Hyperresponsiveness of Vitamin D Receptor Gene Expression to 1,25-Dihydroxyvitamin D_3

A New Characteristic of Genetic Hypercalciuric Stone–forming Rats

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Abstract

Hypercalciuria in genetic hypercalciuric stone–forming (GHS) rats is accompanied by intestinal Ca hyperabsorption with normal serum 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] levels, elevation of intestinal, kidney, and bone vitamin D receptor (VDR) content, and greater 1,25(OH)$_2$D$_3$-induced bone resorption in vitro. To test the hypothesis that hyperresponsiveness of VDR gene expression to 1,25(OH)$_2$D$_3$ may mediate these observations, male GHS and wild-type Sprague-Dawley normocalciuric control rats were fed a normal Ca diet (0.6% Ca) and received a single intraperitoneal injection of either 1,25(OH)$_2$D$_3$ (10–200 ng/100 g body wt) or vehicle. Total RNAs were isolated from both duodenum and kidney cortex, and the VDR and calbindin mRNA levels were determined by Northern blot hybridization using specific cDNA probes. Under basal conditions, VDR mRNA levels in GHS rats were lower in duodenum and higher in kidney compared with wild-type controls. Administration of 1,25(OH)$_2$D$_3$ increased VDR gene expression significantly in GHS but not normocalciuric animals, in a time- and dose-dependent manner. In vivo half-life of VDR mRNA was similar in GHS and control rats in both duodenum and kidney, and was prolonged significantly (from 4–5 to > 8 h) by 1,25(OH)$_2$D$_3$ administration. Neither inhibition of gene transcription by actinomycin D nor inhibition of de novo protein synthesis with cycloheximide blocked the upregulation of VDR gene expression stimulated by 1,25(OH)$_2$D$_3$ administration. No alteration or mutation was detected in the sequence of duodenal VDR mRNA from GHS rats compared with wild-type animals. Furthermore, 1,25(OH)$_2$D$_3$ administration also led to an increase in duodenal and renal calbindin mRNA levels in GHS rats, whereas they were either suppressed or unchanged in wild-type animals. The results suggest that GHS rats hyperrespond to minimal doses of 1,25(OH)$_2$D$_3$ by an upregulation of VDR gene expression. This hyperresponsiveness of GHS rats to 1,25(OH)$_2$D$_3$ (*a*) occurs through an increase in VDR mRNA stability without involving alteration in gene transcription, de novo protein synthesis, or mRNA sequence; and (*b*) is likely of functional significance, and affects VDR-responsive genes in 1,25(OH)$_2$D$_3$ target tissues. This unique characteristic suggests that GHS rats may be susceptible to minimal fluctuations in serum 1,25(OH)$_2$D$_3$, resulting in increased VDR and VDR-responsive events, which in turn may pathologically amplify the actions of 1,25(OH)$_2$D$_3$ on Ca metabolism that thus contribute to the hypercalciuria and stone formation. (*J. Clin. Invest.* 1998. 101:2223–2232.) Key words: vitamin D receptor • gene expression • rat • 1,25(OH)$_2$D$_3$ • hypercalciuria

Introduction

Hypercalciuria is a common metabolic abnormality among Ca oxalate stone formers (1–3) that may enhance their risk of nephrolithiasis through an increase in urine Ca oxalate supersaturation (2–4). Idiopathic hypercalciuria (IH),[1] the most common cause of Ca oxalate nephrolithiasis (4, 5), is characterized by normocalcemia and intestinal Ca overabsorption (4–8). In some patients, increased intestinal calcium absorption is due to overproduction (9, 10) and elevated serum levels of 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] (9–16). However, the cause of intestinal calcium hyperabsorption in a significant portion of IH patients who have normal serum 1,25(OH)$_2$D$_3$ levels (4, 5, 10–15) remains unknown. A primary renal tubule Ca transport defect or renal Ca leak, with secondary increases in parathyroid hormone, 1,25(OH)$_2$D$_3$, and intestinal Ca absorption, is a less common cause of IH (4, 5, 7, 17). These three causes of IH (4, 5, 7, 13, 17) suggest that it may be a heterogenous disorder.

Genetic hypercalciuric stone–forming (GHS) rats have normal serum Ca and 1,25(OH)$_2$D$_3$ levels and increased duodenal Ca transport (18–20) and therefore may be analogous to some forms of human IH. A two- to fourfold increase in vitamin D receptor (VDR) content has been found in GHS rat duodenum (19), kidney (19), and bone (21). The majority of the excess urine Ca excretion in GHS rats is from VDR mediation of both intestinal Ca hyperabsorption (18, 20) and enhanced bone resorption (20, 21). Renal tubule Ca reabsorption is reduced in isolated perfused tubular segments from GHS rat kidney (22); however, it is unclear what role the increased renal VDR content plays in the Ca transport defect. 1,25(OH)$_2$D$_3$ exerts its actions through genomic events that involve binding of the steroid hormone to the intracellular VDR,

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1. *Abbreviations used in this paper:* 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; BW, body wt; GHS, genetic hypercalciuric stone–forming; IH, idiopathic hypercalciuria; RT, reverse transcription; VDR, vitamin D receptor.

and the biological actions of $1,25(OH)_2D_3$ are strongly correlated with target tissue VDR number and occupancy (23–25). Thus, the excess VDR content in duodenum and bone plays a primary role in the pathologic increases in intestinal and bone Ca transport characteristic of the GHS rats.

Evidence that GHS rats are hypersensitive to $1,25(OH)_2D_3$ has been suggested from several observations, including the following: duodenal Ca active transport is increased in the presence of normal circulating $1,25(OH)_2D_3$ levels (18, 19); a steep dose-dependent increase in $1,25(OH)_2D_3$-induced in vitro fetal calvarial bone resorption (21); and a marked increase in intestinal Ca transport with modest rises in serum $1,25(OH)_2D_3$ during low Ca diet (20).

The cause of the elevated VDR content in GHS rats is unknown, but $1,25(OH)_2D_3$-induced upregulation of the VDR, which may occur in normal rats and VDR-containing cells in culture (26, 27), may be involved. Lower duodenal VDR mRNA levels in GHS rats (19) suggest that regulation of VDR gene expression in GHS animals may differ from that of normocalciuric rats. Therefore, the potential role of $1,25(OH)_2D_3$ in the control of target tissue VDR was explored in this study by testing the hypothesis that the hyperresponsiveness of GHS rats to $1,25(OH)_2D_3$ results from abnormalities in the regulation of VDR gene expression. These studies were conducted in GHS rats after administration of small doses of $1,25(OH)_2D_3$.

Methods

Animals and diets. A colony of GHS rats was created by mating spontaneously hypercalciuric male and female Sprague-Dawley rats (18, 28, 29). Classification of rats as hypercalciuric was done at completion of weaning, when animals were placed in individual metabolic cages and fed 13 g/d of a diet containing 0.6% calcium, 0.65% phosphorus, 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. GHS animals were defined as those with urine Ca excretion of > 2SD above the mean of control rats (usually > 1.5 mg/24 h) on two successive 24-h urine collections after the animals had been equilibrated on the diet for 5 d. Male and female rats with the highest Ca excretion value were chosen for breeding to propagate the colony. The remaining male hypercalciuric rats from generations 42–44 with 160 ± 15 g body wt (BW) were used for these studies. Normocalciuric male Sprague-Dawley rats obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN) were used as wild-type controls and were fed a similar 0.6% Ca diet for at least 5 d before study. Previous studies have shown that normal and GHS rats grow at the same rate. Therefore, animals matched for weight are also age matched. Care and use of the animals were approved by the University of Chicago Animal Care Committee.

RNA isolation. Animals were killed by exsanguination via the abdominal aorta while under light ether anesthesia. Kidneys and duodenal segments were then removed rapidly for RNA isolation. For duodenum, the proximal 10 cm of duodenum was removed and washed three times with ice-cold PBS (pH 7.4). The mucosa was scraped from underlying coats with a chilled glass slide and placed immediately in 4 M guanidine buffer on ice. For the kidneys, the capsule was trimmed, the medulla was removed, and the remaining cortical tissue was rinsed with ice-cold PBS buffer. The tissue was minced, put immediately into 4 M guanidine buffer, and homogenized for 30 s on ice (Polytron® homogenizer; Brinkmann Instruments, Inc., Westbury, NY). Total RNA isolation was conducted using the guanidine-phenol-chloroform procedure (30) with minor modifications as described previously (31). Briefly, after cell lysis in 4 M guanidine buffer, an equal volume of phenol was added, and the mixture was centrifuged (Sorvall® centrifuge; DuPont-NEN, Boston, MA) at

10,000 g in 4°C for 20 min. The aqueous phase containing the RNA was transferred to a fresh tube. Phenol extraction was repeated once, and the RNA in the phenol-extracted aqueous phase was further purified by two steps of precipitation with 70% ethanol. RNA was redissolved in Tris-EDTA buffer (Tris-Cl$_2$ 10 mM, EDTA 1 mM, pH 7.4). The quantity and purity of total RNA were measured spectrophotometrically at 260/280 nm, assuming 40 μg/ml RNA per unit of absorbance.

Northern blot hybridization. Steady state levels of VDR mRNA were determined by Northern blot hybridization analysis. Approximately 15 μg of total RNA was denatured in 50% formamide, 17.5% formaldehyde, and 1× Mops buffer (20 nM 3-[*N*-morpholino]propane-sulfonic acid at pH 7.0, 5 mM sodium acetate, and 1 mM Na$_2$-EDTA, pH 8.0), electrophoresed in 1% agarose gel, transferred to GeneScreen Plus membranes (New England Nuclear, Boston, MA), and baked at 80°C in a vacuum oven for 2 h. Blots were prehybridized and then hybridized overnight with a radiolabeled specific cDNA probe at a concentration of 3×10^6 cpm/ml. The cDNA probes were labeled with [^{32}P]dCTP (specific activity 3,000 Ci/mM; Amersham Corp., Arlington Heights, IL) by random primer extension using the Multiprim DNA labeling system (Amersham Corp.). The specific activity of labeled probe ranged from 2 to 6×10^8 cpm/μg of cDNA. After hybridization, the blots were washed and exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70°C. Conditions of prehybridization, hybridization, and washing procedures were the same as described previously (31–33). The cDNA probes in this study included the following: (*a*) VDR, as either a 1.7-kb cDNA probe for avian VDR (34) (kindly provided by Dr. J.W. Pike, University of Cincinnati, Cincinnati, OH) or an 845-bp fragment of pure PCR product for rat VDR, which was amplified via reverse transcription (RT) PCR from rat duodenal mRNA using a pair of specific primers and confirmed by sequencing (see below). Preliminary experiments in which rat total RNAs were used demonstrated that both probes detected a 4.4-kb single transcript of VDR mRNA without any distinguishable difference between them. Thus, subsequent hybridizations were done with the 1.7-kb cDNA probe; and (*b*) a 180-bp cDNA probe for calbindin-9kd (35) (a gift from Dr. E. Bruns, University of Virginia, Charlottesville, VA) and a 1.2-kb cDNA probe for calbindin-28kd (36) (from Dr. S. Christakos, New Jersey Medical School, Newark, NJ) were used for the detection of calbindin mRNAs.

In vivo VDR mRNA half-life. In preliminary experiments, a single dose of actinomycin D by intraperitoneal injection of 400 μg/100 g BW efficiently suppressed VDR mRNA transcription, with a decline in VDR mRNA to almost undetectable levels by 8 h. The majority of rats treated with this dose of actinomycin D survived for $\sim$ 8–9 but not 24 h. Thus, normocalciuric control and GHS rats were treated with actinomycin D (400 μg/100 g BW) and were killed at various intervals over the ensuing 8 h. Duodenal and kidney cortical total RNAs were isolated at each time point, and VDR mRNA levels were determined by Northern blot hybridization. After hybridization, the radioactivity in the bands of VDR mRNA on Northern blot was quantified by scanner (AMBIS; Scanalytics, Inc., Billerica, MA), and values were normalized to the amount of corresponding RNAs loaded and blotted onto the blot. The in vivo VDR mRNA half-life was then calculated according to an established mathematical model as described (32). Significance of differences among the experimental groups was determined by examining the group versus time interaction term using ANOVA (37, 38).

Amplification of VDR mRNA by RT-PCR. Duodenal total RNA was obtained from normocalciuric and GHS rats (four animals per group). Approximately 5 g of total RNA from each rat was reverse-transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT) at 42°C for 45 min and at 95°C for 10 min to inactivate MuLV reverse transcriptase (Perkin-Elmer Corp.). The reaction was carried out in a final volume of 0.02 ml containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM MgCl$_2$, 1 mM of each dNTP, 20 U of RNase inhibitor, and 50 U of MuLV reverse transcriptase. Two minus primers (see below) were used in the reactions at concentrations

of 110 nM. The cDNA fragments for VDR mRNA were further amplified by PCR with specific PCR primers for VDR mRNA in a GeneAmp PCR system (2400; Perkin-Elmer Corp.) using the "hot-start" approach. The PCR reaction was carried out in a final volume of 0.1 ml containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 200 μM of each dNTP, 2.5 U of AmpliTaq DNA polymerase, and 20 nM of plus/minus specific primers. The conditions used for PCR amplification were one cycle of 4 min at 94°C, 2.5 min at 60°C, and 2 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C; and a final cycle of 1 min at 94°C, 1 min at 60°C, and 10 min at 72°C. Two pairs of RT-PCR primers were designed using the PC/GENE program (IntelliGenetics, Inc., Campbell, CA) based on the rat VDR mRNA sequence (39). The sequence of the first pair of primers was, for minus primer (852–831 bp), 5′-TCGGAGGTGAGATCCCT-GAATC-3′, and for plus primer (8–29 bp), 5′-CGCCAGACCA-GAGTTCTTTTGG-3′. The second pair was minus primer (2041–2023 bp), 5′-CCCATGGCAAAACCTCAAA-3′, and plus primer (457–478 bp), 5′-GCCCAAGCTATCTGAAGAACAA-3′. After amplification, the PCR products were electrophoresed in a 1.5% agarose gel with 1× TAE buffer (Tris/acetic acid/EDTA), and VDR cDNA bands were excised. The fragments of VDR cDNA were further purified using the Wizard® PCR Preps DNA purification system following the manufacturer's protocol (Promega Corp., Madison, WI). The quantity and purity of VDR cDNA fragments were determined by spectrophotometry and gel electrophoresis.

Sequencing the duodenal VDR cDNA. The pure VDR cDNA fragments obtained as above were sequenced by the thermocycling sequence approach using the *fmol* DNA sequencing system (Promega Corp.) with direct incorporation procedure as described by the manufacturer. Briefly, 50 fmol of pure VDR cDNA fragment, 3 pmol of sequence primer, and 10 μCi of [α-^{35}S]dATP (specific activity > 1,000 Ci/mol; Amersham Corp.) were included in each primer/template mixture. After adding sequencing grade *Taq* DNA polymerase (Perkin-Elmer Corp.), the mixture was distributed into each sequencing reaction tube which contained the d/dNTP mixture. Reaction tubes were subjected to the PCR cycling program with hot-start. The cycling profile included one cycle of 4 min at 94°C, 2.5 min at 60°C, and 2.5 min at 72°C; 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C; and ended with an additional 8 min at 72°C, then soaking at 4°C. The *fmol* sequencing stop solution was added after cycling. The reactions were heated at 70°C for 3 min immediately before loading onto a sequencing gel (S2 sequencing gel apparatus; GIBCO BRL, Gaithersburg, MD) containing 8% polyacrylamide/8 M urea in 1× TBE buffer (Tris/boric acid/EDTA, pH 8.3) (40). After electrophoresis, the gel was fixed in 15% methanol/7% acetic acid and rehydrated before drying with a gel drying system (Fisher Scientific Co., Pittsburgh, PA). The gel was then exposed to Biomax film (Eastman Kodak Co.) at room temperature. The sequencing primers were designed according to the published VDR cDNA sequence (39) at intervals of $\sim$ 250 bp. The sequencing gels were read, and the VDR cDNA sequences of the normocalciuric and GHS rats were compared with each other and to the published VDR cDNA sequence (39).

Materials. 1,25(OH)$_2$D$_3$ was a generous gift from Dr. Milan Uskokovic (Hoffman-La Roche Laboratories, Nutley, NJ). All reagents and chemicals used in this study, except where indicated, were purchased either from Sigma Chemical Co. (St. Louis, MO) or GIBCO BRL, including chemicals of molecular biology grade for gene expression studies.

Quantitative analysis. Radioactivities of VDR bands on the blots of Northern hybridizations were quantified by an AMBIS scanner. The intensity of ribosomal RNA bands transferred onto GeneScreen membranes was visualized by ethidium bromide staining and analyzed densitometrically (LKB, Uppsala, Sweden) to ensure equal RNA sample loading. To permit accurate analyses under identical conditions, samples of RNA extracted from GHS and control rats were always electrophoresed in the same gel and transferred onto the same GeneScreen membrane (31–33). Quantitative data were obtained from at least three individual experiments.

Statistical analysis. Data are presented as mean±SEM. The significance of difference between the means of two groups was analyzed by Student's t test, and statistically significant differences were taken when P values were < 0.05.

Results

GHS rats have a hyperresponsiveness of VDR gene expression to 1,25(OH)$_2$D$_3$. Under basal conditions, duodenal VDR mRNA levels were readily detectable in all animals, and were 30% lower in GHS compared with normocalciuric control rats (Fig. 1), as described previously (19). 24 h after the administration of a single intraperitoneal injection of 1,25(OH)$_2$D$_3$ of 30 ng/100 g BW, VDR mRNA levels increased two- to threefold in duodenum from GHS rats, whereas VDR gene expression in control rats was not affected (Fig. 1).

VDR mRNA levels were $\sim$ 7–10-fold lower in kidney compared with duodenum in both normocalciuric and GHS rats. In
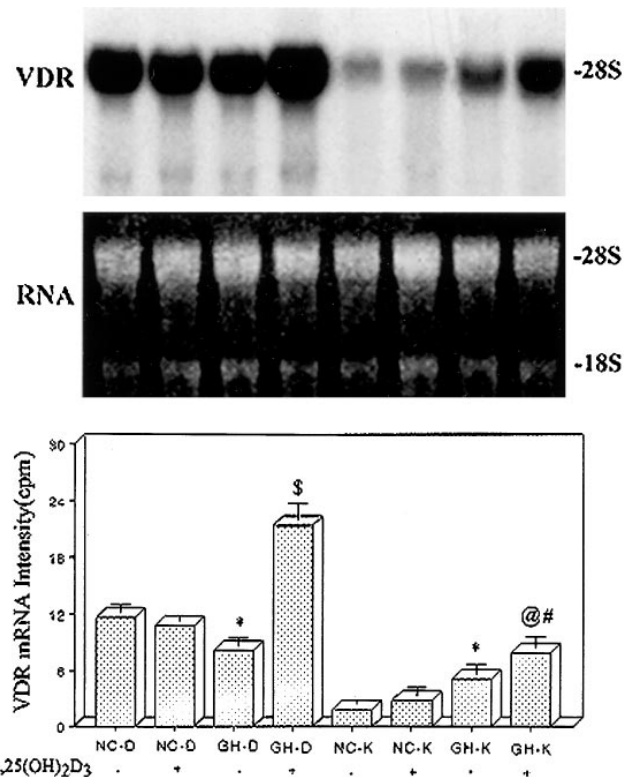

Figure 1. VDR gene expression and regulation by 1,25(OH)$_2$D$_3$. (*Top*) Autoradiogram of Northern blot hybridization showing 4.4 kb VDR mRNA in duodenum (*D*) and kidney (*K*) of normocalciuric (*NC*) and GHS (*GH*) rats treated intraperitoneally with either vehicle (−) or a single 30 ng/100 g BW dose of 1,25(OH)$_2$D$_3$ (+) 24 h before killing. Total RNA was isolated from duodenum and kidney, and RNA electrophoresis, transfer, and hybridization were performed as described in Methods. Representative of three independent experiments. (*Middle*) Corresponding RNAs bound onto the blot are visualized by ethidium bromide staining. (*Bottom*) Quantitative analysis of VDR mRNA bands on the blots by AMBIS scanning. The data are expressed as mean±SEM for three to four individual experiments per group. Statistical significance of differences between group means are as follows: NC-D vs. GH-D and NC-K vs. GH-K, *P < 0.05; +NC-K vs. +GH-K, @P < 0.005; −GH-K vs. +GH-K, #P < 0.05; +NC-D vs. +GH-D, $$P$ < 0.001.
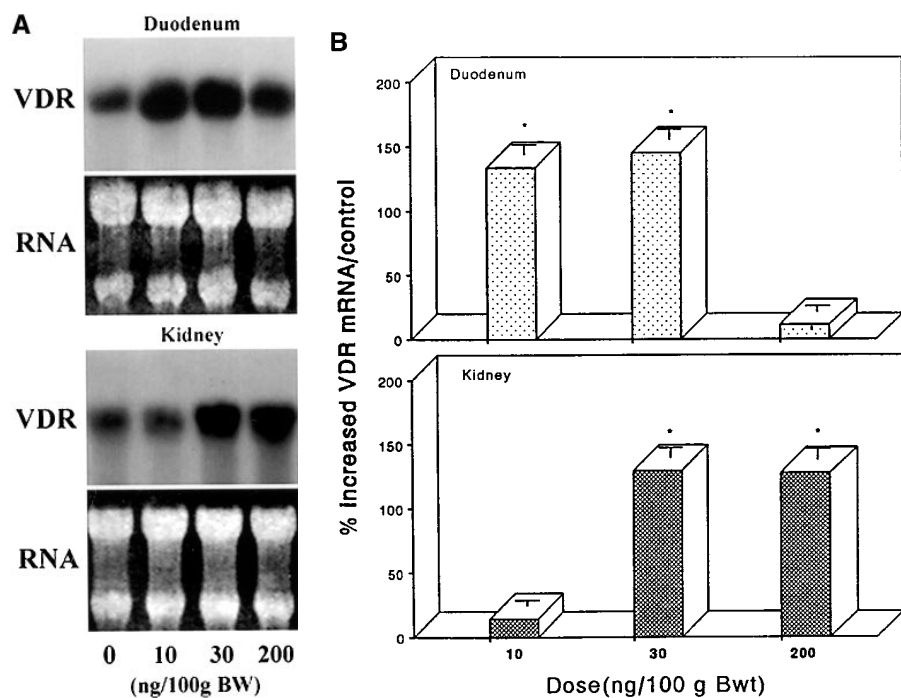
A Duodenum

VDR

RNA

Kidney

VDR

RNA

0   10   30   200
(ng/100g BW)

B

% Increased VDR mRNA/control

Duodenum

200

150

100

50

0

Kidney

200

150

100

50

0

10   30   200

Dose(ng/100 g Bwt)

Figure 2. Dose-dependent changes in VDR gene expression in duodenum and kidney of GHS rats treated with 1,25(OH)$_2$D$_3$. Autoradiogram of Northern blot hybridization showing VDR gene expression. Animals received either vehicle (0) or 10, 30, or 200 ng/100 g BW 1,25(OH)$_2$D$_3$ intraperitoneally 24 h before killing. Corresponding RNAs bound onto the blot and visualized by ethidium bromide staining are shown below. Representative of three independent experiments. (*B*) Quantitative analysis of VDR mRNA levels in duodenum and kidney. Data are mean±SEM of three independent experiments. Statistical significance is as follows: duodenum, for 10 and 30 ng vs. control, *$P < 0.005$; kidney, for 30 and 200 ng vs. control, *$P < 0.05$.

contrast to duodenum, the basal levels of renal VDR gene expression was about threefold greater in GHS rats (Fig. 1) compared with normocalciuric controls. As in duodenum, a single dose of 1,25(OH)$_2$D$_3$ induced about a twofold increase in renal VDR mRNA levels above baseline in GHS rats and did not change VDR mRNA levels in control animals (Fig. 1). Thus, in GHS rats the expression of both duodenal and renal VDR mRNA was highly responsive to a dose of 1,25(OH)$_2$D$_3$ that exerted no significant effect on VDR gene expression in normocalciuric rats.

The hyperresponsiveness to 1,25(OH)$_2$D$_3$ is dose- and time-dependent and tissue specific. To further evaluate the pattern of 1,25(OH)$_2$D$_3$-stimulated VDR gene expression in GHS rats, VDR mRNA was measured 24 h after administration of a wide range of intraperitoneal doses (10–200 ng/100 g BW) of 1,25(OH)$_2$D$_3$. Duodenal VDR gene expression was upregulated approximately threefold by the lowest dose (10 ng/100 g BW) of 1,25(OH)$_2$D$_3$, with maximal increases at intermediate (30 ng/100 g BW) doses, and a small increase at the highest dose of 200 ng/100 g BW (Fig. 2, *A* and *B*). In the kidney, the rise in VDR mRNA after 1,25(OH)$_2$D$_3$ administration was also dose-dependent, with a rightward shift compared with duodenum (Fig. 2, *A* and *B*). The lowest dose, 10 ng of 1,25(OH)$_2$D$_3$, increased VDR gene expression minimally, whereas both 30 and 200 ng increased VDR mRNA about threefold.

The time course of VDR gene expression was determined at various time points up to 48 h after administration of a single dose of 1,25(OH)$_2$D$_3$ (30 ng/100 g BW) in GHS rats. In duodenum, VDR mRNA levels increased 67% by 2 h after treatment, reached the maximal response by 8 h (171%), and returned to 9% above baseline by 48 h (Fig. 3, *A* and *B*). The rise in renal VDR mRNA levels was slower, with significant elevation at 4 h (99% increase over baseline) and maximal response of 167% at 16 h. At 48 h after treatment, renal VDR

gene expression was still 70% greater than that in untreated controls (Fig. 3, *A* and *B*).

1,25(OH)$_2$D$_3$-induced VDR gene expression involves post-transcriptional regulation. To determine whether transcriptional regulatory mechanisms are involved in the upregulation of VDR gene expression in GHS rats, actinomycin D, a blocker of DNA-dependent RNA synthesis, was administered as a single dose of 400 μg/100 g BW to inhibit RNA transcription. 8 h of exposure to actinomycin D efficiently blocked VDR mRNA transcription in both duodenum and kidney, and remaining VDR mRNA levels were < 10% of untreated controls (Fig. 4, compare lane *1* with lane *4*). Despite the presence of actinomycin D, VDR mRNA levels in duodenum and kidney were increased by either an additional 8-h treatment with 1,25(OH)$_2$D$_3$ (30 ng/100 g BW) 1 h after administration of actinomycin D (lane *3*), or a 24-h pretreatment with 1,25(OH)$_2$D$_3$ before actinomycin D administration (lane *5*). The elevation of VDR mRNA levels (lanes *3* and *5*) was similar to that observed in rats treated with 1,25(OH)$_2$D$_3$ alone (lane *2*). Thus, inhibition of gene transcription failed to prevent the upregulation of VDR mRNA induced by 1,25(OH)$_2$D$_3$ (Fig. 5, compare lanes *2*, *4*, and *5*). These results suggest that transcriptional regulation is unlikely involved in 1,25(OH)$_2$D$_3$-stimulated VDR gene expression in GHS rats, but regulation is likely through posttranscriptional mechanisms.

To further understand the regulatory mechanisms of VDR gene expression in GHS rats, VDR mRNA in vivo half-life in duodenum and kidney was assessed after nascent RNA transcription was halted by actinomycin D administration. Under basal conditions, in vivo half-lives of duodenal and renal VDR mRNA were comparable for GHS rats and normocalciuric controls (Fig. 5, *A* and *B*). As anticipated, both duodenal and renal VDR mRNA half-lives in GHS rats were prolonged significantly by 1,25(OH)$_2$D$_3$ administration. However, the in vivo VDR mRNA half-life in 1,25(OH)$_2$D$_3$-treated normocal-

A

Duodenum

VDR

RNA

Kidney

VDR

RNA

0 2 4 8 16 24 48
Time (hours)

B

Duodenum

Kidney

% Increased VDR mRNA/control

2 4 8 16 24 48
Time(hour)

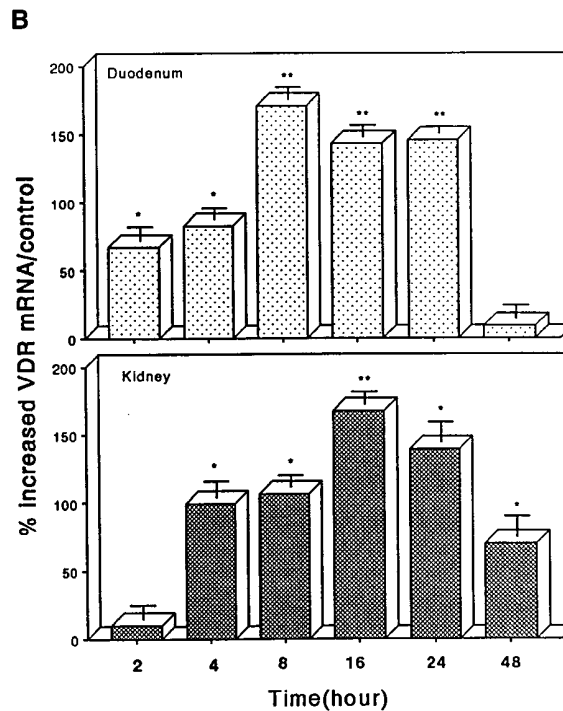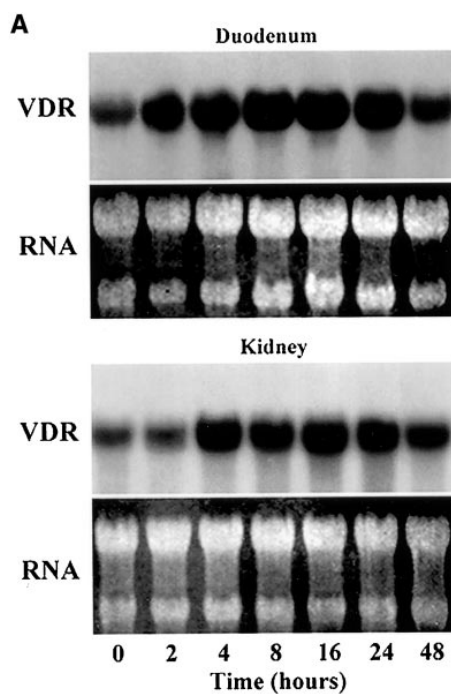*Figure 3.* Time-dependent stimulation of VDR gene expression by $1,25(OH)_2D_3$ in GHS rats. Animals received either vehicle (0) or $1,25(OH)_2D_3$ (30 ng/100 g BW) and were killed at times indicated. (*A*) Northern blot hybridization analysis of VDR mRNA levels in duodenum and kidney. Corresponding RNAs are shown below. Representative of three independent experiments. (*B*) Quantitative analysis of VDR mRNA levels. Data are mean±SEM of three independent experiments for each time point expressed as a percentage of vehicle-treated controls. Statistical significance is as follows: duodenum, *$P < 0.01$ vs. control, and **$P < 0.001$ vs. control; kidney, *$P < 0.02$ vs. control, and **$P < 0.005$ vs. control.

ciuric rats was not changed from baseline (Fig. 5, *A* and *B*). As calculated by an established mathematical model (32), duodenal VDR mRNA half-life was 4.3±0.9 and 4.5±1.4 h in normocalciuric and GHS rats, respectively. Kidney VDR mRNA half-life for controls (5.2±0.7 h) and GHS rats (5.3±0.7 h) was

Figure 4. Effect of actinomycin D pretreatment on $1,25(OH)_2D_3$-stimulated VDR gene expression in GHS rats. Animals were treated with vehicle or $1,25(OH)_2D_3$ (30 ng/100 g BW) in the presence or absence of actinomycin D (400 μg/100 g BW). VDR gene expression in duodenum and kidney was determined by Northern blot hybridization. Lane *1*, Vehicle-treated control; lane *2*, $1,25(OH)_2D_3$ treatment and killing 8 h later; lane *3*, treated with actinomycin D 1 h before $1,25(OH)_2D_3$ and killed 8 h later; lane *4*, treated with actinomycin D and killed 8 h later; and lane *5*, treated with $1,25(OH)_2D_3$ then actinomycin D 24 h later, then killed 8 h thereafter. Representative of three independent experiments.

Duodenum

VDR

RNA

Kidney

VDR

RNA

1 2 3 4 5

also similar. After $1,25(OH)_2D_3$ administration, duodenal and renal VDR mRNA half-lives were prolonged to > 8 h in GHS rats ($P < 0.001$ for decay slopes vs. baseline), whereas half-life of VDR mRNA in wild-type controls did not change significantly from baseline (4.7±1.1 h for duodenum and 5.5±1.4 h for kidney).

De novo protein synthesis is not required for $1,25(OH)_2D_3$-induced VDR gene expression. To address the role of de novo protein synthesis in $1,25(OH)_2D_3$-induced regulation of VDR gene expression, GHS rats were treated with cycloheximide (4 mg/100 g BW) 1 h before treatment with either $1,25(OH)_2D_3$ (30 ng/100 g BW) or vehicle alone. The presence of cycloheximide strongly suppressed duodenal VDR gene expression in the absence and presence of $1,25(OH)_2D_3$ (Fig. 6, *top*). In contrast, cycloheximide did not interfere with renal VDR gene expression in either vehicle-treated or $1,25(OH)_2D_3$-stimulated GHS rats (Fig. 6, *bottom*). Thus, $1,25(OH)_2D_3$-induced upregulation of renal VDR gene expression seems not to require de novo protein synthesis. However, the role, if any, of new protein synthesis in $1,25(OH)_2D_3$-induced upregulation of VDR in duodenum is not conclusive from these experiments, as cycloheximide alone strongly suppressed VDR gene expression. The latter effect may be due in part to a more severe cytotoxicity of cycloheximide on the rapidly turning over intestinal epithelial cells compared with the more stable kidney cells.

VDR mRNA sequence in GHS rats. Virtually the entire sequence of VDR cDNA (8–2041 bp) was amplified by RT-PCR from total RNA isolated from GHS and normocalciuric rat duodenum (four individual rats per group) using two pairs of specific primers. Two fragments (845 and 1584 bp, respec-
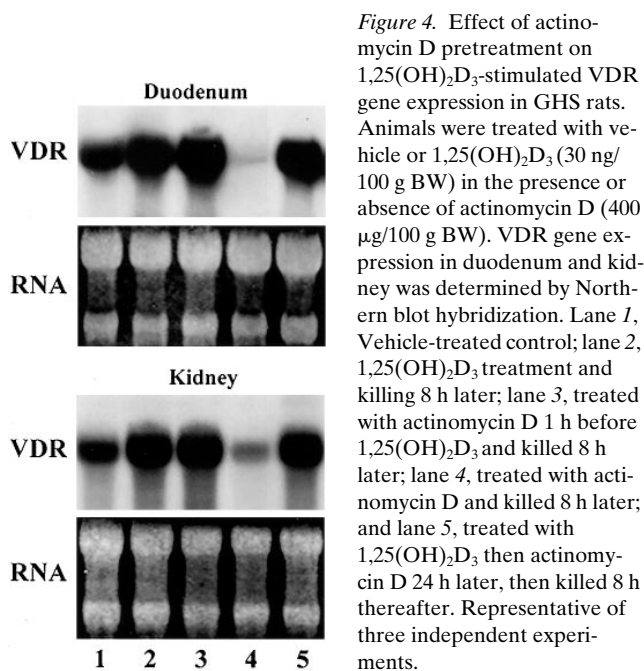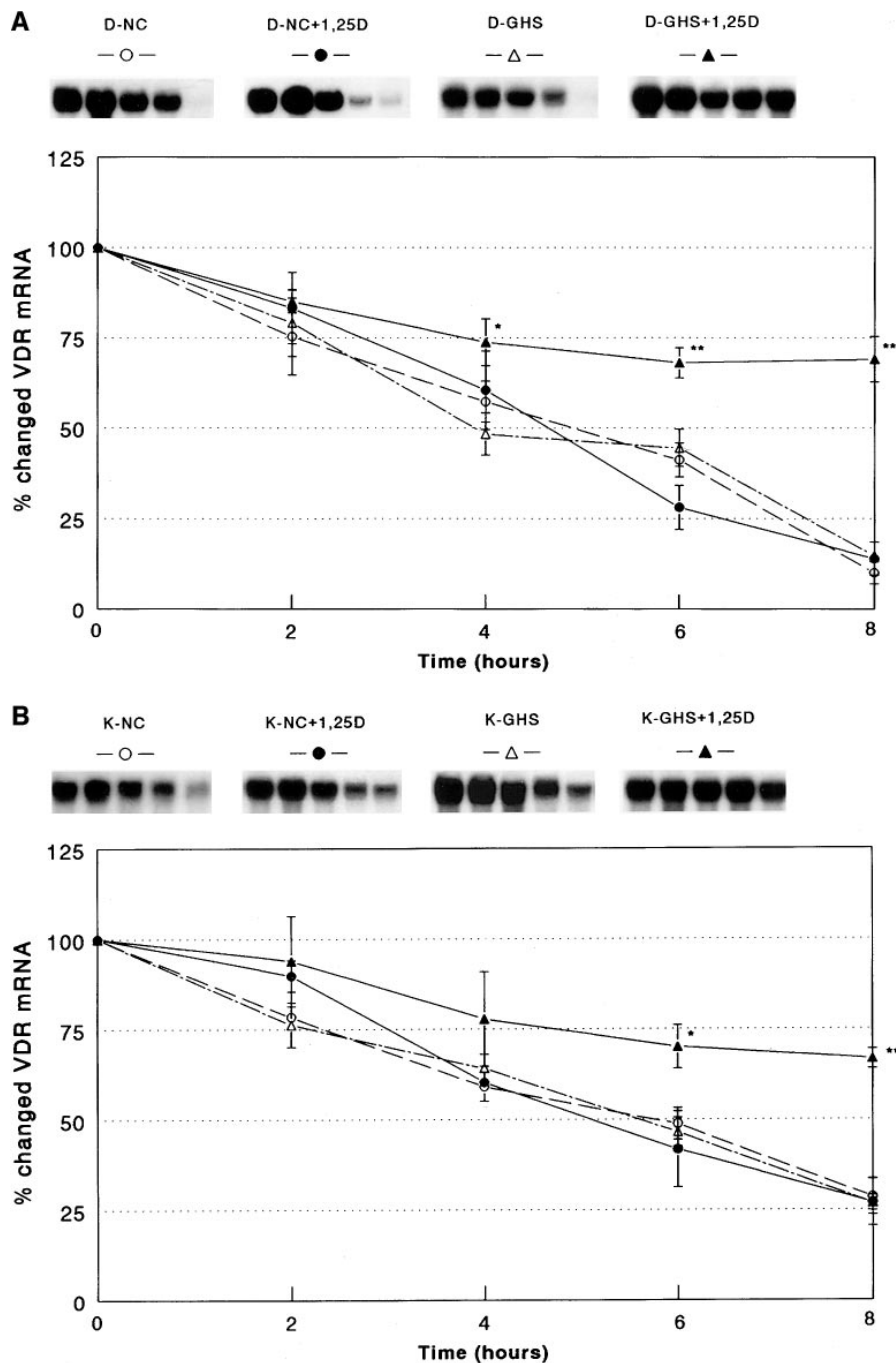
A

B

Figure 5. VDR mRNA in vivo half-life in (*A*) duodenum (*D*) and (*B*) kidney (*K*) from normocalciuric (*NC*) and GHS rats. Animals received a single dose of either $1,25(OH)_2D_3$ (*+1,25D*) or vehicle intraperitoneally, 24 h later were treated with actinomycin D (400 μg/100 g BW), and were killed thereafter at times indicated. VDR mRNA levels were measured by Northern blot hybridization and quantified by scanning. VDR mRNA levels (mean±SEM) after actinomycin D administration are expressed as percentage of baseline (100%). *Insets* (*top*), A representative autoradiogram from three independent experiments. VDR mRNA decay slopes for duodenum GHS plus $1,25(OH)_2D_3$ and kidney GHS plus $1,25(OH)_2D_3$ groups differ from the other groups, $P < 0.001$. Differences in time points are as follows: for duodenum, $*P < 0.005$, and $**P < 0.001$; for kidney, $*P < 0.01$ and $**P < 0.001$.

tively) of PCR products were obtained and further purified (Fig. 7 *A*). These VDR cDNA fragments from four sets of rats were then sequenced by the d/dNTP thermocycling method. The VDR cDNA sequences of the GHS and normocalciuric rats were identical, without any evidence of alteration. Differences in basepairs were discovered at three sites when the sequences of VDR cDNA from both normocalciuric and GHS rats were compared with the published rat intestinal VDR cDNA sequence (34). As shown in Fig. 7 *B*, the first difference encountered at bp 253 is C instead of G; the second at bp 569 is G instead of A; and the third difference is at bp 1658, an A for a G. The substitution of G for A at bp 569 will change the pu-

tative amino acid VDR sequence from methionine located at 159 to valine, whereas the difference at bp 253 may not change the putative amino acid sequence. The third substitution at bp 1658 is in the 3′-untranslated region, and has no influence on VDR amino acid sequence.

Calbindin-9kd and -28kd mRNA levels are elevated after upregulation of VDR gene expression in GHS rats. To determine whether upregulated VDR gene expression leads to a biological response in target tissues, gene expression of the VDR-responsive genes calbindin-9kd and calbindin-28kd were measured in rats after administration of $1,25(OH)_2D_3$ or vehicle. As shown in Fig. 8, under basal conditions, calbindin-9kd
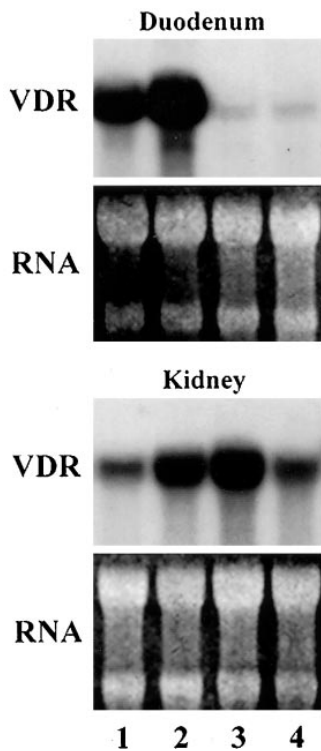
Duodenum

VDR

RNA

Kidney

VDR

RNA

1 2 3 4

Figure 6. Effect of cycloheximide on 1,25(OH)$_2$D$_3$-induced VDR gene expression. GHS rats were treated with vehicle or cycloheximide (4 mg/100 g BW) and/or 1,25(OH)$_2$D$_3$ (30 ng/100 g BW). Duodenal and kidney VDR mRNA levels were identified by Northern blot hybridization. Lane *1*, Vehicle control; lane *2*, 1,25(OH)$_2$D$_3$ treatment and killing 8 h later; lane *3*, cycloheximide given 1 h before 1,25(OH)$_2$D$_3$ treatment and animals killed 8 h later; and lane *4*, cycloheximide-treated 8 h before killing. Representative of three individual experiments.

mRNA levels were fourfold greater in duodenum of wild-type rats, whereas renal calbindin-28kd levels were comparable to GHS rats. A single intraperitoneal dose of 1,25(OH)$_2$D$_3$ (30 ng/100 g BW) increased duodenal calbindin-9kd mRNA levels five- to sixfold ($P < 0.002$) and renal calbindin-28kd mRNA levels two- to threefold ($P < 0.002$). In contrast, treatment of wild-type animals with the same dose of 1,25(OH)$_2$D$_3$ decreased calbindin-9kd mRNA levels by 63% ($P < 0.005$), whereas calbindin-28kd mRNA levels did not change.

Discussion

Several lines of evidence suggest that GHS rats have exaggerated in vivo and in vitro responses to 1,25(OH)$_2$D$_3$. GHS rats have a fivefold increase in vitamin D–dependent duodenal Ca active transport with normal circulating 1,25(OH)$_2$D$_3$ levels (18); duodenal Ca transport increases out of proportion to the modest increase in serum 1,25(OH)$_2$D$_3$ during dietary Ca restriction (20); and 1,25(OH)$_2$D$_3$-stimulated in vitro bone resorption in GHS calvaria follows a steeper dose–response curve than in wild-type controls (21). The present study confirms that GHS rats hyperrespond to 1,25(OH)$_2$D$_3$ administration, and demonstrates that the 1,25(OH)$_2$D$_3$ action is mediated by enhanced VDR gene expression. First, the hyperresponsiveness to 1,25(OH)$_2$D$_3$ administration is time- and dose-dependent. Second, 1,25(OH)$_2$D$_3$ upregulates duodenal and kidney VDR gene expression by prolongation of the VDR mRNA in vivo half-life rather than via transcriptional events. Third, 1,25(OH)$_2$D$_3$-induced upregulation of VDR gene expression seems not to require de novo protein synthesis. Fourth, hyperresponsiveness to 1,25(OH)$_2$D$_3$ is not due to a sequence alteration or mutation in the VDR mRNA. Fifth, 1,25(OH)$_2$D$_3$ administration leads to a parallel increase in VDR gene expression and calbindin-9kd and -28kd mRNA levels. In addition, by comparing duodenum and kidney, the study demonstrates tissue-specific VDR mRNA levels and turnover rates, and differential responses to 1,25(OH)$_2$D$_3$.

The hyperresponsiveness to 1,25(OH)$_2$D$_3$ is a unique feature of GHS rats, as doses of 1,25(OH)$_2$D$_3$ which could upregulate GHS rat VDR gene expression three- to fourfold in both duodenum and kidney did not alter duodenal or kidney VDR mRNA levels in wild-type control animals. The increased VDR binding capacity in GHS rat duodenum, bone, and kidney in the basal state (19, 21) and the hyperresponsiveness to 1,25(OH)$_2$D$_3$ predict that modest fluctuations in serum 1,25(OH)$_2$D$_3$ levels would significantly increase VDR gene expression through increased numbers of VDR–1,25(OH)$_2$D$_3$ complexes. As the biologic actions of 1,25(OH)$_2$D$_3$ are directly related to target tissue VDR content (23–25), such increases in VDR–1,25(OH)$_2$D$_3$ complexes may result in pathologic amplification of the biologic actions of 1,25(OH)$_2$D$_3$, including increased intestinal Ca transport and bone resorption, and decreased renal tubular Ca reabsorption. Each of these changes in Ca transport has been demonstrated in GHS rats (18, 20, 21) and may contribute to the hypercalciuria.

Studies suggest that 1,25(OH)$_2$D$_3$ regulation of VDR may occur through one of several mechanisms. In vitro studies using a variety of cell types show that 1,25(OH)$_2$D$_3$ or 24,25(OH)$_2$D$_3$ upregulates VDR either by enhancing VDR gene expression or through translational events (41). Prolongation of VDR protein half-life may also occur, as observed in cultures of guinea pig kidney cells in which VDR half-life was lengthened from 4.3 to 8.9 h by 24,25(OH)$_2$D$_3$ (19). The mechanisms whereby 1,25(OH)$_2$D$_3$ regulates VDR in vivo are less clear. Strom et al. (42) reported that 1,25(OH)$_2$D$_3$ administration increases intestinal VDR protein detected by an immunoradiometric assay without an accompanying increase in ligand binding capacity, suggesting that the increase in receptor protein might be nonfunctional or functional but occupied. Naveh-Many et al. (43) found that 1,25(OH)$_2$D$_3$ increased parathyroid cell VDR content through enhanced VDR gene expression. Using a low P diet to stimulate endogenous 1,25(OH)$_2$D$_3$ production and upregulation of VDR, Sriussadaporn et al. (44) describe increases in rat duodenal VDR and VDR mRNA content that were detectable within 24 h of starting the low P diet. By day 5 of the low P diet, VDR gene expression had declined to below normal, while duodenal VDR content remained three- to fourfold greater than in controls fed a normal P diet. Thus, in normal animals, a complex regulation of VDR may occur with an initial period of enhanced gene expression, followed by events that control receptor protein half-life.

In this study, 1,25(OH)$_2$D$_3$-mediated increases in VDR mRNA levels in GHS rats may have resulted from either increased VDR gene transcription or decreased degradation, or from a combination of both. The former is very unlikely because the 1,25(OH)$_2$D$_3$-induced VDR mRNA was not prevented by inhibiting nascent RNA synthesis due to the presence of actinomycin D. Therefore, the upregulation of the VDR mRNA must have been due to a posttranscriptional action of 1,25(OH)$_2$D$_3$ to increase stability of VDR mRNA. This is demonstrated by the observation that VDR mRNA half-life is prolonged significantly, from ~4 to >8 h. Furthermore, this regulation seemed to be independent of de novo protein synthesis. Thus, the control of VDR mRNA clearance appears to
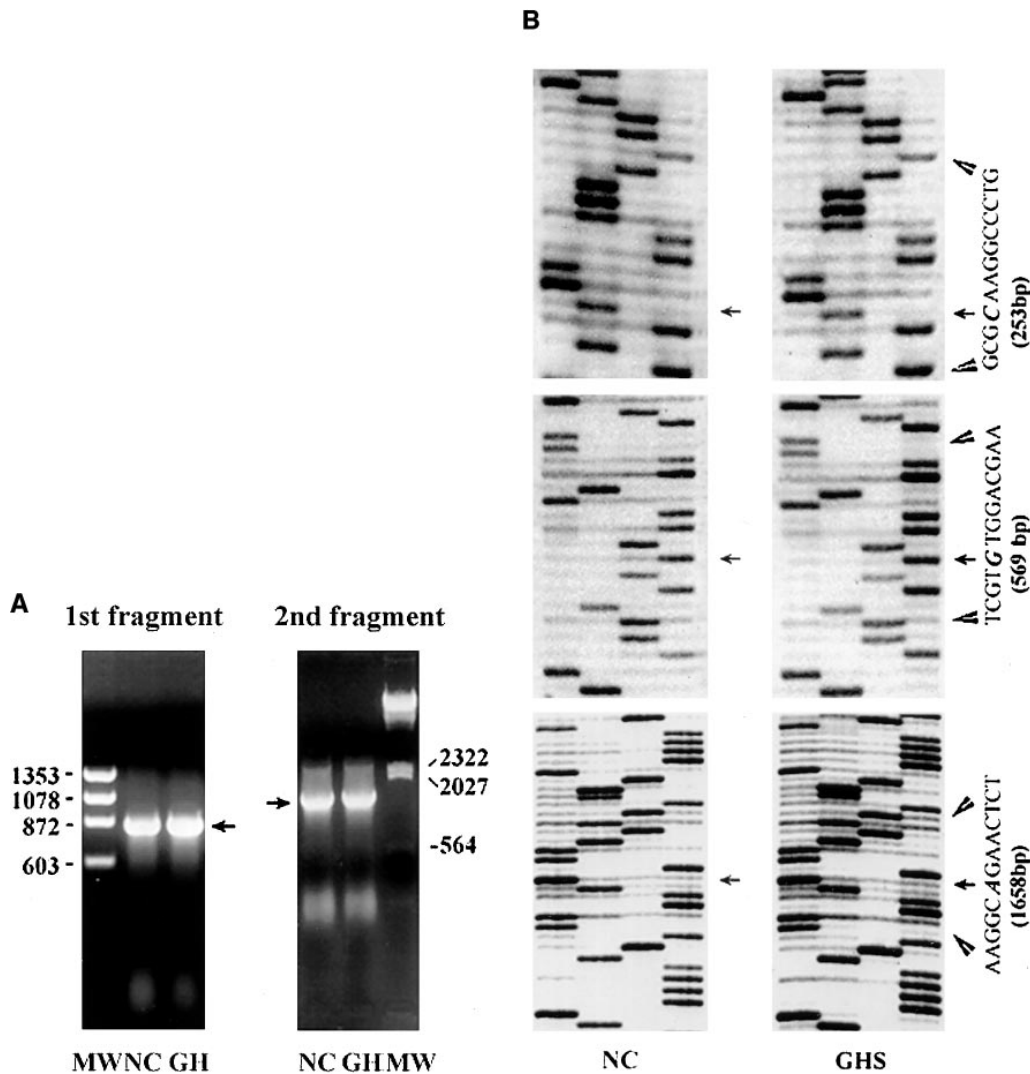
Figure 7. Sequence of duodenal VDR cDNA prepared from VDR mRNA from normocalciuric and GHS rats. (*A*) RT-PCR product of VDR mRNA. Duodenal VDR cDNA from four sets of normocalciuric (*NC*) and GHS (*GH*) rats was amplified by RT-PCR using two pairs of specific primers. PCR products were electrophoresed on agarose/ TAE gel (see Methods), and two fragments of VDR cDNA with expected size of 845 and 1584 bp were obtained (*arrows*). Molecular weight (*MW*) markers are indicated. (*B*) Autoradiograms of sequence gels of VDR cDNA. A representative of four sets from each group of rats is shown. Samples from normocalciuric (*NC*) and GHS duodenum contain three consistent differences in basepair sequences compared with published VDR cDNA sequence (*arrows*). 13-bp reading sequence around the substituted basepairs (**bold**) and their locations are indicated.

be due to certain as yet unidentified mechanisms, which will require additional investigation to define.

This study also explored the possibility that mutations in the VDR mRNA sequence may lead to an altered expression in this gene in response to $1,25(OH)_2D_3$. However, no difference in duodenal VDR mRNA sequences between GHS and wild-type rats was discovered. Thus, altered VDR mRNA sequence and structure have been excluded as potential causes of the hyperresponsiveness of GHS rats to $1,25(OH)_2D_3$. Three basepair differences were discovered when VDR mRNA sequences from GHS and wild-type rats were compared with the published sequences obtained previously from Sprague-Dawley rats (39). The PCR thermocycling approach used to sequence VDR mRNA in this study is commonly used to detect mutations. While mispriming might occur occasionally in PCR cycling when cycling temperature is too low, we obtained identical sequences from eight individual animals (four GHS rats and four normocalciuric controls) at relatively high priming temperature (60°C). Thus, it is unlikely that the basepair alterations in VDR mRNA sequence observed in this study are due to technical error. Whether the basepair substitutions and potential single amino acid sequence change have

any apparent significance in VDR structure and/or function needs further clarification.

Current concepts of VDR–$1,25(OH)_2D_3$ action include binding of the complex to *cis*-acting, enhancer-like DNA sequences (VDR response elements) or other transcriptional factors located in the vicinity of $1,25(OH)_2D_3$-regulated genes (41). This interaction would lead to conformational changes in chromatin structure, which might then alter the expression of responsive genes. Although this study has not excluded the participation of other vitamin D response elements or other regulatory factors in the control of VDR in GHS rats, the role of other genes appears less likely, as upregulation of VDR mRNA by $1,25(OH)_2D_3$ did not require new protein synthesis. The current observations are also consistent with the possibility that GHS rat VDR gene sequences may contain vitamin D response elements that could exert a "self-amplifying" effect in the presence of VDR–$1,25(OH)_2D_3$ complexes. This study does not exclude the possibility that VDR–$1,25(OH)_2D_3$ complexes or changes in cellular Ca transport are involved in regulating the degradation of VDR mRNA.

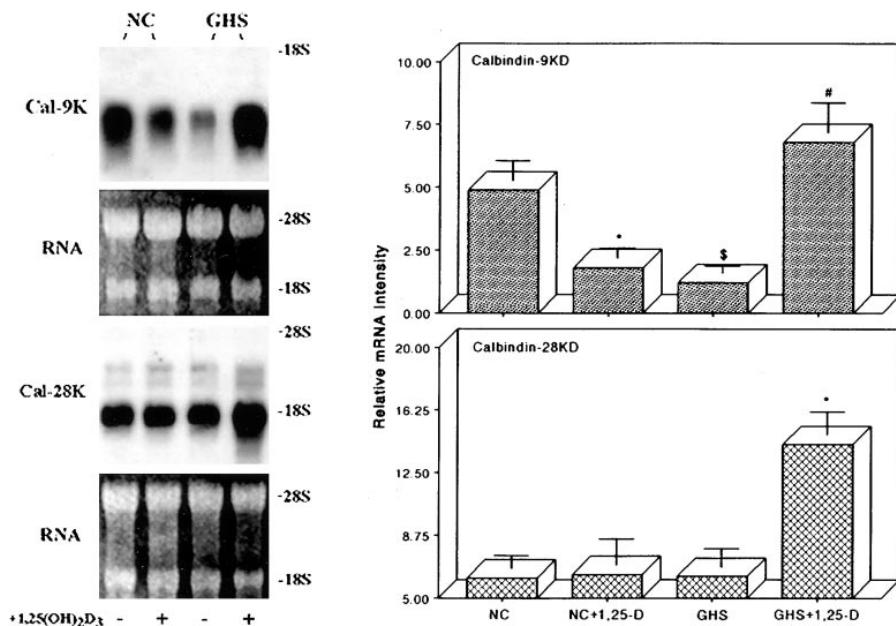Calbindins are products of VDR-responsive genes, and are regulated by $1,25(OH)_2D_3$ (45, 46). The increases in mRNA

Figure 8. Regulation of calbindin mRNA levels in normocalciuric (*NC*) and GHS rats. Animals received 1,25(OH)$_2$D$_3$ (+, 30 ng/100 g BW) or vehicle (−) 24 h before killing. The mRNA levels of calbindin-9kd (*Cal-9K*) in duodenum and calbindin-28kd (*Cal-28k*) in kidney were determined by Northern blot hybridization. *Left panel*, A representative autoradiogram from three independent experiments. *Right panel*, Quantitative analysis. For calbindin-9kd, $^*P < 0.005$ vs. NC, $^\$P < 0.002$ vs. NC, and $^\#P < 0.002$ vs. GHS; for calbindin-28kd, $^*P < 0.002$ vs. GHS.

levels of calbindin-9kd and -28kd in GHS but not normocalciuric rats after 1,25(OH)$_2$D$_3$ administration reveal important differences in vitamin D–dependent gene expression between GHS and wild-type rats and serve as functional indicators of 1,25(OH)$_2$D$_3$-induced upregulation of VDR gene expression. Other experiments show that VDR protein is also increased (data not shown) in GHS rats in response to 1,25(OH)$_2$D$_3$ treatment. These data suggest that upregulated VDR gene expression in GHS rats after 1,25(OH)$_2$D$_3$ is functional and may elicit biologic actions in target tissues.

Previously, we have reported elevated duodenal Ca active transport rates and calbindin-9kd protein levels in GHS rats (19) with lower calbindin-9kd mRNA levels compared with wild-type animals (47). The results of this study are consistent with our earlier observations, and demonstrate that the low calbindin-9kd mRNA levels can be increased dramatically by a dose of 1,25(OH)$_2$D$_3$ that slightly decreased calbindin-9kd mRNA levels in wild-type animals. The latter change in calbindin-9kd mRNA differs from the actions of 1,25(OH)$_2$D$_3$ in vitamin D–deficient rats, where it increases intestinal calbindin-9kd mRNA (46, 48, 49). The decrease in calbindin-9kd mRNA levels in vitamin D–sufficient rats after 1,25(OH)$_2$D$_3$ administration may be part of a feedback mechanism that minimizes the effects of excess 1,25(OH)$_2$D$_3$ on intestinal Ca absorption in normal animals. The increase in calbindin-9kd mRNA in response to 1,25(OH)$_2$D$_3$ in GHS rats demonstrates the differences in regulation of this gene between the normocalciuric and GHS rats, and may reflect an impairment or loss of the feedback mechanism that normally protects against excess intestinal Ca absorption during pathologic or pharmacologic increases in 1,25(OH)$_2$D$_3$. The same explanation may be applied to the lack of an increase in renal calbindin-28kd mRNA in wild-type rats treated with 1,25(OH)$_2$D$_3$. These studies of calbindins indicate the complexity of the mechanisms involved in the tissue-specific regulation of calbindin gene expression (46, 49, 50). Although the precise regulatory mechanisms remain unclear, this study strongly suggests that the usual control mechanisms for the expression of calbindin genes are altered in GHS rats.

In summary, this study has characterized a unique hyperresponsiveness of VDR gene expression to 1,25(OH)$_2$D$_3$ in GHS rats. The results strongly support the hypothesis that amplification of 1,25(OH)$_2$D$_3$ action may occur through posttranscriptional upregulation of duodenal, renal, and bone cell VDR content, which in turn provokes biological responses in VDR target tissues. This unique characteristic might predict that in GHS rats, minimal fluctuation of serum 1,25(OH)$_2$D$_3$ levels significantly increases VDR content, which pathologically intensifies the biological actions of 1,25(OH)$_2$D$_3$. As a consequence, enhanced Ca transport in these tissues contributes to hypercalciuria and formation of kidney stones in the GHS rat.

Acknowledgments

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