Biochemical Pharmacology, Vol. 58, pp. 465–470, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00106-9

SHORT COMMUNICATION

Establishment of Permanent Cell Lines Exhibiting Vitamin D-dependent Expression of β-Galactosidase Activity

Yoichi Negishi,†‡ Naoki Kajiyama,* Kohtaro Kawashima,† Toshie Fujishima,§ Hiroaki Takayama,§ Hiroyoshi Endo† and Ryuji Kawaguchi**

*Center for Molecular Biology and Cytogenetics, SRL, Inc., Hino-shi, Tokyo 191; †Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01; and §Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa, 199-01, Japan

ABSTRACT. The active hormonal form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), has been described as a principal mediator of skeletal homeostasis. Treatment of rat osteosarcoma (ROS)17/2.8, an osteoblast-like cell line, with 1α,25$(OH)_2D_3$ results in a ligand-dependent increase in transcription of the bone-specific osteocalcin gene. We isolated permanent cell lines that were established by transfecting ROS 17/2.8 cells with plasmids consisting of the human osteocalcin gene promoter containing the vitamin D responsive element linked to a bacterial β-galactosidase gene. In one of many cell lines, especially in clone NK-31, 1α,25$(OH)_2D_3$ strongly stimulated β-galactosidase activity. Reverse transcription-polymerase chain reaction analysis also showed endogenous osteocalcin gene expression and β-galactosidase gene expression in clone NK-31 cells, which paralleled the increase in β-galactosidase activity. Using a synthetic analogue of 1α,25$(OH)_2D_3$, 24,24-difluoro-1α,25-dihydroxyvitamin D_3, we found that the levels of this activity and these gene expressions were nearly parallel to those of 1α,25$(OH)_2D_3$. 24*R*,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 at high doses (concentration: 10^{-7} M) also induced β-galactosidase activity in clone NK-31. These cell lines, harboring the plasmid-carrying β-galactosidase gene under the control of the osteocalcin gene promoter, may contribute to studies on the regulation by 1α,25$(OH)_2D_3$ or to the development of synthetic analogues of 1α,25$(OH)_2D_3$. BIOCHEM PHARMACOL **58**;3:465–470, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. vitamin D; VDRE; osteocalcin; permanent cell lines; β-galactosidase

A large number of analogues of active vitamin D_3 [1–3] have been synthesized for the treatment of osteoporosis. However, these drugs have weak effects on bone and do not completely reverse the loss of bone mineral caused by osteoporosis. Therefore, finding new analogues of active vitamin D_3 to alleviate osteoporosis is still important. Active vitamin D_3, 1α,25$(OH)_2D_3$[‖], initially stimulates bone resorption resulting in an increase in the serum calcium level, but at the same time also increases the level of bone formation, with the result that the rate of bone formation exceeds that of bone resorption. Thus, it regulates the balance of bone metabolism in whole bones. Therefore, synthetic analogues of 1α,25$(OH)_2D_3$ are attractive candidates as new drugs for the treatment of osteoporosis.

Osteocalcin is specifically synthesized by the osteoblast and is believed to be the best marker of bone formation [4–8]. Osteocalcin gene expression and its synthesis are stimulated by 1α,25$(OH)_2D_3$ in human and rat osteoblasts, but not in mouse osteoblasts. Therefore, determination of the level of osteocalcin activity is a good method of investigating the bone-forming effect of 1α,25$(OH)_2D_3$ analogues [7]. However, measuring the level of osteocalcin synthesis is complex, time-consuming, and expensive. In this study, we sought to establish permanent cell lines that exhibit vitamin D hormone-dependent β-galactosidase activity and to evaluate the effect of different synthetic analogues of 1α,25$(OH)_2D_3$ on these cell lines. The 1α,25$(OH)_2D_3$ responsive element (VDRE), which is within the promoter for osteocalcin, is unresponsive to other steroid hormones, can function as a heterologous promoter, and includes a doubly palindromic DNA sequence. Active vitamin D_3 activates an intranuclear receptor that binds to the VDRE of the promoter region of the osteocalcin gene and induces the promoter activity of osteocalcin with a 7- to 9-fold elevation [8]. As β-galactosidase activity is easily quantified, we constructed a plasmid composed of the human osteocalcin gene promoter, includ-

‡ Corresponding author: Yoichi Negishi, Ph.D., Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa, 199-01, Japan. Tel. 81-426-85-3744; FAX 81-426-85-3745.

‖ *Abbreviations:* 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; ROS, rat osteosarcoma; RT, reverse transcription; RT-PCR, reverse transcription-polymerase chain reaction; VDRE, vitamin D responsive element; and β-gal, β-galactosidase.

Received 3 April 1998; accepted 2 February 1999.

ing the VDRE, linked to a bacterial β-galactosidase gene. This vector (pOST-β-gal) was used to transfect osteoblastic ROS17/2.8 cells, and we then isolated permanent cell lines that expressed β-galactosidase in response to exposure to $1\alpha,25(OH)_2D_3$. In one of many cell lines, especially in clone NK-31, a synthetic analogue of $1\alpha,25(OH)_2D_3$, 24,24-difluoro-1α,25-dihydroxyvitamin D_3, induced endogenous osteocalcin gene expression and β-galactosidase expression in a similar manner as $1\alpha,25(OH)_2D_3$. These cell lines may contribute to studies on regulation by $1\alpha,25(OH)_2D_3$ or to the development of synthetic analogues of $1\alpha,25(OH)_2D_3$.

MATERIALS AND METHODS

Cell Culture

ROS17/2.8 is a rat osteosarcoma cell line [9, 10]. ROS17/2.8 cells respond to $1\alpha,25(OH)_2D_3$ and parathyroid hormone with a reduction in the level of type I collagen synthesis [11–13]. ROS17/2.8 cells were maintained in minimum essential Earle's medium supplemented with non-essential amino acids and 10% fetal bovine serum in a humidified atmosphere of 5% CO_2. When several permanent cell lines were incubated with $1\alpha,25(OH)_2D_3$ or when the NK-31 cell line was incubated with analogues of $1\alpha,25(OH)_2D_3$ or other steroids, the medium was changed to 1% fetal bovine serum. All compounds were dissolved in ethanol, whose concentration in the assay medium did not exceed 0.1%.

Isolation of the Human Osteocalcin Gene Promoter

The human osteocalcin gene promoter (−574/+22; nucleotide positions are numbered relative to the cap site, with negative numbers indicating nucleotides extending in the 5′ direction) including the VDRE [8] was isolated from human genomic DNA by use of the PCR. The amplified DNA fragment was subcloned using a TA-cloning kit (Invitrogen), and the resulting plasmid was termed pOST-plasmid. DNA sequencing was performed by the dideoxy method, and entire sequences were analyzed using DNASIS software (Hitachi Soft).

Plasmid Constructions

A *Hind* III/*Pst* I fragment containing the β-galactosidase gene (lacZ) of plasmid pCH110 (Pharmacia Biotech) was initially cloned into pUC19, and the resulting plasmid was termed pβ-gal. A *Hind* III/*Xho* I fragment containing the human osteocalcin gene promoter of pOST-plasmid was ligated into the *Sma* I/*Sal* I sites of pβ-gal (pOST-β-gal). The plasmids used in this study were purified by centrifugation on a cesium chloride gradient.

Stable Cell Lines

Cells were transfected with 20 μg of pOST-β-gal and 1 μg of the plasmid pChmBp1 [14] containing the hygromycin resistance gene by the calcium phosphate co-precipitation method [15]. After incubation for 4 hr, the cells were shocked with 15% glycerol for 1 min. Following incubation for 48 hr after transfection, cells were selected in hygromycin B medium (70 μg/mL). Approximately forty clones resistant to hygromycin B were isolated. The isolated clones were placed into 24-well plates, grown to confluence, and then placed in minimum essential Earle's medium containing $1\alpha,25(OH)_2D_3$ (10^{-8}M) for 48 hr. Subsequently, the cells were stained at 37° with a solution of the following composition: 0.1% X-gal, 5 mM $K4[Fe(CN)_6]$, 5 mM $K3[Fe(CN)_6]$, 2 mM $MgCl_2$, 0.02% Nonidet P-40 and 10% sodium deoxycholate in PBS(−). The staining reactions were performed at 37° until the contents of the wells had turned blue. Staining was stopped by washing the cells with PBS (−) [16]. The vitamin D responsive clones were selected for further use.

β-Galactosidase Assay

Cells were washed once with PBS (−) and disrupted in 100 μL of lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol, 10% glycerol, 1% Triton-X100), followed by incubation for 10 min at room temperature. Protein was measured by the Bradford protein assay kit (BioRad) for normalization of the β-galactosidase activity, and enzyme activity was determined in a reaction mixture consisting of 30 μL of cell extract and 270 μL of the following solution: 1mM $MgCl_2$, 45 mM 2-mercaptoethanol, 100 mM sodium phosphate (pH 7.8), 0.26 mg *o*-nitrophenyl-β-D-galactopyranoside). The reaction mixtures were incubated at room temperature until a faint yellow color had developed. Subsequently, the reaction was stopped by adding 500 μL of 1M Na_2CO_3 to each reaction mixture, and the optical density of the reaction mixture at a wavelength of 420 nm was recorded [17].

Primers for RT-PCR

Osteocalcin, β-galactosidase, and β-actin gene primers for RT-PCR were synthesized by Sawady Technologies. The primers were RNA-specific in that the recognition sites of the upstream and downstream primers resided in separate exons or at intron/exon boundaries in the genomic sequence. The sequences of these primers are listed as follows:

osteocalcin, forward: 5′-CTGCATTCTGCCTCTCT-GAC-3′
reverse: 5′-TGCCATAGATGCGCTTGTAT-3′
β-galactosidase, forward: 5′-GGTGATGGTGCTGCGT-TGGAGTGA-3′
reverse: 5′-GTAGTGTGACGCGATCGGCATA-ACC-3′
β-actin, forward: 5′-CACCCTGTGCTGCTCACCGAG-GCC-3′
reverse: 5′-CCACACAGATGACTTGCGCTCAGG-3′

RT-PCR of the Genes for Osteocalcin, β-Galactosidase, and β-Actin

After incubation with $1\alpha,25(OH)_2D_3$ or 24,24-F2-$1\alpha,25(OH)_2D_3$ for 48 hr, total RNAs were extracted from clones using the acid guanidium isothiocyanate method, as previously described [18]. To degrade DNA prior to the RT reaction, 1 μg of total RNA was treated with DNase I in a reaction mixture containing 1 unit of DNase I (Life Technologies), 20 mM Tris–HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl, in a final volume of 10 μL. The RT reaction proceeded at 37° for 60 min with 1 μg of total RNA in a reaction mixture containing 0.1 mM oligo-dT (15mer) primer (Promega), 50 mM Tris–HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 1mM dithiothreitol, 0.5 mM concentration of each of the dNTPs, 40 units of ribonuclease inhibitor (Takara), and 200 units of Super Script II reverse transcriptase (Life Technologies) in a final volume of 20 μL. One microliter of the RT reaction mixture was subjected to PCR using the specific primers for osteocalcin, β-galactosidase, and β-actin genes in separate tubes. The PCR mixture, a total volume of 20 μL, contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.62 mM $MgCl_2$, 50 μM dNTP, 1 μM concentrations of forward and reverse primers, 0.5 units of rTaq polymerase (Takara), and 1μCi [^{32}P]-dCTP. PCRs were performed for 20 cycles (for osteocalcin and β-actin) or 24 cycles (for β-galactosidase) at 94° for 1 min, 55° for 2 min, and 72° for 3 min. Subsequently, PCR products were loaded onto a 5% polyacrylamide gel (29:1, acrylamide/bis-acrylamide). The gel was dried and exposed to x-ray film. The x-ray film was developed, and the radioactive bands were excised from the gel and measured by liquid scintillation counting. The linear range was determined in each case, as shown in Fig. 2B.

RESULTS AND DISCUSSION

To establish a permanent cell line for monitoring the expression of a reporter gene in response to exposure to vitamin D hormone, we used the human osteocalcin gene promoter carrying the vitamin D responsive element, a sequence that has been well studied as a transcriptional element [8, 19] that is associated with the vitamin D receptor activated by $1\alpha,25(OH)_2D_3$ or its analogues. To construct the reporter genes, the expression of which is induced by $1\alpha,25(OH)_2D_3$ analogues, we isolated the human osteocalcin gene promoter (−575/+22) containing the VDRE [8, 19] from human genomic DNA by PCR as described in Materials and Methods. The amplified DNA fragment was ligated to the bacterial β-galactosidase gene (lacZ), and the resulting plasmid was termed pOST-β-gal. Transient transfection assays were performed using ROS17/2.8 cells to determine whether the β-galactosidase gene would be expressed in ROS17/2.8 cells harboring pOST-β-gal under the regulation of the osteocalcin gene promoter in response to $1\alpha,25(OH)_2D_3$. To establish cell lines harboring pOST-β-gal, we transfected ROS17/2.8 cells

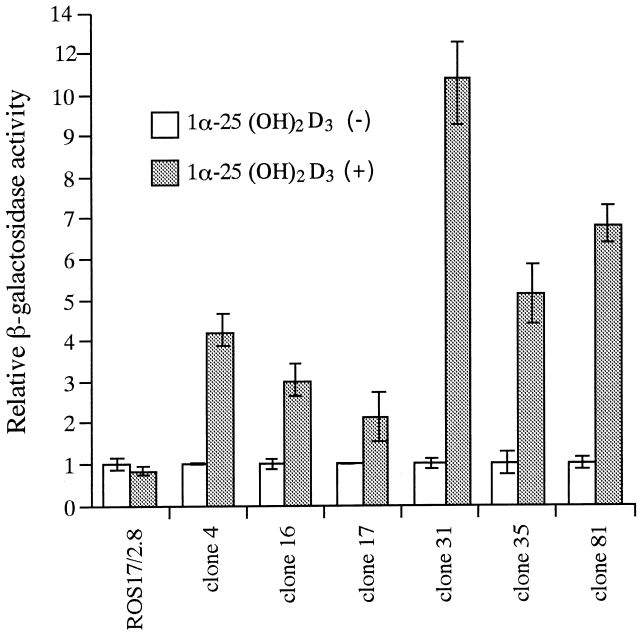

FIG. 1. $1\alpha,25(OH)_2D_3$-induced β-galactosidase activity of established clones. ROS17/2.8 cells were co-transfected with the test plasmid, pOST-β-gal, and pChmBp1 carrying the hygromycin resistance gene and selected for resistance to hygromycin B. The hygromycin B-resistant clones were tested with $1\alpha,25(OH)_2D_3$ (10^{-8} M). β-Galactosidase activities in the presence of $1\alpha,25(OH)_2D_3$ relative to that of each clone in its absence are shown. Values represent the means ± SD from two experiments, each with duplicate determinations.

with pOST-β-gal and pChmBp1 carrying the hygromycin resistance gene and selected for hygromycin B resistance. We determined the level of β-galactosidase activity of each hygromycin-resistant clone, and designated each as a member of an NK clone series. Forty clones that exhibited β-galactosidase activity in the presence of $1\alpha,25(OH)_2D_3$ were selected.

To determine whether these clones responded to $1\alpha,25(OH)_2D_3$ with expression of β-galactosidase activity, we cultured the clones to 80% confluence in 10% serum medium in 24-well plates and then supplemented each well with 1% serum medium containing $1\alpha,25(OH)_2D_3$ (10^{-8}M). After a 48-hr incubation, β-galactosidase activities were measured as described in Materials and Methods. The highest β-galactosidase activity elicited by $1\alpha,25(OH)_2D_3$ was found in clone NK-31. In contrast, the parental cell line, ROS17/2.8, did not yield any β-galactosidase (Fig. 1). These clones responded to $1\alpha,25(OH)_2D_3$ differently, as shown in Fig. 1, with the magnitude of the response corresponding to the number of intact copies of pOST-β-gal introduced into their genomes (data not shown). In clone NK-31, $1\alpha,25(OH)_2D_3$ (10^{-8} or 10^{-9} M) stimulated β-galactosidase activity in a dose-dependent manner (Fig. 2A). Furthermore, we examined, by RT-PCR analysis, expression of the endogenous osteocalcin gene and β-galactosidase gene in clone NK-31 exposed to $1\alpha,25(OH)_2D_3$. The results showed that the expressions of

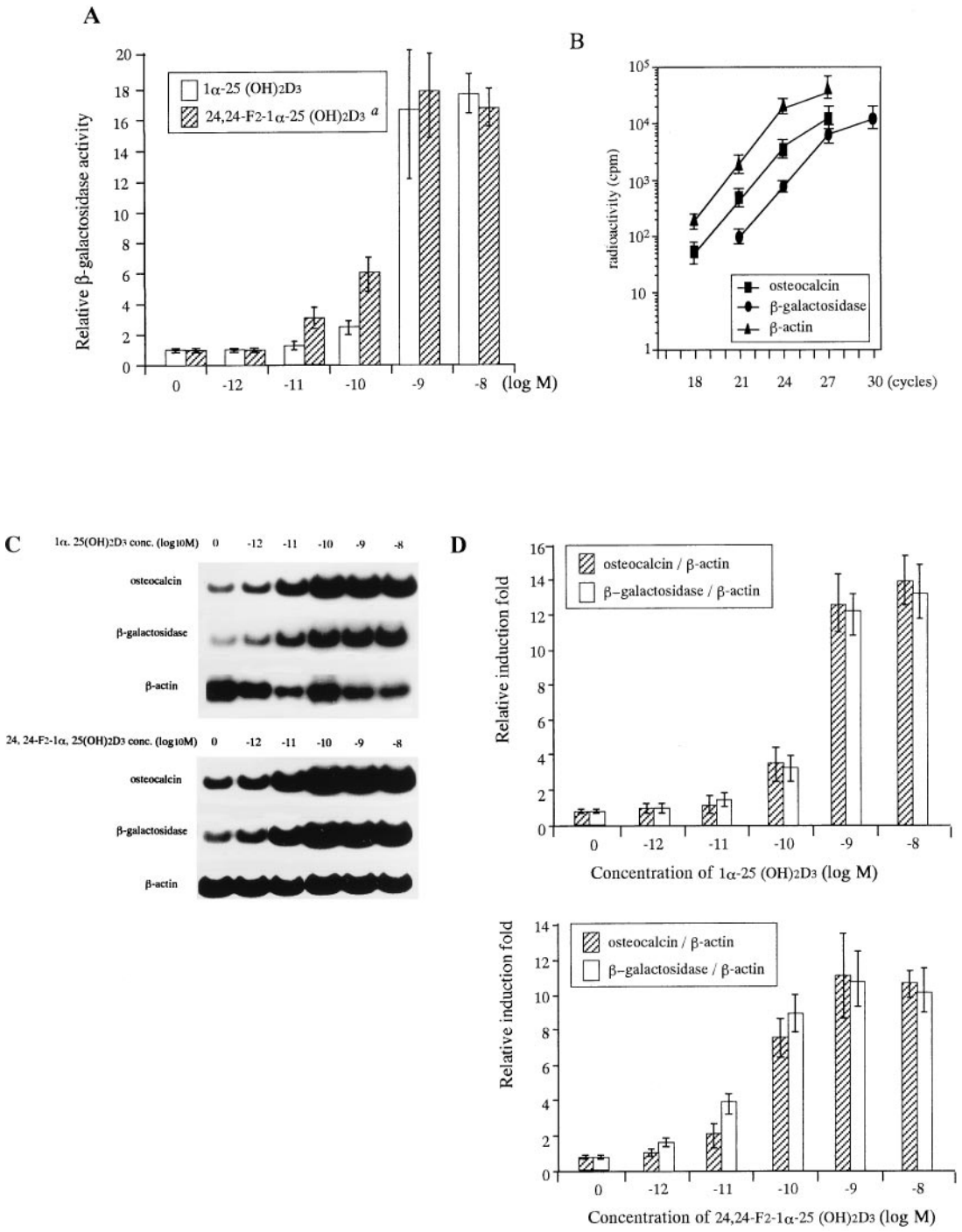

FIG. 2. Effect of $1\alpha,25(OH)_2D_3$ or 24,24-F2-$1\alpha,25(OH)_2D_3$ on clone NK-31. (A) Clone NK-31 was exposed for 48 hr to various concentrations (0, −12, −11, −10, −9, and −8 log M, respectively) of $1\alpha,25(OH)_2D_3$ or 24,24-F2-$1\alpha,25(OH)_2D_3$. β-Galactosidase activity relative to that in the absence of $1\alpha,25(OH)_2D_3$ or 24,24-F2-$1\alpha,25(OH)_2D_3$ is shown. "24,24-F2-$1\alpha,25(OH)_2D_3$, 24,24-difluoro-1α,25-dihydroxyvitamin D_3. (B) RT-PCR analysis was performed for 18 to 30 cycles to determine the linear range in each case. One microgram of total RNA isolated from clone NK-31 that had been exposed for 48 hr to $1\alpha,25(OH)_2D_3$ (10^{-8} M) was reverse transcribed using oligo-dT primers. Each transcript was amplified with the specific primers for osteocalcin-, β-galactosidase, and β-actin genes by PCR with [^{32}P-dCTP]. (C) Expressions of endogenous osteocalcin gene and β-galactosidase gene induced with increasing concentrations (0, −12, −11, −10, −9, and −8 log M, respectively) of $1\alpha,25(OH)_2D_3$ or 24,24-F2-$1\alpha,25(OH)_2D_3$ were analyzed by RT-PCR with [^{32}P-dCTP]. (D) The expression levels of endogenous osteocalcin gene and β-galactosidase gene determined by RT-PCR were quantified as described in Materials and Methods and corrected for the expression in levels of the β-actin gene. Relative induction fold to that in the absence of $1\alpha,25(OH)_2D_3$ or 24,24-F2-$1\alpha,25(OH)_2D_3$ is shown. Values represent the means ± SD from two experiments, each with duplicate determinations.

TABLE 1. Relative β-galactosidase activity of analogues of vitamin D_3 and other steroids

Compounds	Relative β-galactosidase activity
Control	1.02 ± 0.15
1α,25$(OH)_2D_3$	
10^{-10} M	3.10 ± 0.89
10^{-9} M	11.23 ± 2.12
10^{-8} M	14.18 ± 2.24
10^{-7} M	12.56 ± 1.58
24,24-F2-1α,25-$(OH)_2D_3$*	
10^{-10} M	6.41 ± 0.55
10^{-9} M	13.35 ± 1.24
10^{-8} M	12.21 ± 2.14
10^{-7} M	11.22 ± 1.18
24R, 25-$(OH)_2D_3$†	
10^{-10} M	1.39 ± 0.32
10^{-9} M	1.19 ± 0.22
10^{-8} M	1.40 ± 0.16
10^{-7} M	4.63 ± 1.31
25-$(OH)D_3$‡	
10^{-10} M	1.21 ± 0.42
10^{-9} M	1.05 ± 0.30
10^{-8} M	1.24 ± 0.13
10^{-7} M	5.31 ± 2.05
Dexamethasone	
10^{-10} M	0.64 ± 0.29
10^{-9} M	0.62 ± 0.18
10^{-8} M	0.46 ± 0.11
10^{-7} M	0.34 ± 0.25
17-β-Estradiol	
10^{-10} M	0.91 ± 0.41
10^{-9} M	1.11 ± 0.24
10^{-8} M	0.98 ± 0.32
10^{-7} M	1.23 ± 0.17

Clone NK-31 was exposed for 48 hr to various concentrations (−10, −9, −8, and −7 log M, respectively) of analogues of vitamin D_3 and other steroids. Relative β-galactosidase activities to that in the control (in the absence of analogues of vitamin D_3 and other steroids) are shown. Values are means ± SD for triplicate measurements.

*24,24-F2-1α and 25-$(OH)_2D_3$, 24,24-difluoro-1α and 25-dihydroxyvitamin D_3.

†24*R*,25-$(OH)_2D_3$, 24*R*, 25-dihydroxyvitamin D_3.

‡25-$(OH)D_3$,25-hydroxyvitamin D_3.

endogenous osteocalcin and β-galactosidase genes were induced by 1α,25$(OH)_2D_3$ (Fig. 2, C and D to a similar extent) as the increase in the expression of β-galactosidase activity by the reporter gene (Fig. 2A). Using a synthetic analogue of 1α,25$(OH)_2D_3$, 24,24-F2-1α,25$(OH)_2D_3$ [19–21], we found that the level of this activity and these gene expressions were similar to those obtained with 1α,25$(OH)_2D_3$ (Fig. 2, A, C, and D). However, 24,24-F2-1α,25$(OH)_2D_3$ at low doses (concentration: 10^{-11}–10^{-10} M) was about two times more potent than 1α,25$(OH)_2D_3$ in the reporter system. Thus, the potency of 24,24-F2-1α,25$(OH)_2D_3$ is consistent with the known fact that the effect of 24,24-F2-1α,25$(OH)_2D_3$ on bone metabolism is similar to that of 1α,25$(OH)_2D_3$ [20, 21]. 24*R*,25$(OH)_2D_3$ and 25$(OH)D_3$, two naturally occurring products in the metabolism of vitamin D, at high dose (concentration: 10^{-7} M) also induced pOST-β-gal-derived β-galactosidase activity to a degree consistent with their known affinities for the vitamin D receptor [22]; however, dexamethasone and 17-β-estradiol did not induce the activity (Table 1). Therefore, β-galactosidase activity stimulated by 1α,25$(OH)_2D_3$ and analogues of vitamin D_3 are dependent on VDRE in the reporter system. Thus, the expression of β-galactosidase under the control of the osteocalcin gene promoter in these cell lines may contribute to the study of regulation by 1α,25$(OH)_2D_3$ as well as to the development of synthetic vitamin D_3 analogues.

We thank Dr. Hiroshi Iwasaki of the Tsukuba Research Laboratory, NOF Corporation (5-10 Tsukuba, Ibaraki 300-2635, Japan) for kindly providing the 24,24-F2-1α,25$(OH)_2D_3$, members of the Center for Molecular Biology and Cytogenetics, SRL, for helpful discussion and comments, and Dr. Fumiko Kihara-Negishi, Sasaki Institute, for helpful discussion and advice.

References

1. Haussler MR, Vitamin D receptors: Nature and function. *Annu Rev Nutr* **6:** 527–562, 1986.
2. DeLuca HF, The vitamin D story: A collaborative effort of basic science and clinical medicine. *FASEB J* **2:** 224–236, 1988.
3. Bouillon R, Okamura WH and Norman AW, Structure–function relationships in the vitamin D endocrine system. *Endocr Rev* **16:** 200–257, 1995.
4. Ross PD and Knowlton W, Rapid bone loss is associated with increased levels of biochemical markers. *J Bone Miner Res* **13:** 297–302, 1988.
5. Nakayama H, Yano T, Sagara Y, Ando K, Kasai Y and Taketani Y, Clinical usefulness of urinary CrossLaps as a sensitive marker of bone metabolism. *Endocr J* **44:** 479–484, 1997.
6. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A and Karsenty G, Increased bone formation in osteocalcin-deficient mice. *Nature* **382:** 448–452, 1996.
7. Gundberg CM, Hauschka PV, Lian JB and Gallop PM, Osteocalcin: Isolation, characterization, and detection. *Methods Enzymol* **107:** 516–544, 1984.
8. Kerner SA, Scott RA and Pike JW, Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3. *Proc Natl Acad Sci USA* **86:** 4455–4459, 1989.
9. Majeska RJ, Rodan SB and Rodan GA, Maintenance of parathyroid hormone response in clonal rat osteosarcoma lines. *Exp Cell Res* **111:** 465–468, 1978.
10. Rodan GA and Rodan SB, Expression of the osteoblastic phenotype. *J Bone Miner Res* **2:** 244–285, 1984.
11. Rowe DW and Kream BE, Regulation of collagen synthesis in fetal rat calvaria by 1,25-dihydroxyvitamin D_3. *J Biol Chem* **257:** 8009–8015, 1982.
12. Genovese C, Rowe D and Kream B, Construction of DNA sequences complementary to rat α1 and α2 collagen mRNA and their use in studying the regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D. *Biochemistry* **23:** 6210–6215, 1984.
13. Kream BE, Rowe DW, Gworek SC and Raisz LG, Parathyroid hormone alters collagen synthesis and procollagen mRNA levels in fetal rat calvaria. *Proc Natl Acad Sci USA* **77:** 5654–5658, 1980.
14. Blochlinger K and Diggelmann H, Hygromycin B phosphotransferase as a selectable marker for DNA transfer experi-

ments with higher eukaryotic cells. *Mol Cell Biol* **4:** 2929–2931, 1984.
15. Gorman CM, Moffat LF and Howard BH, Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2:** 1044–1051, 1982.
16. Miller JH, Assay of β-galactosidase. In: *Experiments in Molecular Genetics* pp. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1972.
17. Sambrook J, Fritsch EF and Maniatis T, Assay for β-galactosidase in extracts of mammalian cells. In: *Molecular Cloning* (Eds. Sambrook J, Fritsch EF and Maniatis T) pp. 16.66–16.67. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
18. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
19. Kabakoff BD, Kendrik NC, Faber D, Deluca HF, Yamada S and Takayama H, Determination of biological activity of 24,24-difluoro-1,25-dihydroxyvitamin D_3 in the chick using a new method for assessing intestinal calcium uptake. *Arch Biochem Biophys* **215:** 582–588, 1982.
20. Okamoto S, Tanaka I, DeLuca HF, Kobayashi Y and Ikekawa N, Biological activity of 24,24-difluoro-1,25-dihydroxyvitamin D_3. *Am J Physiol* **244:** E159–163, 1983.
21. Rambeck WA, Weiser H and Zucker H, Biological activity of fluorine-substituted 1,25-dihydroxyvitamin D_3 in rats, in chicken and in Japanese quails. *Ann Nutr Metab* **30:** 9–14, 1986.
22. Morrison NA, Shine J, Fragonas JC, Verkest V, McMenemy ML and Eisman JA, 1,25-dihydroxyvitamin D-responsive element and glucocorticoid repression in the osteocalcin gene. *Science* **246:** 1158–1161, 1989.