Increased Intestinal Vitamin D Receptor in Genetic Hypercalciuric Rats

A Cause of Intestinal Calcium Hyperabsorption

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Abstract

In humans, familial or idiopathic hypercalciuria (IH) is a common cause of hypercalciuria and predisposes to calcium oxalate nephrolithiasis. Intestinal calcium hyperabsorption is a constant feature of IH and may be due to either a vitamin D–independent process in the intestine, a primary overproduction of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], or a defect in renal tubular calcium reabsorption. Selective breeding of spontaneously hypercalciuric male and female Sprague-Dawley rats resulted in offspring with hypercalciuria, increased intestinal calcium absorption, and normal serum 1,25$(OH)_2D_3$ levels. The role of the vitamin D receptor (VDR) in the regulation of intestinal calcium absorption was explored in 10th generation male genetic IH rats and normocalciuric controls. Urine calcium excretion was greater in IH rats than controls (2.9±0.3 vs. 0.7±0.2 mg/24 h, $P < 0.001$). IH rat intestine contained twice the abundance of VDR compared with normocalciuric controls (536±73 vs. 243±42 nmol/mg protein, $P < 0.001$), with no difference in the affinity of the receptor for its ligand. Comparable migration of IH and normal intestinal VDR on Western blots and of intestinal VDR mRNA by Northern analysis suggests that the VDR in IH rat intestine is not due to large deletion or addition mutations of the wild-type VDR. IH rat intestine contained greater concentrations of vitamin D–dependent calbindin 9-kD protein. The present studies strongly suggest that increased intestinal VDR number and normal levels of circulating 1,25$(OH)_2D_3$ result in increased functional VDR–1,25$(OH)_2D_3$ complexes, which exert biological actions in enterocytes to increase intestinal calcium transport. Intestinal calcium hyperabsorption in the IH rat may be the first example of a genetic disorder resulting from a pathologic increase in VDR. (*J. Clin. Invest.* 1993. 91:661–667.) Key words: male rats • calcium absorption, 1,25$(OH)_2D_3$, vitamin D receptor • calbindin 9 kD

Introduction

Hypercalciuria may increase urine calcium oxalate supersaturation and contributes to increased risk for calcium oxalate nephrolithiasis (1, 2). Idiopathic hypercalciuria (IH),[1] a common cause of hypercalciuric calcium oxalate nephrolithiasis (3), is accompanied by intestinal calcium overabsorption in almost all patients (4–9) and is the major source of urinary calcium excess during adequate calcium intake (10). The pathogenesis of intestinal calcium hyperabsorption and hypercalciuria in IH are unknown, but clinical evidence suggests the disease is heterogenous with at least three possible pathogenetic mechanisms for the calcium hyperabsorption: inappropriate renal 1,25$(OH)_2D_3$ synthesis with increased serum 1,25$(OH)_2D_3$levels (11–14); a primary increase in enterocyte calcium transport with normal serum 1,25$(OH)_2D_3$ levels (7, 8, 10, 14); and a primary renal tubule defect in calcium reabsorption with compensatory increases in parathyroid hormone, 1,25$(OH)_2D_3$, and intestinal calcium absorption (7, 8, 15).

IH affects 5–10% of the adult population (16, 17) and familial patterns of inheritance suggest a genetic disorder with a dominant mode of inheritance (18–21). Because of the difficulty in separating dietary and genetic factors that may influence calcium metabolism in patients with IH, a colony of genetically hypercalciuric rats was established (22) through breeding spontaneously hypercalciuric male and female Sprague-Dawley rats (23, 24). In this colony, hypercalciuria increased in intensity and frequency among male and female offspring with successive generations (22). Genetic idiopathic hypercalciuric rats have normal serum calcium, increased intestinal calcium absorption, and normal, not elevated, serum 1,25$(OH)_2D_3$ (22). Thus, IH rats may be analogous to some forms of human IH.

There is considerable evidence that 1,25$(OH)_2D_3$ regulates target tissue biological response through genomic events that involve binding of the steroid hormone to the intracellular vitamin D receptor (VDR) (25–27). In addition, studies in vitro using cell cultures (28, 29) and in vivo in rats (30–32) indicate that the biological actions of 1,25$(OH)_2D_3$ are strongly correlated with VDR number and occupancy (33, 34). Therefore, the present study was undertaken to test the hypothesis that increased intestinal calcium transport in IH rats may result from an increase in intestinal cell VDR content.

Methods

Genetic hypercalciuric rats. Male and female rats of the 10th generation of inbreeding of hypercalciuric rats were placed in individual metabolic cages and fed 13 g/d of a diet containing 0.6% calcium, 0.65% phosphorous, 0.24% magnesium, 0.4% sodium, 0.43% chloride, and 2.2 IU vitamin D/g of food. Deionized distilled water was given ad libitum. Two successive 24-h urine collections in 0.25 ml 6 N HCl were obtained after the animals had been equilibrated on the diet (5 d). Those animals with urine calcium excretion of > 2 SD above the mean of control rats (usually > 1.5 mg/24 h) were considered hypercalciuric. Male and female rats with the highest calcium excretion values were chosen for breeding to propagate the colony. The remaining hypercalciuric animals were used in the following studies.

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Received for publication 3 December 1991 and in revised form 23 July 1992.

1. *Abbreviations used in this paper*: Gt, tissue conductance; IH, idiopathic hypercalciuria; Isc, short circuit ccurrent; Jms, mucosal to serosal flux; Jsm, serosal to mucosal flux; VDR, vitamin D receptor.

J. Clin. Invest.

0021-9738/93/02/0661/07 $2.00
Volume 91, February 1993, 661–667

Normal male Sprague-Dawley rats obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) were used as controls and were fed a similar 0.6% calcium diet for ≥ 5 d before study. Previous studies have shown that normal and IH rats grow at the same rate. Therefore, animals matched for weight are also age matched.

In vitro calcium transport. At killing, adjacent segments of proximal duodenum were removed and rinsed in ice-cold saline and mounted as the partition between two Lucite hemi-chambers with an exposed tissue area of 0.49 cm^2. Mucosal and serosal surfaces were bathed in 10 ml of KRB (pH 7.4) with the following composition (mM): 120 NaCl, 25 $NaHCO_3$; 5.0 KCl, 1.25 $CaCl_2$; 1.2 $MgSO_4$, and 11 D-glucose. Mucosal and serosal reservoirs were gassed continuously with O_2/CO_2 (95:5) to maintain pH and adequate mixing. Unidirectional transepithelial calcium fluxes were measured as previously described (35) by the addition of ^{45}Ca as $CaCl_2$ to either mucosal or serosal reservoirs and removal of aliquots from the unlabeled reservoirs at 20-min intervals. ^{45}Ca content was determined using a liquid-scintillation spectrometer (model 4530; Packard Instrument Co., Inc., Downers Grove, IL). Steady state calcium fluxes from mucosa to serosa (Jms) and serosa to mucosa (Jsm) were calculated by standard methods (36). Net flux is the difference between the two unidirectional fluxes with positive values indicating net absorption.

Intrinsic electrical gradients across the intestinal segments were neutralized by passing a continuous short-circuit current as previously described (37). Steady state fluxes for each pair of tissues were used in the data analysis only if their tissue conductances (G_t) agreed within 30%.

Preparation of vitamin D receptor–rich tissues. Animals were placed under ether anesthesia and exsanguinated via the abdominal aorta. The proximal 10 cm of duodenum was removed, rinsed in ice-cold PBS, and the epithelial layer scraped free of the underlying muscle coats. Cells were washed three times with PBS and sonicated on ice in hypertonic buffer with the following composition (mM): 300 KCl, 10 tris, 1 EDTA, 10 Molybdate, and 50 ng/ml Aprotinin for 20 min. The homogenate was centrifuged at 115,000 *g* at 4°C for 60 min, and the vitamin D receptor–rich cytosol supernatant was used in subsequent binding assays.

After exsanguination, kidneys were perfused in situ with ice-cold PBS until the tissue was pale. One kidney was removed, minced into 0.5-cm cubes, and the cells were dispersed using a Polytron for 2 s. The cell suspension was washed three times with ice-cold PBS and sonicated for 20 min while on ice in the same hypertonic buffer used to prepare intestinal receptor. Receptor-rich cytosol fraction is then obtained as described for intestinal homogenate.

Vitamin D receptor saturation assay. $1,25(OH)_2D_3$ binding to the cytosol fraction was performed using [^3H]$1,25(OH)_2D_3$ (specific activity 110 Ci/mmol) in a saturation binding assay by our modification (30) of the method of Feldman et al. (38). Briefly, 0.1-ml aliquots of cytosol fraction containing ~ 150 μg protein were incubated with [^3H]$1,25(OH)_2D_3$ (0.15–2.5 nM) at 4°C for 16 h. Unbound ligand was removed from the cytosol fraction using dextran-charcoal, and radioactivity in the soluble cytosol fraction representing [^3H]$1,25(OH)_2D_3$ bound to receptor was determined by liquid-scintillation spectrometry. Nonspecific binding was determined in a parallel incubation as the residual radioactivity that remained in the bound fraction after incubation in the presence of 250-fold excess nonradioactive $1,25(OH)_2D_3$ and 100-fold excess 25-hydroxyvitamin D_3. Nonspecific binding was usually < 5% of total binding.

Northern analysis. The proximal 15 cm of duodenum was removed, rinsed with PBS buffer, and the epithelial cells were scraped from underlying coats. Total RNA was purified by the method of Chomczynski and Sacchi (39) using guanidine isothiocyanate-phenol-chloroform. mRNA was purified by elution of total RNA over a column of oligo-dT cellulose (40) and subjected to electrophoresis (10 μg mRNA per lane) on 1% denatured agarose gel. Separated mRNA species were transferred to nylon membranes (Nytran), dried, and baked for 60 min at 80°C. The membranes were hybridized overnight with a 1.7 kb cDNA probe for rat VDR (41, 42) kindly provided by Dr. J. W. Pike, (San Diego, CA) or actin (gamma actin supplied by Dr. Harinder Singh, Chicago) uniformly labeled with [^{32}P]dCTP by the hexamer extension method using a kit (Promega Corp., Madison, WI). The rat VDR cDNA contains a major portion of the open reading frame and part of the 3′ untranslated region. Hybridization was performed under standard conditions using SSC buffer containing 5% Denhardt's solution, SDS, and salmon DNA at 37°C, followed by washing at 68°C. High stringent conditions were used to reduce background radioactivity to undetectable. After the filter was washed with 0.1 × SSC at room temperature, liquid was removed using filter paper. The membranes were then subjected to autoradiography and the intensity of the autoradiograms was determined using laser densitometry. VDR mRNA was expressed as the VDR/actin ratio to compensate for potential differences in mRNA loaded onto the gels.

Isolation of intestinal nuclei. Nuclei were isolated by the method of Dupret et al. (43) in which the rats were placed under deep ether anesthesia and the lumen of the proximal 10 cm of duodenum rinsed with cold PBS and removed. The intestine was then opened along a longitudinal incision and placed on ice and the epithelium scraped from the underlying muscle coats. The cells were washed twice with 5 ml PBS and then with ice-cold Krebs-Ringer Phosphate (KRP) buffer (pH 7.4). Cells were resuspended in KRP-6% dextran solution and homogenized for 2 min at 100 rpm while on ice. After filtering through a 200-gauge screen and being washed, the cells were resuspended in buffer with the following composition (mM): 60 KCl, 15 NaCl, 0.15 spermin, 0.5 spermadin, 14 mercaptoethanol, 0.5 EGTA, 2.0 EDTA, 15 Hepes, 0.3 M sucrose, and Triton X-100 and homogenized for 2 min. The nuclei were then washed three times in the above buffer and resuspended in buffer for a final concentration of 10^7 nuclei/0.1 ml.

Nuclear transcription assay. The transcription run-on assay using nuclei from intestinal cells was performed by the method of Vaulont et al. (44) and Schibler et al. (45). Briefly, nuclei were added to 0.4 ml reaction buffer with the following composition (mM): 20 Tris, 50 NaCl, 4 $MnCl_2$, 1.2 dithiothreitol, 10 creatine-P, 1 mg/ml heparin sulfate, 500 U/ml RNasine, and 20% glycerol in the presence of 1 mM ATP, GTP, and CTP and 250 μCi [^{32}P]UTP (800 Ci/mmol). Preliminary studies showed linear incorporation of [^{32}P]UTP for 40 min, therefore subsequent incubations were continued for 30 min. The reaction was stopped by the addition of SDS in Tris buffer, DNAase I, and proteinase K for 30 min. RNA was separated from unincorporated nucleotides by precipitation with phenol-chloroform. The RNA was then dissolved in TES solution and placed on membranes with 2× SSC for 1 h for hybridization with VDR cDNA, actin cDNA, or calbindin 9-kD cDNA. Background was determined from the radioactivity remaining on the membranes after hybridization with rat insulin cDNA, which is not expressed by enterocytes. After washing with 2× SSC, the dried membranes were subjected to autoradiography and laser scanning densitometry.

Western blot. VDR in 100 μg of duodenal epithelial cell cytosolic protein was resolved by 12% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA) by electroblotting (46). Blocking was accomplished using Tris-buffered saline with 5% bovine serum albumin for 60 min. Membranes were then incubated in the presence of monoclonal anti-chick VDR antibody at 1:1,500 dilution (41) followed by rabbit anti-rat IgG second antibody (1:1,000) and I^{125}-protein A. Membranes were then washed and subjected to autoradiography. VDR or Calbindin 9 kD in duodenal cytosolic fraction (10 μg protein) was detected using 15% SDS-PAGE electrophoresis and transferred to Immobilon-P membranes by elecroblot. The membranes were reacted with anti-chick VDR monoclonal antibody 9A7 gamma kindly provided by Dr. J. W. Pike (41) or polyclonal anti-rat calbindin 9-kD antisera (provided by Dr. Elizabeth Bruns, University of Virginia; reference 47), and then goat anti-rabbit IgG followed by development by the alkaline phosphatase method (48, 49).

Serum and urine chemistries. Serum calcium was measured by modified colorimetric microanalysis (Sigma Kits; Sigma Chemical

Table I. Effects of Hypercalciuria on In Vitro Duodenal Bidirectional Ca Fluxes and Electrical Parameters

Group	*n*	Jms	Jsm	Jnet	Isc	Gt
			nmol/cm² per h		*μA/cm²*	*mmhos/cm²*
NM	9	51±12	11±2	40±11	45±6	37±4
HM	7	264±27*	19±2*	245±28*	195±22*	13±1*
NF	11	29±9	14±2	14±8	50±6	30±3
HF	5	258±40*	23±2*	235±40*	189±26*	11±1*

Values are mean±SE for *n* rats per group. NM and HM are normal and hypercalciuric males; NF and HF are normal and hypercalciuric females. Jnet is Jms − Jsm. * vs. normal of same sex, $P < 0.01$.

Co., St. Louis, MO) and serum phosphorus by autoanalyzer methodology. Serum $1,25(OH)_2D_3$ was measured in duplicate on 1.0-ml aliquots from individual rats using a radioreceptor assay as previously described (50, 51). All samples were analyzed in a single assay in which the sample recovery was 67±1.9% (mean±SEM).

Materials. $1,25(OH)_2$-[23,24(*n*)-[^{3}H]vitamin D_3 (110 Ci/mmol) and $^{45}CaCl_2$ were purchased from Amersham Searle Co. (Arlington Heights, IL). Crystalline $250HD_3$ was a gift from Organon Inc. (West Orange, NJ), and $1,25(OH)_2D_3$ was a gift from Dr. Milan Uskokovic (Roche Laboratories, Nutley, NJ). Other reagent grade chemicals were purchased from Sigma Chemical Co.

Statistical analyses. Values are presented as group mean and standard error of the mean (mean±SE) for *n* observations per group. Significance of differences between two group means was assessed by Student's *t* test. Variances within groups were not assumed to be equal. Regressions were calculated by least squares. All calculations used standard methods (BMDP, University of California, Los Angeles, CA) written for a digital computer (PDP 11/44; Digital Equipment Corp., Maynard, MA).

Results

At time of killing, mean weights of animals in both groups were not different (range from 188 to 247 g). Serum calcium and phosphorus from IH rats ($n = 12$) were not different from normocalciuric controls ($n = 8$) (serum calcium, normal vs. IH, 9.63±0.34 vs. 10.24±0.22 mg/dl, P = NS; serum phosphorus, normal vs. IH, 7.93±0.17 vs. 8.28±0.32 mg/dl, P = NS). Serum $1,25(OH)_2D_3$ levels were slightly lower in the IH rats but not significantly different from normocalciuric controls (135±12 vs. 174±19 pg/ml, P = NS). 24-h urine calcium excretion was significantly greater in IH rats (2.9±0.3 vs. 0.7±0.2 mg/24 h, $P < 0.0001$).

In vitro intestinal calcium transport. Duodenal net calcium absorption (Jms > Jsm) was present in normal and IH rats (Table I), and calcium Jnet (Jms − Jsm) was greater in hypercalciuric males and females compared with normocalciuric controls. The increase in Jnet was due to increased Jms. Calcium secretory flux, Jsm, was significantly greater in hypercalciuric rats, but the increase in Jsm had a small effect on Jnet, as Jms was 10 to 12 times greater than Jsm (Table I).

Tissue conductance (Gt) was lower and short-circuit (Isc) was greater in hypercalciuric males and females than normocalciuric controls (Table I), and Gt and Isc were comparable in normocalciuric males and females.

Intestinal VDR number. VDR-rich cytosol fraction prepared from proximal duodenum from normal and IH male rats bound progressively larger amounts of [^{3}H]$1,25(OH)_2D_3$ at greater [^{3}H]$1,25(OH)_2D_3$ concentrations and then approached a plateau (Fig. 1). Nonspecific binding was < 5% of total binding for all concentrations of $1,25(OH)_2D_3$ in all bind-

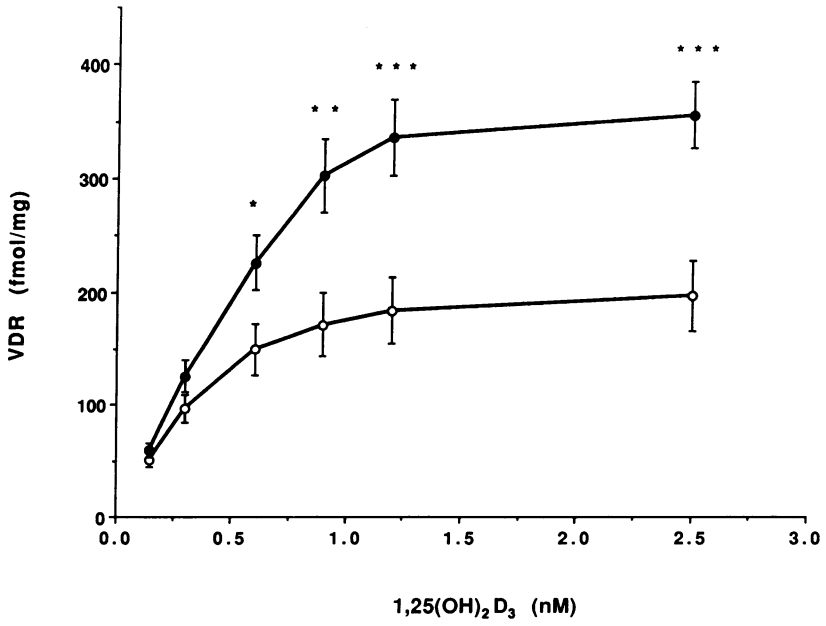


Figure 1. Specific binding of [^{3}H]$1,25(OH)_2D_3$ to duodenal cytosolic fraction (see Methods) prepared from normocalciuric control (*open circles*) and genetic hypercalciuric male rats (*closed circles*) fed a normal calcium diet. Values are mean±SEM for four observations per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; IH vs. control.

ing assays. Duodenal VDR-rich cytosol fractions prepared from hypercalciuric rats bound more [^{3}H]1,25(OH)$_2$D$_3$ than cytosolic fractions from normocalciuric control animals (Fig. 1).

To determine whether the increase in [^{3}H]1,25(OH)$_2$D$_3$ binding by IH rats was due to an increase in the number of available binding sites or to a change in the affinity of the vitamin D receptor for 1,25(OH)$_2$D$_3$, Scatchard analysis of the binding data was performed. For normocalciuric controls, duodenum (Fig. 2 *A*) contained a single class of binding sites. Specific VDR 1,25(OH)$_2$D$_3$ binding in cytosol from IH rats also contained a single class of binding sites (Fig. 2 *A*). The affinity of the intestinal VDR for its ligand (kD) was not different from controls (Table II). The maximal number of intestinal binding sites in IH rats was twofold greater than normocalciuric controls (Table II).

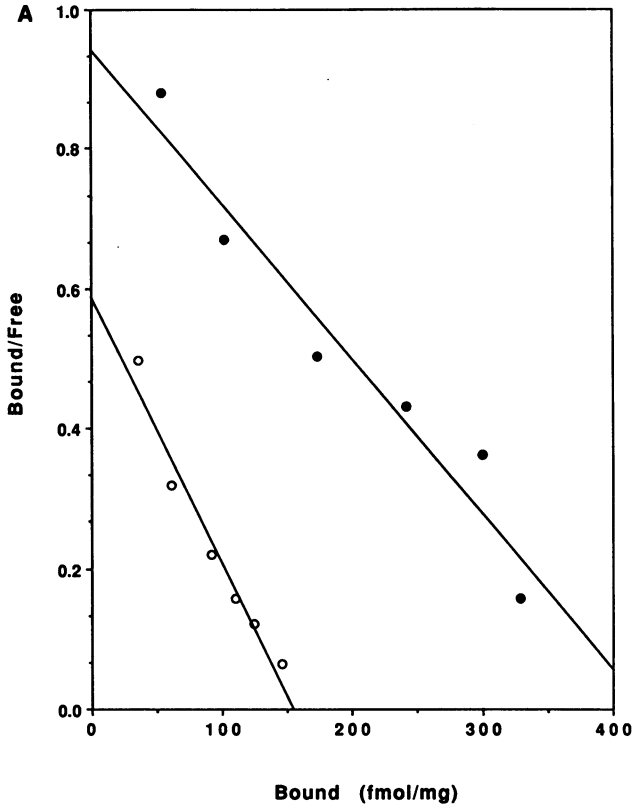
Kidney cytosolic fraction from IH and normocalciuric control rats contained fewer specific ligand binding sites than observed in duodenum (Fig. 2 *B*). However, like intestine, kidney from IH rats contained twice the number of binding sites as found in cytosol prepared from controls (Table II). Affinity of the receptor for 1,25(OH)$_2$D$_3$ was not statistically different between the two groups.

Intestinal VDR gene expression. Northern analysis revealed one species of intestinal VDR mRNA for IH and controls with no difference in migration of VDR mRNA between the two groups (Fig. 3). Using laser densitometry to quantify the hybridization of VDR cDNA probe to the receptor mRNA and actin mRNA as an internal control, the densitometry ratio of VDR/actin (1.162±0.03 vs. 0.299±0.05, $n = 4$, $P < 0.001$) showed a significant suppression of VDR mRNA from hypercalciuric rats (Fig. 3). There was no overlap in intensity of the VDR bands by laser densitometry scanning or of the calculated VDR/actin ratios between the IH (values range from 0.222 to 0.534) and normocalciuric controls (from 0.849 to 0.928). Thus, the present evidence suggests that the increased intestinal VDR number in IH rats is not result of increased expression of the VDR gene.

Intestinal nuclear transcription in vitro. In initial studies, the rate of enterocyte nuclear RNA synthesis determined from radiolabeled nucleotide incorporation was constant for 40 min and then declined. Therefore, subsequent transcription run-on assays were performed for 30 min. RNAs extracted from the in vitro intestinal nuclear incubation were hybridized to the rat VDR cDNA and the level of hybridization was quantified by scanning laser densitometry. VDR gene transcription was not different between IH and control groups (6.61±1.1 vs. 6.49±1.3 mm area under the curve, mean±SE for $n = 4$ nuclei per group, $P = 0.948$).

Hybridization of newly synthesized radiolabeled RNA with calbindin 9-kD cDNA showed that synthesis of the vitamin D–dependent calcium-binding protein was not different in the intestinal nuclei from IH and normocalciuric control rats (5.73±1.6 vs. 5.64±1.3, $n = 4$, $P = 0.945$).

Western analysis of intestinal VDR and calbindin 9-kD proteins. A monoclonal antibody raised against chick VDR, which cross-reacts with the rat VDR, has identified the 50-kD VDR in intestinal extracts from both IH and normal animals (Fig. 4 *A*). Migration of the VDR bands are equivalent, with more intense staining in the extract from the IH rats despite the loading of identical amounts of protein. In the absence of the pri-



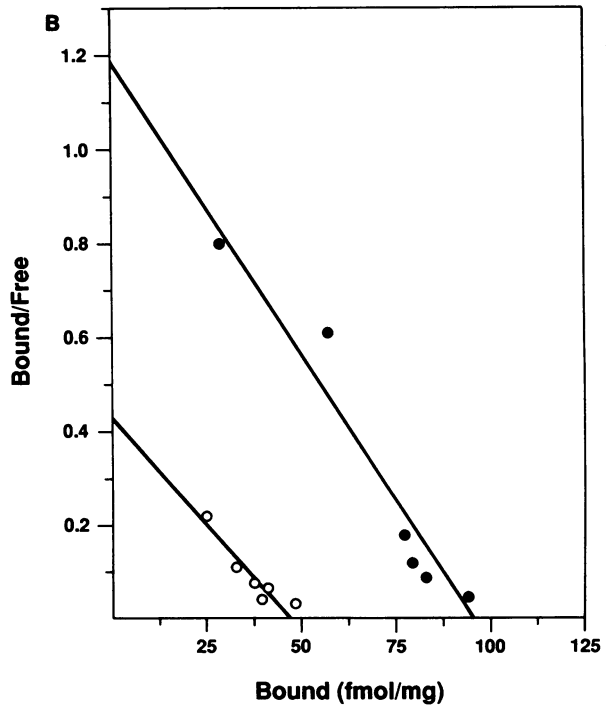


Figure 2. Representative Scatchard plot of specific binding derived from saturation assay of the VDR using (*A*) duodenal cytosol fraction and (*B*) kidney cytosolic fraction from normocalciuric (*open circles*) and IH (*closed circles*) male rats fed a normal calcium diet. Bound/free and bound refer to 1,25(OH)$_2$D$_3$ bound.

Table II. Vitamin D Receptor Binding Sites in Intestine and Kidney from Normal and Hypercalciuric Rats

Group	*n*	Nmax	K_d
		fmol, vitamin D_3 bound/mg protein	*nM*
Intestine			
NM	14	243±42	0.33±0.01
HM	8	536±73*	0.49±0.01
Kidney			
NM	7	34±4	0.27±0.16
HM	8	87±4*	0.45±0.28

Values are mean±SE for *n* rats per group. NM and HM are normocalciuric and hypercalciuric male rats. Nmax is maximum number of VDR binding sites and K_d is affinity of ligand for receptor, derived from Scatchard analysis. * $P < 0.001$, NM vs. HM.

mary antibody (Fig. 4 *B*), the 50-kD band in IH and normal rat intestine disappears. The anti-chick VDR monoclonal antibody also recognized an 80-kD protein that is not present in the absence of the primary antibody. This 80-kD band may contain the protein found in activated lymphocytes that has cross-reactivity with the VDR monoclonal antibody (52). Several bands that persist in the absence of the primary antibody are light and heavy chain immunoglobulins of intestinal origin (41).

A polyclonal anticalbindin 9-kD antiserum has identified calbindin 9 kD in intestinal extracts from both IH and normal animals, with a more intense staining of the IH intestinal extract (Fig. 5).

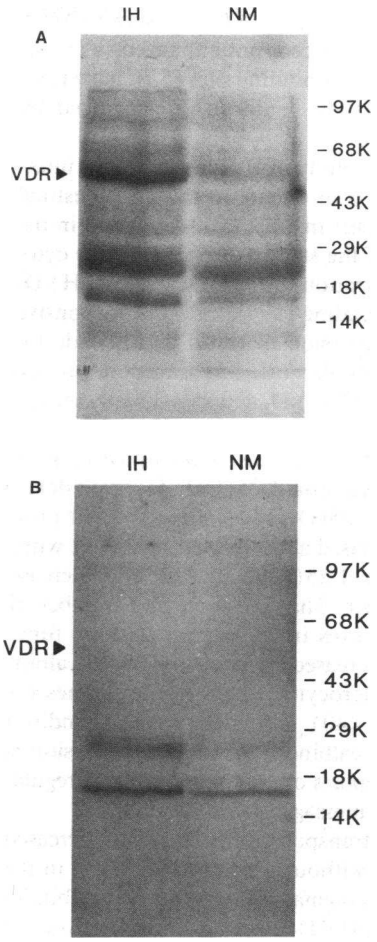


Figure 4. (*A*) Western blot of VDR contained in 100 μg intestinal mucosal protein. After SDS-PAGE, proteins were exposed to monoclonal anti-chick VDR antibody (see Methods). Note the equivalent migration of the VDR band in genetic idiopathic hypercalciuric (*IH*) and normocalciuric control (*NM*) male rats and the more intense staining of the VDR band in the cytosol fraction from the IH rat. (*B*) Western analysis performed as in *A* except that the primary antibody has been omitted. The several bands recognized by the secondary antibody are consistent with light and heavy chain immunoglobulins of intestinal origin.

Discussion

Binding analyses of VDR–1,25$(OH)_2D_3$ using duodenal mucosa from IH rats demonstrated a clear increase in the concentration of high affinity VDR binding sites compared with intestinal mucosa from normocalciuric control animals. The Western immunoblots support the results of the kinetic studies that duodenal mucosa of IH rats contain more VDR. Several possibilities may be considered for the increased VDR content in the IH rat intestine. Normal enterocyte VDR mRNA synthesis by nuclear run-on assay and reduced VDR mRNA levels indicate that the increase in VDR must occur by mechanisms that do not involve VDR gene expression. Increased VDR mRNA translation efficiency could increase VDR synthesis, but this possibility was not addressed in the present study. Reduced VDR mRNA with increased VDR by kinetic and immunoblot analyses are consistent with prolongation of the VDR half-life. Direct measurements of VDR turnover are required to test this possibility.

A missense mutation of the VDR could result in enhanced affinity of VDR for its ligand and could show apparently higher binding capacity at subsaturating concentrations of ligand. However, binding affinity by Scatchard analysis of kinetic data revealed no difference in VDR kD between groups. Mobility of the IH VDR on Western blot and VDR mRNA migration by Northern analysis were not different from normal, suggesting that it is unlikely that IH rat intestine contained a structurally

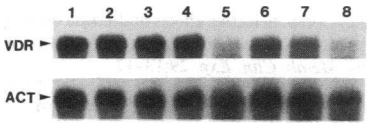


Figure 3. Northern analysis of VDR mRNA. Polyadenylated RNA (10 μg per lane) from individual duodenal segments from normocalciuric controls (lanes *1–4*) and male IH rats (lanes *5–8*) was isolated (see Methods), subjected to electrophoresis on 1% denatured agarose-formaldehyde gel, and electrotransferred to nylon filter. The filter was hybridized with radiolabeled rat VDR cDNA followed by autoradiography for 72 h. After stripping, rehybridization was performed with a gamma-actin cDNA probe.

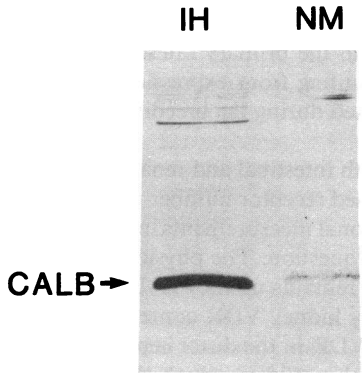


Figure 5. Western blot of intestinal calbindin 9 kD (*Calb*). Intestinal protein (10 μg protein per lane) from genetic idiopathic hypercalciuric (*IH*) and normocalciuric control (*NM*) male rats were separated using SDS-PAGE. Calbindin 9 kD was identified using polyclonal rabbit anti-rat calbindin 9-kD antisera (see Methods). Note the more intense calbindin 9-kD band in the lane from IH rat intestine.

altered VDR. Also, previous examples of even single amino acid substitution resulted in reduced, not enhanced, VDR ligand binding or affinity for DNA binding sites (53). Nevertheless, a single missense mutation could go unrecognized by Northern or Western analyses.

We propose that, in this colony of IH rats, increased intestinal VDR abundance results in enhanced rates of intestinal calcium transport. Early events in $1,25(OH)_2D_3$ action in the intestine include binding of the steroid hormone to the cytosolic VDR, followed by migration of the VDR–$1,25(OH)_2D_3$ complex into the nucleus, binding to specific VDR-responsive elements and increased expression of genes that encode for proteins involved in the cellular calcium transport process (26). The magnitude of $1,25(OH)_2D_3$ action is directly related to VDR content and functional VDR–$1,25(OH)_2D_3$ complexes or receptor occupancy (26, 27, 29). Under physiological conditions, variations in circulating $1,25(OH)_2D_3$ levels determine the number of VDR–$1,25(OH)_2D_3$ complexes. The present study suggests that increased abundance of the VDR without a change in circulating $1,25(OH)_2D_3$ may also increase VDR–$1,25(OH)_2D_3$ number. That the increased number of VDR–$1,25(OH)_2D_3$ complexes in IH rat intestine are functional is supported by the increased intestinal content of calbindin 9 kD, an abundant enterocyte protein whose synthesis is regulated by $1,25(OH)_2D_3$ (40). The increase in calbindin 9 kD without an increase in calbindin 9 kD gene expression is consistent with previous reports of posttranscriptional regulation of the calbindin 9 kD message (43).

Duodenal net calcium transport in the IH rats is increased due to an increase in Jms without a significant change in the secretory flux, Jsm. Both duodenal calcium Jms and calbindin 9 kD are regulated by $1,25(OH)_2D_3$ (54). Thus, increases in these vitamin D–dependent processes in the presence of increased VDR support the concept that intestinal calcium hyperabsorption in IH rats is a VDR-mediated process.

Increased intestinal VDR content in the IH rats may be a phenotypic marker for primary intestinal calcium hyperabsorption (22) or part of a secondary adaptation of the intestinal calcium transport process to urinary calcium losses. VDR up-regulation accompanies the intestinal adaptive response during chronic dietary calcium restriction (30) and may also increase or decrease in response to serum calcium (32) or PTH (33), respectively. In vitro, $1,25(OH)_2D_3$ itself (28, 29) induces an homologous up-regulation of the VDR. However, in the present study, rats were fed a normal, not low, calcium diet; serum $1,25(OH)_2D_3$ was not increased and there is no evidence for increased PTH (22). Therefore, in these IH rats there is no evidence that increased VDR and calcium transport are secondary, adaptive events in response to urinary calcium losses but rather are more proximate to the primary intestinal calcium overabsorption, perhaps resulting from expression of one or more of the genotypes selected during the breeding for hypercalciuria.

The accumulation of both intestinal and renal VDR in IH rats suggests that the increased receptor number may be more generalized, although additional measurements in other tissues are required to answer this question. The physiological function of VDR in the kidney remains unknown. The increased total cortical and medullary kidney VDR content in IH rats may result from increased VDR in the distal nephron. This is supported by autoradiographic studies, which show selective nuclear localization of tritium-labeled $1,25(OH)_2D_3$ in distal tubules (55).

The phenotypic expression of human genetic hypercalciuria is that of a state of $1,25(OH)_2D_3$ excess, with normocalcemia and intestinal calcium hyperabsorption. However, increased $1,25(OH)_2D_3$ renal production rates and circulating levels are found in some patients whereas others have calcium hyperabsorption with normal serum $1,25(OH)_2D_3$ levels (6, 8, 10–14). Despite normal serum $1,25(OH)_2D_3$ levels in some patients, there is evidence for increased $1,25(OH)_2D_3$ action. Dietary calcium restriction results in negative calcium balance (10), with a large portion of urinary calcium resulting from increased bone resorption. These same metabolic changes have been reproduced by the administration of $1,25(OH)_2D_3$ to healthy subjects (56, 57) and rats (58) fed low calcium diet. Thus, IH rats share some mineral metabolic features with some forms of human IH in that serum $1,25(OH)_2D_3$ levels are normal and negative calcium balance may develop during dietary calcium restriction (10, 59). Whether the same pathogenetic process involving increased intestinal VDR found in genetic IH rats is present in some forms of humans IH remains to be determined.

Acknowledgments

The authors thank Dr. J. Wesley Pike (Ligand Pharmaceuticals, San Diego, CA) for the generous supplies of VDR cDNA and anti-VDR monoclonal antibody (9A7 gamma). We also acknowledge the technical assistance of Karen Ambrosic.

This research was supported by grants DK 33949 from the National Institutes of Health.

References

1. Pak, C. Y. C., C. O. Waters, L. Arnold, K. Holt, C. Cox, and D. Barilla. 1977. Mechanism for calcium urolithiasis among patients with hyperuricosuria. Supersaturation of urine with respect to monosodium urate. *J. Clin. Invest.* 59:426–431.

2. Weber, D. V., F. L. Coe, J. H. Parks, M. S. L. Dunn, and V. Tembe. 1979. Urinary saturation measurements in calcium nephrolithiasis. *Ann. Intern Med.* 90:180–184.

3. Coe, F. L., and J. H. Parks. 1988. Nephrolithiasis: Pathogenesis and Treatment. Yearbook Medical Publishers, Chicago. 315 pp.

4. Caniggia, A., C. Gennari, and L. Cesari. 1965. Intestinal absorption of ^{45}Ca in stone-forming patients. *Br. Med. J.* 1:427–429.

5. Birge, S. J., W. A. Peck, M. Berman, and G. D. Whedon. 1969. Study of calcium absorption in man. A kinetic analysis and physiologic model. *J. Clin. Invest.* 48:1705–1713.

6. Pak, C. Y. C., D. A. East, L. J. Sanzenbacher, C. S. Delea, and F. C. Bartter. 1972. Gastrointestinal calcium absorption in nephrolithiasis. *J. Clin. Endocrinol. Metab.* 35:261–270.

7. Pak, C. Y. C., M. Ohata, E. C. Lawrence, and W. Snyder. 1974. The hypercalciurias. Causes, parathyroid functions, and diagnostic criteria. *J. Clin. Invest.* 54:387–400.

8. Zerwekh, J. E., and C. Y. C. Pak. 1980. Selective effects of thiazide therapy on serum 1 alpha, 25-dihydroxyvitamin D and intestinal calcium absorption in renal and absorptive hypercalciurias. *Metab. Clin. Exp.* 29:13–17.

9. Pak, C. Y. C., J. McGuire, R. Peterson, F. Britton, and M. J. Harrod. 1981. Familial absorptive hypercalciuria in a large kindred. *J. Urol.* 126:717–719.

10. Coe, F. L., M. J. Favus, T. Crockett, A. L. Strauss, J. H. Parks, A. Porat, C. L. Gantt, and L. M. Sherwood. 1982. Effects of low calcium diet on urine calcium excretion, parathyroid function and serum $1,25(OH)_2D_3$ levels in patients with idiopathic hypercalciuria and in normal subjects. *Am. J. Med.* 72:25–32.

11. Broadus, A. E., K. L. Insogna, R. Lang, A. F. Ellison, and B. E. Dreyer. 1984. Evidence for disordered control of 1,25-dihydroxyvitamin D production in absorptive hypercalciuria. *N. Engl. J. Med.* 311:73–80.

12. Shen, F. H., D. J. Baylink, R. L. Nielsen, D. J. Sherrard, J. L. Ivey, and

M. R. Haussler. 1977. Increased serum 1,25-dihydroxyvitamin D in idiopathic hypercalciuria. *J. Lab. Clin. Med.* 90:955–962.

13. Insogna, K. L., A. E. Broadus, B. E. Dreyer, A. F. Ellison, and J. M. Gertner. 1985. Elevated production rate of 1,25-dihydroxyvitamin D in patients with absorptive hypercalciuria. *J. Clin. Endocrinol. Metab.* 61:490–495.

14. Kaplan, R. A., M. R. Haussler, L. J. Deftos, H. Bone, and C. Y. C. Pak. 1977. The role of 1-alpha, 25-dihydroxyvitamin D in the mediation of intestinal hyperabsorption of calcium in primary hyperparathyroidism and absorptive hypercalciuria. *J. Clin. Invest.* 59:756–760.

15. Sutton, R. A. L., and V. R. Walker. 1980. Responses to hydrochlorothiazide and acetazolamide in patients with calcium stones. Evidence suggesting a defect in renal tubular function. *N. Engl. J. Med.* 302:709–713.

16. Hodgkinson, A., and L. N. Pyrah. 1958. The urinary excretion of calcium and inorganic phosphate in 344 patients with calcium stones of renal origin. *Br. J. Surg.* 46:10–18.

17. Robertson, W. G., and D. B. Morgan. 1972. The distribution of urinary calcium excretions in normal persons and stone-formers. *Clin. Chim. Acta.* 37:503–508.

18. Coe, F. L., J. H. Parks, and E. S. Moore. 1979. Familial idiopathic hypercalciuria. *N. Engl. J. Med.* 300:337–340.

19. Mehes, K., and Z. S. Szelid. 1980. Autosomal dominant inheritance of hypercalciuria. *Eur. J. Pediatr.* 133:239–242.

20. Pak, C. Y. C., J. McGuire, R. Peterson, F. Britton, and M. J. Harrod. 1981. Familial absorptive hypercalciuria in a large kindred. *J. Urol.* 126:717–719.

21. Favus, M. J. 1989. Familial forms of hypercalciuria. *J. Urol.* 141:719–722.

22. Bushinsky, D. A., and M. J. Favus. 1988. Mechanism of hypercalciuria in genetic hypercalciuric rats. *J. Clin. Invest.* 82:1585–1591.

23. Favus, M. J., and F. L. Coe. 1979. Evidence for spontaneous hypercalciuria in the rat. *Miner. Electrolyte Metab.* 2:150–154.

24. Lau, K., and B. K. Eby. 1982. Tubular mechanism for the spontaneous hypercalciuria in laboratory rat. *J. Clin. Invest.* 76:420–425.

25. Haussler, M. R., and A. W. Norman. 1969. Chromosomal receptor for a vitamin D metabolite. *Proc. Natl. Acad. Sci. USA.* 62:155–162.

26. Reichel, H., P. Koeffler, and A. W. Norman. 1989. The role of the vitamin D endocrine system in health and disease. *N. Engl. J. Med.* 320:980–991.

27. Ozono, K., T. Sone, and J. W. Pike. 1991. The genomic mechanism of action of 1,25-dihydroxyvitamin D_3. *J. Bone Miner. Res.* 6:1021–1027.

28. Costa, E. M., M. A. Hirst, and D. Feldman. 1985. Regulation of 1,25-dihydroxyvitamin D analogs in cultured mammalian cells. *Endocrinology.* 117:2203–2210.

29. Chen, T. L., P. V. Hauschka, S. Cabrales, and D. Feldman. 1986. The effects of 1,25-dihydroxyvitamin D_3 and dexamethasone on rat osteoblast-like primary cell cultures: receptor occupancy and functional expression patterns for three different bioresponses. *Endocrinology.* 118:250–259.

30. Favus, M. J., D. J. Mangelsdorf, V. Tembe, B. J. Coe, and M. R. Haussler. 1988. Evidence for in vivo upregulation of the intestinal vitamin D receptor during dietary calcium restriction in the rat. *J. Clin. Invest.* 82:218–224.

31. Strom, M., M. E. Sandgren, T. A. Brown, and H. F. DeLuca. 1989. 1,25-dihydroxyvitamin D_3 up-regulates the 1,25-dihydroxyvitamin D_3 receptor in vivo. *Proc. Natl. Acad. Sci. USA.* 86:9770–9773.

32. Sandgren, M., and H. F. DeLuca. 1990. Serum calcium and vitamin D regulate 1,25-dihydroxyvitamin D_3 receptor concentration in rat kidney in vivo. *Proc. Natl. Acad. Sci. USA.* 87:4312–4314.

33. Pols, H. A. P., J. C. Birkenhager, J. P. Schlite, and T. J. Visser. 1988. Evidence that self-induced metabolism of 1,25-dihydroxyvitamin D-3 limits the homologous up-regulation of its receptor in rat osteosarcoma cells. *Biochim. Biophys. Acta.* 970:122–129.

34. Reinhardt, T. A., and R. L. Horst. 1989. Self-induction of 1,25-dihydroxyvitamin D_3 metabolism limits receptor occupancy and target tissue responsiveness. *J. Biol. Chem.* 264:15917–15921.

35. Favus, M. J., S. C. Kathpalia, F. L. Coe, and A. E. Mond. 1980. Effects of diet calcium and 1,25-dihydroxyvitamin D_3 on colon calcium active transport. *Am. J. Physiol.* 238:G75–G78.

36. Schultz, S. G., and R. Zalusky. 1964. Ion transport in isolated rat ileum: short-circuit current and Na fluxes. *J. Gen. Physiol.* 47:567–587.

37. Field, M., D. Fromm, and I. McColl. 1971. Ion transport in rabbit ileal mucosa. I. Na and Cl fluxes and short-circuit current. *Am. J. Physiol.* 220:1388–1396.

38. Feldman, D., T. A. McCain, M. A. Hirst, T. L. Chen, and K. W. Colston. 1979. Characterization of a cytoplasmic receptor-like binder for 1,25-dihydroxycholecalciferol in rat intestinal mucosa. *J. Biol. Chem.* 254:10378–10384.

39. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

40. Davis, L. G., Dibner, M. D., and Battey, J. F. 1986. Basic Methods in Molecular Biology. Elsevier, New York. 388 pp.

41. Pike, J. W. 1984. Monoclonal antibodies to chick intestinal receptors for 1,25-dihydroxyvitamin D_3. Interaction and effects of binding on receptor function. *J. Biol. Chem.* 259:1167–1173.

42. McDonnell, D. P., D. J. Mangelsdorf, J. W. Pike, M. R. Haussler, and B. W. O'Malley. 1987. Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science (Wash. DC).* 235:1214–1217.

43. Dupret, J.-M., P. Brun, C. Perret, N. Lomri, M. Thomasset, and P. Cuisinier-Gleizes. 1987. Transcriptional and post-transcriptional regulation of vitamin D-dependent calcium-binding protein gene expression in the rat duodenum by 1,25-dihydroxycholecalciferol. *J. Biol. Chem.* 262:16553–16557.

44. Vaulont, S., A. Munnich, J. Marie, G. Reach, A.-L. Pichard, M.-P. Simon, C. Besmond, P. Barbry, and A. Kahn. 1984. Cyclic AMP as a transcriptional inhibitor of upper eukaryotic gene transcription. *Biochem. Biophys. Res. Commun.* 125:135–141.

45. Schibler, U., O. Hagenbuchle, P. K. Wellauer, and A. C. Pittet. 1983. Two promoters of different strengths control the transcription of the mouse alpha-amylase gene amy-1[a] in the parotid gland and the liver. *Cell.* 33:501–508.

46. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current Protocols in Molecular Biology. John Wiley & Sons, Inc, New York.

47. Bruns, M. E. H., A. Fausto, and L. V. Avioli. 1978. Placental calcium-binding protein in rats. *J. Biol. Chem.* 253:3186–3190.

48. Leary, J. J., D. J. Brigati, and D. C. Ward. 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. *Proc. Natl. Acad. Sci. USA.* 80:4045–4049.

49. McGadey, J. 1970. A tetrazolium method for non-specific alkaline phosphatase. *Histochemie.* 23:180–184.

50. Favus, M. J., and C. B. Langman. 1986. Evidence for calcium-dependent control of 1,25-dihydroxyvitamin D_3 production by rat kidney proximal tubules. *J. Biol. Chem.* 261:11224–11229.

51. Reinhardt, T. A., R. L. Horst, J. W. Orf, and B. W. Hollis. 1984. A microassay for 1,25-dihydroxyvitamin D not requiring HPLC: application to clinical medicine. *J. Clin. Endocrinol. Metab.* 58:91–98.

52. Yu, X.-P., F. G. Hustmyer, W. T. Garvey, and S. C. Manolagas. 1991. Demonstration of a 1,25-dihydroxyvitamin D_3-responsive protein in human lymphocytes: immunologic crossreactivity and inverse regulation with the vitamin D receptor. *Proc. Natl. Acad. Sci. USA.* 88:8347–8351.

53. Malloy, P. J., Z. Hochberg, D. Tiosano, J. W. Pike, M. R. Hughes, and D. Feldman. 1990. The molecular basis of hereditary 1,25-dihydroxyvitamin D_3 resistant rickets in seven related families. *J. Clin. Invest.* 86:2071–2079.

54. Favus, M. J. Factors that influence absorption and secretion of calcium in the small intestine and colon. 1985. *Am. J. Physiol.* G147–G157.

55. Stumpf, W. E., M. Sar, R. Narbaitz, F. A. Reid, H. F. DeLuca, and Y. Tanaka. 1980. Cellular and subcellular localization of 1,25-$(OH)_2$-vitamin D_3 in rat kidney: comparison with localization of parathyroid hormone and estradiol. *Proc. Natl. Acad. Sci. USA.* 77:1149–1153.

56. Maierhofer, W. J., R. W. Gray, H. S. Cheung, and J. Lemann, Jr. 1983. Bone resorption stimulated by elevated serum 1,25-$(OH)_2$-vitamin D concentrations in healthy men. *Kidney Int.* 24:555–560.

57. Maierhofer, W. J., J. Lemann, Jr., R. W. Gray, and H. S. Cheung. 1984. Dietary calcium and serum 1,25-$(OH)_2$-vitamin D concentrations as determinants of calcium balance in healthy men. *Kidney Int.* 26:752–759.

58. Favus, M. J., F. L. Coe, S. C. Kathpalia, A. Porat, P. K. Sen, and L. M. Sherwood. 1982. Effects of chlorothiazide on 1,25-dihydroxyvitamin D_3, parathyroid hormone, and intestinal calcium absorption in the rat. *Am. J. Physiol.* 242:G575–G581.

59. Kim, M., N. E. Sessler, M. J. Favus, and D. A. Bushinsky. 1990. Response of genetic hypercalciuric rats to a low calcium diet. *J. Am. Soc. Nephrol.* 1:576a. (Abstr.)