Glucocorticoid regulation of the murine PHEX gene

ERIC R. HINES,1*, JAMES F. COLLINS,1*, MARCI D. JONES,2 SAMANTHA H. SEREY,1 AND FAYEZ K. GHISHAN1
1Departments of Pediatrics and Physiology and 2 Department of Orthopedic Surgery, Steele Memorial Children’s Research Center, University of Arizona Health Sciences Center, Tucson, Arizona 85724
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Hines, Eric R., James F. Collins, Marci D. Jones, Samantha H. Serey, and Fayez K. Ghishan. Glucocorticoid regulation of the murine PHEX gene. Am J Physiol Renal Physiol 283: F356–F363, 2002.—The phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) is a member of the neutral endopeptidase family, which is expressed predominantly on the plasma membranes of mature osteoblasts and osteocytes. Although it is known that the loss of PHEX function results in X-linked hypophosphatemic rickets, characterized by abnormal bone matrix mineralization and renal phosphate wasting, little is known about how PHEX is regulated. We therefore sought to determine whether the murine PHEX gene is regulated by glucocorticoids (GCs), which are known to influence phosphate homeostasis and bone metabolism. Northern blot analysis revealed increased PHEX mRNA expression in GC-treated suckling mice (1.5-fold) and in rat osteogenic sarcoma (UMR-106) cells (2.5-fold). An increase was also seen in PHEX promoter activity in transiently transfected UMR-106 cells with GC treatment. Analysis of nested promoter deletions revealed that an atypical GC response element was located between –337 and –315 bp. Mutational analysis and electrophoretic mobility shift assays further identified –326 to –321 bp as a site involved in GC regulation. Supershift analyses and electrophoretic mobility shift assay competition studies indicated that the core binding factor α1-subunit transcription factor is able to bind to this region and may therefore play a role in the GC response of the murine PHEX gene.

dexamethasone; core binding factor α1-subunit; Hyp mouse; transcriptional regulation

THE PHOSPHATE-REGULATING gene with homologies to endopeptidases on the X chromosome (PHEX) is a neutral endopeptidase found predominantly on the plasma membrane of osteoblasts and osteocytes (1, 8, 16, 20). Loss of function mutations in the PHEX gene result in X-linked hypophosphatemic rickets (XLH) in humans and, in the animal model of XLH, the Hyp mouse (28). Manifestations of XLH can vary widely and include vitamin D-resistant rickets, which results in growth retardation, lower body skeletal abnormalities, bone and joint pain, and decreased range of motion (28). Biochemical characteristics include high serum alkaline phosphatase levels (28) and hypophosphatemia resulting from decreased phosphate reabsorption in the renal proximal tubules (5, 22, 23, 34). Although the precise physiological function of PHEX is unclear, studies on the Hyp mouse have implicated PHEX in phosphate homeostasis and bone mineralization.

The role of PHEX in phosphate homeostasis has been characterized by means of the Hyp mouse model. The renal phosphate leak seen in the Hyp mouse and in XLH is a result of decreased expression of the sodium-dependent phosphate cotransporter (NaPi-IIa) in the proximal tubules (5, 21, 34). Additional experiments with the murine model of XLH revealed that the phosphate wasting is the result of a humoral factor, rather than a primary renal defect (15, 24), and that this circulating factor must be modified by PHEX.

Conversely, the role of PHEX in bone mineralization is not as clear. Clinically, skeletal features of XLH in humans and in Hyp mice include shortened stature, osteomalacia in trabecular and cortical bone, recurrent dental abscesses, and late dentition (28). The osteomalacia seen is a result of slowed bone remodeling, with a significant delay in osteoid mineralization and reduced resorption (28). This defective mineralization seen in XLH has also been seen in vitro in cultured, immortalized osteoblasts (38) and in primary cultures of bone marrow cells (20). Recent studies by Miao et al. (20) on Hyp and normal mice demonstrate that the observed osteomalacia is associated with altered expression of several bone matrix proteins. This altered expression occurred both transcriptionally, with Hyp mice having decreased levels of bone sialoprotein and vitronectin mRNA, and posttranscriptionally, with biglycan and fibrillin immunoreactive protein levels being elevated in Hyp mice while mRNA levels remained unchanged (20).

Although the physiological results of inactivating mutations in PHEX are known and it is clear that PHEX is involved in regulating bone mineralization and phosphate homeostasis, little is known about how the PHEX gene and protein are regulated. Ecarot et al. included this fact.

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* E. R. Hines and J. F. Collins contributed equally to this work.

Address for reprint requests and other correspondence: F. K. Ghishan, Dept. of Pediatrics, Steele Memorial Children’s Research Ctr., Univ. of Arizona Health Sciences Ctr., 1501 N. Campbell Ave., Tucson, AZ 85724 (E-mail: fghishan@peds.arizona.edu).

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GLUCOCORTICOID REGULATION OF THE MURINE PHEX GENE

We recently cloned and characterized the murine PHEX gene promoter and now have the ability to use this as a tool to investigate transcriptional regulation of the PHEX gene (12). Computer analysis of the promoter region predicted several putative glucocorticoid (GC) response elements (12). GCs are known to regulate renal sodium-dependent phosphate reabsorption (GC) response elements (12). GCs are known to regulate renal sodium-dependent phosphate reabsorption and to exert both positive and negative effects on bone growth and metabolism. In the present investigation, we sought to determine the following: 1) whether PHEX is regulated by GCs in vivo and in an in vitro cell culture model (UMR-106 cells), 2) whether this regulation is at the level of gene transcription, and 3) whether the cloned murine promoter contains cis-acting elements that mediate this response to GC.

MATERIALS AND METHODS

Experimental animals. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used in groups of five. Twelve-day-old suckling mice were subcutaneously injected with methylprednisolone (MP; Solu-Medrol, Upjohn, Kalamazoo, MI) at a dose of 30 μg/g body wt or equal volumes of saline once every 12 h for a total of four injections. Animals were supplied with food and water ad libitum. The animals were killed 3 h after the last injection (at 14 days of age) by CO2 narcosis followed by cervical dislocation. Calvaria were removed, flash frozen in liquid nitrogen, and stored at −70°C. All animal procedures were approved in advance by the University of Arizona Institutional Animal Care and Use Committee.

Chemicals and reagents. Lipofectamine, 10× Tris-boric acid-EDTA (TBE), 20× SSC, 100 mM sodium pyruvate, and T₄ DNA ligase were purchased from GIBCO BRL (Bethesda, MD). High-glucose DMEM and fetal bovine serum were from Irvine Scientific (Santa Ana, CA). Taq polymerase, restriction enzymes, and dual luciferase assay kit were from Promega (Madison, WI). DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). All other reagents, unless otherwise indicated, were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO).

Northern blot analyses. Total RNA was prepared from mouse calvaria or UMR-106 cells with TRIzol Reagent (GIBCO) according to the manufacturer’s protocol. Ten micrograms of total RNA were fractionated on a 1% formaldehyde-acrylamide gel and downward transferred to a Zeta-Probe GT nylon membrane. Hybridization with radiolabeled probe in Ultraspeed buffer (Ambion), washed according to the manufacturer’s protocol, and then exposed to X-ray film (Pierce, Rockford, IL) at −70°C. Resulting films were analyzed with a Bio-Rad GS-700 Imaging Densitometer with Quantity One software. PHEX hybridization band intensities were normalized for 18 s on the ethidium bromide-stained membrane.

Reporter gene constructs. Nested promoter deletions, with the same 3’ ends, were generated between −542 and −133 bp of the PHEX promoter (12) by using the Exo-Size Deletion Kit (New England Biolabs, Beverly, MA). All deletion constructs were confirmed by sequencing. Site-directed scanning mutations were introduced five bases at a time between −336 and −317 bp by two-step PCR, as previously described (13). The resulting mutant constructs were cloned into pGL3-basic (Promega), as previously described (12). The primers used to construct the mutant promoter constructs were forward −542 primer, 5’-ACTAGTGGTACATTAGCCTACAC-3’; reverse +104 primer, 5’-AGGCAATCTAGTGTGGAATT-3’; mutant 1 forward and reverse, 5’-ACTAGTAGTCTGAGGAT-3’ and 5’-GTTATTTCAG-CTGAGGAT-3’, respectively; mutant 2 forward and reverse, 5’-CAGGAGCTGTAGCTATACAACCACCTT-3’ and 5’-AAGTGGTTATGGAACGTACCTGAGTC-3’, respectively; mutant 3 forward and reverse, 5’-AGCTGTCTAGAGGACCCACACTTTT-3’ and 5’-CCCTAAGGTGTGGCGTTGACACAGCT-3’, respectively; and mutant 4 forward and reverse, 5’-CTGAAATAACACAGGTTAGGGAAAACA-3’ and 5’-TGGTTCTCCTAGGCTTATACAG-3’, respectively. Bold type indicates mutated bases, which were mutated A→C and G→T.

Cell culture and transient transfection. Rat osteogenic sarcoma cells (UMR-106) were obtained from the American Type Culture Collection (CRL-1661) and were cultured according to its guidelines. The cells (passages 7–13) were seeded on 24-well plates and cotransfected, by means of liposome-mediated transfection, at 70–80% confluence with 0.5 μg of reporter vector DNA and 0.015 μg of pRL-TK vector (encoding Renilla luciferase, used as an internal standard; Promega) in serum-free media. Twelve hours after transfection, cells were allowed to recover for 24 h in medium with 10% fetal calf serum followed by 12 h in serum-free medium. Cells were then treated with 10−7 M dexamethasone (DEX; Sigma), 10 μM mifepristone (RU-486, an antagonist of the GC receptor; Sigma) (36), DEX+RU-486, or equal amounts of vehicle (ethanol) in medium with charcoal-stripped serum. The next day four hours after treatment, cells were harvested, and dual luciferase assays were performed with equal amounts of cellular lysate. To assess the effect of DEX on endogenous PHEX expression in UMR-106 cells, untransfected cells were cultured in 100-mm plates and treated as described above. These experiments were repeated at least three times with separate cell populations on different days. In each experiment, two (for 100-mm plates) or three dishes (for 24-well plates) were considered as n = 1, and the results from these dishes were averaged. Additionally, firefly luciferase activity was normalized for Renilla luciferase activity in each cell extract.

Electrophoretic mobility shift assays. Nuclear protein for electrophoretic mobility shift assays (EMSAs) was prepared from UMR-106 cells as previously described (33). Double-stranded synthetic oligonucleotides were end labeled with [γ-32P]dATP (NEN). For each probe used for EMSAs, 20,000 counts/min of probe was incubated with 4 μg of nuclear protein, 4 μl of 5× binding buffer (in mM) 100 HEPES, pH 7.5, 5 EDTA, 50 (NH₄)₂SO₄, 5 dithiothreitol, and 150 KCl, as well as 1% Tween 20 (wt/vol); Roche Molecular Biochemicals, Indianapolis, IN), 1 μg poly (dI-C), Roche Molecular Bio-
GC regulation of PHEX in vivo. To determine the ability of exogenous GCs to regulate PHEX in vivo, 12-day-old mice were treated with pharmacological doses of MP or an equal volume of saline (vehicle). Total RNA was extracted from calvaria and subjected to Northern blot analysis with a PHEX cDNA-specific probe. Results revealed a 1.5-fold increase in PHEX mRNA expression in MP-treated mice compared with vehicle-treated controls (Fig. 1A).

GC regulation of PHEX in UMR-106 cells. Northern blot analysis of rat osteogenic sarcoma UMR-106 cells treated with 10^{-7} M DEX or vehicle revealed a 2.51-fold increase in PHEX mRNA expression in DEX-treated cells compared with ethanol (vehicle)-treated cells (P < 0.005, n = 3; Figs. 1, B and C).

Responsiveness of the murine PHEX promoter to GCs. We have previously cloned and shown functionality of the murine PHEX promoter in UMR-106 cells (12). Preliminary experiments designed to test for GC responsiveness of the previously characterized PHEX promoter fragments (12) showed that the −542/+104 construct was responsive to DEX whereas the −133/+104 construct was not. To better define the region of the promoter responsible for the observed DEX response, a series of nested deletions were constructed between −542/+104 and −133/+104. DEX treatment of transiently transfected UMR-106 cells showed no increase in luciferase activity with the −133/+104 and −315/+104 constructs; however, the remaining three constructs, −338/+104, −354/+104, and −542/+104 showed a 60–85% increase compared with pGL3-basic (P < 0.05, n = 7–10; Fig. 2). Data are expressed as “fold” increase due to variation in basal promoter activity levels within individual constructs among experiments. However, the fold increases in promoter activity with DEX treatment remained consistent among experiments.

To determine whether this increase in promoter activity by DEX treatment was mediated through the GC receptor, the −542/+104 construct and pGL3-basic were treated with 10^{-7} M DEX plus 10 \mu M RU-486 or vehicle or 10 \mu M RU-486 only. Addition of 10 \mu M RU-486 to the DEX treatment regime reduced the DEX...
response from the twofold increase previously seen in the −542/+104 construct (P < 0.0001, n = 4–5) to levels that were not different from the RU-486 treated or untreated pGL3-basic levels (Fig. 3). No difference was seen between treated vehicle and vehicle plus 10 μM RU-486 in either of the constructs (data not shown).

Mutational analysis of the PHEX promoter region −338 to −315 bp. To determine which base pairs between −338 and −315 bp were responsible for the observed DEX response, A→C and G→T mutations were introduced five bases at a time between −336 and −316 bp by PCR. All the mutations were introduced in the −542/+104 construct for ease of construction. The four mutant promoter constructs, mutant 1, mutant 2, mutant 3, and mutant 4, (Fig. 4 and Table 1) along with the −542/+104 construct and pGL3-basic were transfected into UMR-106 cells, which were then treated with DEX or vehicle. Analysis of luciferase activity revealed that the DEX response of the mutant 3 construct was significantly reduced (P < 0.02, n = 4) from the twofold increase in the −542/+104 construct to levels not significantly different from pGL3-basic (Fig. 4). The remaining constructs, mutant 1, mutant 2, and mutant 4, showed trends toward reduction in DEX-stimulated activity, compared with the wild-type −542/+104 construct but were different from pGL3-basic (P < 0.05, n = 4–5; Fig. 4).

EMSA analysis of the PHEX promoter region from −332 to −306 bp. To assess the ability of transcription factors to bind to the region between −326 and −321 bp (encompassing mutant 3), which when mutated showed a loss of DEX response, EMSAs were conducted. Nuclear extracts obtained from UMR-106 cells were incubated with an oligonucleotide probe spanning −332 to −306 bp, which resulted in two shifted bands (Fig. 5). Both of these bands were competed with a 100-fold excess of cold probe (Fig. 5, lane 3). However, these bands were not competed with an excess of cold mutant 3 probe (Fig. 5, lane 4). Comparison of −325 to −315 bp (the region encompassing mutant 3) of the
PHEX promoter with known osteoblast-specific transcription factor binding sites showed that mutations 3 and 4 contain a putative osteoblast-specific, cis-acting element (OSE2) [6] to which the transcription factor Cbfa1 has been shown to bind [7]. Therefore, to determine whether any of the observed shifted bands corresponded to Cbfa1, we competed the PHEX wild-type probe with an excess of cold probe specific for Cbfa1. The Cbfa1 probe was able to compete out both bands seen with the PHEX wild-type probe (Fig. 5, lane 6). To further confirm the binding of Cbfa1 to the PHEX probe, an anti-Cbfa1 antibody was used. The addition of the anti-Cbfa1 antibody completely removed the highest, shifted band (Fig. 5, lane 7), indicating that Cbfa1 was binding to the probe. Additionally, incubation of the Cbfa1-specific oligonucleotide probe with nuclear extract shifted bands of identical molecular weight to those shifted by the PHEX-specific probe, with the upper band being supershifted by the anti-Cbfa1 antibody (Fig. 5, lane 12). Additionally, because it appeared that Cbfa1 was capable of binding to the OSE2 site spanning mutations 3 and 4, competition with 100× cold mutant 4 probe, which contains the three 3′ bases of the OSE2 site, was conducted. Results of this competition revealed that mutations made between −321 and −316 bp (mutant 4) could only partially compete the PHEX wild-type probe (Fig. 5, lane 5). Furthermore, the affinity of the nuclear protein for the PHEX probe was seen to be lower than that for the Cbfa1 probe (Fig. 5) when equal amounts of protein were incubated with equal probe activities.

**DISCUSSION**

We undertook the present study to determine whether GCs, which are known to have important implications in phosphate homeostasis bone and metabolism, are capable of regulating the PHEX gene. We found that PHEX mRNA isolated from calvaria of suckling mice treated with methylprednisolone was elevated 1.5-fold compared with that of vehicle-treated mice (Fig. 1). Additionally, PHEX mRNA expression in the rat osteogenic sarcoma cell line UMR-106 treated with DEX was seen by Northern blot analysis to be elevated ~2.5-fold (Fig. 1). The physiological relevance of this observed upregulation of PHEX by GCs is unclear. Studies on the Hyp mouse showed that PHEX is involved in phosphate homeostasis through transcriptional regulation of the renal NaPi-IIa gene. Furthermore, NaPi-IIa has been shown to be downregulated by GCs at a posttranscriptional level in an age-dependent fashion [9]. Because of these differences in the way that gene expression is effected (up- vs. downregulation) and at what level GC regulation occurs (transcriptional vs. posttranscriptional), it seems unlikely that GC-mediated effects on PHEX expression play a role in NaPi-IIa gene expression. However, GC studies in Hyp and normal littersmates will ultimately determine whether GC regulation of the PHEX gene, in turn, alters renal phosphate reabsorption by means of changes in NaPi-IIa expression.

GCs have been reported to exert both positive and negative effects on osteoblasts, the predominant site of PHEX expression. It is well characterized that long-term clinical use of GCs results in serious skeletal side effects, most notably osteoporosis. Weinstein et al. [37] and Silvestrini et al. [32] have both indicated a role for osteoblast apoptosis in GC-induced osteoporosis. However, no correlation has yet been made between PHEX expression and osteoporosis. Additionally GCs were administered over a much longer time period in the studies showing GC-induced osteoblast apoptosis than we used in the present study, making precise comparisons difficult.

GCs have also been shown to exert positive effects on cells of the osteoblast lineage. The addition of GCs to cultured osteoblasts has been shown to promote differ-

**Table 1. Luciferase activity of site-directed mutants of PHEX promoter**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Sequence</th>
<th>n</th>
<th>Fold-Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>−336 bp 5′-AGCTGCTGAAAT[AACCAC]TT-3′ −317 bp</td>
<td>4</td>
<td>2.07 ± 0.21</td>
</tr>
<tr>
<td>Mutant 1</td>
<td>5′-CTAGTCCTGAAT[AAACCA]TT-3′</td>
<td>4</td>
<td>1.83 ± 0.29</td>
</tr>
<tr>
<td>Mutant 2</td>
<td>5′-AGCTGAGTCAT[AAACCA]TT-3′</td>
<td>4</td>
<td>1.69 ± 0.13</td>
</tr>
<tr>
<td>Mutant 3</td>
<td>5′-AGCTGCTGAA[CGCCA]CTT-3′</td>
<td>4</td>
<td>1.36 ± 0.06*</td>
</tr>
<tr>
<td>Mutant 4</td>
<td>5′-AGCTGCTGAA[TTACACAGCCT-3′</td>
<td>5</td>
<td>1.61 ± 0.19</td>
</tr>
</tbody>
</table>

DNA sequence of the wild-type PHEX promoter between −336 and −317 bp. The bases mutated in each mutant construct are indicated in bold. Luciferase activity for each construct in terms of fold-induction ± SE, DEX vs. EtOH, and n value are given for each construct. The putative OSE2 site sequence is indicated by an open box in the wild-type construct. *Significant difference from the wild-type construct (P = 0.017).

**Fig. 5.** Electrophoretic mobility shift assay. For all EMSAs, 4 μg of nuclear protein was incubated with 20,000 counts/min of 32P-labeled probe. Lanes 1–9 were shifted with PHEX −332 to −306 bp probe. Lanes 10–12 were shifted with Cbfa1-specific probe. Lanes 1, 8, and 10: probe without nuclear protein. Lanes 2, 9, and 11: probe with nuclear protein. Lane 3: competed with 100× cold PHEX −332 to −306 bp probe. Lane 4: competed with 100× cold mutant 3 probe. Lane 5: competed with 100× cold mutant 4 probe. Lane 6: competed with 100× cold Cbfa1-specific probe. Lane 7: PHEX probe with 2 μg anti-Cbfa1 antibody. Lane 12: Cbfa1-specific probe with 2 μg anti-Cbfa1 antibody. SC indicates specific complex and SSC indicates super-shifted complex.
entiation (25, 31), along with increasing the size and amount of bone nodule formation (2, 11, 19, 31). This increase in nodule formation has further been shown to be greater in less mature than in more mature osteoblastic cells, with the former requiring GCs to differentiate (35). Therefore, it is likely that the increase in endogenous PHEX mRNA expression seen in UMR-106 cells and the activity of the longer promoter constructs is the result of GC-induced maturation of the relatively immature UMR-106 cells. This is consistent with the observations that PHEX expression increases as osteoblasts differentiate (8) and that DEX increases nodule formation in UMR-106 cells (11). The observation that there are two populations of osteoblastic cells that respond differently to GCs would also explain the difference in GC response seen between the in vitro and in vivo studies. UMR-106 cells, which are a homogenous population of relatively immature osteoblasts, show a 2.5-fold increase in PHEX expression with GC treatment, whereas a more modest 1.5-fold increase in expression is seen in mice calvaria, which contain a more heterogeneous population of osteoblasts at different stages of maturation.

Analysis of PHEX promoter constructs transfected into UMR-106 cells showed that treatment with $10^{-7}$ M DEX significantly increased ($P \leq 0.006$) promoter-driven luciferase activity from the $-542/+104$ construct compared with the promoterless luciferase vector pGL3-basic. This twofold increase in PHEX expression with GC treatment, whereas a more modest 1.5-fold increase in expression is seen in mice calvaria, which contain a more heterogeneous population of osteoblasts at different stages of maturation.

To determine whether this putative Cbf1-binding site was capable of binding Cbf1, an EMSA was conducted utilizing a probe from $-332$ to $-306$ bp, encompassing the bases mutated in the mutant 3 construct. The EMSA revealed that the PHEX $-332$ to $-306$ bp probe shifted two bands when incubated with UMR-106 nuclear protein. Both of these bands were competed with 100× cold PHEX and Cbf1-specific probes but neither were competed with the PHEX mutant 3 probe. Furthermore, the Cbf1-specific probe shifted bands identical to those of the PHEX $-332$ to $-306$ bp probe. Additionally, the affinity of the nuclear proteins for the Cbf1-specific probe was much greater than for the PHEX probe. To determine whether one or more of the shifted bands contained the Cbf1 protein, supershift analyses were conducted with an anti-Cbf1 antibody. With addition of anti-Cbf1 antibody, the upper shifted band seen with the PHEX probe was abolished, whereas the same band with the Cbf1-specific probe was shifted to a higher molecular weight. The inability of the anti-Cbf1 antibody to supershift the upper PHEX probe band is likely the result of disruption of the much weaker interaction of Cbf1 with the PHEX probe compared with the Cbf1-specific probe.

Taken together, the EMSA data and the results with promoter mutant 3 suggest that Cbf1 may play a role in the GC response of the PHEX gene. This would appear to be in contrast with the results of Liu S et al. (17), who reported that overexpression of Cbf1 had no effect on luciferase activity driven by the murine PHEX promoter. However, these authors only evaluated the ability of Cbf1 to stimulate promoter expression and did not appear to consider the possibility of repression of PHEX expression by Cbf1. The ability of Cbf1 to repress PHEX expression would be consistent with the recent observations by Liu et al. (18), which showed that late-stage osteoblast maturation was blocked in transgenic mice overexpressing Cbf1, along with those of Ecarot et al. (8) and Miao et al. (20), who showed PHEX expression is highest in fully differentiated osteoblasts and osteocytes. In light of these findings, it is possible that Cbf1 may serve to repress PHEX expression in nonmature osteoblasts. Therefore, attempts to alter PHEX promoter expression, in an osteoblastic cell line that endogenously expresses Cbf1, by overexpressing Cbf1 would likely result in no change in promoter activity. Additionally, studies by Chang et al. (4) showed that GCs decreased immu-
noreactive Cbfα1 protein in primary rat osteoblasts. Therefore, our findings along with those of Liu et al. (18) and Chang et al. (4) suggest that the GC-induced increase in PHEX expression in suckling mice and UMR-106 cells, along with increased PHEX promoter activity in GC-treated UMR-106 cells, may result from removal of Cbfα1 from the promoter, which may act to repress PHEX expression.

In conclusion, we have demonstrated that the rodent PHEX gene is regulated by GCs through the GC receptor and that this regulation is, at least in part, mediated at the transcriptional level. Furthermore, we have identified position –326 to –321 bp in the murine PHEX promoter as a site involved in this regulation. Additionally EMSA analysis of the GC responsive region indicates that the osteoblast transcription factor Cbfα1 is likely involved. Future studies will define the precise role Cbfα1 plays in the expression of the PHEX gene and its regulation by GCs.

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