1,25-Dihydroxyvitamin D₃ Down-regulation of PHEX Gene Expression Is Mediated by Apparent Repression of a 110 kDa Transfactor That Binds to a Polyadenine Element in the Promoter*

The PHEX gene encodes an endopeptidase expressed in osteoblasts that inactivates an uncharacterized peptide hormone, phosphatonin, which suppresses bone mineralization as well as renal phosphate reabsorption and vitamin D bioactivation. We demonstrate that 1a-25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active renal vitamin D metabolite, decreases PHEX mRNA in the rat osteoblastic cell line, UMR-106, as well as in mouse calvaria. Promoter/reporter construct analysis of the murine PHEX gene in transfected UMR-106 cells localized the repressive effect of 1,25(OH)₂D₃ to the −133 to −74 bp region, and gel mobility shift experiments revealed that 1,25(OH)₂D₃ treatment of the cells diminished the binding of a nuclear protein(s) to a stretch of 17 adenines from bp −116 to −100 in the proximal PHEX promoter. Either overexpression of a dominant-negative vitamin D receptor (VDR) or deletion of this sequence of 17 A-T base pairs abolished the repressive effect of 1,25(OH)₂D₃ by attenuating basal promoter activity, indicating that this region mediates the 1,25(OH)₂D₃ response and is involved in basal transcription. Southwestern blot analysis and DNA affinity purification show that an unidentified 110 kDa nuclear protein binds to the poly(A) element. Because 1,25(OH)₂D₃-liganded VDR neither binds to the polyadenine region of the PHEX promoter nor directly influences the association of the 110 kDa transfactor, we conclude that 1,25(OH)₂D₃ indirectly decreases PHEX expression via VDR-mediated repression (or modification) of this novel transactivator. Thus, we have identified a cis-element required for PHEX gene transcription that participates in negative feedback control of PHEX expression and thereby modulates the actions of phosphatonin.

Bone mineral homeostasis is achieved by the concerted actions of several hormones that control the circulating levels of calcium and phosphate, as well as directly affect osteoblast-mediated mineralization and/or osteoclast-driven resorption (1, 2). Thus, skeletal mineral integrity is maintained by the availability of calcium and phosphate ions and by a coupled balance between the activities of osteoblasts and osteoclasts. Prominent among the endocrine factors that regulate bone mineral is the sterol, 1α25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active renal metabolite of vitamin D₃ (3). 1,25(OH)₂D₃ functions via its nuclear vitamin D receptor (VDR) to induce intestinal calcium and phosphate absorption, as well as renal phosphate reabsorption, thereby preventing rickets/osteomalacia by ensuring adequate blood concentrations of these ions to facilitate bone mineralization (4, 5). Osteoblasts represent another target for 1,25(OH)₂D₃, where the sterol acts via VDR to induce bone remodeling proteins such as osteocalcin (6) and osteopontin (7), as well as the receptor activator of NF-κB ligand (RANKL), which is a paracrine signal for osteoclastogenesis (8). Therefore, as a calcemic and phosphaturic hormone, 1,25(OH)₂D₃ operates directly to resorb/remodel bone, and we show in the present communication that this steroid paradoxically also inhibits mineralization by cultured osteoblasts. The mechanism whereby 1,25(OH)₂D₃ limits bone mineralization is herein characterized as the apparent up-regulation of a novel phosphaturic peptide(s), termed phosphatonin, via a repression in osteoblasts of the expression of a gene encoding a neutral endopeptidase PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) that is hypothesized to normally inactivate phosphatonin by proteolysis.

Phosphatonin, an uncharacterized phosphaturic hormone that may include in part FGF23 (9), is an inhibitor of osteoblastic mineralization, which also blocks renal 25-OH-vitamin D₃ bioactivation to 1,25(OH)₂D₃, and elicits hypophosphatemia by repressing the renal type IIa sodium phosphate cotransporter (NaP₂-H₃a) (10). The PHEX gene encodes an endopeptidase that is predominantly expressed in osteoblasts and osteocytes (11, 12). Phenotypically, inactivating mutations in the PHEX gene results in vitamin D-resistant, X-linked hypophosphatemic rickets (X-linked hypophosphatemia; XLH) (11). This familial disorder manifests as hypophosphatemia, low circulating...
ing 1,25(OH)_{2}D_{3} levels for the prevailing blood phosphate, high serum alkaline phosphatase, and osteomalacia (13). A theoretical model postulates that phosphatonin regulates P, reabsorption through changes in renal NaP_{2}-IIa cotransporter expression. PHEX normally inactivates phosphatonin, limiting P excretion; in XLH, the mutant PHEX fails to inactivate phosphatonin, resulting in phosphaturia and hypophosphatemia (10). The defects in bone associated with XLH are not fully attributable to hypophosphatemia, and there is evidence for an intrinsic abnormality in the osteoblast (14). This hypothesis is supported by the observation that osteoblasts from Hyp mice (the murine homologue of XLH, deficient in PHEX), fail to mineralize in media supporting mineralization of normal osteoblasts. Further, when co-cultured with Hyp osteoblasts, normal osteoblasts exhibit a significant decrease in mineralization (14). Finally, targeted expression of PHEX in Hyp mice osteoblasts is not sufficient to rescue the Hyp phenotype (15, 16). These observations highlight the significance of the PHEX endopeptidase gene product in promoting osteoblastic mineralization, presumably by inactivating the phosphatonin anti-matrix metalloproteinase.

Regulation of PHEX both in vivo and in vitro by several hormones important for skeletal homeostasis has been reported. Up-regulation of PHEX expression was observed with IGF-I (17, 18) and growth hormone (GH) (18) treatment of laboratory rodents, and with glucocorticoid exposure of cultured cells (19). Conversely, PHEX was found to be down-regulated by parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) in UMR-106 osteoblastic cells (20). 1,25(OH)_{2}D_{3} down-regulation of PHEX has been shown at the mRNA and protein levels in both cultured mouse osteoblasts (21) and more recently in 1,25(OH)_{2}D_{3}-treated rats (22). In primary osteoblasts and MC3T3-E1 cells, 1,25(OH)_{2}D_{3}-mediated PHEX down-regulation was independent of the mineralization state of the cells (21). Additionally, 1,25(OH)_{2}D_{3} has also been reported to decrease mineralization in osteoblasts grown in culture (21, 23). Finally, an altered mineralization response to 1,25(OH)_{2}D_{3} was observed in osteoblasts isolated from Hyp mice compared with those isolated from normal mice (24). These studies highlight the physiological relevance of PHEX in osteoblast function and point to the important effects of 1,25(OH)_{2}D_{3} on PHEX expression in these bone-mineralizing cells.

We have previously cloned ~3000 bp of the 5’-flanking region of the murine PHEX gene and have shown that it is sufficient to drive reporter gene expression in UMR-106 rat, osteoblast-like, osteosarcoma cells (25). We thus undertook the current investigation to verify that 1,25(OH)_{2}D_{3} represses both PHEX expression and mineralization, and to determine the molecular mechanism by which 1,25(OH)_{2}D_{3} decreases PHEX expression. To this end, we examined the effect of 1,25(OH)_{2}D_{3} on PHEX mRNA levels in mice, and on the expression and stability of PHEX mRNA in UMR-106 cells. Reporter gene assays and electrophoretic mobility shift assays (EMSA) were used to identify and characterize the PHEX promoter region involved in 1,25(OH)_{2}D_{3} regulation of gene expression. Our results demonstrate a novel 1,25(OH)_{2}D_{3} transcriptional regulatory mechanism involving VDR-mediated repression of an unidentified transacting factor(s) that binds to a polyadenylyle element in the 5’-flanking region of the murine PHEX gene. Our data further indicate that this transfactor is required for optimal transcriptional initiation of the PHEX gene.

**EXPERIMENTAL PROCEDURES**

*Experimental Animals—* 4-5-week-old male C57BL/6 mice in groups of 3–4 were subcutaneously injected once with 1,25(OH)_{2}D_{3} (Sigma) at a dosage of 6 μg/kg b.w. or with an equal volume of vehicle (1:1, ethanol:propylene glycol) (26). Animals were supplied with food and water ad libitum. The mice were sacrificed 24 h after injection by CO₂ narcosis followed by cervical dislocation. Calvaria were removed, flash frozen in liquid nitrogen, and stored at –70 °C.

*Chemicals and Reagents—* 10× TBE, 20× SSC, 100 mM sodium pyruvate, 100× antibiotic-antimycotic, restriction enzymes, SuperScript II, 6% DNA retardation gels, dNTPs, random hexamers, TRizol reagent, and Taq DNA ligase were purchased from Invitrogen (Carlsbad, CA). DME high glucose medium and fetal bovine serum were from Irvine Scientific (Santa Ana, CA). [α-32P]dATP and [γ-32P]ATP were from PerkinElmer Life Sciences. Taq polymerase was from Continental Laboratory Products (San Diego, CA). DNA oligonucleotides were synthesized by Integrated DNA Technologies (Corvalle, IA). PHEX and 18S rRNA primer/probe sets, and TaqMan universal PCR master mix were purchased from Applied Biosystems (Foster City, CA). Streptavidin MagneSphere Paramagnetic Particles (SA-PMP) and polynucleotide kinase were purchased from Promega (Madison, WI). Gels and reagents for SDS-PAGE were from Bio-Rad. All other reagents, unless otherwise indicated, were purchased from Fisher Scientific (Santa Cruz, CA).

**Northern Blot Analyses—** Total RNA was prepared from mouse calvaria or UMR-106 cells using TRizol reagent according to the manufacturer’s protocol. 10 μg of total RNA were fractionated on a 1% formaldehyde gel and downward transferred to a Zeta-Probe GT nylon membrane (Bio-Rad). Antisense PHEX and GAPDH radiolabeled, single-stranded DNA probes were generated using the PCR EZ kit (Stratagene, Austin, TX). The PHEX probe was synthesized using the PHEX reverse primer 5’TTCATGTTGCAGCTGGAGAG-3’, with a mouse PHEX cDNA template from +816 bp to +1443 bp (97% homology between mouse and rat) (GenBank™ accession number NM_011077), and [α-32P]dATP. The GAPDH probe was synthesized using GAPDH reverse primer 5’-TCTGAGCTTCGTCCTGTC-3’, with a mouse GAPDH DNA template from +1080 bp to +1635 bp (GenBank™ accession AF108680), and [α-32P]dATP. Membranes were hybridized with radiolabeled probe and washed as previously described (19), and then exposed to x-ray film (Firce) at –70 °C. Resulting films were analyzed using a Bio-Rad GS-700 Imaging Densitometer with Quantity One software (Bio-Rad).

PHEX hybridization band intensities were normalized for GAPDH hybridization band intensities on the same membranes. Experiments were repeated three times with different groups of mice or with cells grown at different times.

**Real-time PCR—** 10 μg of total RNA were treated with DNase I according to the DNA free kit protocol (Ambion). The resulting RNA was evaluated by agarose gel electrophoresis and concentrations were adjusted according to densitometric analysis of the 18 S rRNA band. 250 ng of DNase I-treated RNA were reverse-transcribed using SuperScript II and random primers (Invitrogen; according to the manufacturer's protocol). 20 μl PCR reactions were set up in 96-well plates containing 10 μl of TaqMan universal PCR master mix, 1 μl of TaqMan primer/probe set, 2 μl of cDNA synthesis reaction (out of a 20-μl total volume), and 7 μl of molecular grade water. Reactions were run and analyzed on a Bio-Rad iCycler iQ Real-Time PCR detection system. Cycling parameters were determined, and resulting data were adjusted according to ABL. Briefly, data were analyzed using the comparative Ct method as means of relative quantitation, normalized to an endogenous reference (18 S ribosomal RNA) and relative to a control (normalized Ct value obtained from vehicle-treated UMR-106 cells or mice) and expressed as 2^(-ΔΔCt), according to Applied Biosystems User Bulletin 2: Rev B Relative Quantitation of Gene Expression.

**Reporter Gene Constructs—** Progressive promoter deletions, with the same 3’-ends, were generated between –1064 and –135 bp of the PHEX promoter (25) by PCR, and cloned into pGlo-Basic (Clontech; Palo Alto, CA) as previously described (25). A poly(A) deletion construct was generated by two step PCR using primers with the polyadenyline region deleted, forward 5’-CTCTCTCTCTCTCTCTCGTCAAACCC-3’ reverse 5’-GTTTGGACTGGAACAGGAAGACGAAG-3’, as previously described (19), and the resulting PCR product was cloned into pGlo-Basic. All promoter reporter gene constructs were sequenced to confirm fidelity.

**Cell Culture and Transient Transfection—** Rat osteogenic sarcoma cells (UMR-106) were obtained from the American Type Culture Collection (ATCC CRL-1661) and were cultured in DME high glucose media containing 10% fetal bovine serum, 1 mM sodium pyruvate, and 1% antibiotic-antimycotic at 37 °C with 5% CO₂. For analysis of endogenous PHEX gene expression, cells were seeded on 100-mm plates at 2 × 10^6 cells per plate, and treated with 10 μM of 1,25(OH)_{2}D_{3}, or vehicle 48 h post-seeding. Where indicated, 1 μg/ml of actinomycin D (Calbiochem; San Diego, CA) was added 30 min prior to 1,25(OH)_{2}D_{3} vehicle treatment and continued throughout the treatment period. For transfection experiments, cells were seeded onto 24-well plates (62,500 cells/well), and each well was transfected with 0.3 μg of reporter vector DNA.
Mechanism of PHEX Repression by 1,25(OH)₂D₃ in Osteoblasts

We first determined whether the endogenously expressed mouse osteoblasts isolated from calvaria and in the clonal, murine preosteoblast cell line MC3T3-E1, upon differentiation. We first determined whether the endogenously expressed PHEX gene was similarly repressed by 1,25(OH)₂D₃ in rat UMR-106 cells (25). Northern blot analysis of UMR-106 cells treated with 10⁻⁹ M 1,25(OH)₂D₃ or vehicle revealed a time-dependent decrease in PHEX mRNA expression, with a maximal decrease of −75% after 48 h of treatment (Fig. 1, A and B). Furthermore, real-time PCR analysis of the same RNA used for Northern blot analysis displayed virtually identical decreases in PHEX mRNA expression at 24, 48, and 72 h of 1,25(OH)₂D₃ treatment (Fig. 1C), i.e. significant repression at 24 h and a near maximal decrease at 48 h.

The potential biological relevance of 1,25(OH)₂D₃ down-regulation in UMR-106 cells was investigated by assessing the effect of the steroid hormone on mineralization. As seen in Fig. 2, the mineralization of cultured UMR-106 osteoblasts is dramatically attenuated by treatment of the cells with 10⁻⁷ M 1,25(OH)₂D₃, to −80% by 72 h. The temporal association between PHEX repression (Fig. 1) and inhibition of mineralization at 24–72 h by 1,25(OH)₂D₃ (Fig. 2) suggests, but does not prove, that the repression of PHEX endopeptidase activity results in higher effective concentrations of phosphatonin to inhibit mineralization. Thus, UMR-106 cells are a biologically valid osteoblast model in which to investigate PHEX regulation, and we have previously shown that the murine PHEX promoter is active when transfected into these cells (25).

1,25(OH)₂D₃ Suppression of PHEX mRNA Expression in Mouse Calvaria—In order to demonstrate the physiological relevance of 1,25(OH)₂D₃-mediated repression of PHEX gene expression, we next verified that the endogenous PHEX gene was also repressed by 1,25(OH)₂D₃ in mice, in vivo. 4–5-week-old mice were treated with 1,25(OH)₂D₃ (or vehicle) and then total RNA was isolated from calvaria. Real-time PCR analysis revealed an approximate 35% decrease in PHEX mRNA expression after 24 h of 1,25(OH)₂D₃ treatment (data not shown), which was similar to the decrease observed with 24 h of 1,25(OH)₂D₃ treatment of UMR-106 cells (Fig. 1, B and C). Furthermore, this in vivo decrease in PHEX mRNA expression is in agreement with the approximate 40% decrease in PHEX mRNA and protein expression reported by Brewer et al. (22) in the thibia of 1,25(OH)₂D₃-treated rats. Therefore, both murine and rat PHEX genes are repressed by 1,25(OH)₂D₃ in bone, in vivo.

Transcriptional Regulation of the PHEX Gene by 1,25(OH)₂D₃—To determine whether the repression of PHEX by 1,25(OH)₂D₃, in vitro and in vivo, occurs at the transcriptional or post-transcriptional level, we assessed the effect of 1,25(OH)₂D₃ on PHEX mRNA transcript stability. UMR-106 cells were pretreated with actinomycin D, exposed to 1,25(OH)₂D₃ or vehicle, and then assayed for PHEX mRNA levels at various time points up to 24 h. Real-time PCR indicated no difference in the rate of PHEX mRNA degradation between 1,25(OH)₂D₃ and vehicle-treated samples (Fig. 3), thus demonstrating that 1,25(OH)₂D₃ regulation of PHEX gene expression likely occurs at the transcriptional level.

Responsiveness of the Murine PHEX Promoter to 1,25(OH)₂D₃—We have previously cloned the murine PHEX gene promoter and shown functionality and glucocorticoid-responsiveness in UMR-106 cells (19, 25). Preliminary experi-
Mechanism of PHEX Repression by 1,25(OH)₂D₃ in Osteoblasts

Fig. 1. Quantitation of PHEX mRNA levels in UMR-106 cells treated with 1,25(OH)₂D₃. A, representative Northern blot of total RNA isolated from UMR-106 cells treated with 1,25(OH)₂D₃ or vehicle (EtOH). Upper bands are PHEX-specific 6.6-kb transcripts showing a decrease with 1,25(OH)₂D₃ treatment. Lower bands are unregulated GAPDH, which was used to normalize for loading and transfer efficiency. B, densitometric analysis of Northern blot data (n = 3–4). Values shown are means ± S.E. * indicates significantly different from EtOH treatment (p < 0.001); **, different from EtOH treatment (p < 0.005). C, real-time PCR analysis of PHEX mRNA expression in UMR-106 cells with EtOH or 1,25(OH)₂D₃ treatment. Values are means ± S.E. of 3–4 independent experiments; *, p < 0.001 for EtOH versus 1,25(OH)₂D₃; **, p < 0.05 for EtOH versus 1,25(OH)₂D₃.

Fig. 2. Inhibition of UMR-106 osteoblastic mineralization by 1,25(OH)₂D₃. Quantitation of calcein staining of calcium phosphate (Ca₃(PO₄)₂) deposition in UMR-106 monolayers is expressed as fraction of EtOH control, which is shown on the y-axis. a, p < 0.01 for 24 h 1,25(OH)₂D₃ treatment versus vehicle (n = 4). b, p < 0.001 for 48 h 1,25(OH)₂D₃ treatment versus 24 h 1,25(OH)₂D₃ treatment and vehicle (n = 4). c, p < 0.05 for 72 h 1,25(OH)₂D₃ treatment versus all other treatments (n = 4).

Fig. 3. Analysis of PHEX mRNA decay in UMR-106 cells treated with vehicle or 1,25(OH)₂D₃. Open squares represent EtOH treatment in the presence of actinomycin D, and filled squares represent 1,25(OH)₂D₃ treatment in the presence of actinomycin D. At all time points there was no significant difference in PHEX expression levels between groups (n = 3).

Our experiments revealed that the promoterless vector pGL3-Basic (Promega) was itself regulated by 1,25(OH)₂D₃ in UMR-106 cells (data not shown). We therefore reengineered the murine PHEX constructs in the promoterless β-galactosidase reporter vector pβGal-Basic (Clontech), which was not 1,25(OH)₂D₃ responsive (data not shown). Next, in the pβGal-Basic vector, we tested a series of 5’ progressive deletions of the PHEX promoter from bp −542 to bp −74 for 1,25(OH)₂D₃ responsiveness. Transient transfection of these constructs into UMR-106 cells, and subsequent treatment with 10⁻⁷ M 1,25(OH)₂D₃ for 48 h, revealed a 50–55% percent decrease in promoter activity with the −542/−104 and −133/−104 promoter constructs (Fig. 4). However, no effect on reporter gene activity was observed with 1,25(OH)₂D₃ treatment of cells transfected with the −74/−104.
The reporter plasmid with no promoter fragment. A construct (Fig. 5), 24-hydroxylase (24-OHase)-positive VDRE-luciferase reporter, of VDR/RXR to either protein partner. Gel shift analysis revealed no detectable binding of VDR/RXR heterodimers interacted directly with the 74 region. Visual inspection of this region, pictured in Fig. 6, indicating of VDR/RXR to either protein partner. Gel shift analysis revealed no detectable binding of VDR/RXR heterodimers interacted directly with the 74 region. Visual inspection of this region, pictured in Fig. 6, along with purified hVDR and its hRXR heterodimeric protein partner. Gel shift analysis revealed no detectable binding of VDR/RXR to either PHEX probe, whereas strong binding was observed with a positive control oligonucleotide containing the mouse osteopontin (MOP) VDRE (data not shown) (31). Therefore, unless VDR requires an unknown DNA-binding protein tether to associate with this region of the PHEX promoter, VDR-mediated repression of PHEX may be executed via an indirect mechanism.

To determine whether other nuclear proteins interact with this region of the PHEX promoter, EMSA was conducted using synthetic double-stranded oligonucleotides –134/–85 GS, and –102/–71 GS (Fig. 6), and N.P. obtained from UMR-106 cells. Incubation of the –134/–85 GS probe with N.P. revealed a single shifted band (Fig. 6, lane 2), whereas no N.P. binding was observed with the –102/–71 GS probe (data not shown). Computer analysis of the –134/–85 GS probe region for putative transcription factor binding sites using MatInspector pro (www.genomatix.de/cgi-bin/matinsepator_profomat.fam.pl), indicated putative binding sites for BCL6, STAT, and MEF2 (which overlap), as well as MYT1 (Fig. 6). To determine if the specific, shifted complex was the result of binding to one or more of these four predicted cis-elements, N.P. binding to the –134/–85 GS probe was competed with a 100-fold excess of cold probes with single mutations in these sites or mutations in all four of the predicted cis-elements (Fig. 6). These experiments revealed that mutations abolishing all four cis sites, singly and in combination, generated oligonucleotides in the –126 to –88 region that still competed the shifted band, indicating a lack of N.P. binding to these putative sites (Fig. 6A, lanes 4-6).

Further EMSAs with three overlapping competitor probes (Comp 1, Comp 2, Comp 3), spanning the –141 to –88 region, suggested partial competition by Comp 2 and Comp 3, but no competition with Comp 1 (Fig. 6A, lanes 7-9). Comp 2 and Comp 3 each contain 10 A-T base pairs of the 17-base pair polyadenine stretch in the murine PHEX promoter (Fig. 6). We therefore assessed directly if the poly(A) region bound N.P. from UMR-106 cells. EMSAs demonstrated that a 28-base pair poly(A) oligonucleotide probe was able to obliterate the shifted complex (Fig. 6A, lane 10). To confirm that this polyadenine stretch was the site of protein binding, the labeled –134/–85 GS probe was competed with excess nonradioactive –128/–85Δ/–116/–100 GS, in which the 17-bp poly(A) stretch was deleted. This oligonucleotide failed to compete the shifted complex (Fig. 6A, lane 11), pinpointing the polyadenine stretch as the region of nuclear protein binding. Moreover, the mobility of a 32P-labeled polyadenine probe retarded as a single band when incubated with UMR-106 N.P., whereas no shift was observed when labeled –128/–85Δ/–116/–100 GS was used as a probe (data not shown).
**FIG. 6.** EMSA analysis of the $1,25(OH)_2D_3$ responsive region of the PHEX promoter. Top, nucleotide sequences of the $1,25(OH)_2D_3$-responsive region of the PHEX promoter (−133 to −74 bp) and derived gel shift (GS) probes used for EMSA are listed above A–C. Putative cis-elements are depicted with brackets, and mutations in EMSA probes are indicated by *underlining*. MOP designates a mouse osteopontin probe containing a known, positive direct repeat 3 (DR3) VDRE, with brackets below each half-element. A, EMSA analysis of nuclear protein(s) interactions with the −134 to −85 PHEX promoter region. For all experiments, UMR-106 nuclear protein (N.P.) was incubated with $^{32}$P-labeled −134/−85 GS probe. All competition assays were performed with 100-fold excess of unlabeled probe. Lane 1, probe without N.P. Lanes 2–11, probe with N.P. The specific sequences of the probes are listed above. SC indicates shifted complex. B, EMSA analysis of protein binding from N.P. isolated from vehicle and $1,25(OH)_2D_3$-treated UMR-106 cells. When UMR-106 cells are treated with $1,25(OH)_2D_3$, there is an average reduction in the intensity of the SC of 40 ± 0.08% (n = 3). NS indicates nonspecific shifted complex. C, EMSA analysis of N.P. binding to the −134/−85 GS probe in the presence of exogenous VDR/RXR/$1,25(OH)_2D_3$. Based upon six independent experiments, a representative of which is shown in the two far right lanes, there is no significant change (average 102 ± 3%) in the intensity of the SC when VDR/RXR/$1,25(OH)_2D_3$ is incubated with UMR-106 N.P. and the labeled −134/85 GS probe. In both panels the identity of labeled probe used, and the presence of N.P. and/or competitor are shown above the respective lanes. Competition was carried out with the same probe that was labeled and used for the EMSA.
Therefore, we conclude that an apparently unique nuclear protein(s) in UMR-106 osteoblasts binds to the 17 A-T bp element in the proximal PHEX promoter.

1,25(OH)2D3 Treatment of UMR-106 Cells Reduces the Association of Nuclear Protein with the Poly(A) Element in the PHEX Promoter—To evaluate whether 1,25(OH)2D3 treatment of UMR-106 cells associates specifically with the poly(A) element in the PHEX promoter, EMSA was carried out with N.P. isolated from UMR-106 cells treated with 1,25(OH)2D3 or vehicle for 48 h. Fig. 6B, representative of three independent EMSAs, reveals an ~40–50% decrease in binding with protein isolated from 1,25(OH)2D3 treated cells. The less retarded, nonspecific (NS) band appearing in Fig. 6B was neither reproducible nor influenced by 1,25(OH)2D3 treatment of the cells. To test whether liganded VDR-RXR interacts directly with the poly(A)-binding protein, we added purified hVDR, hRXR, and 10−7 M 1,25(OH)2D3 to UMR-106 N.P. prior to incubation with the radiolabeled probe. The data in Fig. 6C show that inclusion of excess liganded VDR-RXR did not result in a decrease in binding to the PHEX poly(A) region. This suggests that the decrease in binding is likely not the result of direct negative interaction of liganded VDR-RXR with the nuclear protein that binds to the polyadenine region of the PHEX promoter, and points instead to indirect repression of the poly(A)-binding protein.

Southwestern Blot Analysis of the Protein(s) Interacting with the Poly(A) Region of the PHEX Promoter—Southwestern blot analysis was next performed to determine the approximate molecular mass of the protein(s) that binds to the poly(A) stretch from −116 to −100 bp of the murine PHEX promoter. This experiment revealed that the 32P-labeled −134/−85 GS probe bound to a protein of approximate molecular mass of 110 kDa, which was not observed with addition of 100× excess cold probe to the hybridization reaction (Fig. 7A). Furthermore, a dramatic decrease in binding was found when the −134/−85 GS probe was hybridized to nuclear protein extracted from 10−7 M 1,25(OH)2D3 treated UMR-106 cells (Fig. 7A). Membranes were stained with Ponceau S to confirm equal protein loading prior to hybridization with the DNA probe. The results shown in Fig. 7A strengthen the conclusion from Fig. 6B that 1,25(OH)2D3 treatment of UMR-106 cells markedly represses interaction of the 110 kDa poly(A)-binding protein with the promoter.

To further investigate the protein(s) that interacts with the poly(A) region, we incubated a biotin labeled −134/−85 GS probe with N.P. under the same conditions used for EMSA, then separated the probe/protein complex from the remaining N.P. with SA-PMP. Although SDS-PAGE and subsequent Sypro Ruby staining of the gel revealed nonspecific binding of protein to the SA-PMP, an enriched band was observed at ~110 kDa (Fig. 7B). Furthermore, this band was not present when an excess of non-biotin-labeled −134/−85 GS probe was added to the binding reaction (Fig. 7B). This affinity purification independently confirms that the 110 kDa poly(A)-binding protein from UMR-106 cells associates specifically with the poly(A) element in the PHEX promoter, and extensions of this procedure should permit the eventual isolation and identification of this transactivator.

Functional Analysis of the Poly(A) Region of PHEX Promoter—As a final test of the relevance of the 110 kDa nuclear protein to PHEX transcription and to examine whether its poly(A) cis element docking site is involved in the 1,25(OH)2D3-dependent regulation of PHEX promoter activity, a promoter/reporter construct was created with the 17 base polyadenine region deleted (−133/+104Δ−116/−100Δ). Analysis of β-galactosidase activity from this construct revealed a decrease in promoter activity to ~50% of the wild-type −134/+104 construct (Fig. 8). Furthermore, no difference in transcription was observed between vehicle and 1,25(OH)2D3 treatment of cells transfected with the −134/+104Δ−116/−100 construct (Fig. 8), demonstrating that PHEX proximal promoter repressibility is abolished when the −116 to −100 poly(A) element is deleted. The essentially identical results of suppressing basal activity and eliminating the 1,25(OH)2D3 repressive effect, achieved with either deletion of the PHEX poly(A) element (Fig. 8) or overexpression of a dominant-negative VDR (Fig. 5B), provide compelling evidence that 1,25(OH)2D3 controls PHEX transcription via the poly(A) cis element, and likely does so by VDR-mediated attenuation of its uncharacterized 110 kDa transactivator.

DISCUSSION

The present results support and extend previous data suggesting that 1,25(OH)2D3 suppresses the expression of PHEX in bone. Chronic exposure to 1,25(OH)2D3 significantly represses PHEX transcription in cultured rat osteoblasts (Fig. 1) and mouse calvaria (data not shown), as well as mouse osteoblasts (21) and rat tibia (22). The magnitude of this repression (35–75%) is indicative of pathophysiologic relevance because the disease phenotype of impaired skeletal mineralization in X-linked hypophosphatemia caused by an inactivating PHEX mutation is manifested in heterozygous females with a 50% decrease in PHEX expression (32).

Temporally correlated with PHEX repression by 1,25(OH)2D3...
Mechanism of PHEX Repression by 1,25(OH)_2D_3 in Osteoblasts

FIG. 8. Functional analysis of the polyadenine region in the PHEX promoter. Schematic representations of the promoter constructs are shown to the left on the y-axis. Promoter activity is indicated on the x-axis. *p < 0.01 between EtOH-treated -133/+104 construct and all others (n = 7).

in osteoblasts (Fig. 1) is inhibition of mineralization of these bone cells (Fig. 2). It may seem paradoxical that 1,25(OH)_2D_3 would exert an antimineralization effect considering that vitamin D is recognized instead as an anabolic principle. However, 1,25(OH)_2D_3 is known to suppress Cbfal, a gene involved in osteoblast development (33), as well as repress al1 (1) collagen expression to limit bone matrix (34). Moreover, knockout in mouse bone of either the genes encoding VDR (35) or osteocalcin (36), a bone remodeling protein induced by 1,25(OH)_2D_3, results in greater mineralized bone than in wild-type littermates. Therefore, although 1,25(OH)_2D_3-VDR promotes mineralization indirectly via stimulation of intestinal calcium and phosphate absorption, its direct effect on the mineralization process is counterintuitively negative. In other words, the role of 1,25(OH)_2D_3 -VDR at the level of bone is to limit mineralization (Fig. 2, (21, 23)). We propose that the mechanism whereby 1,25(OH)_2D_3 curtails osteoblastic mineralization is via repression of PHEX, which in turn potentiates the antimineralization action of phoshatonin.

Our observation of 1,25(OH)_2D_3 down-regulation of murine PHEX promoter activity in UMR-106 osteoblasts is in contrast to the results of Liu et al. (37) who reported no modulation of a −2736/+54 murine PHEX promoter/reporter gene construct by 1,25(OH)_2D_3 in a different line of transfected rat osteosarcoma (ROS 17/2.8) cells. This apparent discrepancy may be the result of different concentrations of 1,25(OH)_2D_3 used (10^{-8} M (37) versus 10^{-7} M in the current studies), or the fact that Liu et al. (37) only examined the early 24-h time point when osteocalcin was induced by 1,25(OH)_2D_3. Importantly, Liu et al. (37) did not show 1,25(OH)_2D_3 down-regulation of the endogenous PHEX gene in ROS 17/2.8 cells under their conditions. Thus, because of cell-type plus 1,25(OH)_2D_3 dose differences, and potential differential catabolism rates for 1,25(OH)_2D_3 in UMR-106 versus ROS 17/2.8 cells, as well as the fact that Liu et al. only tested the 24-h time period for what we propose is a secondary repression response that may require protracted time, it is not incongruous that they found no repression of the −2736/+54 murine PHEX construct. Finally, we have not generated murine PHEX promoter constructs larger than −1064/+104, which 1,25(OH)_2D_3 repressed in UMR-106 cells (data not shown) similarly to the −133/+104 construct (Figs. 4 and 8). Therefore, it is possible that the results of Liu et al. can also be explained by an unknown element between −1064 and −2736 that suppresses the response to 1,25(OH)_2D_3. Nevertheless, our observed 50% decrease in murine PHEX promoter activity caused by 1,25(OH)_2D_3 treatment (Fig. 4) is similar to the decrease in endogenous PHEX gene expression in UMR-106 cells (Fig. 1) and the in vivo decrease observed in rats (22) and mice, further suggesting similar regulation in vitro and in vivo. Moreover, this observed −50% decrease in PHEX expression by 1,25(OH)_2D_3 (Figs. 1, 4, 5, and 8) is in agreement with the reported decreases seen in expression of other genes known to be down-regulated by 1,25(OH)_2D_3, including Id1 (38), hANP (39), HES-1 (40), Cbfal (33), CD95 Ligand (41), 25(OH)D_3-1α-hydroxylase (42), GM-CSF (43), and PTH (44). Finally, as stated above, a 50% decrease in PHEX promoter activity elicited by 1,25(OH)_2D_3 is in concert with a significant biologic effect, because the defective bone mineralization phenotype in XLIH is manifest with mutation of only one of the two PHEX gene copies in heterozygous females (32). Therefore, although 1,25(OH)_2D_3-VDR promotes mineralization indirectly via stimulation of intestinal calcium and phosphate absorption, its direct effect on the mineralization process is counterintuitively negative. In other words, the role of 1,25(OH)_2D_3 -VDR at the level of bone is to limit mineralization (Fig. 2, (21, 23)). We propose that the mechanism whereby 1,25(OH)_2D_3 curtails osteoblastic mineralization is via repression of PHEX, which in turn potentiates the antimineralization action of phoshatonin.

To date, we have been unable to locate in the scientific literature or in DNA sequence/transcription factor databases any description of a polyadenine-binding nuclear protein, much less one that has a molecular weight of −110 kDa. We thus conclude that the protein which apparently mediates 1,25(OH)_2D_3 control of PHEX is likely novel, and we have named it PHEX-activating protein 110 (PAP110). It should be noted that there is one newly recognized family of DNA-binding proteins that is perhaps the most closely related to PAP110, because members of this family bind mixed AT-rich DNA sequences. These proteins contain a novel DNA binding domain referred to as an AT-rich interacting domain (ARID), which is comprised of a series of six α-helices that contact both major and minor groove residues in an AAT(T/A)AA core hexamer consensus (45). Members of the ARID protein family function in embryonic development, cell lineage gene regulation, and cell cycle control, and they mediate both positive and negative transcriptional control, likely involving modification of chromatin structure (46). Recently, it has been revealed that several ARID proteins possess relaxed DNA binding specificity (45), raising the possibility that PAP110 constitutes a unique member of the ARID family, or is at least related to this class of transcription factors in structure and/or function.

Considering the several potential modes of 1,25(OH)_2D_3-mediated down-regulation of gene expression, it is possible to integrate the current results into the framework of known negative actions of 1,25(OH)_2D_3 to construct a hypothetical molecular mechanism for down-regulation of PHEX gene expression. We have demonstrated that 1,25(OH)_2D_3 exposure or DNVR expression (with or without 1,25(OH)_2D_3) decreased PHEX promoter activity to identical levels (Fig. 5B). It should be noted that the DNVR utilized for these studies contains a mutation that abolishes its ability to transactivate (glutamic acid to alanine substitution at position 420, Ref. 27), but its ability to function in a repressive manner and to bind corepressors is unaffected. When considered together, these observations suggest an indirect mode of action, whereby VDR/RXR/1,25(OH)_2D_3 interacts with a nVDRE on another gene that encodes an activator of PHEX gene expression. Furthermore, we show that a polyadenine region in the PHEX promoter is responsible for 1,25(OH)_2D_3 mediated down-regulation and that this region specifically binds a novel 110 kDa nuclear protein (PAP110). We thus postulate that the most likely mechanism explaining these results would be for the gene encoding
PAP110 to possess a nVDRE that is responsible for decreasing PAP110 protein expression with 1,25(OH)2D3 exposure. This protein is presumed necessary for basal transcription of the PHEX gene, and when its expression is decreased (Figs. 6B and 7) or its binding is abolished by mutation of the 17 A-T base pair element (Fig. 8), PHEX promoter activity decreases to subbasal levels. Thus, in the absence of 1,25(OH)2D3, osteoblastic mineralization.

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