Vitamin D and phosphate regulate fibroblast growth factor-23 in K-562 cells

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FGF-23 reproduces hypophosphatemic osteomalacia and the (28, 31). In addition, continuous exposure to recombinant growth factor (FGF)-23 as a likely candidate for phosphatonin parathyroid hormone (PTH) levels are usually normal (8, 9). Patients with these diseases because their plasma calcium and hypophosphatemic rickets (ADHR; see Refs. 28, 31, and 32), bone are shared by X-linked hypophosphatemic rickets (XLH), from renal phosphate wasting and impaired mineralization of the largely based RT-PCR analysis suggested that FGF-23 products. However, it is still unclear whether FGF-23 expression is regulated by 1,25(OH)2D3 because of the decreased expression of renal 1α-OHase and increased expression of renal 24-OHase (26). Collectively, these findings further support the idea that FGF-23 acts physiologically to reduce plasma phosphate and 1,25(OH)2D3 levels.

Recent advances have led to a better understanding of the function of FGF-23. The largely based RT-PCR analysis suggests that FGF-23 is produced in liver, lymph nodes, thymus, heart, K-562, and Raji cell lines (28, 31, 34). In a recent report, Liu et al. (16) demonstrated that bone is the predominant tissue for FGF-23 products. However, it is still unclear whether FGF-23 expression is regulated by 1,25(OH)2D3, PTH, calcitonic, plasma calcium, and plasma phosphate. In the current report, we show that the FGF-23 promoter is activated in the erythroleukemia cell line K-562 and regulated by 1,25(OH)2D3 and plasma phosphate.

MATERIALS AND METHODS

Animals and diet. Vitamin D receptor (VDR) (−/−) mice were generated by gene targeting as previously described (10, 36). VDR genotypes were confirmed by analyzing the DNA obtained from each mouse ∼3 wk after birth. Genomic DNA was extracted from a tail
clipping and amplified by PCR using primers specific for VDR (+/+)
exon 2 or the neomycin resistance gene, as described previously (24).
VDR(+/+) and VDR(−/−) mice were weaned at 3 wk of age and
given free access to water and a control diet containing 0.5% phos-
phate and 0.5% calcium for 5 wk (24). Male C57BL mice were
purchased from SLC (Shizuoka, Japan) and housed in plastic cages.
The 6-wk-old animals were fed standard mouse chow (Oriental,
Osaka, Japan) ad libitum for the 1st wk. After this period, they
received a diet containing 0.6% calcium and 0.6% phosphate for 5
days. On the 6th day, three groups of six mice each were established:
a control phosphate group, which was fed a diet containing 0.6% phos-
phate; a low-phosphate group, which was fed a diet containing
0.02% phosphate; and a high-phosphate group, which was fed a diet
containing 1.2% phosphate. After 7 days of diet administration, all
mice were used for the experiments.

Cell culture. Human chronic myelogenous leukemia K-562 cells,
human hepatocarcinoma cells (Hep G2), human Burkitt lymphoma
cells (Raji), and MC3T3-E1 cells derived from mouse calvaria were
obtained from the RIKEN Bioresource Center (Tokyo, Japan). K-562
and Raji cells were cultured in Ham’s F-12 medium (Sigma) supple-
mented with 10% FCS and 2 mM L-glutamine and antibiotics.
Hep G2 and MC3T3-E1 cells were cultured in DMEM (Invitrogen) sup-
pplemented with 100 units/ml penicillin/streptomycin; Invitrogen,
Carlsbad, CA) for growth media. Hep G2 and MC3T3-E1 cells were
cultured in DMEM (Invitrogen) supplemented with 10% FCS and 2 mM L-glutamine and antibiotics.
Subcultivations were performed every 3–4 days. All cell cultures
were kept at 37°C in a humidified environment (5% CO2-95% air).
For the experiments of 1.25(OH)2D3 and other hormones, FCS was
replaced with charcoal-treated FCS to remove endogenous steroids.
Phosphate-free DMEM (Invitrogen) was used for phosphate concen-
tration experiments.

Isolation of the promoter region of the mouse FGF-23 gene. To
characterize the promoter region of the FGF-23 gene, we performed a
gov/BLAST/) using a segment of DNA encompassing the FGF-23
promoter region on chromosome 6 (GenBank accession no.
NT_039356) as a probe. The mouse FGF-23 genes were cloned by
PCR using genomic DNA isolated from the mouse kidney, the primers
listed in Table 1, and pfu polymerase (Promega, Madison, WI). The
PCR amplifications were performed using primers pro1 and pro7 as
follows: 3 min at 94°C; followed by 25 cycles of 94°C for 30 s, 63°C for
30 s, and 72°C for 90 s; and a final extension at 72°C for 3 min.
The amplification products were detected on a 0.8% agarose gel and
visualized by ethidium bromide staining. PCR products <2 kb were
purified and subcloned into pCR2.1. Genomic FGF-23 promoter
regions were sequenced by an automated dye termination method
(Amersham Biosciences, Piscataway, NJ).

PCR analysis to detect FGF-23 expression in cells. Total mRNA
was purified using Isogen reagent (Wako Pure Chemicals, Tokyo,
Japan) from K-562, Raji, and Hep G2 cells cultured in growth media.
RT-PCR was performed using total RNA and the RT system from a
first-strand synthesis kit (Invitrogen) according to the manufacturer’s
protocol. The cDNA generated was used for nested PCR along with
human FGF-23-specific primers (Table 2). The first PCR was carried
out as follows: initial melting for 3 min at 94°C; followed by 20 cycles
of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C; and a final extension
for 3 min at 72°C. A second PCR, using the first PCR products (1:100
diluted) as a template and the second set of PCR primers, was
performed to detect FGF-23 as follows: initial melting for 3 min at
94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s
at 72°C; and a final extension step for 3 min at 72°C. The amplifica-
tion products were detected on a 1.5% agarose gel. All PCR fragments
were subcloned into the pCR2.1 vector (Invitrogen), and the expected
amplification products were confirmed by sequencing. The position of
the primers and the expected size of the amplification product from
the FGF-23 mRNA is shown in Fig. 2A. As an internal control,
glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was amplified
using the following primers: 5’-CTGACACACCAACTGCTTAGC-3’
(forward) and 5’-GCTGCCTGACACCCCTTCTTG-3’ (reverse).

Construction of reporter plasmids. The reporter plasmids used
in this study were derived from the pGL3-Basic vector (Promega), which
contains the firefly luciferase reporter gene. The mouse FGF-23
promoter-reporter constructs pGL1–0.4, pGL1–0.9, pGL1–0.6, pGL1–
0.4, pGL1–0.13, and pGL1+1 were generated by PCR followed by
digestion with restriction enzymes. A 1,430-bp genomic fragment was
amplified by PCR from the 2,000-bp subfragment using an antisense
primer starting at bp –1,403 (pro2, see Table 1) and a sense primer
starting at bp +98 (pro6) on the FGF-23 gene. The fragment was
digested with KpnI and HindIII, subcloned into KpnI/HindIII-digested
pBluescript SK− to create pBlu1430, and sequenced. To generate the
luciferase reporter gene construct pGL1–1.4, the insert was excised
from pBlu1430 with KpnI and HindIII within the primer sequence and
then subcloned into the KpnI/HindIII-digested pGL3 basic vector to
create pGL1–1.4. The gene constructs pGL1–0.9, pGL1–0.4, pGL1–
0.3, and pGL1+1 were generated by PCR with a sense primer (pro3,
pro4, pro10, or pro9) and antisense primer pro6. These fragments
were also subcloned in the same way as pGL1–1.4 into the pBlue-
script SK− and pGL3 basic vectors. A 554-bp and a 128-bp genomic
DNA fragment were generated by enzyme digestion of pBlu1430
within the primer using HindIII or within the genome sequences using
SacI. These fragments were subcloned into SacI/HindIII-digested pGL3
basic vector and sequenced on both strands. The 3′-end of all
constructs ends at bp +98 bp in the mouse FGF-23 gene. We used the
PCR to delete the sequence of the TATA-like box (pGL1/DATA)
with primer pro13 and a M13 primer RV (5’-CAGGAAAACGCTAT-
GAC-3’: Takara Bio, Tokyo, Japan) from the pBluescript SK− vector.
The PCR fragment was inserted in pGL1–128 with SmaI-digested

Table 1. Oligonucleotides used in PCR cloning and deletion
constructs of mouse FGF-23 genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense/Antisense</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro1</td>
<td>Sense</td>
<td>TGAAGTGGCTGCTGATCTTACTC</td>
</tr>
<tr>
<td>pro2</td>
<td>Sense</td>
<td>ggtaccAGAGAACGTAGACAGACAG</td>
</tr>
<tr>
<td>pro3</td>
<td>Sense</td>
<td>KpnI</td>
</tr>
<tr>
<td>pro4</td>
<td>Sense</td>
<td>ggtaccCTGAGAGGGTATGAGTTACG</td>
</tr>
<tr>
<td>pro5</td>
<td>Antisense</td>
<td>HindIII</td>
</tr>
<tr>
<td>pro6</td>
<td>Antisense</td>
<td>ctgcaAGACGACAGACAG</td>
</tr>
<tr>
<td>pro7</td>
<td>Sense</td>
<td>ggtaccATATCGCGAGATCCOCC</td>
</tr>
<tr>
<td>pro10</td>
<td>Sense</td>
<td>KpnI</td>
</tr>
<tr>
<td>pro11</td>
<td>Sense</td>
<td>ATTCTGCGAGAGGTAGTCAGACAG</td>
</tr>
<tr>
<td>pro12</td>
<td>Sense</td>
<td>SmaI</td>
</tr>
<tr>
<td>pro13</td>
<td>Sense</td>
<td>GACGACCATGTTGCTGAGTACAG</td>
</tr>
<tr>
<td>pro14</td>
<td>Sense</td>
<td>GACGACCATGTTGCTGAGTACAG</td>
</tr>
<tr>
<td>pro15</td>
<td>Sense</td>
<td>HindIII</td>
</tr>
<tr>
<td>pro16</td>
<td>Sense</td>
<td>CCGCCTCGATTTGAGATAGTGG</td>
</tr>
<tr>
<td>pro17</td>
<td>Sense</td>
<td>ATTATCGATTTGAGATAGTGG</td>
</tr>
</tbody>
</table>

FGF, fibroblast growth factor. Small letters indicate restriction enzyme
recognition sites. Underlines depict additional sequences for deletion constructs.

Table 2. Oligonucleotides used in nested PCR cloning of human FGF-23

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense/Antisense</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hFGF</td>
<td>Sense</td>
<td>CTCTGCGATGATTTGAGAGC</td>
</tr>
<tr>
<td>hFGF23</td>
<td>Sense</td>
<td>GAGCTCTCTGUGATACAGAGG</td>
</tr>
<tr>
<td>hFGF23</td>
<td>Antisense</td>
<td>CCGTCGATGATTTGAGAGC</td>
</tr>
<tr>
<td>hFGF23</td>
<td>Sense</td>
<td>AACAGCGAGGTCGTCTTCG</td>
</tr>
<tr>
<td>hFGF23</td>
<td>Antisense</td>
<td>ATATTGCGCTATTGAGTTTC</td>
</tr>
<tr>
<td>hFGF23</td>
<td>Sense</td>
<td>CACAGCGAGGTCGTCTTCG</td>
</tr>
<tr>
<td>hFGF23</td>
<td>Antisense</td>
<td>GACGACCATGTTGCTGAGTACAG</td>
</tr>
</tbody>
</table>

hFGF, human FGF.
plasmid. For the pGLΔMCM construct in which the C-Ets-1 and MZF1 motifs were deleted, we performed three rounds of PCR with pro11 and the M13 primer RV (first round) and pro12 and pro6 (second round) using a 0.6-kb fragment from the pBluescript SK− vector for template. After these PCR, the PCR fragments were diluted and mixed, and the third round PCR was performed using the M13 primer RV and pro6 primers. We also performed three rounds of PCR to generate constructs lacking the vitamin D response element (VDRE)-like motif (VLM): for pGLΔVLM-1, the first round of PCR was carried out using the M13 primer RV and pro14, and the second round with pro6 and pro15 using a 0.6-kb fragment from the pBluescript SK− vector for template; and for pGLΔVLM-2, the first round of PCR was carried out using primer pro16 and pro17 using a 0.6-kb fragment from the pBluescript SK− vector for template. The third round PCR was performed using the M13 primer RV and pro6 primers. The fragments were digested with SacI and HindIII and then inserted in the pGL3 basic vector. All constructs were confirmed by sequencing on both strands.

Transient transfection and functional promoter analysis. K-562, Hep G2, and MC3T3-E1 cells were cultured in 24-well plates in culture medium without antibiotics. After 24 h (when cells reached 80–90% confluence), liposome-mediated transfection was performed. Promoter vector DNA (1 μg), 0.05 μg of phRL-TK (Renilla luciferase reporter construct used as an internal standard; Promega), and 4 μl Lipofectamine2000 (Invitrogen) were mixed with 100 μl of Opti-MEM medium (Invitrogen) for 15 min at room temperature. The mixture was then added to the cells and incubated for 48 h, after which the cells were harvested for reporter gene assays. Promoter reporter assays were performed using the Dual Luciferase assay kit (Promega) according to the manufacturer’s instructions. Luciferase activities were measured with a Lumat LB9507 luminometer (Berthold Detection Systems, Pforzheim, Germany), and all assays provided data that were well within the linear range of the instrument. The experiments were performed in triplicate wells and were repeated at least three times.

For studies related to the effect of phosphate concentration, the K-562 cells were plated in 24-well plates in triplicate wells, and the cells were kept for 24 h in appropriate medium containing 10% FCS and without antibiotics. Subsequently, cultured cells were transfected with the plasmid constructs and incubation for 4 h, followed by the addition of hormones or phosphate. To determine the effects of hormones, the cells were incubated for 24 h in 1.25(OH)2D3 (10−9 to 10−6 M) or in vehicle in Ham’s F-12 medium containing 10% charcoal-treated FCS. The cells were then harvested for reporter gene assays. For studies of the effect of different phosphate concentrations, the cells were kept in phosphate-free DMEM (Invitrogen) supplemented with 0.1, 0.9, or 1.5 mM Na2HPO4-NaH2PO4 (pH 7.4) or in Ham’s F-12 medium containing 1 mM phosphate supplemented to 3 or 6 mM phosphate and then incubated with 24 h in 10−8 M 1.25(OH)2D3. To determine the effect of different concentrations of calcium, cells were kept in Ham’s F-12 medium containing 0.3 mM calcium supplemented to 1 or 6 mM with CaCl2 and then incubated for 24 h with 10−8 M 1.25(OH)2D3.

Quantitative real-time PCR for endogenous FGF-23 gene expression. K-562 cells were treated for 24 h with 10−8 M 1.25(OH)2D3 in high-phosphate or normal growth medium. The cDNA synthesized from 1 μg of total RNA was used for real-time PCR (LightCycler; Roche Diagnostics, Indianapolis, IN) using a Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA). Each sample was analyzed in triplicate. For amplification of a 147-bp fragment of the FGF-23, the primers (hFGF23-p3 and 5′-TCCAGCGTCTGGTGGTTGGAAAC-3′) were used. The mRNA levels were normalized to the GAPDH mRNA level. Primers for amplification of a 114-bp GAPDH fragment were the GAPDH forward primer and 5′-CTTCTGGGTGGACAGTGGAT-GGC-3′ (reverse 2), and the annealing temperature was set at 55°C.

Transfection and detection of endogenous VDR. The human VDR (hVDR) expression vector pSG5/hVDR (2) was used for cotransfection experiments. The pSG5 empty vector (Invitrogen) was used for control experiments. After 24 h (when cells reached 80–90% confluence), cells were transfected for 4 h with 1 μg promoter vector DNA, 0.05 μg phRL-TK, and 0.2 μg pSG5/hVDR using Lipofectamine2000. Next, cells were treated for 24 h with phosphate, calcium, and/or 1,25(OH)2D3 and then harvested for reporter gene promoter assays. Gene expression of VDR was analyzed by real-time PCR. Primer sequences (5′-GGTGGACTGAAGCTGACACACGC-3′ and 5′-TGTGTTGGACAGCCGGTCTCTG-3′) for amplification of a 135-bp fragment of the VDR were generated based on GeneBank sequence number J03258.

Detection of plasma FGF-23. The plasma concentration of the endogenous FGF-23 (mouse) was determined using a FGF-23 ELISA kit (Kainos Laboratories, Tokyo, Japan).

Statistical analysis. Results are reported as means ± SE for at least three samples. The statistical significance of differences between
measured values was determined by ANOVA using GraphPad InStat (GraphPad Software, San Diego, CA). Kinetic parameters of uptake were determined by linear regression analysis of Lineweaver-Burk plots.

RESULTS

Isolation and structure of the mouse FGF-23 5'-flanking region. The mouse FGF-23 gene was cloned and characterized using PCR and the primers listed in Table 1. Primer sequences were derived from the mouse FGF-23 cDNA sequences (GenBank accession no. NM_022657). The transcription initiation site of the mouse FGF-23 gene was determined by 5'-RACE (data not shown). The 5'-flanking DNA sequence (~1400 nucleotides) is shown in Fig. 1 and was analyzed for putative trans-acting factor binding sites using the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html). The promoter region of the mouse and human FGF-23 gene has ~58% identity (data not shown). There are two highly conserved regions in the promoter region of the mouse FGF-23 promoter, one of which is a classic TATA-like box (TFIID-binding element). The TATA-like box was identified 27 bp upstream of the transcription start site and is located at a similar position within the human FGF-23 promoter. However, neither AP1 or Sp1 binding sites nor glucocorticoid T3 or VDRE were found. However, the 1.4-kb FGF-23 promoter fragment contains a number of MZF-1, GATA-1, GATA-2, and C-Ets-1 motifs that are recognized by well-characterized transcription factors in lymphocytes and T cells.

Cell specificity and abundance of FGF-23 mRNA. Before proceeding with the functional analysis of the 5'-flanking region of the FGF-23 gene, we identified cells expressing or lacking FGF-23 mRNA. Recent reports, largely based on RT-PCR analysis, suggest that FGF-23 is produced in liver, lymph nodes, thymus, heart, K-562, and Raji cell lines (28, 31, 34). We confirmed expression in the cell lines by nested-PCR analysis using various primers (Table 2 and Fig. 2A). We detected FGF-23 mRNA expression in the human leukemia cell line K-562 but failed to detect FGF-23 transcripts in Hep G2 and Raji cells (Fig. 2B). We also examined the expression of FGF-23 in other cell lines, including primary osteoblasts derived from mouse calvaria, MC3T3-E1, ST2, and Saos-2 cells, but failed to detect it (data not shown).

Functional characterization of the FGF-23 promoter in K-562 cells. To investigate the transcription of the FGF-23 gene, we evaluated the effect of several deletions of the FGF-23-luciferase promoter constructs in K-562 cells (Fig. 3). Progressive 5'-deletion mutants of the mouse FGF-23 promoter revealed a bimodal pattern of functional activity in transiently transfected K-562 cells (Fig. 3). The pGL−0.6 promoter construct (~554 to +98) caused a nearly a sixfold increase in FGF-23 mRNA expression K-562 cells. Progressive 5'-deletions relative to the transcription start site caused a gradual reduction in promoter activity. Further deletions from −1430 to −554 bp, however, led to an increase in promoter activity, although it did not exceed the activity of the pGL−0.6 promoter. Successive
deletions from −128 bp (pGL/−0.13) lead to a clear reduction in promoter activity in K-562 cells, resulting in a level activity close to that of the basic plasmid alone.

To elucidate whether the two motifs from −275 bp to −256 bp (C-Ets-1 and MZF-1) are essential for promoter activity, we examined the activity of deletion mutant pGL/−0.3 (−246 to +98) and pGL/ΔMC constructs lacking only the two motif sequences in pGL/−0.6 (Fig. 4). The promoter activities of these deletion mutants were lower than that observed with pGL/−0.6. These results suggested that the two motifs are important for promoter activity in K-562 cells. The construct with a 10-bp deletion lacked the TATA-like box (pGL/ΔTATA) in pGL/−0.6 and had luciferase activity that was the same as the basic plasmid alone, suggesting that it functions as a TFIID-binding element.

We next examined the specificity of the FGF-23 promoter activity in Hep G2, Raji, and osteoblastic MC3T3-E1 cells (data not shown). Although the various FGF-23 promoter constructs were transiently transfected in cells, promoter activity was not detected. These findings were consistent with the results of the RT-PCR and suggested that the promoter activities in K-562 are specifically regulated.

Effect of phosphate- and calcium-regulating hormone on FGF-23 promoter activity. We used a transient expression assay to determine whether 1,25(OH)2D3 enhance FGF-23 gene promoter (pGL/−0.6) activity in K-562 cells (Fig. 5A). Transfected cells were treated with 1.25(OH)2D3 at a dose ranging from 10−9 to 10−6 M, and luciferase activity was recorded after 24 h. We observed a dose-dependent effect of 1,25(OH)2D3 to increase luciferase activity in K-562 cells transfected with pGL/−0.6.

The VDRE consensus sequence (A/GGG/TCTA/G nng A/GGTTCA/G, where n is optional nucleotide) was not found in the 1.4-kb FGF-23 promoter region (Fig. 1), but there were two similar VDRE half-site regions (VLML1 and VLM2) between −275 and −182. To test whether these motifs function as VDRES, deletion constructs for VLML1 and VLM2 were transfected, and then their response to 1,25(OH)2D3 was examined. As shown in Fig. 5B, the two constructs still responded to 1,25(OH)2D3 in high-phosphate medium.

Effect of phosphate concentration on FGF-23 promoter activity. To test whether phosphate level regulates FGF-23 expression, we assayed the promoter activity at different phosphate concentrations in the medium. High-phosphate (3 mM) medium caused a significant increase (1.7-fold) in the promoter activity of pGL/−0.6 (Fig. 6A). The FGF-23 promoter did not respond to low-phosphate (0.1 mM) or normal phosphate (1.5 mM) medium (data not shown). In addition, FGF-23 promoter activity was increased 2.7-fold after treatment with 10−8 M 1,25(OH)2D3 in the presence of 1.0 mM phosphate, and it

Fig. 5. Effect of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] and VLM on FGF-23 promoter activity. A: cells were transiently transfected and then treated with 1,25(OH)2D3 (10−8 M to 10−6 M) or vehicle 4 h after transfection. After 24 h, the cells were collected in cell lysis buffer and analyzed for luciferase activity. Values represent means ± SE from 3 separate experiments. *P < 0.05 for treatment of 1,25(OH)2D3 vs. vehicle for pGL/−0.6. B: K-562 cells were transfected with the promoter constructs pGL/−0.6 and two deletion constructs of VLM-1 or -2 (pGL/ΔVLM-1, VLM-2). Cells were treated with 10−8 M 1,25(OH)2D3 and 1 mM phosphate for 24 h. Luciferase activity is related to pGL3 basic vector alone and to cells treated with 1 mM phosphate but without 1,25(OH)2D3 for each construct. Values represent means ± SE from 6 separate experiments. *P < 0.05.
synergistically enhanced (4.0-fold) the promoter activity in the high-phosphate medium.

**Effect of phosphate and 1,25(OH)_2D_3 on endogenous FGF-23 gene expression in K-562 cells.** K-562 cells were treated with 1,25(OH)_2D_3 in high-phosphate medium for 24 h, and the endogenous expression of FGF-23 was detected by RT-PCR (Fig. 6B). The quantitative real-time PCR analysis clearly showed that the abundance of FGF-23 mRNA was significantly increased (3.3-fold) in 1,25(OH)_2D_3-treated cells in the presence of 3 mM phosphate, but there was no change in 6 mM phosphate (Fig. 6C). Also, there was no change in GAPDH mRNA abundance upon treatment with vehicle or 1,25(OH)_2D_3 in high-phosphate medium. To test whether calcium affects FGF-23 promoter activity, we assayed the promoter activity in medium containing 0.3–5 mM CaCl_2 (data not shown). The promoter activity of FGF-23 was unaffected in this range of calcium concentrations, and calcium had no synergistic effect in combination with 1,25(OH)_2D_3 (Fig. 7).

**Activation of VDR dependent on FGF-23 promoter.** To test whether exogenous VDR affects FGF-23 promoter activity, we examined the effect of cotransfection of hVDR and FGF-23 promoter construct pGL/-0.6 in K-562 cells (Fig. 8A). Enhancement of promoter activity with 1,25(OH)_2D_3 was greater when VDR was cotransfected. This suggests that elevation of VDR stimulates FGF-23 promoter activity.

We next examined whether 1,25(OH)_2D_3 and phosphate affect the level of the VDR mRNA. As shown in Fig. 8B, endogenous VDR levels were stimulated by addition of 1,25(OH)_2D_3 but not by the addition of phosphate. These results suggested that the cellular VDR levels are an important determinant of FGF-23 promoter activity. In contrast, the synergistic effect of phosphate and 1,25(OH)_2D_3 was not dependent on the level of the VDR.
Effect of dietary phosphate on the levels of plasma FGF-23 in vivo. To further investigate whether dietary phosphate affects the levels of plasma FGF-23 in normal mice, we measured the concentration of plasma FGF-23 in low (0.02%), normal (0.6%), and high (1.2%) inorganic phosphate diets. The plasma phosphate levels were decreased in mice fed a low-phosphate diet (3.5 ± 0.4 mg/dl) and increased in mice fed a high-phosphate diet (9.8 ± 2.1 mg/dl). Moreover, as shown in Fig. 9A, the levels of plasma FGF-23 were significantly increased in mice fed a high-phosphate diet compared with those receiving normal and low-phosphate diets. In contrast, the levels of plasma FGF-23 in mice fed a low-phosphate diet were significantly decreased compared with those receiving the normal phosphate diet.

Effect of plasma FGF-23 on the VDR knockout mice in vivo. Finally, we examined the levels of FGF-23 in VDR knockout mice (VDR-KO). VDR-KO mice displayed hypophosphatemia and hypocalcemia as described previously (10, 36). As shown in Fig. 9B, the levels of plasma FGF-23 in 8-wk-old VDR-KO mice were significantly decreased compared with in the levels in wild-type mice.

DISCUSSION

In the present study, we characterized the 5′-flanking region of the mouse FGF-23 and indicated the regulation by phosphate and 1,25(OH)2D3. FGF-23 is overexpressed in several tumors that cause TIO. In TIO patients, the most frequent histological diagnosis has been in mesenchymal tissues, such as in hemangioepicytomas, mesenchymal tumors with vascular elements, epidermal naevi, osteosarcomas, and chondrosarcomas (14, 20, 30). K-562 is a human erythroleukemic cell line derived from human chronic myelogenous leukemia and the proliferation of erythroid progenitor cells in human bone marrow culture. The leukemias are now also considered to be angiogenesis-dependent malignancies (1). Thus we suggest that the regulation of expression and physiological roles of FGF-23 in erythroleukemia K-562 cells can provide new insights into the homeostatic control of phosphate metabolism.

Plasma FGF-23 levels were increased in mice fed a high-phosphate diet, and the FGF-23 promoter activity was upregulated in high-phosphate medium containing 1,25(OH)2D3. This is the first report that dietary phosphate affects the levels of plasma FGF-23 and that phosphate in the medium affects the transcription of the FGF-23 gene. A connection between phosphate levels and FGF-23 has also been shown in mutant mice lacking FGF-23, which exhibit severe hyperphosphatemia, and in transgenic FGF-23 animals, which show hypophosphatemia (27, 29). In the present study, we further demonstrated that plasma phosphate levels are important regulators of the transcription via the FGF-23 gene promoter.

In a previous study, we identified the phosphate-responsive element (PRE) of the human type IIa Na-Pi cotransporter gene (12, 17). The corresponding DNA-binding protein was identified as the transcription factor TFE3. In this study, we showed that a low-phosphate diet increases the transcription of the type IIa Na-Pi cotransporter gene and that TFE3 binds the PRE in
the type IIa Na-Pi cotransporter gene (12, 17). However, in the current study, the PRE was not detected in the mouse FGF-23 gene promoter region. Other reports demonstrated that phosphate can act as a signaling molecule capable of altering gene expression in osteoblast development, which prompted us to conduct a microarray analysis of phosphate-treated osteoblasts (4, 5). Beck et al. (5) showed that the basic region leucine zipper transcription factor Nrf2 (NF-E2-related factor 2) was upregulated in the osteoblast response to the high-phosphate medium. We found several binding motifs for leucine zipper transcription factors, such as cAMP regulatory element binding protein, in the mouse FGF-23 gene promoter. We are currently investigating the roles of Nrf2 in the regulation of FGF-23 gene promoter.

In addition to showing that phosphate regulates FGF-23 expression, we also demonstrated that 1,25(OH)2D3 controls the transcription of the mouse FGF-23 gene in K-562 cells. Several reports indicate that leukemia cell lines, including U-937 and HL-60, are differentiated to an erythroid phenotype by 1,25(OH)2D3 but that the activation pathways may be different.

FGF-23 promoter. Indeed, the levels of plasma FGF-23 in the VDR-KO mice were significantly lower than in wild-type mice. Thus the upregulation of the FGF-23 promoter by 1,25(OH)2D3 is not the result of nonspecific differentiation of K-562 cells.

In the promoter region of the mouse FGF-23 gene, we did not find the consensus VDRE. In K-562 cells cotransfected with VDR and FGF-23 promoter construct pGL–0.6, 1,25(OH)2D3 markedly increased the activity of the mouse FGF-23 promoter. Indeed, the levels of plasma FGF-23 in the VDR-KO mice were significantly lower than in wild-type mice. Thus the upregulation of the FGF-23 promoter by 1,25(OH)2D3 may be because of the presence of cellular VDR.

The FGF-23 promoter activity was also upregulated by high phosphate and 1,25(OH)2D3; however, high phosphate did not increase the activity. These results suggested that the activation of FGF-23 promoter was regulated by phosphate and 1,25(OH)2D3 but that the activation pathways may be different. For the 24-OHase gene promoter, the action of 1,25(OH)2D3 is mediated by Ras-dependent activation of ERK1/ERK2 and ERK5 (7). The 24-OHase promoter has two VDREs and an Ets-1 binding site. The induction of the 24-OHase expression by 1,25(OH)2D3 is mediated by ERK5 phosphorylation of Ets-1. We found two c-Ets-1-binding motifs in the mouse FGF-23 promoter region. Therefore, the activation of the FGF-23 promoter by vitamin D may be mediated by the ERK/ERK2 and ERK5 signal pathway. A recent report indicates that the regulation of osteopontin expression by inorganic phosphate is mediated by ERK1/2 and protein kinase C signaling pathways (4). These results suggest that high phosphate affects the activity of the VDR, but further studies are needed to clarify the regulation of the VDR by 1,25(OH)2D3 and phosphate.

Several recent studies have demonstrated that FGF-23 is an important factor in vitamin D metabolism (15, 22, 27, 29). Injection of FGF-23 in mice reduces 1,25(OH)2D3 in plasma (26, 29). In FGF-23 null mice, Shimada et al. (27) showed that there is an elevation in plasma 1,25(OH)2D3 that is the result of the enhanced expression of renal 1α-OHase. FGF-23 is decreased by 1,25(OH)2D3 because of downregulation of 1α-OHase and induction of renal 24-OHase in vivo (26). It is well known that 1,25(OH)2D3 has a negative feedback mechanism in which increased 1,25(OH)2D3 directly regulates 1α-OHase and 24-OHase gene expression via a VDR. The present study also suggests a negative feedback pathway involving FGF-23 because 1,25(OH)2D3 enhanced FGF-23 promoter activity and mRNA expression. We are currently investigating the role of VDR on the expression of FGF-23 in K-562 cells.

PTH is also well known as a phosphate-regulating hormone that inhibits renal phosphate reabsorption by affecting the type IIa Na-Pi, cotransporter and by indirectly increasing intestinal phosphate absorption by stimulating the synthesis of 1,25(OH)2D3 (11, 19). In renal proximal tubules, long-term treatment (5–9 h) with PTH causes downregulation of FGF-23 (26), but the type IIa Na-Pi, cotransporter proteins are decreased within 2 h (11, 19). Therefore, FGF-23 seems to participate in a PTH-independent pathway (26).

In summary, we demonstrated that vitamin D and plasma phosphate are important regulators of the transcription of the mouse FGF-23 gene. These two factors have a synergistic effect on FGF-23 expression. Further studies are needed to clarify the physiological roles of FGF-23 expression in erythroleukemic cell lines.

**REFERENCES**


