., Koyama, Y., Baba, A., and Matsuda, T. (2000)

T588 inhibits astrocyte apotosis via mitogen-activated protein kinase signal pathway. *Eur. J. Pharmacol.* **399,** 1–8.

14. Fujita, T., Fukuyama, R., Izumo, N., Meguro, T., Nakamuta, H., and Koida, M. (2000) Enhanced Cbfa1 transactivation as a basic mechanism to trigger bone anabolic action of PTH (1-34). *J. Bone. Miner. Res.* **15**(Suppl. 1), S170.
15. Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S., and O'Shea, E. K. (1998) The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature* **396,** 482–486.
16. Nakielny, S., and Dreyfuss, G. (1999) Transport of proteins and RNAs in and out of the nucleus. *Cell* **99,** 677–690.
17. Anh, D. J., Dimai, H. P., Hall, S. L., and Farley, J. R. (1998) Skeletal alkaline phosphatase activity is primarily released from human osteoblasts in an insoluble form, and the net release is inhibited by calcium and skeletal growth factors. *Calcif. Tissue Int.* **62,** 332–340.