

# CYP27B1 null mice with *LacZ* reporter gene display no 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase promoter activity in the skin

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The hormonally active form of vitamin D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], is synthesized in the kidney through a tightly regulated reaction catalyzed by 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase), the product of the *CYP27B1* gene. Through gene targeting in embryonic stem cells, we engineered a mouse strain in which the coding region of the 1 $\alpha$ -hydroxylase gene is replaced by the genes for  $\beta$ -galactosidase (*lacZ*) and neomycin resistance. Null mice produced no detectable 1 $\alpha$ -hydroxylase transcript. The mice grew normally when maintained on a balanced diet containing 1,25(OH)<sub>2</sub>D<sub>3</sub> but rapidly developed rickets when phosphorus and 1,25(OH)<sub>2</sub>D<sub>3</sub> were restricted. Rickets was curable through administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> but not its biological precursor, 25-hydroxyvitamin D<sub>3</sub>. Upon administration of a diet low in calcium and devoid of any form of vitamin D<sub>3</sub>,  $\beta$ -galactosidase activity was detected in the kidneys of the  $-/-$  and  $+/-$  mice and in placentas harvested from  $-/-$  females bred with  $-/-$  males. No  $\beta$ -galactosidase activity was detected in skin sections or in primary keratinocyte cultures from  $-/-$  animals. Our results demonstrate we have generated 1 $\alpha$ -hydroxylase null mice that display phenotypes characteristic of vitamin D-dependency rickets type I. From the histochemical analysis of reporter gene expression in these mice, we conclude that acute 1,25(OH)<sub>2</sub>D<sub>3</sub> deficiency in otherwise healthy animals does not stimulate local production of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the skin. These findings stand in contrast to previously published reports of 1,25(OH)<sub>2</sub>D<sub>3</sub> production in keratinocytes.

extrarenal 1,25-dihydroxyvitamin D<sub>3</sub>

The vitamin D endocrine system plays an integral role in maintaining calcium and phosphorus homeostasis and modulates growth and differentiation of certain cell types (1). The major circulating form of vitamin D, 25-hydroxyvitamin D<sub>3</sub> (25-OH-D<sub>3</sub>), is produced upon 25-hydroxylation of vitamin D<sub>3</sub> in the liver. Subsequent 1 $\alpha$ -hydroxylation of 25-OH-D<sub>3</sub> in the kidney produces the physiologically active metabolite, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>]. The second hydroxylation is catalyzed by the 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase), a mitochondrial cytochrome P450 enzyme that is the product of the *CYP27B1* gene (2–6). Activity of 1 $\alpha$ -hydroxylase is tightly regulated through complex mechanisms that depend on the circulating levels of calcium, phosphorus, parathyroid hormone, and 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Mutations in the 1 $\alpha$ -hydroxylase gene are known to cause vitamin D-dependency rickets type I (VDDR-I) (2, 7–9). Patients afflicted with this disease are unable to maintain normal serum calcium and suffer from secondary hyperparathyroidism, rickets, and osteomalacia (10). VDDR-I is cured by administration of physiological doses of 1,25(OH)<sub>2</sub>D<sub>3</sub> (11). Physiological doses of 25-OH-D<sub>3</sub> are noncurative, but high dose administration can be effective (11), presumably due to the ability of 25-OH-D<sub>3</sub> to bind and activate the vitamin D receptor when present in vast excess.

The experiments reported by Fraser and Kodicek in 1970 (12) were the first to demonstrate the kidney as the major, if not the only, tissue in which 1,25(OH)<sub>2</sub>D<sub>3</sub> is produced under normal physiological conditions. Over the next several years, extrarenal production of 1,25(OH)<sub>2</sub>D<sub>3</sub> was convincingly demonstrated in pregnant nephrectomized rats and in an anephric patient suffering from sarcoidosis (13–15). In these cases, synthesis was localized to the placenta and the sarcoid macrophages (14, 16, 17). Production of 1,25(OH)<sub>2</sub>D<sub>3</sub> at other sites has remained a subject of much investigation. A number of research groups have reported 1 $\alpha$ -hydroxylase activity in cultured cells, including those of the skin, bone, cartilage, intestine, prostate, and vascular epithelium (18–25). Bikle *et al.* (26) have also reported 1,25(OH)<sub>2</sub>D<sub>3</sub> production in perfused flaps of porcine skin. Local production of 1,25(OH)<sub>2</sub>D<sub>3</sub> has been proposed to regulate cellular function and/or differentiation in an autocrine or paracrine fashion (18, 19, 24, 27, 28), and it has been suggested that keratinocytes could supply 1,25(OH)<sub>2</sub>D<sub>3</sub> to the systemic circulation when renal production of the hormone is impaired (26, 29). Production of extrarenal 1,25(OH)<sub>2</sub>D<sub>3</sub> in these experiments is not supported, however, by *in vivo* metabolic studies in nephrectomized nonpregnant rats. In these studies, two independent research groups were unable to detect <sup>3</sup>H-1,25(OH)<sub>2</sub>D<sub>3</sub> in the tissue or plasma after administering a dose of <sup>3</sup>H-25-OH-D<sub>3</sub> of high specific radioactivity (30, 31). These conflicting results demonstrate a need for further investigation of the *in vivo* expression of the 1 $\alpha$ -hydroxylase. We have approached such an investigation by using gene targeting to replace the 1 $\alpha$ -hydroxylase coding sequence with a bacterial *lacZ* gene controlled by the 1 $\alpha$ -hydroxylase promoter. The *lacZ* gene codes for  $\beta$ -galactosidase, whose activity is readily detected *in situ* through histochemical staining with X-Gal (32). Herein we report the successful production of 1 $\alpha$ -hydroxylase null mice harboring the *lacZ* gene and present our analysis of *in vivo* 1 $\alpha$ -hydroxylase expression determined through histochemical assay of the reporter.

## Materials and Methods

**Targeting Vector Construction.** The mouse 1 $\alpha$ -hydroxylase gene was isolated from a P1 clone through PCR screening of a 129 strain mouse embryonic stem cell genomic library (33). *SacI*

Conflict of interest statement: No conflicts declared.

Abbreviations: 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; 1 $\alpha$ -hydroxylase, 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase; 25-OH-D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>; VDDR-I, vitamin D-dependency rickets type I.

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digestion of the clone yielded a 16-kb fragment containing the entire gene, which was subcloned in pBluescript KS (Stratagene). This *SacI* subclone was subjected to restriction mapping and used for determination of the gene sequence (33). A targeting construct was designed to replace the coding region of the  $1\alpha$ -hydroxylase with a cassette containing the *lacZ* and neomycin resistance genes. To create the construct, a 1.7-kb *EcoRI*-*XhoI* fragment containing the  $1\alpha$ -hydroxylase promoter (34) was subcloned into pBluescript KS, creating *EcoRI*-*XhoI*pBSPT. The *lacZ*-neo cassette (6.1 kb) was isolated from pHM2 (35) by *XhoI*-*SaI* digestion after introducing an *XhoI* site upstream of the *lacZ* gene (digestion with these two enzymes generated a cassette with compatible cohesive ends). *KpnI* digestion of the above-mentioned *SacI* subclone yielded a 6.4-kb fragment that included exon 9 of the  $1\alpha$ -hydroxylase gene and additional mouse genomic DNA (*KpnI* to *SacI* site), as well as the multiple cloning site of the parent vector (*SacI* to *KpnI*). The *lacZ*-neo cassette and the *KpnI* fragment were subcloned in *EcoRI*-*XhoI*pBSPT (*XhoI* and *KpnI* sites, respectively) to make pCYP1*alacZ*neo8.3. A 2.3-kb fragment containing the Herpes simplex virus thymidine kinase gene was isolated from the plasmid TK1-TK2C by *XhoI*-*SaI* digestion. This negative selection cassette (36) was subcloned in pCYP1*alacZ*neo8.3 at the *SaI* site in the multiple cloning sequence introduced with the *KpnI* fragment, to yield the final construct, pCYP1*alacZ*neoTK. Diagrams of pCYP1*alacZ*neoTK and the exchange between this vector and the wild-type allele to yield the  $1\alpha$ -hydroxylase mutant allele are illustrated in Fig. 6, which is published as supporting information on the PNAS web site.

**Stem Cell Transfection and Generation of Null Mice.** The targeting vector was amplified in *Escherichia coli* XL1-Blue (Stratagene) and purified with the EndoFree Plasmid Mega Kit (Qiagen, Valencia, CA). Embryonic stem cells (AB2.2) were transfected with linearized vector (*SaI* digestion), and positive transfectants were selected with the aminoglycoside G418. A total of 288 transfectants were chosen and clonally expanded. DNA from the clones was screened by PCR for homologous recombination by using 5'-GGCGGAGGAACAGGGAGATAAGC-3' as the forward primer, and 5'-TG TAGAGGGCCCAGGGATGTCAG-3' (wild-type allele) and 5'-CGGGCCTCTTCGCTATTACGC-3' (mutant allele) as the reverse primers. Because the wild-type and mutant PCR products generated were approximately equal in size (Fig. 6*b*), performing one PCR reaction with both reverse primers present was not possible, thus two separate reactions were performed for each clone. Thirteen embryonic stem cell clones were identified as homologous recombinants.

One of the recombinant clones was injected into C57BL/6J blastocysts, which were subsequently implanted into pseudopregnant females (B6CBAF1/J). Four male chimeric founders were obtained and backcrossed with C57BL/6J females. Germline transmission of the mutation was obtained from three of the four founders. Null mice were produced by crossing  $F_1$  heterozygotes. Offspring from generations  $F_3$  through  $F_8$  were used in studies to confirm the expected phenotype, and the  $F_{10}$  through  $F_{12}$  generations were used in the analysis of the reporter gene. Mouse lines descended from the chimeras were maintained separately; no phenotypic differences between the lines were observed. Mice were maintained on a C57BL/6J background. Genotyping was performed by PCR by using the primers listed above.

**Confirmation of Rachitic Phenotype.** Breeding pairs (+/-) were maintained on LabDiet Mouse Diet 5015 (PMI Feeds, St. Louis), which contains 0.8% Ca, 0.5% P, and 3.3 international units of vitamin  $D_3$  per gram. Offspring were weaned at 3 weeks of age and fed a purified maintenance diet containing 0.47% Ca, 0.3% P, 5 ng of  $1,25(\text{OH})_2\text{D}_3$  (Tetronics, Madison, WI) per 4 g

of diet, and vitamins A, E, and K; the composition of the basal diet is described in Yang *et al.* (37). At 5 weeks of age, the mice were switched to a high-calcium (1.2%) low-phosphorus (0.02%) diet, with supplementary vitamins A, E, D, and K. Egg-white albumin (Teklad, Madison, WI) served as the protein source. Weight measurements and blood collection were performed weekly. Mice were killed by cervical dislocation after 3 weeks on the regimen. Femurs and radii-ulnae units were removed for analysis of bone ash and epiphyseal plate width, respectively, and kidneys were collected for analysis of the  $1\alpha$ -hydroxylase transcript.

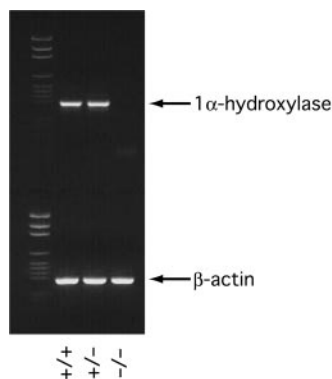
**Serum Chemistry Measurements.** Serum calcium was determined by atomic absorption spectroscopy (Perkin-Elmer Model 3110 spectrometer) after dilution of the serum in 0.1%  $\text{LaCl}_3$ . Serum phosphorus was determined by spectrophotometric assay by using the Sigma Diagnostics Phosphorus Reagent (Sigma Aldrich). Test values were compared with reference standards measured on the same day.

**Bone Ash Determination.** Femurs were cleaned of adherent tissue and soaked for 24–48 h in absolute ethanol. The ethanol was decanted, and the femurs were soaked for 24–48 h in chloroform to complete lipid removal. Dry weight was determined after heating at  $100^\circ\text{C}$  for at least 12 h. Femurs were then ashed by heating in a muffle furnace at  $600^\circ\text{C}$  for 24 h. Percent bone ash was determined as [(ashed weight/dry weight)  $\times$  100]. All weights were measured after cooling the samples in a desiccator.

**Analysis of Epiphyseal Plate Width.** Radii-ulnae units were cleaned of most of the adherent tissue. The bones were split longitudinally with a scalpel and soaked overnight in deionized water. Calcified regions were stained by briefly soaking the bones in 1.5%  $\text{AgNO}_3$ . Photographic imaging of the sections was performed with a Spotcam digital camera system (Diagnostic Instruments, Sterling Heights, MI) and METAMORPH Software (Universal Imaging, West Chester, PA).

**Detection of  $1\alpha$ -Hydroxylase Transcript.** Total RNA from the kidneys was isolated by using Tri Reagent (Molecular Research Center, Cincinnati, OH). Samples containing 2 mg of RNA were treated with RQ-1 RNase-Free DNase (Promega) and reverse-transcribed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR was performed in duplicate on the cDNA with the LightCycler System (Roche Applied Sciences). The oligonucleotides 5'-CCGCGGGCTATGCTGGAAC-3' (exon 3) and 5'-CTCTGGGCAAAGCAAACATCTGA-3' (exons 4 and 5) were used as the forward and reverse primers, respectively. Amplification of  $\beta$ -actin cDNA was performed for housekeeping purposes; 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TA-AAACGCAGCTCAGTAACAGTCCG-3' were the forward and reverse primers, respectively. PCR products were electrophoresed in a 2% agarose gel and visualized after staining with ethidium bromide.

**Rescue of VDDR-I.** Five-week-old -/- mice were placed on the high-calcium low-phosphorus diet to induce rickets. After 3 weeks, the mice were divided into four treatment groups and administered daily 0.1-ml s.c. injections of either vehicle (5% ethanol in propylene glycol), 25-OH- $D_3$  (250 ng per dose), or  $1,25(\text{OH})_2\text{D}_3$  (10 or 25 ng per dose) for a total of 6 days. After 6 additional days of no treatment, the mice were killed, and radii-ulnae units were removed for analysis of epiphyseal plate width as described above. The mice remained on the experimental diet throughout the experiment. Each treatment group contained at least four mice.



**Fig. 1.** Agarose electrophoresis of products generated from RT-PCR of total kidney RNA from  $+/+$ ,  $+/-$ , and  $-/-$  mice by using  $1\alpha$ -hydroxylase- and  $\beta$ -actin-specific primers. pGEM DNA markers (Promega) are shown in the first lane. PCR product sizes are 345 bp for  $1\alpha$ -hydroxylase, and 350 bp for  $\beta$ -actin.

**$\beta$ -Galactosidase Induction.** Offspring ( $+/+$ ,  $+/-$ , and  $-/-$ ) from  $+/-$  breeders were weaned at 3 weeks of age and fed the maintenance diet described earlier. At 6 weeks of age, the mice were transferred to a room with UV-filtered light, and calcium and  $1,25(\text{OH})_2\text{D}_3$  were removed from the diet ( $[\text{Ca}] = 0.02\%$ ). Animals were killed beginning at day 1, continuing on even days through day 12. Whole kidneys and skin sections were harvested at death for reporter gene analysis. Skin sections measuring  $\approx 2 \times 1$  cm were taken from the sides of the trunk; fur was shaved from the area before skin harvest.

To induce  $\beta$ -galactosidase expression in the placenta, 7-week-old  $-/-$  mice receiving the maintenance diet were mated. At day 10 of gestation, the dams were put in UV-filtered lighting, and calcium and  $1,25(\text{OH})_2\text{D}_3$  were removed from the diet. At gestation day 19, the dams were killed, and the fetuses and placentas were harvested for analysis. Fetuses and placentas from  $+/+$  dams (bred with  $+/+$  males and treated in the same manner) were used for negative controls.

**Tissue Preparation and  $\beta$ -Galactosidase Assay.** Samples were rinsed in ice-cold PBS and fixed in 4% paraformaldehyde in PBS at pH 7.0–7.5 (kidneys were bisected to facilitate penetration of the fixative). After fixation, the samples were permeabilized at room temperature by rinsing with 0.1 M phosphate buffer at pH 7.3, containing 2 mM  $\text{MgCl}_2$ , 0.01% sodium deoxycholate, and 0.02% Nonidet P-40. Some samples were stained directly at this point; others were used to prepare frozen sections for subsequent staining. For the latter procedure, samples were cryoprotected by incubation in 30% sucrose and frozen in Neg-50 section medium (Richard-Allan Scientific, Kalamazoo, MI). Sections measuring 10–14 microns in thickness were made with a Cryostat HM 505 E (Microm, Walldorf, Germany). Histochemical staining with X-Gal was carried out at pH 7.3 according to published procedures (38). A uniform staining time of 4 h at room temperature was used throughout, after which the remain-

ing substrate was removed by rinsing with PBS. All tissue sections were counterstained with eosin.

**Keratinocyte Culture and Assay.** Newborn  $-/-$  and  $+/+$  pups (1–2 days, genotype confirmed by PCR of tail DNA) were killed and rinsed in 70% ethanol. After decapitation and limb amputation, the bodies were rinsed briefly in betadine, followed by a rinse in 70% ethanol and two rinses in PBS. Keratinocytes were isolated and cultured in serum-free nondifferentiating conditions according to the manufacturer's protocol supplied with Defined Keratinocyte-SFM (Invitrogen). Briefly, the skin was removed and placed dermal side down in culture plates containing 25 caseinolytic units of dispase (Invitrogen) and 5 mg/ml gentamycin in PBS. After overnight incubation at  $4^\circ\text{C}$ , the epidermal layer of keratinocytes was separated from the dermis, minced, and digested in 0.05% trypsin in 0.53 mM EDTA for 20 min at  $37^\circ\text{C}$ . Digestion was quenched by addition of soybean trypsin inhibitor in PBS to a final concentration of 10 mg/ml. The keratinocyte suspension was centrifuged at  $40 \times g$  for 5 min, gently resuspended, and plated at  $\approx 3 \times 10^6$  cells per T-75 flask in 20 ml of Defined Keratinocyte-SFM. Cells were cultured at  $37^\circ\text{C}$ , in 5%  $\text{CO}_2$ -air with  $>95\%$  humidity. The medium was changed 48–72 h after plating; cells were passed when they reached 60–70% confluency. Keratinocytes were analyzed for  $\beta$ -galactosidase activity at low passage number (2–5), and assays were done in 6- and 12-well plates. Before addition of X-Gal, cells were washed twice in PBS, fixed for 10 min in 4% paraformaldehyde, and washed again with PBS. Incubation in X-Gal solution was performed for either 16 h at  $4^\circ\text{C}$  or 3 h at  $37^\circ\text{C}$ , after which the cells were rinsed with PBS and counterstained with eosin.

## Results

**Obliteration of the  $1\alpha$ -Hydroxylase Gene.** Fig. 1 illustrates the knockout of the  $1\alpha$ -hydroxylase gene. Activation of the  $1\alpha$ -hydroxylase promoter was stimulated by phosphorus deprivation (39–41). Quantitative RT-PCR of kidney RNA with  $1\alpha$ -hydroxylase-specific primers demonstrated that  $1\alpha$ -hydroxylase transcript was present only in the  $+/+$  and  $+/-$  mice.

**Induction of VDDR-I in  $1\alpha$ -Hydroxylase Null Mice.**  $+/+$ ,  $+/-$ , and  $-/-$  mice were fed a purified diet high in calcium, low in phosphorus, and supplemented with fat-soluble vitamins, including vitamin  $\text{D}_3$ . Diets low in phosphorus create a state of hypophosphatemia, which can rapidly lead to rickets when  $1,25(\text{OH})_2\text{D}_3$  is not available (42). The biochemical response of the mice to the diet is shown in Table 1, and the effect on the long bones of the wrist is illustrated in Fig. 2A. As expected, serum calcium remained in the normal range for all mice in the study, whereas serum phosphorus values declined rapidly. At death, all mice were hypophosphatemic. Silver nitrate staining of the wrist sections indicated that the epiphyseal plates of the  $+/+$  and  $+/-$  mice remained narrow, but those of the  $-/-$  mice were extremely wide, a condition that arises due to improper mineralization of the cartilage within the plate and is a hallmark of

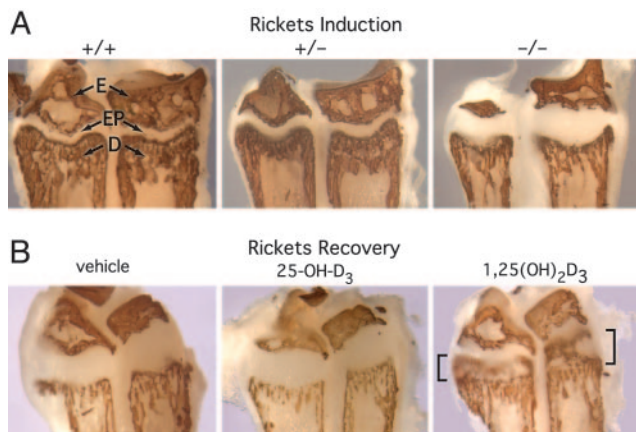
**Table 1. Biochemical response to 1.2% Ca, 0.02% P diet**

Genotype	n	Serum Ca, mg%*		Serum P, mg%*		Bone ash, <sup>†</sup> %
		Initial	At death	Initial	At death	
$-/-$	17	$9.9 \pm 0.8$	$9.1 \pm 1.2$	$8.4 \pm 1.4$	$2.0 \pm 0.7$	$51.2 \pm 0.6^\ddagger$
$+/-$	23	$9.5 \pm 0.7$	$11.0 \pm 1.2$	$7.9 \pm 1.1$	$2.9 \pm 0.7$	$53.9 \pm 0.5$
$+/+$	17	$10.3 \pm 0.6$	$10.4 \pm 0.6$	$7.4 \pm 0.9$	$2.7 \pm 0.4$	$54.4 \pm 0.6$

\*Expressed as mean  $\pm$  SD.

<sup>†</sup>Expressed as mean  $\pm$  SEM.

<sup>‡</sup>Significance at  $P < 0.003$  vs.  $+/-$ ;  $P < 0.001$  vs.  $+/+$ .

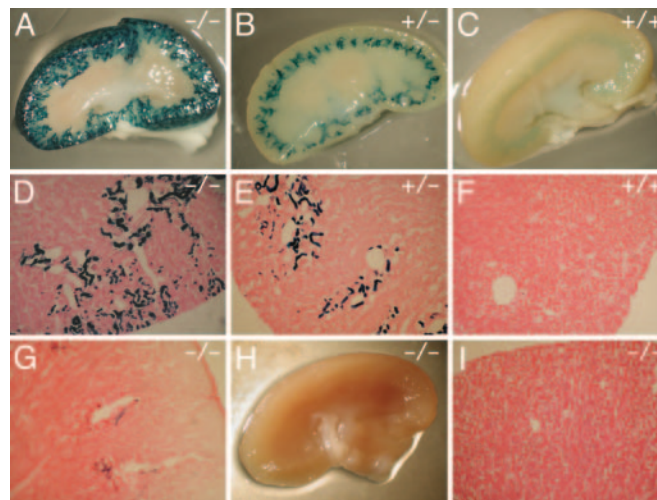


**Fig. 2.** Silver nitrate staining of wrist long bones for analysis of rickets induction and rescue. (A) Wrists of  $+/+$ ,  $+/-$ , and  $-/-$  animals fed a high-calcium phosphorus-free diet. E, EP, and D indicate the epiphysis, epiphyseal plate, and diaphysis of the growing bone, respectively. (B) Wrists of rachitic  $-/-$  mice after 6-day treatment with vehicle, 25-OH-D<sub>3</sub> (250 ng/day) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (25 ng/day). The zone of newly calcified cartilage in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mouse is indicated by the brackets.

rickets. Close inspection also revealed that the diaphyses in the wrists of the  $-/-$  mice were narrower and more diffuse than those of the  $+/+$  and  $+/-$  mice. The difference in mean bone ash percentage between groups was not as large as expected based on earlier work with B6C3Fe mice (43), and although statistical analysis (ANOVA with three post hoc tests of significance) indicated the mean value of the  $-/-$  group was significantly different from that of the  $+/-$  and  $+/+$  groups, individual bone ash measurements were a poor indicator of rickets development.

**Reversal of VDDR-I.** Fig. 2B illustrates the effect of 25-OH-D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment on the growth plates in the wrists of rachitic  $-/-$  mice. These mice were made rachitic through administration of the high-calcium low-phosphorus diet and then given a series of daily s.c. injections of either vehicle or the above compounds. Wrist epiphyseal plates from mice treated with 25-OH-D<sub>3</sub> (250 ng per day) were indistinguishable from those treated with vehicle. Treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> at 25 ng per day was observed in all cases to stimulate mineralization of the epiphyseal cartilage and thus reverse the rickets. This phenomenon is demonstrated in Fig. 2B Right, where a zone of new calcification within the growth plate is clearly obvious. This effect was also observed in  $\approx 25\%$  of mice treated with daily injections of 10 ng of 1,25(OH)<sub>2</sub>D<sub>3</sub> (data not shown).

**Tissue Distribution of  $\beta$ -Galactosidase.** The *E. coli lacZ* gene in the mutant mice is controlled by the endogenous 1 $\alpha$ -hydroxylase promoter. The promoter was stimulated by switching the mice to a diet low in calcium and devoid of 1,25(OH)<sub>2</sub>D<sub>3</sub>, after which tissues were prepared and stained with X-Gal to reveal the location of the expressed gene product. Representative kidney specimens are shown in Fig. 3.  $\beta$ -Galactosidase activity well above the background level of the  $+/+$  mice was clearly evident in the renal cortex of both the  $+/-$  and  $-/-$  mice after administration of the experimental diet. Enzyme activity was detectable in the  $-/-$  mice after 1 day on the diet and was maximally stimulated by day 10.  $\beta$ -Galactosidase activity became detectable in the  $+/-$  mice considerably later (day 6), and these mice expressed the enzyme to a significantly lower extent. Under conditions of complete vitamin D deficiency, the pattern of  $\beta$ -galactosidase expression in the heterozygotes would be ex-



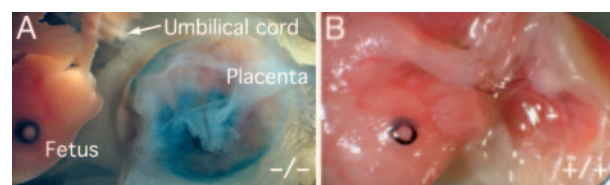
**Fig. 3.** Reporter gene activity in kidneys of 1 $\alpha$ -hydroxylase mutant mice. (A–C) Gross stains of kidneys (longitudinal bisection) from  $-/-$ ,  $+/-$ , and  $+/+$  mice after 8-day administration of diet lacking calcium and any form of vitamin D<sub>3</sub>. (D–F) Stains of thin kidney sections after 8 days of experimental diet ( $\times 40$ ). (G) Stains of kidney from a  $-/-$  mouse after 1 day on the experimental diet. (H and I) Kidney stains of a  $-/-$  mouse that received the maintenance diet only.

pected to closely mimic that of the null mice. Complete vitamin D deficiency was not achieved in this experiment, however, because the mice received vitamin D from their mothers before weaning. Upon administration of the experimental diet, the  $+/-$  mice were thus capable of producing 1,25(OH)<sub>2</sub>D<sub>3</sub>, which presumably down-regulated further gene expression driven by the 1 $\alpha$ -hydroxylase promoter.

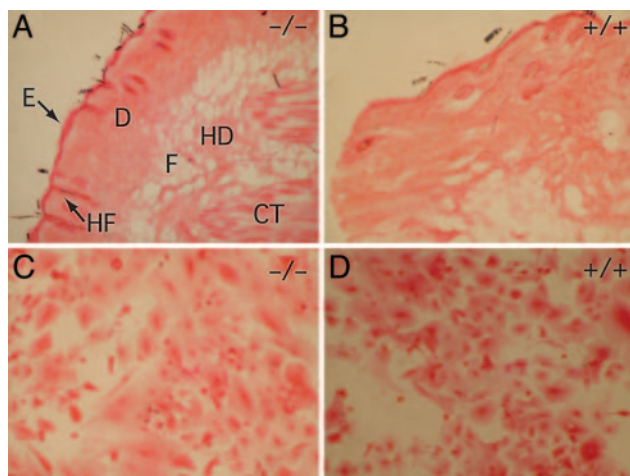
$\beta$ -Galactosidase expression was also observed in placentas harvested from  $-/-$  dams subjected to calcium and 1,25(OH)<sub>2</sub>D<sub>3</sub> restriction during pregnancy (Fig. 4), confirming earlier reports of 1 $\alpha$ -hydroxylase activity in placental tissues. 1 $\alpha$ -Hydroxylase activity in the skin, however, was not confirmed by the reporter gene assay. As shown in Fig. 5,  $\beta$ -galactosidase activity was not detected in frozen skin sections or in primary keratinocyte cultures. Skin sections shown in Fig. 5 were prepared from  $-/-$  mice that received the calcium- and 1,25(OH)<sub>2</sub>D<sub>3</sub>-restricted diet for 8 days; the same results were obtained upon diet restriction for 12 days and with prolonged exposure to X-Gal (data not shown). Keratinocytes were cultured in medium containing low calcium ( $< 0.1$  mM); under these conditions differentiation is minimal, and on the basis of previous reports, the 1 $\alpha$ -hydroxylase promoter should be highly stimulated (44).

## Discussion

The absence of detectable 1 $\alpha$ -hydroxylase gene transcript in the  $-/-$  mice verifies that the gene has been abolished and its



**Fig. 4.** Extrarenal 1 $\alpha$ -hydroxylase promoter activity in placental tissue. X-Gal staining of fetuses and placentas harvested after mating of  $-/-$  (A) and  $+/+$  (B) mice. Promoter activity was stimulated during midgestation by depriving dams of calcium and any form of vitamin D<sub>3</sub>, as described in *Materials and Methods*.



**Fig. 5.** X-Gal stains of skin sections and cultured keratinocytes. (A and B) X-Gal stains ( $\times 100$ ) of frozen skin sections from  $-/-$  and  $+/+$  mice after 8-day administration of diet lacking calcium and any form of vitamin  $D_3$ . The epidermis, dermis, and hypodermis are indicated as E, D, and HD, respectively. Hair follicles (HF) extend into the dermis, and the fat (F) and connective tissue (CT) layers of the hypodermis are clearly visible. (C and D) X-Gal stains of keratinocytes ( $\times 400$ ) from  $-/-$  and  $+/+$  newborns grown in nondifferentiating conditions.

product,  $1\alpha$ -hydroxylase, is not synthesized in the mice. We are able to maintain healthy  $-/-$  mice on a purified diet containing 0.47% Ca, 0.3% P, and 5 ng of  $1,25(OH)_2D_3$  per 4 g of diet. Under these conditions the  $-/-$  mice are fertile.

Upon removal of phosphorus from the diet (thereby creating a severe mineral imbalance) and replacement of  $1,25(OH)_2D_3$  with vitamin  $D_3$ , the  $-/-$  mice rapidly developed rickets. Treatment with low doses of  $1,25(OH)_2D_3$  reversed this condition, but treatment with 25-OH- $D_3$ , the biological precursor of  $1,25(OH)_2D_3$  and substrate for the  $1\alpha$ -hydroxylase, did not [at least not at the dose tested, which was 10 times that of the highest dose of  $1,25(OH)_2D_3$ ]. These results establish that this  $1\alpha$ -hydroxylase knockout is a valid mouse model for VDDR-I. [It should be noted that the low-phosphorus diet will also give rise to the eventual development of rickets in the  $+/+$  and  $+/-$  mice, but their ability to synthesize  $1,25(OH)_2D_3$  from the available vitamin  $D_3$  provides them protection from the disease during the course of the experiment.]

It has been well established that conditions of pregnancy and granulomatous disease result in extrarenal synthesis of  $1,25(OH)_2D_3$ , but the issue of whether  $1,25(OH)_2D_3$  is synthesized in extrarenal tissues under normal physiological circumstances has remained unresolved.  $1,25(OH)_2D_3$  production has been demonstrated *in vitro* in cell culture (18–25); however, these experiments do not necessarily represent actual *in vivo* cellular activity. The  $\beta$ -galactosidase reporter gene has proved an excellent tool for *in situ* detection of transcription driven *in vivo* by endogenous promoters (45–49). We engineered this reporter gene into the *CYP27B1* locus to reveal the normal distribution of  $1\alpha$ -hydroxylase promoter activity. For this initial study, we gave particular focus to the skin, since endogenous production of  $1,25(OH)_2D_3$  in keratinocytes has been proposed to regulate growth and differentiation of these cells *in vivo*, especially under conditions of low circulating levels of  $1,25(OH)_2D_3$  (26, 29). Acute  $1,25(OH)_2D_3$  deficiency in the  $-/-$  mice did not trigger *in vivo* expression of  $\beta$ -galactosidase in the skin, and *in vitro* culture of keratinocytes from  $-/-$  mice also failed to induce the reporter gene. We believe these findings reflect a strict dependence of keratinocytes on  $1,25(OH)_2D_3$

produced in the kidney and delivered through the circulatory system.

We have performed preliminary analyses of reporter gene activity in several other tissues, including lung, intestine, skeletal muscle, liver, and ovary, and in each case, no reporter gene activity was observed. The absence of  $\beta$ -galactosidase expression in the skin and these other tissues is consistent with previous reports that acutely nephrectomized nonpregnant rats produce no  $1,25(OH)_2D_3$  (30, 31), and it reaffirms the previous conclusion from our laboratory and others that the kidney is the sole source of  $1,25(OH)_2D_3$  synthesis under normal physiological conditions (12, 30, 31, 50). We must consider the possibility, however, that  $1\alpha$ -hydroxylase promoter activity in extrarenal tissues could be less than that detectable by the assay, so the present data do not constitute unequivocal proof. Also, in the event that extrarenal synthesis of  $1,25(OH)_2D_3$  in a nondiseased state requires a stimulus other than those known to regulate the renal  $1\alpha$ -hydroxylase, our experimental system may not report promoter activity in extrarenal tissues. Although we cannot discount the possibility of an unknown stimulus, we believe it an unlikely scenario for regulation of  $1,25(OH)_2D_3$  synthesis external to the kidney.

The  $1\alpha$ -hydroxylase promoter activity we report stands in stark contrast to that reported by Hewison and coworkers (28). In their study,  $1\alpha$ -hydroxylase expression was probed in histologically normal human tissue sections with a polyclonal antiserum, and positive staining was reported in the skin, lymph nodes, colon, pancreas, adrenal medulla, brain, and placenta. Using the same antiserum, this group had previously reported  $1\alpha$ -hydroxylase expression in both the cortical and medullary regions of the kidney and proposed that under conditions of vitamin D sufficiency  $1,25(OH)_2D_3$  is synthesized in the distal convoluted tubules and the cortical and medullary collecting ducts, where it serves an autocrine function (51). As shown in Fig. 3, we detected  $\beta$ -galactosidase activity in the renal cortex only. This observation is consistent with previous studies of isolated nephrons from vitamin D-deficient rats, chickens, and rabbits, all of which reported localization of  $1\alpha$ -hydroxylase activity to the proximal tubules (52–55). Given the extensive staining of the distal nephrons reported by Hewison and coworkers (28), our reporter assay system should have easily detected  $1\alpha$ -hydroxylase promoter activity in the renal medulla. No such activity was ever observed in either the vitamin D-deficient or -sufficient state. The results we report thus call into question the specificity of the polyclonal antiserum used by these investigators for detection of the  $1\alpha$ -hydroxylase.

At present, two other independent research groups have produced  $1\alpha$ -hydroxylase knockout mice and established that they are valid mouse models of VDDR-I (56–58). Dardenne *et al.* (57) have engineered a mouse strain carrying floxed (loxP)  $1\alpha$ -hydroxylase alleles for creation of tissue-specific *CYP27B1* knockouts. This strain has been used for specific inactivation the  $1\alpha$ -hydroxylase in chondrocytes (59). A thorough analysis of these mice has not been reported, but the authors (59) have stated their preliminary assessment indicated both mineral ion homeostasis and epiphyseal growth plate appearance remained normal. These findings argue against the necessity of locally produced  $1\alpha,25-(OH)_2D_3$  for chondrocyte growth and maturation. Our continued investigation of the  $\beta$ -galactosidase reporter gene will also include an examination of  $1\alpha$ -hydroxylase promoter function in the cartilage and bone.

The *CYP27B1* null mutation affords us the opportunity to produce  $1,25(OH)_2D_3$ -deficient mice quickly and efficiently. By disrupting the  $1\alpha$ -hydroxylase gene, we have eliminated an element of control for parathyroid hormone regulation of serum calcium. Thus, in addition to our investigation of extrarenal  $1,25(OH)_2D_3$  synthesis, we also envision that these mice will

serve as a valuable research tool for our continued study of calcium homeostasis.

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