dogenous steroids and dietary and xenobiotic compounds (29, 32).

During our original screening experiments that led to the discovery of SXR as a steroid and xenobiotic sensor, we noted that vitamin K_2 could also activate SXR.[2] This observation led us to consider the possibility that vitamin K_2 might act as a *bona fide* SXR ligand to mediate biological processes other than xenobiotic metabolism and clearance. Since vitamin K_2 was previously suspected to have a transcriptional regulatory function in addition to its role as an enzyme cofactor (37), we hypothesized that SXR might be the mediator of this activity. In this report, we demonstrate that vitamin K_2 transcriptionally activates SXR in a dose-dependent manner and binds directly to SXR *in vitro* and *in vivo*. SXR mRNA is expressed in osteosarcoma cell lines, and vitamin K_2 induced the expression of the prototypical SXR target gene *CYP3A4* in these cells. Vitamin K_2 up-regulates the steady state mRNA levels for a panel of osteoblastic bone markers in the osteosarcoma cell lines HOS, MG-63, and Saos-2, demonstrating a mechanistic connection between vitamin K_2 and bone development. The known SXR activators rifampicin and hyperforin induce the same panel of bone markers as does vitamin K_2, further confirming a role for SXR in the regulation of these genes. Finally we found that vitamin K_2 was able to induce bone markers in primary osteocytes isolated from wild-type murine calvaria but not in cells isolated from *PXR* knockout mice. From these data, we conclude that vitamin K_2 modulates the expression of osteoblastic bone markers through SXR and infer that vitamin K_2 activation of SXR could be an important factor favoring the deposition of bone over its resorption. Therefore, SXR is likely to be involved in the maintenance of bone homeostasis in addition to its known role in hormonal homeostasis. This reveals a novel biological function for SXR and suggests that a subset of SXR activators may function as effective therapeutic agents for the management of osteoporosis.

EXPERIMENTAL PROCEDURES

SXR Detection by RT-PCR—HOS, MG-63, Saos-2, LS180, and HeLa cells were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was isolated using TriZol reagent (Invitrogen). For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase according to the manufacturer-supplied protocol (Invitrogen). *SXR* was detected with the following primer set: forward primer, 5′-CAAGCGGAAGAAAAGTGAACG-3′; reverse primer, 5′-CTGGTCCTCGATGGGCAAGT-3′. PCR was carried out at 37 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s.

Cell Culture and Transfection—COS-7 cells were cultured and transfected as described previously (38). Typically COS-7 cells were cultured in phenol red-free DMEM supplemented with 10% FBS. For transient transfection experiments, COS-7 cells were seeded into 96-well plates at a density of 5000 cells/well. The next day cells were transfected with either CMX-GAL-SXR or CMX-GAL4 (control) together with tk(MH100)$_4$-luc reporter (39) and CMX-β-galactosidase transfection control plasmids using standard calcium phosphate precipitation methodology. 22–24 h after transfection, the cells were washed twice with phosphate-buffered saline supplemented with 1 mM $MgCl_2$ or DMEM-ITLB (DMEM containing 5 μg/ml insulin, 5 μg/ml holotransferrin, 5 μg/ml selenium, 0.5% defined lipid mix (Invitrogen), 0.12% (w/v) delipidated bovine serum albumin (Sigma)) (40). Ligands were typically purchased from Sigma and BIOMOL Research Laboratories Inc., made freshly from powder in Me_2SO as 0.1 M stocks, diluted in Me_2SO to appropriate concentrations, and added to media with vigorous vortex mixing. Ligands were added in DMEM-ITLB, and the cells were incubated for an additional 24–48 h. The cells were lysed *in situ*, and extracts were prepared and assayed for β-galactosidase and luciferase activity as described previously (41). Reporter gene activity was normalized to the β-galactosidase transfection controls, and the results were expressed as normalized relative luciferase units per OD β-galactosidase per minute to facilitate comparisons between plates. Fold induction was calculated relative to solvent controls. Each data point represents the average of triplicates ± S.E. The experiments were repeated three times with similar results.

For coactivator recruitment experiments, GAL4-coactivator plasmids were generated by cloning the receptor interaction domains of human *TIF2* (GenBank™ accession number NM_006540, amino acids 563–790), human *SRC-1* (GenBank™ accession number U59302, amino acids 600–800), or human *ACTR* (GenBank™ accession number AF036892, amino acids 600–788) into pCMX-GAL4. The GAL4-PBP construct was described previously (30). To construct herpesvirus VP16 activation domain fusion proteins, full-length *SXR* was PCR-amplified and ligated in-frame into pCDG-VP16 vector (25). All constructs were sequenced to verify that no errors were introduced during the PCR.

Ligand Binding Assays—N-terminal His_6-tagged human SXR ligand binding domain was expressed in *Escherichia coli* together with the SRC-1 receptor interaction domain essentially as described previously (28). Active protein was refolded from inclusion bodies solubilized in denaturation buffer (6 M guanidinium-HCl, 50 mM HEPES, pH 7.4, 0.2 M NaCl, 25 mM dithiothreitol, 1% (w/v) Triton X-100) by rapid 10-fold dilution into binding buffer (50 mM HEPES, pH 7.4, 1 M sucrose, 0.2 M NaCl, 0.1 mM dithiothreitol, 0.1% (w/v) CHAPS) followed by dialysis overnight at 4 °C against binding buffer. Binding assays were performed by coating 96-well nickel chelate FlashPlates (PerkinElmer Life Sciences) with a 10-fold molar excess of protein for 1 h at 22 °C in binding buffer (50 mM HEPES, pH 7.4, 200 mM NaCl, 1 M sucrose, 0.1% CHAPS). Unbound protein was removed from the wells by washing four times with binding buffer. [^{3}H]SR12813 (33) (Amersham Biosciences) was added to a final concentration of 50 nM in each well either alone or together with competitor ligands in binding buffer as indicated. Incubation was continued for 3 h at room temperature. Total counts were measured using a Topcount scintillation counter (Packard Instrument Co.). Counts remaining after the addition of 10 μM clotrimazole were taken as nonspecific background and subtracted from all wells (33). All assays were performed in triplicate and reproduced in independent experiments.

Alkaline Phosphatase (ALP) Activity Assay—ALP activity was measured as described previously (42). Briefly, cells were harvested by washing twice with phosphate-buffered saline, then collected with a cell scraper, and transferred to 1.5-ml microcentrifuge tubes. Cell pellets were obtained by centrifugation at 14,000 rpm at 4 °C, and lysates were prepared with a solution containing 0.2% (v/v) Nonidet P-40 and 1 mM $MgCl_2$. Aliquots of lysate were combined with reaction buffer (1 M diethanolamine, pH 9.8, 1 mM $MgCl_2$, and 10 mM *p*-nitrophenyl phosphate) and incubated at 37 °C for 30 min. Absorbance at 405 nm was measured using a Spectra MAX Plus spectrophotometer (Amersham Biosciences), and the enzyme activity was calculated as described previously (6). ALP activity was corrected for protein content, which was determined using the Bio-Rad protein assay kit.

Quantitative Real Time RT-PCR Analysis of Bone Biomarker Genes in Osteosarcoma Cell Lines—Human osteosarcoma cell lines HOS, MG-63, and Saos-2 were obtained from American Type Culture Collection (Manassas, VA) and cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Cells were treated with vitamin K_2, 1α,25-$(OH)_2$ vitamin D_3, rifampicin, or solvent controls for 48 h. Total RNA was isolated and reverse transcribed as described above. Quantitative real time RT-PCR (QRT-PCR) was performed using the following primer sets: ALP (F, 5′-CATGGCTTTGGGCAGAAGGA-3′; R, 5′-CTAGCCCCAAAAAGAGTTGCAA-3′), osteopontin (OPN) (F, 5′-CAGACGAGGACATCACCTCA-3′; R, 5′-TGGCTGTGGGTTTCAGCA-3′), matrix Gla protein (MGP) (F, 5′-ATCGCTACTTCAGGAAGCGCC-3′; R, 5′-TGACTCTCCTTTGACCCTGACCCTCAC-3′), osteoprotegerin (OPG) (F, 5′-CCTCTCATCAGCTGTTGTGTG-3′; R, 5′-TATCTCAAGGTAGCGCCCTTC-3′), glyceraldehyde-3-phosphate dehydrogenase (F, 5′-TGGACCTCATGGCCCACA-3′; R, 5′-TCAAGGGGTCTACATGGCAA-3′), CYP3A4 (F, 5′-GGCTTCATCCAATGGACTGCATAAAT-3′; R, 5′-TCCCAAGTATAACACTCTACACAGACAA-3′), and the SYBR green PCR kit (Applied Biosystems) in a DNA Engine Opticon-Continuous Fluorescence Detection system (MJ Research). All samples were quantitated by the comparative cycle threshold (Ct) method for relative quantitation of gene expression, normalized to glyceraldehyde-3-phosphate dehydrogenase (43).

Isolation of Calvaria, Culture of Primary Bone Cells, and QRT-PCR—Calvaria were isolated from newborn wild-type and *PXR* knockout mice (postnatal day 1–5) and were digested sequentially with 0.1% collagenase, 0.05% trypsin, 4 mM EDTA in 1× phosphate-buffered saline essentially as described in Refs. 44 and 45. Bone cells released upon digestion were cultured in phenol-red free DMEM, 10% FBS and

[2] B. Blumberg, unpublished observations.

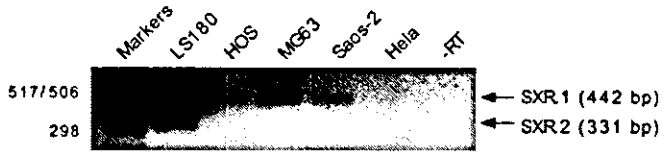

FIG. 1. ***SXR* is expressed in osteosarcoma cell lines.** The human cell lines HOS, MG-63, Saos-2, LS180, and HeLa were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was isolated using TriZol reagent. 1 μg of total RNA from each cell type was reverse transcribed using Superscript II reverse transcriptase and subjected to RT-PCR analysis. RT-PCR products were resolved on a 1% agarose gel. The *hSXR.1* product is 442 bp. The *hSXR.2* product is 331 bp.

treated for 48 h with 10 μM vitamin K_2 or pregnenolone 16α-carbonitrile (PCN). Total RNA was isolated, reverse transcribed, and analyzed using the following mouse primer sets: MGP (F, 5'-TCTCACGAAAGC-ATGGAGTC-3'; R, 5'-ATCTCGTAGGCAGGCTTGTT-3'), OPG (F, 5'-CTGCTGAAGCTGTGGAAACA-3'; R, 5'-AAGCTGCTCTGTGGTGAG-GT-3'), and glyceraldehyde-3-phosphate dehydrogenase (F, 5'-AACTT-TGGCATTGTGGAAGG-3'; R, 5'-GGATGCAGGGATGATGTTCT-3').

Statistical Analysis—Differences between two groups were analyzed using two-sample, two-tailed Student's *t* test. A *p* value less than 0.05 was considered to be significant. All data are presented in the text and figures as the mean ± S.E.

RESULTS

SXR Is Expressed in Osteosarcoma Cell Lines—SXR functions as a xenobiotic sensor and is expressed at high levels in the liver and intestine where it modulates the levels of CYP enzymes and ATP-binding cassette family transporters (31, 46). SXR is expressed at lower levels in normal and neoplastic breast tissues (47) and breast cancer cell lines (MCF-7, T47D, MDA-MB-231, and MDA-MB-435) (47).[3] It is not clear at present what role SXR is playing in other tissues. We were intrigued by the ability of vitamin K_2 to activate SXR in preliminary experiments. To ascertain whether SXR might be mediating the effects of vitamin K_2, we first determined whether SXR was expressed in a panel of osteosarcoma cell lines using RT-PCR. *SXR* expression was observed in the LS180 human colon adenocarcinoma cells and in the osteosarcoma cell lines HOS, MG-63, and Saos-2. SXR mRNA was not detected in HeLa cells or in negative controls (Fig. 1). It has been previously reported that *SXR* is expressed in LS180 cells (28, 30), whereas it is not expressed in HeLa cells (48). *SXR* is expressed at highest levels in LS180 cells with lower levels in the osteosarcoma cell lines (Fig. 1).

Vitamin K_2 Activates SXR—SXR is activated by a diverse array of pharmaceutical agents including Taxol, rifampicin, SR12813, clotrimazole, phenobarbital, and hyperforin. As noted above, our early screening efforts aimed at identifying SXR ligands also demonstrated SXR activation by vitamin K_2. Accordingly we tested the ability of vitamin K_2 to activate SXR in dose-response experiments. As shown in Fig. 2*A*, vitamin K_2 activates CMX-GAL-SXR robustly with the highest levels of activation approximately equivalent to 1 μM rifampicin (RIF). In contrast, no activation was observed using CMX-GAL4 alone, demonstrating that the activation results from a specific interaction with the SXR ligand binding domain.

Next we tested the ability of vitamin K_2 to induce the SXR target gene *CYP3A4* in cultured osteosarcoma cells. It has been reported previously that *CYP3A4* expression is induced by RIF and vitamin D_3 in cultured HepG2 and LS180 cells (49, 50), although the 1α,25-$(OH)_2$ vitamin D_3 induction of *CYP3A4* is mediated by the vitamin D_3 receptor rather than by SXR (51). RIF induced the expression of *CYP3A4* in all three lines (Fig. 2*B*). Vitamin K_2 was able to induce *CYP3A4* expression at both

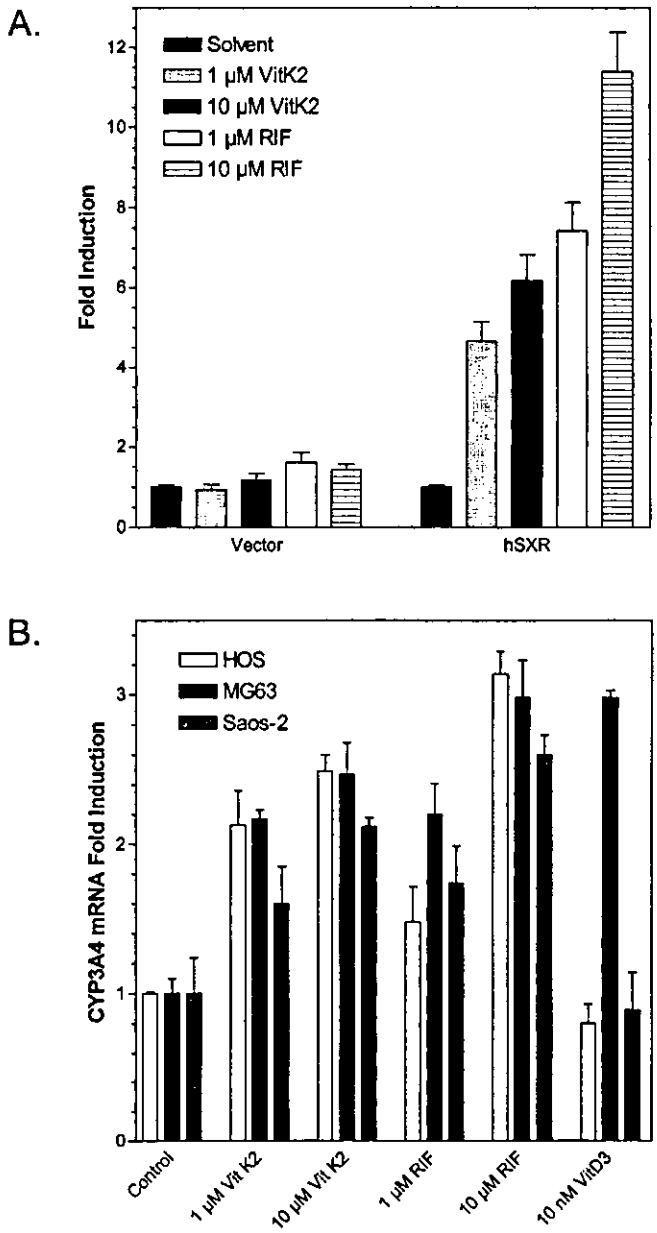

FIG. 2. **Vitamin K_2 activates SXR and induces SXR target genes.** *A*, activation of SXR by RIF and vitamin K_2. COS-7 cells were transiently transfected with either GAL-SXR or CMX-GAL4 (control) together with tk(MH100)4-luc reporter and CMX-β-galactosidase transfection control plasmids. After transfection, cells were treated with control medium or medium containing a dilution series of RIF, vitamin K_2, or 1α,25-$(OH)_2$ vitamin D_3. Only values for 1 and 10 μM rifampicin, 1 and 10 μM vitamin K_2, and 10 nM 1α,25-$(OH)_2$ D_3 are shown for clarity. *B*, *CYP3A4* induction by RIF and vitamin K_2. Human osteosarcoma cell lines HOS, MG-63, and Saos-2 were cultured in the presence or absence of ligands for 48 h. Total RNA was isolated and analyzed by QRT-PCR with primers for human *CYP3A4*. *Vit*, vitamin.

1 and 10 μM in all three lines. 1α,25-$(OH)_2$ vitamin D_3 could not induce *CYP3A4* expression in Saos-2 or HOS cells (Fig. 2*B*).

Vitamin K_2 Specifically Binds to SXR in Vitro and in Vivo—Since vitamin K_2 activates SXR in transient transfections (Fig. 2*A*) and induces the expression of a prototypical SXR target gene in osteosarcoma cells (Fig. 2*B*), we next sought to determine whether vitamin K_2 binds to SXR. One important measure of ligand binding is the ability of a compound to induce a nuclear receptor to interact with coactivator proteins. Accordingly we conducted coactivator recruitment experiments that utilized VP16-SXR together with fusions between the GAL4 DNA binding domain and the receptor-interacting domains of

[3] M. M. Tabb and B. Blumberg, unpublished data.

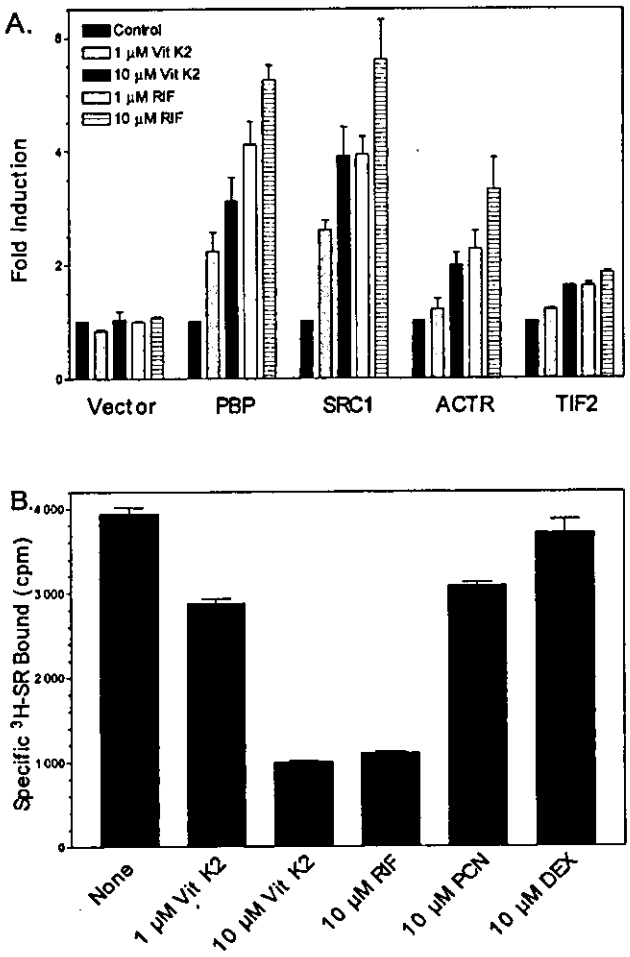

FIG. 3. **Vitamin K_2 specifically binds to SXR *in vitro* and *in vivo*.** *A*, vitamin K_2 induces SXR to recruit coactivators. COS-7 cell were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS and transiently transfected with a GAL reporter together with expression vectors for the GAL4 DNA binding domain (*Vector*) or the GAL4 DNA binding domain linked to the receptor interaction domains of the indicated nuclear receptor coactivators. Cells were treated with 1 and 10 μM RIF or vitamin K_2. Values represent the average of triplicates ± S.E. Experiments were repeated twice with similar results. *B*, vitamin K_2 specifically binds to the purified SXR ligand binding domain. His_6-SXR ligand binding domain was coexpressed with the SRC-1 receptor interaction domain and purified. The receptor complex was bound to nickel chelate FlashPlates and incubated with 50 nM [^{3}H]SR12813 (*^{3}H-SR*) in the presence of the indicated compounds or solvent control. Values represent the average of triplicates ±S.E. and were replicated in independent experiments. *Vit*, vitamin; *DEX*, dexamethasone.

the nuclear hormone receptor coactivators *SRC-1*, *TIF2*, *ACTR*, and *PBP* (30). As shown in Fig. 3*A*, VP16-SXR was able to interact with PBP, SRC-1, and ACTR in the presence of vitamin K_2 or the known SXR ligand RIF. The results from the coactivator recruitment experiments paralleled those of the activation assays. As is the case for other SXR ligands (30), vitamin K_2 and RIF preferentially induced interaction between SXR and PBP or SRC-1 while producing weaker but detectable interactions between SXR and TIF2 or ACTR (Fig. 3*A*). We infer that vitamin K_2 promotes association between SXR and nuclear receptor coactivators SRC-1 and PBP as would be expected from an authentic SXR ligand.

We next tested whether vitamin K_2 binds to purified SXR protein *in vitro* using a sensitive scintillation proximity ligand binding assay similar to that used by other investigators (28, 33). This assay used [^{3}H]SR12813 and recombinant His_6-tagged SXR coexpressed with the SRC-1 receptor-interacting domain (28) and nickel chelate FlashPlates (PerkinElmer Life Sciences). SR12813 interacts specifically with SXR with a dissociation constant of 40 nM (33). As seen in Fig. 3*B*, vitamin K_2 and RIF are able to displace [^{3}H]SR12813 from the SXR ligand binding domain, whereas the control compounds PCN and dexamethasone did not compete effectively for receptor binding. The K_i for vitamin K_2 binding to SXR was determined to be 5.1 μM, a value in the range of other known SXR ligands (28, 34). We infer from these results that vitamin K_2 specifically binds to SXR *in vitro* and *in vivo* and conclude that it acts as a *bona fide* ligand for this receptor.

Effects of Vitamin K_2, Rifampicin, and Hyperforin on Bone Biomarker Genes in Osteosarcoma Cell Lines—Vitamin K_2 is used as a therapeutic agent to treat osteoporosis in Japan and is thought to act by stimulating the deposition of bone (6, 7) and decreasing bone resorption (8–13), although the mechanism of action remained unclear before our experiments. We tested the effect of vitamin K_2 and 1α,25-$(OH)_2$ vitamin D_3, two known therapeutic agents, on the expression of a panel of osteoblast marker genes in the human osteosarcoma cell lines HOS, MG-63, and Saos-2 and compared these with the effects of the SXR activators RIF and hyperforin in the same cells. We chose 1α,25-$(OH)_2$ vitamin D_3 as a positive control compound in this study because it is also used therapeutically to treat osteoporosis and is known to transcriptionally regulate a range of biological processes, including bone growth, bone remodeling, and the expression of osteoblastic markers such as ALP (52–54), osteocalcin (55), and OPN (56, 57).

ALP is a widely distributed glycosylated membrane-bound ectoenzyme. Bone ALP is located on the surface of osteoblasts and is thought to play a major role in bone formation and mineralization (58, 59). ALP levels are considered to reflect osteoblastic activity and can therefore be used as a biochemical marker for assessing metabolic bone disease, including bone metastasis (60). As seen in Fig. 4*A*, 1α,25-$(OH)_2$ vitamin D_3 treatment increased ALP activity 1.7-fold in HOS and Saos-2 cells and 4.0-fold in MG-63 cells. Vitamin K_2 treatment led to a dose-dependent 1.4–2.0-fold increase in ALP levels in these cells, and RIF completely paralleled this effect (Fig. 4*A*). As will be seen with other markers below, there are notable differences in the response of particular cell lines to treatment with vitamin K_2 or 1α,25-$(OH)_2$ vitamin D_3. For example, 1α,25-$(OH)_2$ vitamin D_3 is particularly effective at inducing ALP enzyme activity in MG-63 cells (Fig. 4*A*). The effect of vitamin K_2 treatment on ALP enzyme in HOS cells is in agreement with other reports (6).

Consistent with the effects on ALP enzyme, QRT-PCR analysis showed that ALP mRNA expression levels were significantly increased by vitamin K_2 and 1α,25-$(OH)_2$ vitamin D_3 in a dose-dependent manner in all three cell lines with 1α,25-$(OH)_2$ vitamin D_3 being more potent (Fig. 4*B*). Statistically significant changes in ALP enzyme and mRNA levels were seen at vitamin K_2 concentrations of as little as 1 μM (Fig. 4, *A* and *B*), which is similar to therapeutic levels at which vitamin K_2 is used clinically. RIF and hyperforin produced effects very similar to vitamin K_2 (Fig. 4*B*). The concordance between ALP protein levels (Fig. 4*A*) and ALP mRNA levels (Fig. 4*B*) suggests that QRT-PCR analysis of marker gene expression will be predictive of osteoblastic activity in cultured osteosarcoma cell lines.

OPN is one of the major non-collagenous bone matrix proteins produced by osteoblasts (61, 62). It is an early marker of osteoblast differentiation and a prominent component of the mineralized bone matrix that has been implicated in tissue mineralization and in the attachment of osteoclasts to the bone matrix. Vitamin K_2 elicited a dose-dependent increase in OPN

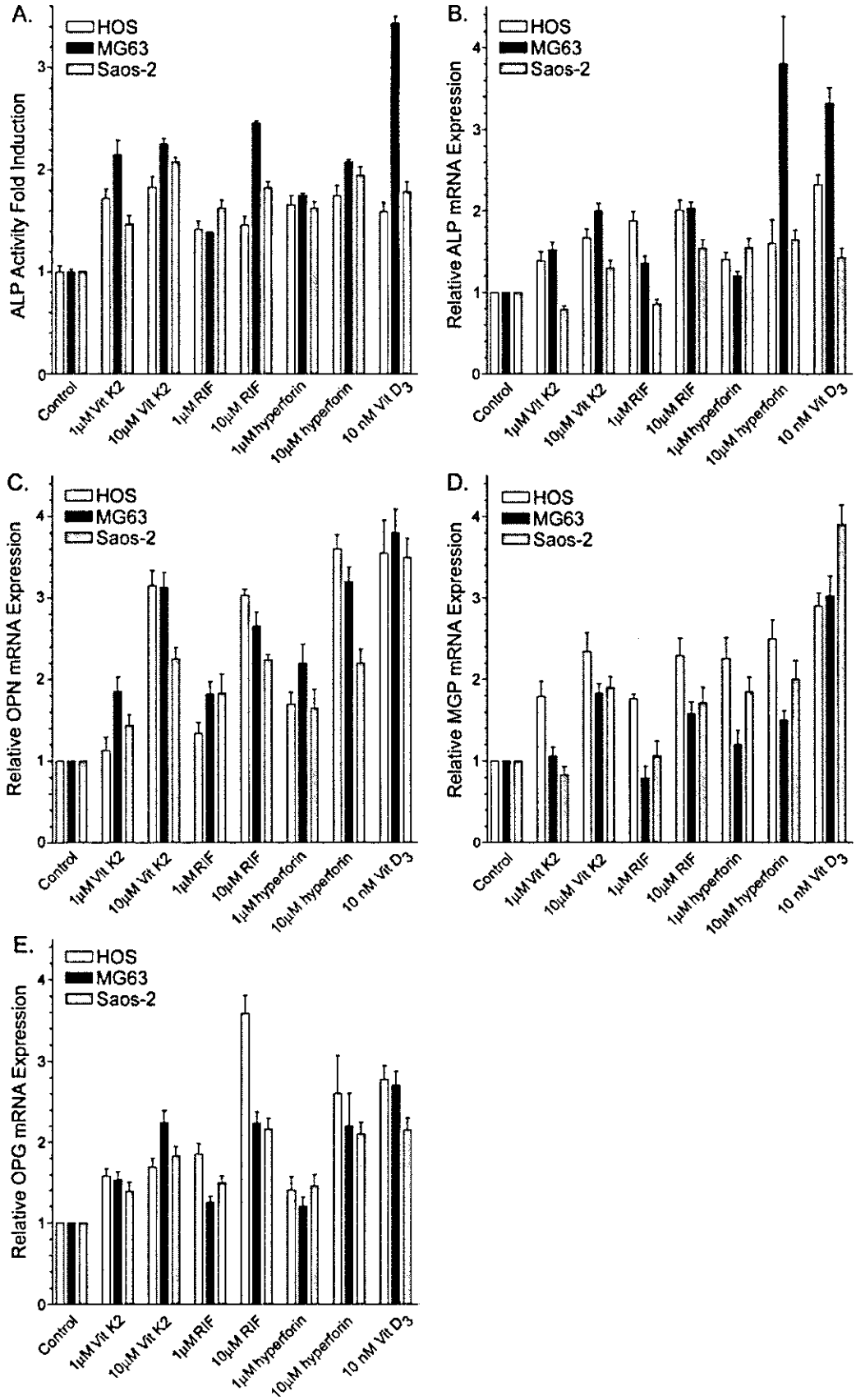

FIG. 4. **Effects of vitamin K_2 and rifampicin on osteoblastic marker genes in osteosarcoma cell lines.** Human osteosarcoma cell lines HOS, MG-63, and Saos-2 were cultured in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS in the presence or absence of ligands for 48 h. ALP activity and bone marker gene expression were determined by ALP activity assay and quantitative real time RT-PCR. Data from quantitative real time RT-PCR are shown as the mRNA expression levels of marker genes normalized to glyceraldehyde-3-phosphate dehydrogenase. *A*, ALP activity assay. *B*, ALP mRNA expression. *C*, OPN mRNA expression. *D*, MGP mRNA expression. *E*, OPG mRNA expression. Data from HOS (*white bars*), MG-63 (*black bars*), and Saos-2 cells (*gray bars*) is shown. Values represent the average of triplicates ± S.E. The experiments were repeated twice with similar results. *Vit*, vitamin.

expression with the 10 μM dose approaching the response observed with 1α,25-$(OH)_2$ vitamin D_3 (Fig. 4*C*). RIF and hyperforin again elicited nearly identical results to those of vitamin K_2 treatment (Fig. 4*C*).

MGP is a vitamin K-dependent extracellular matrix protein with a wide tissue distribution that is particularly abundant in bone and cartilage. MGP is thought to play a key role in the inhibition of tissue calcification (63, 64). 1α,25-$(OH)_2$ vitamin D_3 treatment was previously reported to increase both the level of MGP mRNA expression and the rate of MGP secretion into culture medium in osteosarcoma cells (65). We found that MGP mRNA levels were up-regulated by vitamin K_2, RIF, hyperforin, and 1α,25-$(OH)_2$ vitamin D_3 treatment (Fig. 4*D*). Unlike the other markers studied, MGP mRNA levels were only stimulated by the highest doses of vitamin K_2 and RIF used (10 μM) in MG-63 and Saos-2 cells.

OPG (also known as osteoclastogenesis-inhibitory factor) is a member of the tumor necrosis factor receptor family (66, 67). It is thought to function as a soluble decoy receptor activator of nuclear factor-κB ligand (RANKL). Recombinant OPG has been shown to block osteoclastogenesis *in vitro* and to increase bone density *in vivo* (68). 1α,25-$(OH)_2$ vitamin D_3 (10^{-7} M) increased OPG mRNA levels by 90 and 50% in a fetal osteoblastic cell line (hFOB) and normal trabecular osteoblastic cells (hOB), respectively (69). OPG mRNA levels were up-regulated by both low and high doses of vitamin K_2 as well as by 1α,25-$(OH)_2$ vitamin D_3 and hyperforin (Fig. 4*E*). Rifampicin was an effective inducer of OPG mRNA in all three cell lines, particularly HOS cells (Fig. 4*E*), confirming and extending the published results. In summary, three structurally different human SXR activators, RIF, hyperforin, and vitamin K_2, induce osteoblast bone markers in three different osteosarcoma cell lines supporting a role for SXR in regulation of some osteoblastic bone markers.

We next asked whether the effects of vitamin K_2 or RIF on the expression of the bone markers above were direct transcriptional effects. We first tested induction of the known SXR target gene *CYP3A4* with 10 μM rifampicin at time points from 6 to 48 h. There was very little induction of *CYP3A4* detected before 24 h after treatment in the positive control LS180 cells or in any of the osteosarcoma cells (Fig. 5). Therefore, the osteosarcoma cell lines were treated with vitamin K_2, RIF, or vitamin D_3 in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) for 24 h, and RNA was analyzed by QRT-PCR using primers for *OPN*, *MGP*, and *CYP3A4*. As shown in Fig. 6, the expression of both *MGP* and *CYP3A4* induced by vitamin K_2 and RIF was not decreased by the presence of CHX in either the MG-63 or Saos-2 cell lines. CHX has been demonstrated to increase the stability of a number of mRNAs, explaining the increased expression of *MGP* and *CYP3A4* seen in the presence of CHX. In contrast, the induction of OPN expression by vitamin K_2 and RIF was eliminated in the presence of CHX. No induction of osteoblast marker genes was seen at 6 or 12 h of treatment (data not shown), and no induction of ALP or OPG could be detected even after 24 h of treatment. These results indicate that *CYP3A4* and *MGP* are direct transcriptional targets of activated SXR but that *OPN* requires ongoing protein synthesis for induction by vitamin K_2 or RIF.

Vitamin K_2 Induction of Bone Marker Genes Requires SXR—The data presented above provide strong evidence that the stimulation of osteoblast bone markers by vitamin K_2 occurs via direct transcriptional activation of SXR. To confirm that these effects were indeed mediated by SXR, we utilized primary bone cell cultures derived from collagenase/trypsin-digested calvaria isolated from wild-type (WT) and *PXR* knockout

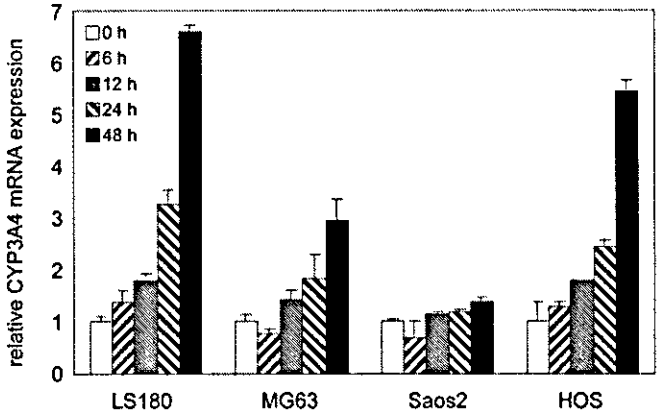

FIG. 5. **Time course of rifampicin induction of *CYP3A4*.** LS180 colon carcinoma cells or MG-63, Saos-2, or HOS osteosarcoma cells were treated for the indicated times with 10 μM rifampicin in phenol red-free DMEM supplemented with 10% resin charcoal-stripped FBS. Total RNA was reverse transcribed and analyzed by QRT-PCR using primers for human *CYP3A4*. Values represent the average of triplicates ± S.E.

mice. Previous studies have shown that the cells isolated from calvaria by this method are primarily osteoblasts (45). *PXR* is the rodent homolog of human SXR, and the *PXR* knockout mouse has been used to demonstrate the key role SXR/PXR plays in the control of xenobiotic metabolism (29, 32). WT or *PXR* knockout cells were treated for 48 h with vitamin K_2, the rodent PXR activator PCN, or vehicle only. Total RNA was then isolated and analyzed using QRT-PCR with primers specific for mouse MGP and OPG. As shown in Fig. 7, vitamin K_2 and PCN were able to induce MGP and OPG expression in WT cells; however, the ability to induce either MGP or OPG was lost in the *PXR* knockout cells. Therefore, vitamin K_2 induction of osteoblast marker genes requires the expression of PXR/SXR in bone cells.

DISCUSSION

The regulation of bone mass in mammals is a complex process that requires a balance between the bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) (70). Vitamin K_2 plays several important roles in bone metabolism. Similar to its role in promoting blood clotting, vitamin K_2 is an essential cofactor for γ-carboxylase, an enzyme that catalyzes the conversion of specific glutamic acid residues to Gla residues. Vitamin K_2 is required for γ-carboxylation of bone matrix Gla-containing proteins such as MGP and osteocalcin. Incomplete γ-carboxylation of osteocalcin and MGP resulting from vitamin K deficiency is associated with osteoporosis and increased risk of fracture (15, 16). In addition to its function as an enzymatic cofactor, vitamin K_2 has a dual role in mediating bone homeostasis. It acts in an anabolic manner to stimulate the synthesis of osteoblastic markers and deposition of bone (6, 7). Vitamin K_2 decreases bone resorption by inhibiting the formation of osteoclasts (10) as well as their bone resorptive activity (11, 12). Vitamin K_2 treatment also induces apoptosis of osteoclasts (13) while inhibiting apoptosis of osteoblasts (71) thereby shifting the balance toward bone formation. Vitamin K_2 was also shown to enhance the induction of osteocalcin mRNA levels mediated by co-administered 1α,25-$(OH)_2$ vitamin D_3 (7, 37). The function of vitamin K_2 was previously only well understood as an enzymatic cofactor.

SXR is expressed at high levels in the liver and intestine where it acts as a xenobiotic sensor that regulates the expression of cytochrome P-450 enzymes such as CYP3A4 and CYP2C8 and ATP-binding cassette family transporters such as MDR1 and MRP2 (28, 30). SXR is thus a master regulator of xenobiotic clearance, coordinately controlling steroid and xeno-

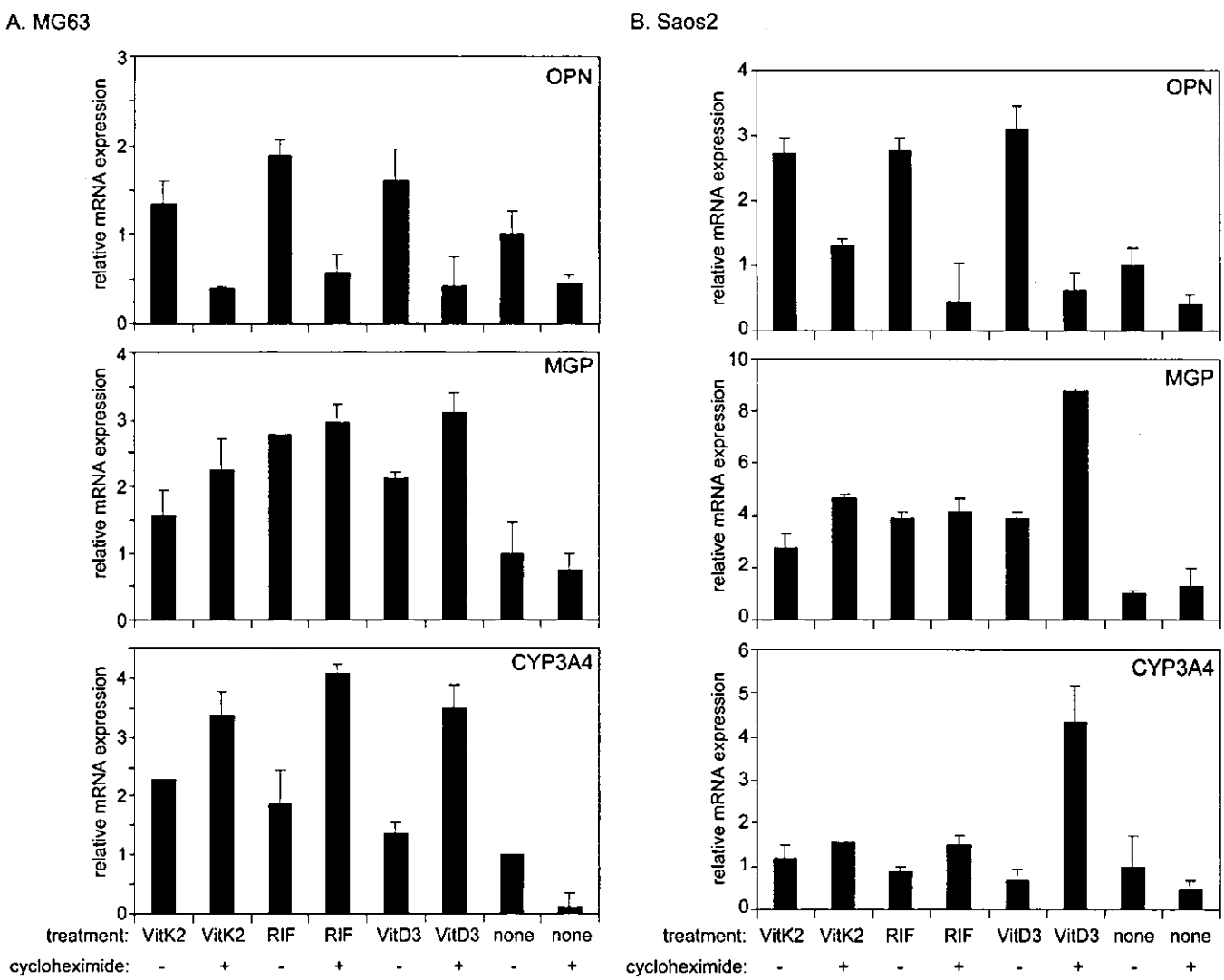

FIG. 6. **Vitamin K_2 has direct transcriptional effects on MGP and *CYP3A4* but not OPN.** Either MG-63 (*A*) or Saos-2 cells (*B*) were pretreated with 10 μg/ml cycloheximide for 30 min prior to addition of 10 μM vitamin K_2, 10 μM rifampicin, 10 nM vitamin D_3, or vehicle only. Total RNA was harvested 24 h later, reverse transcribed, and analyzed by QRT-PCR using primers for OPN, MGP, and *CYP3A4*. Values represent the average of triplicates ± S.E. *Vit*, vitamin.

biotic metabolism (31, 46). *SXR* is also expressed at lower levels in other tissues such as normal and neoplastic breast tissue (47), although no function has yet been described for SXR in these tissues. There are no data available about the expression of *SXR* in normal human bone; however, its mouse ortholog *PXR* is abundantly expressed in bone as measured by *in situ* hybridization (26). Our observation that vitamin K_2 activated SXR-dependent reporter gene constructs led us to hypothesize that vitamin K_2 could regulate the transcription of bone markers through activation of SXR.

In support of this hypothesis, we demonstrated that *SXR* is expressed in three well studied osteosarcoma cell lines, HOS, MG-63, and Saos-2 (Fig. 1). These cell lines are considered to be osteoblastic in nature and are commonly used as models for osteoblast formation (6, 72, 73). Vitamin K_2 activated SXR in a dose-dependent manner (Fig. 2*A*) and up-regulated the known SXR target gene *CYP3A4* (Fig. 2*B*) as well as the osteoblast marker genes ALP, OPN, MGP, and OPG (Fig. 4). Vitamin K_2 stimulated the recruitment of coactivator proteins by SXR and bound directly to the SXR ligand binding domain *in vitro*, identifying it as an SXR ligand (Fig. 3). Although micromolar levels of vitamin K_2 are required for SXR binding (Fig. 3) and activation (Fig. 2), it should be noted that the therapeutic levels of vitamin K_2 in the plasma of patients average about 1 μM (74), and the levels in bone (2.5-fold) and liver (10-fold) are considerably higher (75). Therefore, the concentration of vitamin K_2 required to activate SXR is achieved in clinical use.

Cultured cells derived from sequential collagenase-digested calvaria are routinely used to study bone physiology. Utilizing primary osteocytes isolated from the calvaria of *PXR* knockout mice, we were able to demonstrate a requirement for *PXR* in the induction of MGP and OPG by vitamin K_2. MGP and OPG were induced by vitamin K_2 in the WT cells but not in the PXR knockout cells. The basal levels of both MGP and OPG expression were increased in the *PXR* knockout cells (4- and 3-fold higher expression compared with WT, respectively; data not shown). A similar increase in basal expression was previously noted for *CYP3A11* in the *PXR* knockout mouse (29), further supporting a role for SXR/PXR in the regulation of these bone marker genes.

As described above, prior to this study a growing body of evidence existed linking vitamin K_2 to positive effects on the formation of bone and protection against fractures. Although its role as an enzymatic cofactor that favors the formation of Gla residues is undoubtedly critical for bone development, the anabolic effects of vitamin K_2 on osteoblasts, vitamin K_2-mediated inhibition of osteoclast function, induction of osteoclast apoptosis, and inhibition of osteoblast apoptosis suggest a

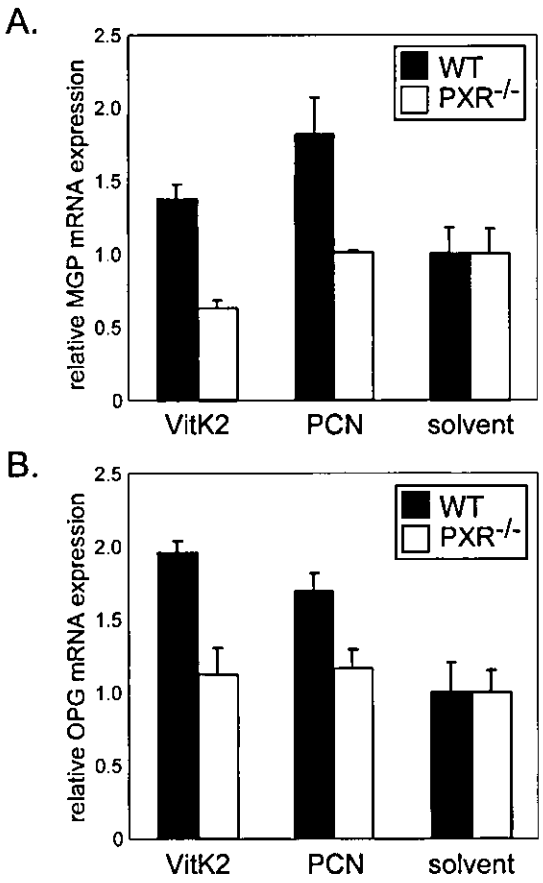

FIG. 7. **Vitamin K_2 induction of bone marker genes in murine primary osteocytes requires PXR.** Osteocyte cultures derived from calvaria isolated from WT (*black bars*) or PXR knockout mice (*white bars*) were treated with 10 μM vitamin K_2 or PCN or vehicle only for 48 h. Total RNA was isolated, reverse transcribed, and analyzed by QRT-PCR using primers specific for mouse MGP or OPG. Values represent the average of triplicates ± S.E. *Vit*, vitamin.

much broader role for vitamin K_2. Aside from its reported ability to increase the production of bone-specific ALP (6), there were previously no data that indicated a direct effect of vitamin K_2 on bone marker genes. Therefore, our demonstration that vitamin K_2 up-regulates the steady state mRNA levels for a broad panel of osteoblastic markers including ALP, MGP, OPN, and OPG (Fig. 4) provides a mechanistic basis for the anabolic effects of vitamin K_2 on osteoblasts and underscores the clinical utility of vitamin K_2 in the treatment of osteoporosis. The SXR activators RIF and hyperforin produce effects on the expression of bone markers very similar to those elicited by vitamin K_2 (Fig. 4). Therefore, we conclude that vitamin K_2 exerts positive effects on the expression of osteoblastic marker genes via transcriptional activation of SXR. The loss of vitamin K_2 inducibility of osteoblast markers in primary bone cell cultures from *PXR* knockout mice confirms that SXR/PXR plays a central role in vitamin K_2 induction of osteoblast marker genes. These results widen the pharmacological implications of SXR action beyond drug interactions and the xenobiotic response and may provide new insight into bone formation and the treatment of osteoporosis.

Acknowledgments—We thank Dr. C. Zilinski and members of the Blumberg laboratory for comments on the manuscript, Hiroshi Umesaka (Eisai Co., Ltd.) for gifts of vitamin K_2 and related compounds, and Dr. Ronald M. Evans at the Salk Institute in whose laboratory this work was initiated.

REFERENCES

1. Orwoll, E. S., Bauer, D. C., Vogt, T. M., and Fox, K. M. (1996) *Ann. Intern. Med.* **124,** 187–196
2. Seeman, E., Tsalamandris, C., Formica, C., Hopper, J. L., and McKay, J. (1994) *J. Bone Miner. Res.* **9,** 739–743
3. Torgerson, D. J., Campbell, M. K., Thomas, R. E., and Reid, D. M. (1996) *J. Bone Miner. Res.* **11,** 293–297
4. Klibanski, A., Adams-Campbell, L., Brassford, T., Blair, S. N., Boden, S. D., Dickersin, K., Gifford, D. R., Glasse, L., Goldring, S. R., Hruska, K., Johnson, S. R., McCauley, L. K., and Russell, W. E. (2000) in *NIH Consensus Statement*, Vol. 17, pp. 1–45, National Institutes of Health, Office of the Director, Bethesda, MD
5. Bouckaert, J. T., and Said, A. T. (1960) *Nature* **185,** 849
6. Akedo, Y., Hosoi, T., Inoue, S., Ikegami, A., Mizuno, Y., Kaneki, M., Nakamura, T., Ouchi, Y., and Orimo, H. (1992) *Biochem. Biophys. Res. Commun.* **187,** 814–820
7. Koshihara, Y., Hoshi, K., Ishibashi, H., and Shiraki, M. (1996) *Calcif. Tissue Int.* **59,** 466–473
8. Hall, J. G., Pauli, R. M., and Wilson, K. M. (1980) *Am. J. Med.* **68,** 122–140
9. Hart, J. P., Shearer, M. J., Klenerman, L., Catterall, A., Reeve, J., Sambrook, P. N., Dodds, R. A., Bitensky, L., and Chayen, J. (1985) *J. Clin. Endocrinol. Metab.* **60,** 1268–1269
10. Akiyama, Y., Hara, K., Tajima, T., Murota, S., and Morita, I. (1994) *Eur. J. Pharmacol.* **263,** 181–185
11. Hara, K., Akiyama, Y., Nakamura, T., Murota, S., and Morita, I. (1995) *Bone* **16,** 179–184
12. Hara, K., Akiyama, Y., Tajima, T., and Shiraki, M. (1993) *J. Bone Miner. Res.* **8,** 535–542
13. Kameda, T., Miyazawa, K., Mori, Y., Yuasa, T., Shiokawa, M., Nakamaru, Y., Mano, H., Hakeda, Y., Kameda, A., and Kumegawa, M. (1996) *Biochem. Biophys. Res. Commun.* **220,** 515–519
14. Weber, P. (1997) *Int. J. Vitam. Nutr. Res.* **67,** 350–356
15. Feskanich, D., Weber, P., Willett, W. C., Rockett, H., Booth, S. L., and Colditz, G. A. (1999) *Am. J. Clin. Nutr.* **69,** 74–79
16. Booth, S. L., Tucker, K. L., Chen, H., Hannan, M. T., Gagnon, D. R., Cupples, L. A., Wilson, P. W., Ordovas, J., Schaefer, E. J., Dawson-Hughes, B., and Kiel, D. P. (2000) *Am. J. Clin. Nutr.* **71,** 1201–1208
17. Orimo, H., Shiraki, M., Fujita, T., Inoue, T., and Kushida, K. (1992) *J. Bone Miner. Res.* **7,** S122–S128
18. Iwamoto, I., Kosha, S., Noguchi, S., Murakami, M., Fujino, T., Douchi, T., and Nagata, Y. (1999) *Maturitas* **31,** 161–164
19. Shiraki, M., Shiraki, Y., Aoki, C., and Miura, M. (2000) *J. Bone Miner. Res.* **15,** 515–521
20. Kaneki, M., Hedges, S. J., Hosoi, T., Fujiwara, S., Lyons, A., Crean, S. J., Ishida, N., Nakagawa, M., Takechi, M., Sano, Y., Mizuno, Y., Hoshino, S., Miyao, M., Inoue, S., Horiki, K., Shiraki, M., Ouchi, Y., and Orimo, H. (2001) *Nutrition* **17,** 315–321
21. Shearer, M. J. (1995) *Lancet* **345,** 229–234
22. Suttie, J. W. (1985) *Annu. Rev. Biochem.* **54,** 459–477
23. Binkley, N. C., and Suttie, J. W. (1995) *J. Nutr.* **125,** 1812–1821
24. Vermeer, C., Jie, K. S., and Knapen, M. H. (1995) *Annu. Rev. Nutr.* **15,** 1–22
25. Blumberg, B., Kang, H., Bolado, J., Jr., Chen, H., Craig, A. G., Moreno, T. A., Umesono, K., Perlmann, T., De Robertis, E. M., and Evans, R. M. (1998) *Genes Dev.* **12,** 1269–1277
26. Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Jones, M. A., McKee, D. D., Oliver, B. M., Willson, T. M., Zetterstrom, R. H., Perlmann, T., and Lehmann, J. (1998) *Cell* **92,** 73–82
27. Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., Ohlsson, R., Postlind, H., Blomquist, P., and Berkenstam, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12208–12213
28. Dussault, I., Lin, M., Hollister, K., Wang, E. H., Synold, T. W., and Forman, B. M. (2001) *J. Biol. Chem.* **276,** 33309–33312
29. Staudinger, J. L., Goodwin, B., Jones, S. A., Hawkins-Brown, D., MacKenzie, K. I., LaTour, A., Liu, Y., Klaassen, C. D., Brown, K. K., Reinhard, J., Willson, T. M., Koller, B. H., and Kliewer, S. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3369–3374
30. Synold, T. W., Dussault, I., and Forman, B. M. (2001) *Nat. Med.* **7,** 584–590
31. Willson, T. M., and Kliewer, S. A. (2002) *Nat. Rev. Drug Discov.* **1,** 259–266
32. Xie, W., Barwick, J. L., Downes, M., Blumberg, B., Simon, C. M., Nelson, M. C., Neuschwander-Tetri, B. A., Brunt, E. M., Guzelian, P. S., and Evans, R. M. (2000) *Nature* **406,** 435–439
33. Jones, S. A., Moore, L. B., Shenk, J. L., Wisely, G. B., Hamilton, G. A., McKee, D. D., Tomkinson, N. C., LeCluyse, E. L., Lambert, M. H., Willson, T. M., Kliewer, S. A., and Moore, J. T. (2000) *Mol. Endocrinol.* **14,** 27–39
34. Moore, L. B., Parks, D. J., Jones, S. A., Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Willson, T. M., Collins, J. L., and Kliewer, S. A. (2000) *J. Biol. Chem.* **275,** 15122–15127
35. Wentworth, J. M., Agostini, M., Love, J., Schwabe, J. W., and Chatterjee, V. K. (2000) *J. Endocrinol.* **166,** R11–R16
36. Moore, L. B., Goodwin, B., Jones, S. A., Wisely, G. B., Serabjit-Singh, C. J., Willson, T. M., Collins, J. L., and Kliewer, S. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7500–7502
37. Koshihara, Y., and Hoshi, K. (1997) *J. Bone Miner. Res.* **12,** 431–438
38. Grun, F., Venkatesan, R. N., Tabb, M. M., Zhou, C., Cao, J., Hemmati, D., and Blumberg, B. (2002) *J. Biol. Chem.* **277,** 43691–43697
39. Forman, B. M., Umesono, K., Chen, J., and Evans, R. M. (1995) *Cell* **81,** 541–550
40. Buck, J., Grun, F., Derguini, F., Chen, Y., Kimura, S., Noy, N., and Hammerling, U. (1993) *J. Exp. Med.* **178,** 675–680
41. Blumberg, B., Sabbagh, W., Juguilon, H., Bolado, J., Jr., Ong, E. S., and Evans, R. M. (1998) *Genes Dev.* **12,** 3195–3205
42. Anonymous (1974) *Scand. J. Clin. Lab. Investig.* **33,** 291–306
43. Livak, K. J., and Schmittgen, T. D. (2001) *Methods* **25,** 402–408
44. Wong, G. L., and Cohn, D. V. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 3167–3171
45. Partridge, N. C., Alcorn, D., Michelangeli, V. P., Kemp, B. E., Ryan, G. B., and

Martin, T. J. (1981) *Endocrinology* **108,** 213–219
46. Xie, W., and Evans, R. M. (2001) *J. Biol. Chem.* **276,** 37739–37742
47. Dotzlaw, H., Leygue, E., Watson, P., and Murphy, L. C. (1999) *Clin. Cancer Res.* **5,** 2103–2107
48. Masuyama, H., Inoshita, H., Hiramatsu, Y., and Kudo, T. (2002) *Endocrinology* **143,** 55–61
49. Schmiedlin-Ren, P., Thummel, K. E., Fisher, J. M., Paine, M. F., and Watkins, P. B. (2001) *Drug Metab. Dispos.* **29,** 1446–1453
50. Sumida, A., Fukuen, S., Yamamoto, I., Matsuda, H., Naohara, M., and Azuma, J. (2000) *Biochem. Biophys. Res. Commun.* **267,** 756–760
51. Makishima, M., Lu, T. T., Xie, W., Whitfield, G. K., Domoto, H., Evans, R. M., Haussler, M. R., and Mangelsdorf, D. J. (2002) *Science* **296,** 1313–1316
52. Majeska, R. J., and Rodan, G. A. (1982) *J. Biol. Chem.* **257,** 3362–3365
53. Manolagas, S. C., Burton, D. W., and Deftos, L. J. (1981) *J. Biol. Chem.* **256,** 7115–7117
54. Mulkins, M. A., Manolagas, S. C., Deftos, L. J., and Sussman, H. H. (1983) *J. Biol. Chem.* **258,** 6219–6225
55. Kerner, S. A., Scott, R. A., and Pike, J. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 4455–4459
56. Noda, M., Vogel, R. L., Craig, A. M., Prahl, J., DeLuca, H. F., and Denhardt, D. T. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9995–9999
57. Prince, C. W., and Butler, W. T. (1987) *Collagen Relat. Res.* **7,** 305–313
58. Sugawara, Y., Suzuki, K., Koshikawa, M., Ando, M., and Iida, J. (2002) *Jpn. J. Pharmacol.* **88,** 262–269
59. Zernik, J., Twarog, K., and Upholt, W. B. (1990) *Differentiation* **44,** 207–215
60. Tamada, T., Sone, T., Tomomitsu, T., Jo, Y., Tanaka, H., and Fukunaga, M. (2001) *J. Bone Miner. Metab.* **19,** 45–51
61. Reinholt, F. P., Hultenby, K., Oldberg, A., and Heinegard, D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4473–4475
62. Young, M. F., Kerr, J. M., Termine, J. D., Wewer, U. M., Wang, M. G., McBride, O. W., and Fisher, L. W. (1990) *Genomics* **7,** 491–502
63. Luo, G., Ducy, P., McKee, M. D., Pinero, G. J., Loyer, E., Behringer, R. R., and Karsenty, G. (1997) *Nature* **386,** 78–81
64. Price, P. A., Faus, S. A., and Williamson, M. K. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 1400–1407
65. Fraser, J. D., and Price, P. A. (1990) *Calcif. Tissue Int.* **46,** 270–279
66. Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., Nguyen, H. Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H. L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T. M., Hill, D., Pattison, W., Campbell, P., Boyle, W. J., *et al.* (1997) *Cell* **89,** 309–319
67. Tsuda, E., Goto, M., Mochizuki, S., Yano, K., Kobayashi, F., Morinaga, T., and Higashio, K. (1997) *Biochem. Biophys. Res. Commun.* **234,** 137–142
68. Yasuda, H., Shima, N., Nakagawa, N., Mochizuki, S. I., Yano, K., Fujise, N., Sato, Y., Goto, M., Yamaguchi, K., Kuriyama, M., Kanno, T., Murakami, A., Tsuda, E., Morinaga, T., and Higashio, K. (1998) *Endocrinology* **139,** 1329–1337
69. Hofbauer, L. C., Dunstan, C. R., Spelsberg, T. C., Riggs, B. L., and Khosla, S. (1998) *Biochem. Biophys. Res. Commun.* **250,** 776–781
70. Rodan, G. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13361–13362
71. Urayama, S., Kawakami, A., Nakashima, T., Tsuboi, M., Yamasaki, S., Hida, A., Ichinose, Y., Nakamura, H., Ejima, E., Aoyagi, T., Nakamura, T., Migita, K., Kawabe, Y., and Eguchi, K. (2000) *J. Lab. Clin. Med.* **136,** 181–193
72. Fedde, K. N. (1992) *Bone Miner.* **17,** 145–151
73. Rodan, S. B., Imai, Y., Thiede, M. A., Wesolowski, G., Thompson, D., Bar-Shavit, Z., Shull, S., Mann, K., and Rodan, G. A. (1987) *Cancer Res.* **47,** 4961–4966
74. Uematsu, T., Nagashima, S., Niwa, M., Kohno, K., Sassa, T., Ishii, M., Tomono, Y., Yamato, C., and Kanamaru, M. (1996) *J. Pharm. Sci.* **85,** 1012–1016
75. Sano, Y., Tadano, K., Kaneko, K., Kikuchi, K., and Yuzuriha, T. (1995) *J. Nutr. Sci. Vitaminol. (Tokyo)* **41,** 499–514

JOURNAL OF BONE AND MINERAL RESEARCH
Volume 18, Number 9, 2003

Association of Molecular Variants, Haplotypes, and Linkage Disequilibrium Within the Human *Vitamin D–Binding Protein* (*DBP*) Gene With Postmenopausal Bone Mineral Density

YOICHI EZURA,[1] TOSHIAKI NAKAJIMA,[1,5] MITSUKO KAJITA,[1] RYOTA ISHIDA,[1] SATOSHI INOUE,[2] HIDEYO YOSHIDA,[3] TAKAO SUZUKI,[3] MASATAKA SHIRAKI,[4] TAKAYUKI HOSOI,[2,3] HAJIME ORIMO,[2,3] and MITSURU EMI[1]

ABSTRACT

Possible contribution of *vitamin D–binding protein* (*DBP*) gene for determination of BMD was tested by characterizing 13 SNPs in 384 adult Japanese women. When the effect of a specific single SNP was tested, five SNPs (−39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, and IVS11+1097G>C) correlated with BMD significantly at various levels. The chromosomal dosage of one haplotype (T-C-C-G-T-C in −39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, D432E, and IVS11+1097G>C) displayed significant correlation with adjusted radial BMD ($r = 0.15$, $p = 0.008$; $n = 331$). Multiple regression analyses revealed a most significant correlation with the combination of IVS1+827C>T and D432E ($r^2 = 0.029$, $p = 0.005$). These results indicate a complex combined effect of several SNPs within the *DBP* gene that might underlie susceptibility to low radial BMD and osteoporosis.

Introduction: Osteoporosis results from the interplay of multiple environmental and genetic determinants. The gene encoding vitamin D–binding protein (*DBP*), a key factor for regulating calcium homeostasis through the vitamin D endocrine system, is a probable candidate for conferring susceptibility to osteoporosis.
Methods: To test a possible contribution of the *DBP* gene for determination of bone mineral density (BMD) of adult women, we have characterized 13 single nucleotide polymorphisms (SNPs) within the *DBP* gene in DNA from 384 adult Japanese women and attempted to correlate specific SNPs with BMD.
Results and Conclusions: Sixteen major haplotypes accounted for 80% of the variations, indicating allelic complexity in this genomic region. Pairwise linkage disequilibrium (LD), measured by the D' and r^2 statistics, demonstrated a general pattern of decline with increasing distance, but individual LD values within small genomic segments were diverse. Regression analysis for adjusted BMD revealed significant correlation with respect to five of them (−39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, and IVS11+1097G>C) at various levels. An intronic SNP (IVS11+1097G>C) with the highest significance of association ($p = 0.006$) showed significant LD with four SNPs located around the first exon (r^2 values >0.18, $D' > 0.5$). A non-synonymous coding SNP, D432E, showed a comparable level of correlation, but it was in a moderate LD only with IVS11+1097G>C. The chromosomal dosage of one haplotype (T-C-C-G-T-C in −39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, D432E and IVS11+1097G>C) estimated in each subject displayed significant correlation with adjusted radial BMD ($r = 0.15$, $p = 0.008$; $n = 331$). Furthermore, multiple regression analyses revealed that the most significant correlation was achieved for the combination of IVS1+827C>T and D432E ($r^2 = 0.029$, $p = 0.005$).

The authors have no conflict of interest.

[1]Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, Kawasaki, Japan.
[2]Department of Geriatric Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan.
[3]Tokyo Metropolitan Institute of Gerontology and Geriatric Hospital, Tokyo, Japan.
[4]Research Institute and Practice for Involutional Diseases, Nagano, Japan.
[5]Division of Genetic Diagnosis, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

These results indicate a complex combined effect of several SNPs within the *DBP* gene that might underlie susceptibility to low radial BMD and osteoporosis.

J Bone Miner Res 2003;18:1642–1649

Key words: single nucleotide polymorphism, vitamin D–binding protein, group-specific component, group-specific component of globulin, bone mineral density, association study, quantitative trait

INTRODUCTION

OSTEOPOROSIS IS A COMMON, multifactorial disease characterized by reduced bone mass, microarchitectual deterioration of bone tissue, and increased risk of fragility fractures. A number of twin studies and family studies have pointed to a strong genetic component, including factors that regulate bone mineral density (BMD) or factors that determine bone geometry.[1,2] Achievement of peak bone mass, as well as postmenopausal maintenance of skeletal homeostasis, is essential for soundness of skeletal bones in elderly individuals. A part of this developmental or homeostatic regulation is accomplished through control of systemic calcium levels, with the skeletal system serving as a calcium reservoir. Thus, a deficiency in this regulation should result in bone mineral loss and increased risk of osteoporosis.

The systemic level of calcium is regulated mainly through a feedback system involving parathyroid hormone and the vitamin D endocrine system.[3–5] Enzymatic activation/inactivation of hormones and competitive binding activities between receptors or transporters and their ligands are the major mechanisms at work.[4–6] Parathyroid hormone, calcitonin, receptors of these ligands, and calcium-sensing receptor have been analyzed in terms of contributions to predisposition to osteoporosis.[1,2,7–9] However, molecules more directly involved in the vitamin D endocrine system must be tested, because calcitriol (1,25-dihydroxyvitamin D_3), the most potent of the active metabolites of vitamin D, is the main effector of this system. For example, a nuclear variant of the vitamin D receptor (VDR) has been investigated intensely since the early 1990s.[10] Vitamin D–binding protein (DBP, also known as group-specific component of globulin [Gc]) is another example, although to date its significance has not been sufficiently defined.[11,12] DBP, a key regulator of the vitamin D endocrine system, is a member of the albumin family of gene products. In serum, DBP stores the preactivated 25-hydroxyvitamin D and other metabolites by directly binding to them[4,13] and transports them to the kidney to be metabolized through additional hydroxylation.[4,13,14] The importance of DBP for the skeletal system has been confirmed by targeted disruption of the *megalin* gene in mice, which encodes an endocytic renal receptor of multiple gene products. This receptor takes up DBP-bound vitamin D in urine for reabsorption into epithelial cells in proximal renal tubules.[15] In vitro studies have suggested that DBP may also mediate differentiation of hematopoietic cells and generate osteoclasts directly.[14,16,17] In view of these features, we considered the *DBP* gene to be a highly likely candidate for involvement in susceptibility to osteoporosis. In the work reported here, we examined the potential involvement of the *DBP* gene in the pathogenesis of osteoporosis by investigating genetic variations, constructing haplotypes, analyzing linkage disequilibrium among the variations, and carrying out multiple regression analyses to examine possible associations with BMD in 384 adult women.

MATERIALS AND METHODS

Subjects

DNA samples were obtained from peripheral blood of 384 adult Japanese women. Clinical data included questions on medical history, including medication, and a survey of the incidence of diseases. Mean ages and body mass indices (body mass index [BMI]) with SDs were, respectively, 58.4 ± 8.6 years (range, 32–69 years) and 23.7 ± 3.61 kg/m^2 (range, 14.7–38.5 kg/m^2). The BMD of radial bone (g/cm^2) of each participant was measured by DXA using a DTX-200 Osteometer (Meditech Inc., Hawthorne, CA, USA). To calculate adjusted BMD, the measured BMD was normalized for differences in age, height, and weight, using the Instat 3 software package (GraphPad Software, San Diego, CA, USA) and multiple regression analysis.[18,19] The adjustment equation for the study samples was as follows: [adjusted BMD (g/cm^2)] = [measured BMD (g/cm^2)] − 0.006375 × {58.39 − [age (years)]} + 0.008961 × {23.65 − [BMI (kg/cm^2)]}. BMD in the radius was measured according to the Guidelines for Osteoporosis Screening established for a health check-up program in Japan.[20] All subjects were nonrelated volunteers who provided written informed consent. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal diseases, or rheumatic diseases, and none were receiving estrogen replacement therapy. The Institutional Review Board approved the study.

Selection of 13 SNPs at the DBP *locus*

Thirteen polymorphic variations in the *DBP* gene were extracted from either the JSNP database (http://snp.ims.u-tokyo.ac.jp/index.html) or the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). The 13 selected SNPs (Table 1) included two non-synonymous substitutions in exon 11 (rs7041 and rs4588 on dbSNP) that respectively replace nucleotide +1295 thymidine with guanosine (nt+1295T/G; D432E) and +1308 cytidine with adenosine (nt+1308C/A; T436K). Combinations of these sites are the traditionally denoted variants Gc1s, Gc1f, and Gc2 that were originally diagnosed by altered electrophoretic mobility of the protein on agarose.[21] To identify further an

TABLE 1. SUMMARY OF POLYMORPHISMS ANALYZED AT THE DBP LOCUS

No.	*Name*	*nt.*	*Location*	*JSNP-ID**	*dbSNP†*
1	−39C > T	C/T	promoter	IMS-JST087014	—
2	IVS1 + 827C > T	C/T	Intron1	IMS-JST054016	rs2298849
3	IVS1 + 1916C > T	C/T	Intron1	IMS-JST017158	rs1352845
4	IVS1 − 1155A > G	A/G	Intron1	IMS-JST017159	rs222020
5	IVS3 − 1283C > T	C/T	Intron3	IMS-JST130682	rs705124
6	IVS3 − 308C > T	C/T	Intron3	IMS-JST082799	—
7	C299C	T/C	Exon8(+896)	IMS-JST082800	rs4752
8	D432E	T/G	Exon11(+1296)	IMS-JST082801	rs7041
9	T436K	C/A	Exon11(+1307)	IMS-JST082802	rs72681
10	C445R	C/T	Exon11(+1333)	IMS-JST082803	—
11	IVS11 + 1097G > C	G/C	Intron11	IMS-JST130685	—
12	IVS11 + 1303C > T	C/T	Intron11	IMS-JST130686	—
13	IVS11 − 2344C > G	C/G	Intron11	IMS-JST054017	rs229850

* Number from Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/index_ja.html).
† Number from dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).

uncharacterized polymorphism in the promoter sequence of the *DBP* gene, 2-kb resequencing of the DNA from 12 selected subjects (24 chromosomes) were conducted, using the ABI Prism BigDye Terminator system. However, there were no uncharacterized SNPs whose minor allele frequencies are greater than 5% in our test subjects. Motifs for binding sites of transcription factors were sought by means of MatInspector V2.2 software (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl) based on the TRANSFAC4.0 database.[22]

Genotyping

All 384 participants were genotyped for the 13 SNPs using the SNP-dependent polymerase chain reaction (Sd-PCR) method, an innovative allele-specific PCR procedure that we had previously developed for discrimination between polymorphic sequences.[19] In brief, two allele-specific primers (AS-primers) and one reverse primer are prepared for each SNP. AS-primers (long and short) each have a five-base difference between them; each has a polymorphic nucleotide of the SNP sequence at its 3′ end and an additional artificial mismatch is introduced near the 3′ end. These primer sets allow distinct discrimination of alleles. The Sd-PCR reaction was carried out using 10 ng of genomic DNA and 250 nM of each primer (two polymorphic forward and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U *Taq*DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA) on a thermal cycler (Gene-amp system 9600; Perkin-Elmer) with initial denaturation at 94°C for 4 minutes, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-minute extension at 72°C. Discrimination of alleles was achieved by electrophoresis and laser scanning of the DNA fragments on the ABI Prism 377 DNA system using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, we carried out direct resequencing using the ABI Prism BigDye Terminator system (Applied Biosystems).

Determination of haplotype frequencies, tests of linkage disequilibrium, and association studies

Haplotype construction and calculation of haplotype frequencies among the 768 alleles investigated were calculated by Arlequin software (Genetics and Biometry Laboratory, Geneva, Switzerland)[23] or by SNPAlyze v2.0 (DYNACOM Co., Ltd., Chiba, Japan) based on EM algorithm and a maximum likelihood approach. The duplotype of each subject was estimated by the SNPAlyze v2.0. We tested linkage disequilibrium of all possible two-way combinations of SNPs with the absolute value of the correlation coefficient, using several widely used methods (D, D', and r^2).[24,25] Quantitative associations between genotypes and adjusted BMD values (g/cm^2) were analyzed using Instat 3 software (GraphPad Software) through ANOVA, with regression analysis as a post-hoc test. The three genotypic categories of each SNP (e.g., T/T, T/G, and G/G for rs7041, D432E = +1296T>G) were converted into incremental values 0, 1, and 2 respectively. These values correspond to the number of chromosomes possessing a minor allele of the SNP. Significant association was defined when the given p value of the ANOVA f test was less than 5% ($p < 0.05$). Dominant effects were tested by the Student's t-test ($p < 0.05$); χ^2 tests ascertained Hardy-Weinberg equilibrium among genotypes ($p > 0.05$).

RESULTS

Molecular variants, haplotypes, and LD

To characterize genetic variations and LD structure within the *DBP* locus, we genotyped 13 SNPs in a total of 768 chromosomes from 384 postmenopausal Japanese women. Distributions of genotypes and allele frequencies are given in Table 2. The observed allelic frequencies at each SNP site were in Hardy-Weinberg equilibrium. We

TABLE 2. SUMMARY OF CORRELATION ANALYSIS

No.	*Name*	*nt.*	*Allele frequency (heterozygosity)*	n*	*Correlation coefficient (r)*†	p *Value*
1	−39C > T	C/T	0.69:0.31 (38%)	381	0.102	0.047‡
2	IVS1 + 827C > T	C/T	0.69:0.31 (38%)	365	0.132	0.019‡
3	IVS1 + 1916C > T	C/T	0.69:0.31 (37%)	365	0.088	0.011‡
4	IVS1 − 1155A > G	A/G	0.70:0.30 (38%)	371	0.060	0.043‡
5	IVS3 − 1283C > T	C/T	0.82:0.18 (28%)	346	0.026	0.626
6	IVS3 − 308C > T	C/T	0.87:0.13 (24%)	329	0.080	0.145
7	C299C	T/C	0.83:0.17 (27%)	362	0.066	0.213
8	D432E	T/G	0.76:0.24 (35%)	383	0.096	0.059
9	T436K	C/A	0.75:0.25 (40%)	368	0.013	0.807
10	C445R	C/T	0.96:0.04 (6.3%)	363	0.015	0.777
11	IVS11 + 1097G > C	G/C	0.82:0.12 (30%)	379	0.140	0.006§
12	IVS11 + 1303C > T	C/T	0.59:0.41 (50%)	375	0.065	0.059
13	IVS11 − 2344C > G	C/G	0.73:0.27 (43%)	358	0.013	0.923

* Number of genotyped subjects.
† Absolute values of correlation efficient.
‡ $p < 0.05$; § $p < 0.01$: p values are calculated for regression analysis with ANOVA F-test.

constructed haplotypes and calculated their frequencies using the Arlequin algorithm and the SNPAlyze v2.0. Although rare SNPs with minor-allele frequencies of less than 1% were excluded, both programs identified 16 distinct haplotypes among the 384 Japanese subjects; the frequencies of six of them were greater than 5%. The 16 major haplotypes accounted for approximately 80% of all alleles present in our study population (Fig. 1A).

Pair-wise LD, measured according to D' and r^2 statistics, was analyzed for all possible two-way comparisons between SNPs. When the extent of LD was evaluated, we saw a general pattern of decline with increasing distance, but individual LD values varied within any given small genomic region. Strong LD was verified among four SNPs localized around the first exon (−39C>T, IVS1+827C>T, IVS1+1916C>T, and IVS1–1154A>G) (Fig. 1B). We also detected relatively strong LD between those four SNPs and three others (C299C, IVS11+1097G>C, and IVS11+2344C>G).

Association of BMD with multiple SNPs in the DBP *gene*

To test whether specific genetic variants of the *DBP* gene might be involved in the pathogenesis of postmenopausal osteoporosis, we tested correlation between adjusted radial BMD and each of the 13 SNPs by ANOVA and linear regression. Five SNPs (39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, and IVS11+1097G>C) displayed significant associations with BMD (Table 2). Among them, IVS11+1097G>C in intron 11 showed the most significant correlation with radial BMD ($p = 0.006$; Fig. 2B). It also showed significant LD (r^2 values >0.18, $D' > 0.5$) with the four significantly correlated SNPs located around the first exon, as well as moderate LD with a non-synonymous coding SNP (D432E) that had shown a comparable level of correlation. No evidence was found for LD between D432E and the four SNPs around the first exon, indicating an independent association of D432E with BMD. Evidence for a dominant effect of the minor E allele was found with Student's t-test (E/E; 0.42 ± 0.003, $n = 26$, D/E and D/D; 0.40 ± 0.003, $n = 357$, $p = 0.02$). The correlation observed between IVS11+1097G>C and adjusted BMD may represent a combined effect of the promoter/enhancer SNPs around the first exon and the non-synonymous coding SNP, D432E. In our cohort at least, the results appear to indicate a combined effect on postmenopausal bone mass of multiple SNPs within the DBP locus. Multiple regression analyses were conducted to detect interactive effects among the SNPs. For every combination that was tested, each panel of two to four SNPs (e.g., −39C>T, IVS1+827 and D432E ($r^2 = 0.033$, $p = 0.008$) or IVS1+827, D432E, and IVS11+1097 ($r^2 = 0.035$, $p = 0.006$) displayed significant correlation with radial BMD in multiple regression analysis. However, the best combination for the lowest p value was obtained with the combination of IVS1+827 and D432E ($r^2 = 0.028$, $p = 0.005$). To evaluate the combined effects, haplotypes consisting of the five significantly correlated SNPs and one marginally correlated SNP were estimated by means of the SNPAlyze. Although we estimated 36 distinctive haplotypes in our subjects, 11 major haplotypes accounted for about 94% of the total (Fig. 2A). To analyze correlation between chromosomal dosage of a distinctive haplotype and adjusted radial BMD, a "duplotype" in each individual was estimated with the SNPAlyze v2.0. This analysis revealed a significant correlation between the genetic dose of one haplotype, T-C-C-G-T-C, and the adjusted BMD ($r = 0.15$, $p = 0.008$, $n = 331$; Fig. 2C).

DISCUSSION

In the study reported here, we investigated haplotypes and linkage disequilibrium among molecular variants of the *DBP* gene and looked for associations of these features with BMD among 384 postmenopausal Japanese women. We

A.

Haplotype No	-36C>T	IVS1+827C>T	IVS1+1916C>T	IVS1-1155A>G	IVS3-1263C>T	IVS3-388C>T	C299C (T>C)	D432E (T>G)	T436K (C>A)	C445R	IVS11+1097G>C	IVS11+1383C>T	IVS11-2344C>G	Frequency	
1	C	T	T	A	C	C	T	T	A	C	G	C	G	20.0%	0.0%
2	C	T	T	A	C	C	T	G	C	C	G	T	C	15.4%	35.4%
3	C	T	T	A	T	C	C	T	C	C	G	T	C	9.5%	44.9%
4	T	C	C	G	C	C	T	T	C	C	G	C	C	6.1%	51.0%
5	T	C	C	G	C	T	T	T	C	C	C	C	C	5.2%	56.3%
6	T	C	C	G	C	C	T	T	C	C	C	C	C	5.0%	61.3%
7	C	T	T	A	C	C	T	T	C	C	G	C	C	3.9%	65.2%
8	T	C	C	G	C	C	T	G	C	C	G	T	C	2.7%	67.9%
9	C	T	T	A	C	C	T	T	C	C	G	T	C	2.1%	70.0%
10	C	T	T	A	T	C	C	T	C	3	G	T	C	2.0%	72.0%
11	C	T	T	A	C	C	T	T	C	C	C	C	C	1.0%	73.0%
12	C	T	T	A	C	C	T	T	C	C	G	C	G	1.4%	74.0%
13	C	T	T	G	C	C	T	T	C	C	G	C	C	1.3%	76.2%
14	T	C	C	G	C	T	T	T	A	C	G	C	G	1.1%	77.2%
15	C	T	T	A	T	C	T	T	A	C	G	C	G	1.1%	78.4%
16	T	C	T	A	C	T	T	T	C	C	C	C	C	1.0%	79.4%

B.

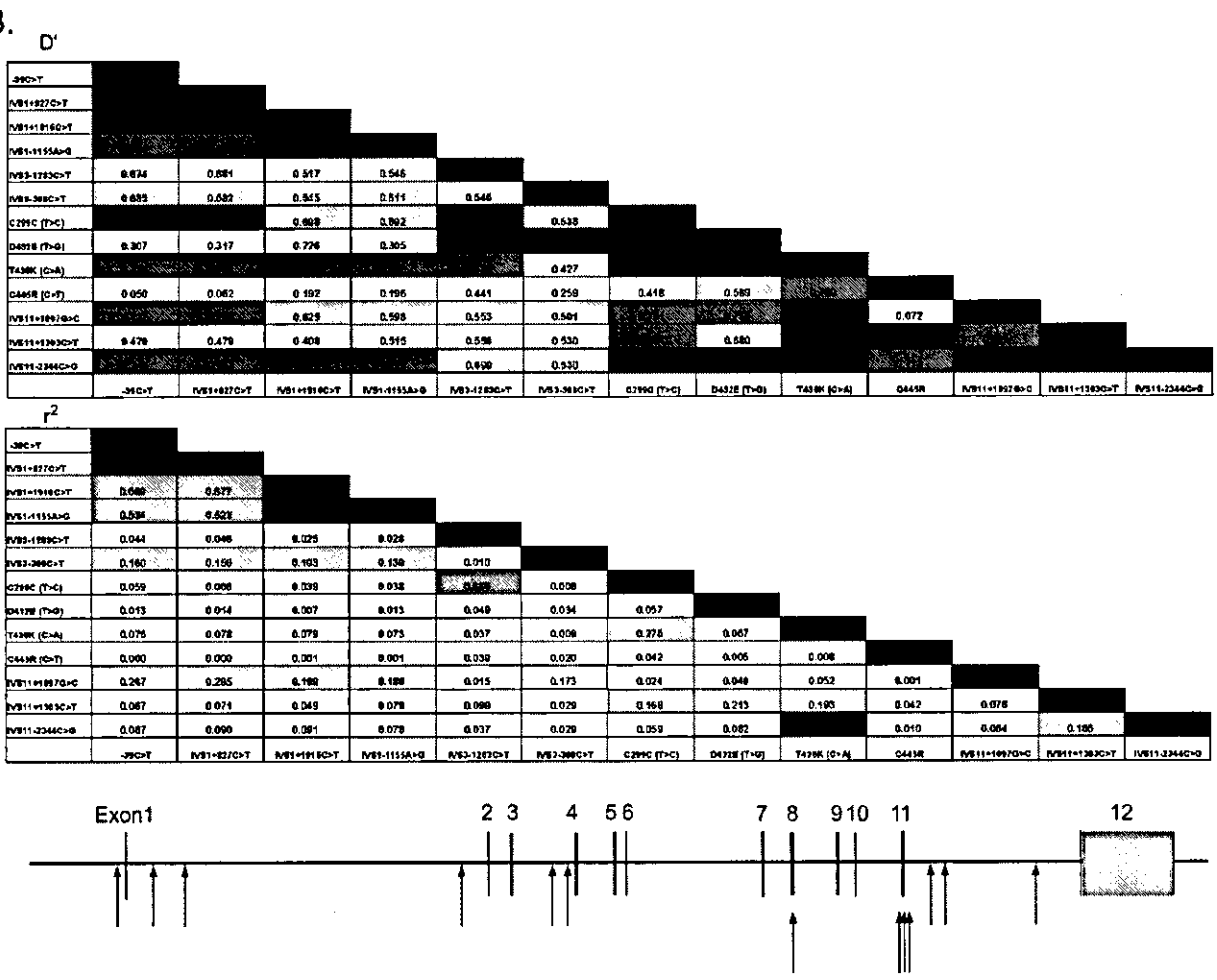

FIG. 1. Analysis of haplotypes and linkage disequilibrium among 13 SNPs at the *DBP* locus. (A) The 16 major predicted haplotypes, ordered according to predicted frequencies indicated in percentages in the second column from the right. The far right column indicates summed occupancy ratios; the sum of predicted frequencies of the 16 major haplotypes accounts for 79.4% of the entire sample. (B) Indices of linkage disequilibrium, D' and r^2, presented in separate tables. D' values greater than 0.4 and r^2 values greater than 0.1 are highlighted with gray halftone. The schematic diagram of the *DBP* gene structure below indicates locations of the 13 tested SNPs by upward arrows.

considered DBP to be a likely candidate for susceptibility to osteoporosis because it is a key regulator of calcium by way of vitamin D endocrine system. It binds to 25-hydroxyvitamin D and other vitamin D metabolites to maintain vitamin D storage in the body and transports these molecules to the kidney for further hydroxylation.[4,13,14] A quantitative-trait locus (QTL) analysis in mice had indicated possible involvement of the chromosomal region around a

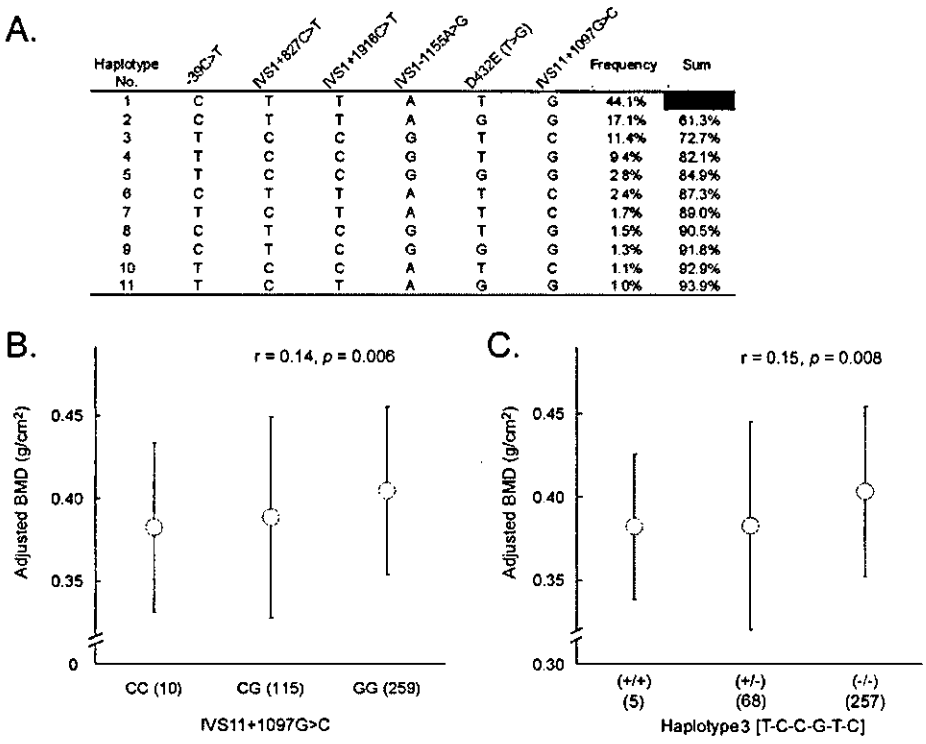

FIG. 2. Haplotype analysis of six SNPs that showed association with adjusted radial BMD. (A) The 11 major predicted haplotypes are presented in the order of their frequencies, as in Fig. 1A. (B) Adjusted BMD levels among 384 subjects plotted against three genotypically classified subgroups for the most significantly associated SNP (IVS11+1097G>C). Open circles indicate mean values; error bars indicate SDs. Correlation between the number of major alleles possessed and the adjusted BMD was tested by linear regression analysis ($r = 0.14$, $p = 0.006$). (C) Adjusted BMD of three duplotypically classified subgroups plotted against the significantly associated haplotype (T-C-C-G-T-C) among our 384 subjects. Open circles indicate mean values and error bars indicate SDs. Correlation between the number of haplotypes possessed (2,1,0) and adjusted BMD was tested by linear regression analysis ($r = 0.14$, $p = 0.006$).

microsatellite marker (Mit112) on murine chromosome 5, suggesting that osteoporosis-susceptibility genes might exist within the corresponding human chromosomal region (4q11–13). This region happens to contain the *DBP* gene.[24]

To determine whether genetic variations in DBP were associated with radial BMD in humans, our correlation studies used age- and BMI-adjusted BMD as a reliable quantitative trait. A hierarchical strategy was adopted to detect association by studying each genetic variant separately, then haplotypes, and finally LD among the SNPs. Previous studies had defined three isoforms of DBP in plasma, Gc1s, Gc1f, and Gc2, and analyzed them for association with common diseases.[14,25–29] Diagnosis was based on altered electrophoretic mobility in agar of isoforms that combined two amino acid variations, D432E and T436K. In addition to those variations, a TAAA-repeat polymorphism in the 8th intron of *DBP* was investigated previously.[12] However, rather than concentrating on this limited number of indirect marker variations, we chose to combine analyses of multiple SNPs spanning the entire 42.4-kb sequence of the *DBP* gene. Using this strategy we selected 13 SNPs, including an additional non-synonymous coding SNP, C445R (nt+1333C/T; IMS-JST082803) for examination in our cohort. Another known coding SNP, R445H (+1334G/A) was not detected in this population sample. Resequencing of the 2-kb 5′ flanking region of 32 chromosomes indicated that no other SNPs with minor-allele frequencies exceeding 3% were present. Combined analyses of the 13 selected SNPs revealed associations of multiple SNPs within the *DBP* gene with radial BMD levels. In general, the alleles affecting radial BMD were common, with frequencies ranging from 12% to 31% (Table 2).

Because multiple SNPs might act in combination to affect BMD, we constructed haplotypes to examine relationships to variations in radial BMD. This procedure increased the significance of any correlation previously found (Fig. 2). In addition, multiple regression analysis was effective to show that several combinations of three or four SNPs from among the "correlation group" consisting of −39C>T, IVS1+827C>T, D432E, and IVS11+1097G>C were significantly associated with radial BMD at the level of $p < 0.01$. Although D432E showed only a marginal tendency for correlation to the adjusted BMD by a separated analysis, we added this SNP to the analysis, because of its implicated functional importance and significant LD with IVS11+1097G>C. Interestingly, this was true when another non-synonymous coding SNP in the exon11, T436K, was included in the analysis; there still remained a significant level of correlation (data not shown).

Of interest, the functional importance of these polymorphic nucleotides of the SNPs that provided better p values was more predictable than the other SNPs. T436K and D432E are non-synonymous coding SNPs that alter protein structure, as evidenced by shifts in electrophoretic mobility of the protein on agarose gels. Although the affected amino acids do not lie within known structural domains,[30] they could affect the affinity of DBP for binding to vitamin D or other proteins. The SNPs in the 5′-flanking region (−39C>T) and in the first intron of *DBP* (IVS1+827C>T) may be in part of enhancer elements. Motif analysis using the MatInspector program revealed that −39C>T, in the

promoter region, lies within a consensus binding site for δ-EF1 (δ-crystallin/E2-box factor 1) [ggtcaccta(*C/T*)a].[31] δ-EF1 is an important transcription factor for differentiation of various types of cells. It is active in many organs, not only in eyes, and may regulate tissue-specific expression of target genes including the crystalline gene in lens and collagen and osteocalcin genes in osteoblasts.[32–34] Of course, in addition to the above-mentioned promoter SNP and coding SNPs, the significantly associated SNPs in noncoding regions (within introns and the 3′ untranslated region) might still influence alternative splicing, splicing efficiency, or mRNA turnover; such mechanisms have been reported for other disease-causing genes. Those possibilities should be tested. Nevertheless, the genetic data reported here, as well as the obvious physiological role of DBP in calcium homeostasis, suggest that alterations in DBP activity or expression level may underlie abnormalities in bone mineral metabolism.

In summary, our genetic analysis of *DBP* variations, haplotypes, and LD, and correlation of those features with radial BMD, suggests that multiple SNPs at the *DBP* locus might act in combination to increase the risk of osteoporosis in postmenopausal Japanese women. The possible involvement of genetic variations in the *DBP* gene may explain, at least in part, the pathogenesis of postmenopausal osteoporosis and may contribute to the establishment of suitable treatment designs and plans for prevention of the disease.

ACKNOWLEDGMENTS

We thank Dr Hironori Iwasaki at Ryukyu University for his advice and discussions, and Mina Kodaira, Kyoko Shimizu, Mayumi Tanaka, and Naoko Tsuruta for their technical assistance. This work was supported in part by a special grant for Strategic Advanced Research on "Cancer" and "Genome Science" from the Ministry of Education, Science, Sports and Culture of Japan, a Research Grant for Research from the Ministry of Health and Welfare of Japan, and a Research for the Future Program Grant of The Japan Society for the Promotion of Science.

REFERENCES

1. Giguere Y, Rousseau F 2000 The genetics of osteoporosis: 'complexities and difficulties'. Clin Genet **57**:161–169.
2. Stewart TL, Ralston SH 2000 Role of genetic factors in the pathogenesis of osteoporosis. J Endocrinol **166**:235–245.
3. Broadus AE 1999 Mineral balance and homeostasis. In: Favus MJ (ed.) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Lippincott Williams and Wilkins, Philadelphia, PA, USA, pp. 74–79.
4. Bouillon R, Okumura WH, Norman AW 1995 Structure-function relationships in the vitamin D endocrine system. Endocr Rev **16**:200–257.
5. Norman AW, Ishizuka S, Okamura WH 2001 Ligands for the vitamin D endocrine system: Different shapes function as agonists and antagonist for genomic and rapid response receptors or as a ligand for the plasma vitamin D binding protein. J Steroid Biochem Mol Biol **76**:49–59.
6. Marcus R, Wong M, Heath H III, Stock JL 2002 Antiresorptive treatment of postmenopausal osteoporosis: Comparison of study designs and outcomes in large clinical trials with fracture as an endpoint. Endocr Rev **23**:16–37.
7. Hosoi T, Miyao M, Inoue S, Hoshino S, Shiraki M, Orimo H, Ouchi Y 1999 Association study of parathyroid hormone gene polymorphism and bone mineral density in Japanese postmenopausal women. Calcif Tissue Int **64**:205–208.
8. Masi L, Becherini L, Colli E, Gennari L, Mansani R, Falchetti A, Becorpi AM, Cepollaro C, Gonneli S, Tnini A, Brandi ML 1998 Polymorphisms of the calcitonin receptor gene are associated with bone mineral density in postmenopausal Italian women. Biochem Biophys Res Commun **248**:190–195.
9. Tsukamoto K, Orimo H, Hosoi T, Miyao M, Ota N, Nakajima T, Yoshida H, Watanabe S, Suzuki T, Emi M 2000 Association of bone mineral density with polymorphism of the human calcium sensing receptor locus. Calcif Tissue Int **66**:181–183.
10. Morrison NA, Yeoman R, Kelly PJ, Eisman JA 1992 Contribution of trans-acting factor alleles to normal physiological variability: Vitamin D receptor gene polymorphism and circulating osteocalcin. Proc Natl Acad Sci USA **89**:6665–6669.
11. Rapado A, Hawkins F, Sobrinho L, Diaz-Curiel M, Galvao-Telles, A, Arver S, Gomes JM, Mazer N, Garcia e Costa J, Horcajada C, Lopez-Gavilanes E, Mascarenhas M, Papapietro K, Lopez Alvarez MB, Pereira MC, Martinez G, Valverde I, Garcia JJ, Carballal JJ, Garcia I 1999 Bone mineral density and androgen levels in elderly males. Calcif Tissue Int **65**:417–421.
12. Papiha SS, Allcroft LC, Kanan RM, Francis RM, Datta HK 1999 Vitamin D binding protein gene in male osteoporosis: Association of plasma DBP and bone mineral density with (TAAA)n-Alu polymorphism in DBP. Calcif Tissue Int **65**:262–266.
13. Verboven C, Rabiins A, Maeyer MD, Van Baelen H, Bouillon R, De Ranter C 2002 A structural basis for the unique binding features of the human vitamin D binding protein. Nat Struct Biol **9**:131–136.
14. White P, Cooke N 2000 The multifunctional properties and characteristics of vitamin D-binding protein. Trends Endocrinol Metab **11**:320–327.
15. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE 1999 An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell **96**:507–515.
16. Benis KA, Schneider GB 1996 The effects of vitamin D binding protein-macrophage activating factor and colony-stimulating factor-1 on hematopoietic cells in normal and osteopetrotic rats. Blood **88**:2898–2905.
17. Schneider GB, Benis KA, Flay NW, Ireland RA, Popoff SN 1995 Effects of vitamin D binding protein-macrophage activating factor (DBP-MAF) infusion on bone resorption in two osteopetrotic mutations. Bone **16**:657–662.
18. Kleinbaum DG, Kupper LL, Muller KE 1988 Applied Regression Analysis and Other Multivariate Methods, 2nd ed. PWS-KENT Publishing, Boston, MA, USA.
19. Iwasaki H, Emi M, Ezura Y, Ishida R, Kajita M, Kodaira M, Yoshida H, Suzuki T, Hosoi T, Inoue S, Shiraki M, Swensen J, Orimo H 2003 Association of a Trp16Ser variation in the Gonadotropin Releasing Hormone (GnRH) Signal Peptide with Bone Mineral Density, revealed by SNP-dependent PCR (Sd-PCR) Typing. Bone **32**:185–190.
20. Orimo H, Hayashi Y, Fukunaga M, Sone T, Fujiwara M, Shiraki M, Kushida K, Miyamoto S, Soen S, Nishimura J, Oh-hashi Y, Hosoi T, Gorai I, Tanaka H, Igai T, Kishimoto H 2001 Diagnostic criteria for primary osteoporosis: Year 2000 revision. J Bone Miner Metab **19**:331–337.
21. Bearn AG, Bowman BH, Kitchin FD 1964 Genetic and biochemical consideration of the serum group-specific component. Cold Spring Harb Symp Quant Biol **29**:435–442.
22. Quandt K, Frech K, Karas H, Wingender E, Werner T 1995 MatInd and MatInspector: New fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res **23**:4878–4884.
23. Schneider S, Roessli D, Excoffier L 2000 Arlequin ver. 2.000: A Software for Population Genetics Data Analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.
24. Beamer WG, Shultz KL, Churchill GA, Frankel WN, Baylink DJ, Rosen CJ, Donahue LR 1999 Quantitative trait loci for bone density in C57BL/6J and CAST/EiJ inbred mice. Mamm Genome **10**:1043–1049.
25. Szathmary EJE 1987 The effect of Gc genotype on fasting insulin level in Dogrib Indians. Hum Genet **75**:368–372.
26. Baier LJ, Dobberfuhl AM, Pratley RE, Hanson RL, Bogardus C 1998 Variations in the vitamin D-binding protein (Gc locus) are

associated with oral glucose tolerance in nondiabetic Pima Indians. J Clin Endocr Metab **83**:2993–2996.
27. Hirai M, Suzuki S, Yoshinori H, Hirai A, Chiba M, Akai H, Suzuki C, Toyota T 2000 Variations in vitamin D-binding protein (group-specific component protein) are associated with fasting plasma insulin levels in Japanese with normal glucose tolerance. J Clin Endocr Metabol **85**:1951–1953.
28. Klupa T, Malecki M, Hanna L, Sieradzka J, Frey J, Warram JH, Sieradzki J, Krolewski AS 1999 Amino acid variants of the vitamin D-binding protein and risk of diabetes in white Americans of European origin. Eur J Endocrinol **141**:490–493.
29. Schmidt-Gayk H, Grawunder C, Tschope W, Schmitt W, Ritz E, Pietsch V, Andrassay K, Bouillon R 1977 25-hydroxy-vitamin-D in nephrotic syndrome. Lancet **16**:105–108.
30. Verboven C, Rabijns A, Maeyer MD, Van Baelen H, Bouillon R, De Ranter C 2002 A structural basis for the unique binding features of the human vitamin D-binding protein. Nat Struct Biol **9**:131–136.
31. Sekido R, Murai K, Funahashi J, Kamachi Y, Fujisawa-Sehara A, Nabeshima Y, Kondoh H 1994 The delta-crystallin enhancer-binding protein delta EF1 is a repressor of E2-box-mediated gene activation. Mol Cell Biol **14**:5692–5700.
32. Funahashi J, Sekido R, Murai K, Kamachi Y, Kondoh H 1993 Delta-crystallin enhancer binding protein delta EF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis. Development **119**:433–446.
33. Sooy K, Demay MB 2002 Transcriptional repression of the rat osteocalcin gene by EF1. Endocrinology **143**:3370–3375.
34. Terraz C, Toman D, Delauche M, Ronco P, Rossert J 2001 EF1 binds to a far upstream sequence of the mouse pro-(I) collagen gene and represses its expression in osteoblasts. J Biol Chem **276**:37011–37019.

Address reprint requests to:
Mitsuru Emi, MD, PhD
Department of Molecular Biology
Institute of Gerontology
Nippon Medical School
1-396 Kosugi-cho, Nakahara-ku
Kawasaki 211-8533, Japan
E-mail: memi@nms.ac.jp

Received in original form January 13, 2003; in revised form February 11, 2003; accepted March 19, 2003.

ELSEVIER

Bone 32 (2003) 185–190

BONE

www.elsevier.com/locate/bone

Association of a Trp16Ser variation in the gonadotropin releasing hormone signal peptide with bone mineral density, revealed by SNP-dependent PCR typing

Hironori Iwasaki,[a] Mitsuru Emi,[a,*] Yoichi Ezura,[a] Ryota Ishida,[a] Mitsuko Kajita,[a] Mina Kodaira,[a] Hideyo Yoshida,[b] Takao Suzuki,[b] Takayuki Hosoi,[b] Satoshi Inoue,[c] Masataka Shiraki,[d] Jeff Swensen,[e] and Hajime Orimo[b]

[a] *Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, 1-396, Kosugi-cho, Nakahara-ku, Kawasaki 211-8533, Japan*
[b] *Tokyo Metropolitan Institute of Gerontology and Geriatric Hospital, Itabashi-ku, Tokyo, Japan*
[c] *Department of Geriatric Medicine, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo, Japan*
[d] *Research Institute and Practice for Involutional Diseases, Nagano, Japan*
[e] *Myriad Genetics, Salt Lake City, UT, USA*

Received 10 June 2002; revised 16 October 2002; accepted 16 October 2002

Abstract

Osteoporosis is believed to result from interplay among multiple environmental and genetic determinants, including factors that regulate bone mineral density (BMD). Among those factors, adequate estrogen is essential for achievement of peak bone mass as well as for postmenopausal maintenance of skeletal homeostasis. Gonadotropin-releasing hormone (GnRH) from the hypothalamus is the primary determinant in the hypothalamic–pituitary–gonadal feedback system. In genetic studies of 384 postmenopausal Japanese women, we found a significant association between BMD and an amino acid variation (Trp16Ser) located within the signal peptide of GnRH ($r = 0.143$, $P = 0.005$). These results were achieved by genotyping all subjects using a newly developed SNP-dependent PCR method. This automated, high-throughput, and inexpensive procedure is suitable for typing large numbers of samples. BMD was lowest among 16Ser/Ser homozygotes, highest among 16Trp/Trp homozygotes, and intermediate among heterozygotes. A case-control study involving 125 osteoporosis patients and 92 healthy controls revealed a significant association between the presence of a 16Ser GnRH allele and affected status ($\chi^2 = 4.74$, $P = 0.041$). The results suggested that variation of the GnRH signal peptide may be an important risk factor for postmenopausal osteoporosis.

Keywords: Single nucleotide polymorphism; GnRH; Bone mineral density; Association study; Quantitative trait

Introduction

Osteoporosis is a common, multifactorial disease characterized by reduced bone mass and microarchitectural deterioration of bone tissue, typically in association with postmenopausal estrogen deficiency. Osteoporosis is believed to result from interplay among multiple environmental and genetic determinants, including factors that regulate bone mineral density (BMD) or determine bone geometry [1,2].

Adequate estrogen is essential for achievement of peak bone mass [3] as well as for postmenopausal maintenance of skeletal homeostasis; estrogen deficiency in the postmenopausal period or after ovariectomy results in bone mineral loss and increased risk of osteoporosis [4,5] and estrogen replacement therapy is a common choice for treatment of osteoporosis [6,7]. Homeostasis of the sex hormones is controlled through a feedback system involving the hypothalamic–pituitary–gonadal axis [8,9]. Recently an amino acid variation (Trp16Ser, W16S) was identified in the 16th

* Corresponding author. Fax: +81-44-733-5192.
E-mail address: memi@nms.ac.jp (M. Emi).

8756-3282/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S8756-3282(02)00949-3

residue of the signal peptide of gonadotropin-releasing hormone (GnRH), the primary determinant in the hierarchical regulatory system. Kallmann syndrome [10] and anorexia nervosa [11] are clinical examples of hypogonadism due to dysfunction of GnRH. We considered that the genetic variation in this hormone could make it one of the most likely candidates for involvement in susceptibility to osteoporosis.

A number of efforts have been expended for identifying genetic factors involved in the onset of osteoporosis [3,5]. An accurate, automated, high-throughput, and inexpensive method is desirable if one is to carry out large-scale genotyping of human DNAs for single-nucleotide polymorphisms (SNPs) as a potentially effective way of determining disease susceptibility or drug sensitivity among individuals. Numerous methods have been introduced, each based on different technologies, including various types of hybridization, enzymatic reactions, electrophoretic separation, sequence-specific incorporation of nucleotides, and oligonucleotide ligation. Allelic discrimination based on PCR priming has the advantages of simplicity and within-assay control for exclusion of false-negative results. We took advantage of the basic principles of mutagenically separated PCR, a method developed originally by Rust et al. for detection of disease-causing mutations [12], and refined those principles to meet the requirements of large-scale genomewide SNP screening. In this way we defined an automated, high-throughput, and inexpensive SNP typing procedure suitable for hundreds of samples at once, which we named SNP-dependent PCR (Sd-PCR).

In the work reported here we examined the potential involvement of the GnRH gene in pathogenesis of osteoporosis by investigating a possible association between the Trp16Ser amino acid variation and BMD in 384 postmenopausal women. Subsequently we undertook a case-control comparison using the Sd-PCR method.

Materials and methods

Subjects

DNA samples for our association study were obtained from peripheral blood of 384 postmenopausal Japanese women. Mean ages and body mass indices (BMI) with standard deviations (SD) were 58.4 ± 8.6 (range 32–69) years and 23.7 ± 3.61 (range 14.7–38.5) kg/m^2 [3]. The BMD of radial bone (expressed in g/cm^2) of each participant was measured by dual energy X-ray absorptiometry (DEXA) using DTX-200 (Osteometer Meditech Inc., Hawthorne, CA). To calculate adjusted BMD, the measured BMD was normalized for differences in age, height, and weight, using the Instat 3 software package (GraphPad Software, San Diego, CA) and logistic regression analysis [13]. The adjustment equation for the study samples was {adjusted BMD (g/cm^2)} = {measured BMD (g/cm^2)} − 0.006375 × [58.39 − {age (years)}] + 0.008961 × [23.65 − {BMI (kg/cm^2)}] [10,12]. BMD in the radius was measured according to the Guidelines for Osteoporosis Screening in a health checkup program in Japan [14]. All were nonrelated volunteers and gave their informed consent prior to the study. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal diseases, or rheumatic diseases, and none was receiving estrogen replacement therapy.

For case-control correlation, two groups of adult Japanese female subjects were ascertained: (1) osteoporosis patients, consisting of 125 women who exhibited low lumbar BMD (Z score ≤ -1), (2) healthy controls, consisting of 92 women with sufficient lumbar BMD (Z score ≥ 1). The BMD of lumbar vertebral bodies (expressed in g/cm^2) was measured in each participant by DEXA using DPX-L. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20,000 Japanese women [15]. The patients and the control subjects were ascertained from the Research Institute and Practice for Involutional Diseases, and all gave their informed consent prior to the study. This project was approved by the IRB ethical committee of the Institute.

SNP-dependent PCR (Sd-PCR) procedure

Using the Sd-PCR method that we had defined for large-scale SNP genotyping, we placed the polymorphic nucleotide of the SNP sequence at the 3′ end of each polymorphic (forward) primer, and an additional artificial mismatch was introduced near the 3′ end, after being selected to be the best choice by a computer algorithm, a program that attempts to balance the strength of the mutations on the two allele-specific primers. This procedure allowed distinct discrimination of alleles, due to almost exclusive amplification of one allele over the other. Two allele-specific primers (AS primers), each specific for respective allelic sequences, were prepared, with a five-base difference between them, i. e., the TTTTT sequence is placed at the 5′ end of one AS primer to make one allele longer than the other. Either a GG or a CC sequence is also placed at the 5′ portion of each AS primer in order to stop the short product from unfavorable annealing to long product and filling in the five-base extension.

For the C allele (16Ser) of the GnRH gene, a specific forward primer was constructed having at its 5′ end a five-T stretch and double G: 5′-TTTTTGGGCTGGCCTTATTCTACTGAATTG-3′. The forward primer specific to the G allele (16Trp), having only double C at the 5′ end, was designed as 5′-CCGCTGGCCTTATTCTACTGACCTC-3′; polymorphic, discriminating nucleotides are marked here by single and double underlines. The nonpolymorphic reverse primer (R) having a four-base stretch of GTTT at its 5′ end was 5′-GTTTTTTTCGGCATCTCTCTTTCC-3′. Two AS primers were mixed with a single nonpolymorphic

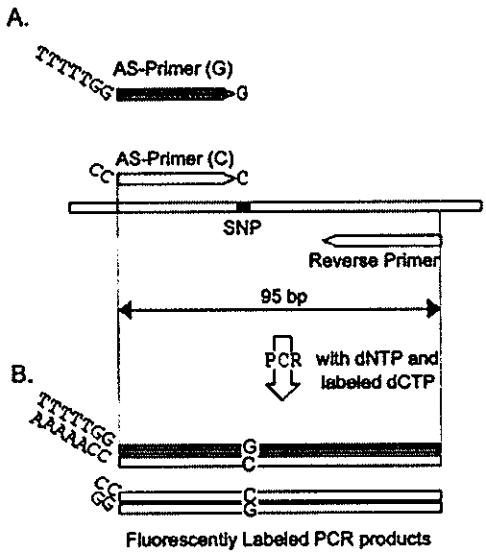

Fig. 1. Schematic representation of the Sd-PCR method. (A) Two allele-specific forward primers having a seven-base or two-base stretch of TTTTTGG or CC at their 5′ ends amplify a 95-bp flanking sequence of the SNP with a specific reverse primer. (B) Allele-specific PCR products are labeled fluorescently by incorporation of dye-conjugated dCTP.

reverse primer for each PCR reaction. The C allele was designed to produce a 102-bp fluorescent DNA fragment and the G allele a 97-bp fragment.

Each genomic DNA sample (10 ng) was amplified with 250 nM each primer (two polymorphic forward and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris–HCl, 1.5 mM $MgCl^2$, 50 mM KCl, 1 U of *Taq* DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin–Elmer, Norwalk, CT). The Sd-PCR reaction was carried out on a thermal cycler (Gene-Amp System 9600, Perkin–Elmer) with initial denaturation at 94°C for 4 min, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s), and then 25 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system using GeneScan Analysis Software version 2.1 (Applied Biosystems, Foster City, CA). To confirm the accuracy of the Sd-PCR method, direct resequencing was carried out using the ABI Prism BigDye Terminator system.

Statistical analysis

Quantitative associations between genotypes and adjusted BMD values (g/cm^2) or between genotypes and *Z* scores were analyzed using Instat 3 software (GraphPad Software) via analysis of variance (ANOVA), with regression analysis as a post-hoc test. The three genotypic categories of GnRH-W16S (+47C/G; rs6185), C/C, C/G, and G/G, were converted into incremental values 0, 1, and 2 respectively. These values correspond to the number of chromosomes possessing a G allele of the SNP. Significant association was defined when the given *P* value of the ANOVA *F* test was less than 5% ($P < 0.05$). A χ^2 test was used to ascertain Hardy-Weinberg equilibrium among genotypes ($P > 0.05$).

Results

Genotyping by Sd-PCR

The Sd-PCR is a method that transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles. It is based on the "mutagenically separated PCR" technique proposed by Rust [12] and has been refined by us into an automated, high-throughput, and inexpensive SNP typing procedure for large-scale, genomewide SNP screening. A flow chart outlining the procedure is shown in Fig. 1. We first genotyped 24 samples of test genomic DNAs (48 alleles) using the Sd-PCR method, achieving a success rate of 100%. In parallel, the same 24 DNAs were examined at the SNP site by direct resequencing using the ABI Prism BigDye Terminator system, to confirm the accuracy of the Sd-PCR method. This experimental comparison proved the complete accuracy (48 correspondence/48 typings) of the Sd-PCR method. We used the validated system to genotype 384 individual DNAs for the W16S SNP (G/C at nt +47) of the GnRH gene. In those experiments the C allele was transformed into a fluorescent DNA fragment of 102 bp, and the G allele at the same nucleotide position was transformed into a fluorescent fragment 97 bp long. The two sizes were well separated and easily distinguishable by electrophoresis on a polyacrylamide gel (Fig. 2).

To confirm the reproducibility of the Sd-PCR assay we prepared two forward allele-specific primers harboring different artificial mismatches for each variant allele. A computerized algorithm was used to design these four primers, enabling us to test four different combinations of primer sets. We genotyped the 24 test samples (48 alleles) in quadruplicate and compared the results with each other and to the results from direct resequencing; all were completely concordant. Based on this satisfactory experience, we made it a rule to routinely carry out Sd-PCR with four different combinations of primer sets for 24 alleles as a preliminary test before choosing the most suitable pair of primers.

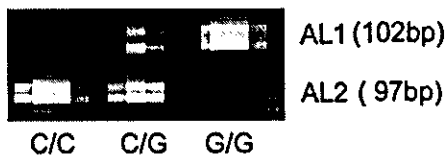

Fig. 2. Representative gel image of three genotypes for GnRH-W16S SNP using the Sd-PCR method. A 3-μl aliquot of denatured labeled PCR products was discriminated by a five-base mobility difference on electrophoresis using an automated ABI Prism 377 DNA sequencing system.

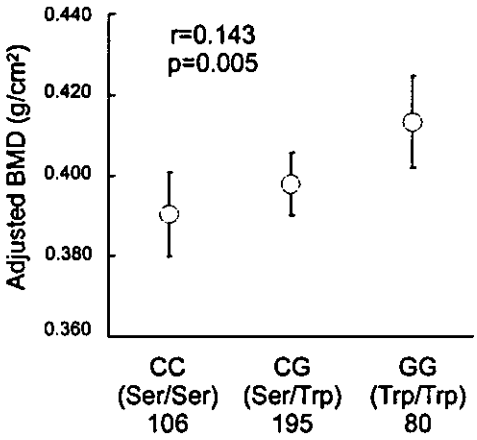

Fig. 3. Effect of GnRH alleles on quantitative adjusted BMD. Open circles indicate mean values; error bars indicate 95% confidence interval (95% C.I.); *r*, correlation coefficiency; p, *P* values, calculated by ANOVA *F* test.

Association of 16S alleles with low radial BMD in Japanese women

To test whether potentially functional amino acid variants of GnRH might be involved in the pathogenesis of postmenopausal osteoporosis, we selected for study an amino acid-substituting SNP in the GnRH gene (rs6185 in the NCBI dbSNP database). This SNP is located within the signal peptide domain and causes substitution of serine for tryptophan at residue 16 (W16S); the change is likely to affect processing of the precursor GnRH signal peptide on account of its location. As mentioned above, we applied the Sd-PCR method to determine genotypes at this site in DNA from 384 Japanese women, who were then classified into one of the three possible genotypic groups. Among our subjects, the C allele (corresponding to the second nucleotide of a serine codon) was a major variant, with a frequency of 54%; the proportions of different genotypes were in Hardy-Weinberg equilibrium.

Correlation between the genotypes and the adjusted values of BMD, examined by logistic regression analysis, was significant (Fig. 3). Homozygous C allele carriers (16S/S) had the lowest BMD values (0.390 ± 0.054 g/cm^2), homozygous G allele carriers (16W/W) had the highest (0.413 ± 0.052 g/cm^2), and heterozygous individuals (16W/S) had intermediate adjusted BMD levels (0.398 ± 0.055 g/cm^2), indicating a dosage effect ($r = 0.143$, $P = 0.005$).

Correlation of 16Ser alleles with low BMD in a case-control comparison

To examine the above correlation in a case-control study, we compared distributions of 16Ser alleles between 125 osteoporosis patients (low BMD; *Z* scores, −1.0) and 92 control individuals (sufficient BMD levels; *Z* scores, 1.0); χ^2 tests revealed a significant association between GnRH genotype and affected status, i.e., the 16S allele was more frequent among patients than among controls ($\chi^2 = 4.74$, $P = 0.041$; Table 1). A statistically significant association was ascertained by analyzing allele frequencies in both groups using Fisher's exact test (relative risk, 1.22, 95% confidence interval; 1.01–1.47, $P = 0.041$; Table 1). The results supported a consistent contribution of the SNP GnRH-16Ser to a lowering of bone mineral density not only in the general population but also in the comparison of cases with controls among Japanese women.

Discussion

We examined the effect of an amino acid-substituting SNP (rs6185), within the signal peptide-encoding domain of the GnRH gene, GnRH-W16S, on bone mineral density (BMD) and development of osteoporosis in a population-based quantitative analysis and in a case-control comparison involving osteoporosis patients. BMD levels and the frequency of osteoporosis were significantly correlated with the presence of 16Ser alleles encoded at nt +47, when we applied an Sd-PCR method of SNP genotyping.

Our SNP genotyping method can be summarized as follows: the Sd-PCR transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles. It is based on the "mutagenically separated PCR" proposed by Rust [12] and has been refined by us into an automated, high-throughput, and inexpensive SNP typing procedure for large-scale, genomewide SNP screening. This procedure incorporates double-nucleotide mismatches at the 3′ end of polymorphic (forward) primers representing each allele, one mismatch corresponding to the natural SNP to be tested and the other designed to allow distinct allelic discrimination through almost exclusive amplification of one allele over the other.

Innovations that were incorporated into the Sd-PCR method presented here are (1) addition of a five-thymidine stretch at the 5′ end of one of the two polymorphic (forward) primers, for discriminating the two alleles according to size; (2) incorporation of fluorescent dyes into the prod-

Table 1
Distribution of Trp and Ser alleles among cases (low *Z* scores) versus distribution among controls (high *Z* scores)

	Low *Z* score	High *Z* score	Total	*P* value
Genotype				
CC (Ser/Ser)	42 (33.6%)	24 (32.2%)	64	
CG (Ser/Trp)	60 (48.0%)	39 (32.3%)	103	
GG (Trp/Trp)	23 (18.4%)	29 (32.4%)	49	
Total	125 (100%)	92 (100%)	216	0.041[a]
Allele				
C Allele (Ser)	144 (57.6%)	87 (47.3%)	231	
G Allele (Trp)	106 (42.4%)	97 (52.7%)	201	
Total	250 (100%)	184 (100%)	432	0.041[b]

[a] *P* value was calculated by χ^2 test of trend ($P < 0.05$).
[b] *P* value was calculated by Fisher's exact test ($P < 0.05$).