JCI The Journal of Clinical Investigation

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J Clin Invest. 1990;86(6):1968-1975. https://doi.org/10.1172/JCI114931.

Research Article

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Regulation of 1,25-Dihydroxyvitamin D_3 Receptor Gene Expression by 1,25-Dihydroxyvitamin D_3 in the Parathyroid In Vivo

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Abstract

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) dramatically decreases parathyroid hormone (PTH) gene transcription. We have now studied the effect of $1,25(OH)_2D_3$ on the $1,25(OH)_2D$ receptor (VDR) in the parathyroid in vivo. Rats were injected with $1,25(OH)_2D_3$ and the parathyroid-thyroid tissue analyzed for PTHmRNA and VDRmRNA. $1,25(OH)_2D_3$ (50 and 100 pmol ip) decreased PTHmRNA at 6 h with a maximum at 48 h (< 4% of basal), whereas VDRmRNA was increased only after 6 h with a 1.7-fold increase at 24 h. VDRmRNA levels peaked at 25 pmol $1,25(OH)_2D_3$ with a twofold increase. Serum calcium did not affect VDRmRNA. Parathyroid VDRmRNA ran at 2.2 and 4.4 kb, whereas duodenum VDRmRNA had a single band, all of which increased after $1,25(OH)_2D_3$. Weanling rats on a vitamin D–deficient diet for 3 wk had a more intense 2.2-kb transcript, whereas vitamin D–replete rats had a more intense 4.4-kb band.

Dispersed parathyroid-thyroid cells were separated by a flow cytometry (FACS) into a parathyroid cell peak containing PTHmRNA and a second peak with cells positive for thyroglobulin mRNA and calcitonin mRNA. VDRmRNA was concentrated in the parathyroid cell peak. In situ hybridization of parathyroid-thyroid and duodenum for VDRmRNA showed its localization to the parathyroid cells and the duodenal mucosa. Therefore, the VDRmRNA in the parathyroid-thyroid tissue represents predominantly parathyroid cell and not C-cell VDRmRNA which is also a $1,25(OH)_2D_3$ target organ. The increased VDR gene expression in the parathyroid cell would amplify the effect of $1,25(OH)_2D_3$ to decrease PTH gene transcription. (*J. Clin. Invest.* 1990. 86:1968–1975.) Key words: calcium • flow cytometry • in situ hybridization • calcitonin transcription

Introduction

The vitamin D sterol's biologically active metabolite 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) regulates mineral homeostasis by acting on its classical target organs, the intestine, bone, and kidney (1, 2). It also regulates mineral homeostasis by decreasing the transcription of the calciotrophic hormones, parathyroid hormone (PTH)[1] (3–5) and calcitonin (6, 7). $1,25(OH)_2D_3$ acts on its diverse target tissues by binding to the $1,25(OH)_2D$ receptor (VDR), a 48-kD protein which has been cloned, and shown to be structurally similar to other steroid receptors (8). The DNA sequences in the rat and human osteocalcin genes that bind the $1,25(OH)_2D_3$ receptor and confer responsiveness to $1,25(OH)_2D_3$ have now been determined (9–11), thus providing further evidence that $1,25(OH)_2D_3$ binds to its receptor which activates gene transcription analogous to that of other small nonpeptide hormones (12). Mutations in the VDR gene result in $1,25(OH)_2D_3$-resistant rickets, further confirming the importance of the VDR (13, 14).

The VDR concentration in the $1,25(OH)_2D_3$ target sites would allow a modulation of the $1,25(OH)_2D_3$ effect. Ligand-dependent upregulation of the VDR has been shown in vivo in rat intestine (15–17) at the levels of VDRmRNA and VDR protein content (18). In vitro ligand-dependent upregulation has been shown on VDRmRNA in 3T6 cells (8), human osteosarcoma cell lines (19), and human leukemia cells (HL 60) (20, 21).

We have shown previously that in vivo in the rat $1,25(OH)_2D_3$ dramatically decreased PTH gene transcription (4), and we now demonstrate that this is associated with an upregulation of the VDRmRNA levels in the parathyroids.

Dot blots of RNA extracts of parathyroid-thyroid tissue from normal rats which had been hybridized with PTHcDNA and actin cDNA were now washed and rehybridized for VDRmRNA. Other rats were injected with $1,25(OH)_2D_3$ and their parathyroid-thyroid extracts run on agarose gels for Northern blots for PTHmRNA and VDRmRNA. These results were supplemented by studies which demonstrated the cellular localization of the VDRmRNA to the parathyroids.

Methods

Animals. Male Hebrew University (Jerusalem, Israel) strain rats (150–180 g) were maintained on a normal diet and injected ip with vitamin D metabolites dissolved in propylene glycol (100 μl), or with 0.6 M sodium phosphate monobasic (0.2–2 mmol/100 g body wt), calcium gluconate lactate (2 ml), or calcitonin (synthetic human calcitonin, CIBA-Geigy, Basel, Switzerland) as previously described (4, 6, 22).

In one series of experiments weanling male rats were housed in an ultraviolet free environment for 3 or 8 wk and fed ad libitum one of the following diets (Teklad, Madison, WI): normal vitamin D, 0.4% calcium (NDNCa) (TD 88304); normal vitamin D, 0.02% calcium (ND–Ca) (TD 88346); vitamin D deficient, 0.4% calcium (–DNCa) (TD 85049); vitamin D deficient, 0.02% calcium (–D–Ca) (TD 85048); vitamin D deficient, 2% calcium (–D+Ca) (TD 89155).

At timed intervals the parathyroid-thyroid tissue was excised under

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Received for publication 4 June 1990 and in revised form 30 July 1990.

J. Clin. Invest.

0021-9738/90/12/1968/08 $2.00
Volume 86, December 1990, 1968–1975

1. *Abbreviations used in this paper:* PTH, parathyroid hormone; VDR, $1,25(OH)_2D$ receptor.

pentobarbital anesthesia, and blood samples taken. In some experiments also duodenum was excised (first 10 cm) after flushing with ice-cold PBS, and the mucosa was separated from the serosa with a glass microscope slide on ice. The excised tissue was flash frozen in liquid nitrogen and stored at −70°C until extraction. Serum calcium was measured with an atomic absorption spectrophotometer (Perkin-Elmer Corp., Pomona, CA).

Measurement of cellular mRNA levels. Total cellular RNA was prepared from frozen tissue by homogenization in guanidium thiocyanate followed by deproteinization using guanidine hydrochloric acid and ethanol precipitation as previously described (4). For the dot blot hybridization assay, total tissue RNA extracts were prepared by formaldehyde denaturation followed by serial dilutions with 15× SSC (1× = 0.15 M NaCl, 0.01 M Na citrate), and blotted onto nitrocellulose filters (0.45 μm; Schleicher & Schuell, Keene, NH) soaked in 10× SSC, using a manifold apparatus (Bethesda Research Laboratories, Gaithersburg, MD). For Northern blot analysis RNA and markers were fractionated under denaturing conditions on a 1.25 or 1.5% formaldehyde-agarose gel and transferred to nitrocellulose filters by diffusion blotting. The mRNA size was determined from the migration of 28S and 18S ribosomal RNA. The filters were baked at 80°C for 120 min in a vacuum oven.

Hybridization. An 833-bp rat preproPTHcDNA was obtained by restriction enzyme (Kpn I, Pst I) digestion of plasmid PT 43, and labeled by nick translation to a specific activity of 2–5 × 10^8 cpm. Hybridization was carried out over night at 42°C, autoradiographed, and the films scanned with a densitometer.

$1,25(OH)_2D_3$ receptor mRNA levels were measured by hybridization with the human $1,25(OH)_2D_3$ receptor cDNA (2.1-kb insert) cloned in the p GEM 7 Z f (+) vector (23). The plasmid serves as a standard cloning vector and a template for in vitro transcription of the receptor mRNA which is labeled to a high-specific activity RNA probe. A modification of the method of Kreig et al. (24) was used as follows. 100 ng Hind III–linearized template DNA was used in 10 μl reaction volume. An incubation of 10 mM DTT, 500 μM each of ATP, CTP, GTP, 3,000 Ci/mmol ^{32}P-UTP, 40 mM Tris HCl, pH 7.5, 60 mM $MgCl_2$, 2 mM spermidine-HCl, 100 U/ml T 7 polymerase, 1 U/ml human placental ribonuclease inhibitor was carried out at 37°C for 20 min. The DNA template was removed by the addition of 1 μl ribonuclease-free DNase (1 U/μg DNA) and incubated at 37°C for 10 min. The reaction mix was diluted with water to a total volume of 100 μl. The specific activity of the RNA transcript was ~ 6 × 10^8 cpm/μg. 5–10 μl was used for each 1 ml hybridization mix.

For in situ hybridization the anti-sense VDR RNA probe was synthesized, by linearizing the p GEM 7 Zf (+) plasmid with Hind III digestion. [^{35}S]-labeled RNA was synthesized in the antisense orientation as described above using the T7 polymerase. Before in situ hybridization the probe was fragmented by a mild alkaline treatment to 100–200-bp fragments.

Flow cytometry of thyroparathyroid cells. Thyroparathyroid tissue was minced in 5% FCS at 4°C, and then incubated with collagenase CLS1 (500 U/ml) (Worthington Diagnostics, Freehold, NJ) at 37°C for 1 h in a shaking water bath. The glands were digested completely into isolated cells. The cell suspension was filtered through a nylon mesh, centrifuged at 400 *g* for 5 min at 4°C, washed twice in HBSS and the pellet resuspended in 500 μl of HBSS. The membrane integrity of the cell suspension was demonstrated by incubating the cells with 3',6'-diacetyl fluorescein (FDA) (Sigma Chemical Co., St. Louis, MO) (1 μl per 100–μl cell suspension). Intact cells have nonspecific esterases which cleave the FDA, which readily crosses cell membranes, into free fluorescein which is highly fluorescent (24–26). 80% of the cell suspension exhibited positive fluorescence. The cell suspension was analyzed by a FACS 440 cell sorter (Becton-Dickinson & Co., Paramus, NJ) using 300 mV excitation at 488 nm from an argon ion laser source. The diameter of the nozzle used was 70 μm. The fluorescent angle light scatter was at 520 nm. The integrity of the cells collected from the cell sorter have also been demonstrated by propidium iodide staining (> 90% intact cells) and by microscopy of cells after concentration and staining with Papanicoula stain (27). In some experiments RNA was extracted from the cells collected from the cell sorter and analyzed for mRNA content as before.

In situ hybridization analysis. Sections of parathyroid-thyroid and duodenum were prepared for in situ hybridization and hybridized as described by Hogan et al. (28). Briefly, 10-μm-thick frozen sections were collected on poly-L-lysine-coated glass slides, refixed in 4% paraformaldehyde, and dehydrated in graded ethanol solutions. Before hybridization sections were pretreated successively with 0.2 N HCl, 2× SSC, 0.125 mg/liter pronase, 4% paraformaldehyde, and acetic anhydride in triethanolamine buffer. Hybridization was carried out at 50°C overnight in 50% formamide, 0.3 M NaCl containing 10% dextran sulfate, 1× Denhardt's solution, 1 mg/ml carrier tRNA, 10 mM DTT, 5 mM EDTA, and 2× 10^8 cpm/ml ^{35}S-labeled $1,25(OH)_2D_3$ receptor riboprobe. Organs were immediately fixed in 4% paraformaldehyde in PBS for 16 h at 4°C, then incubated for 24 h in 0.5 M sucrose in PBS, after which they were embedded in OCT embedding medium (Miles Scientific Inc., Naperville, IL) and frozen. Posthybridization washing was performed under stringent conditions that included an incubation at 50°C for > 4 h in 50% formamide/0.3 M NaCl and a 30-min incubation at 37°C in 20 μg/ml RNase A (28, 29). Autoradiography was performed using Kodak NTB-2 nuclear track emulsion.

Results

After a single injection of $1,25(OH)_2D_3$ (50 or 100 pmol) to normal rats we had demonstrated a decrease in PTHmRNA levels at 6 h, reaching < 4% of basal at 48 h after 100 pmol $1,25(OH)_2D_3$ (4). These same filters were extensively washed and then rehybridized with the VDR riboprobe (Fig. 1). At 6 h after both 50 and 100 pmol $1,25(OH)_2D_3$ VDRmRNA levels in these rat parathyroid-thyroid RNA extracts were less but not significantly different from basal levels. At 24 h there was a

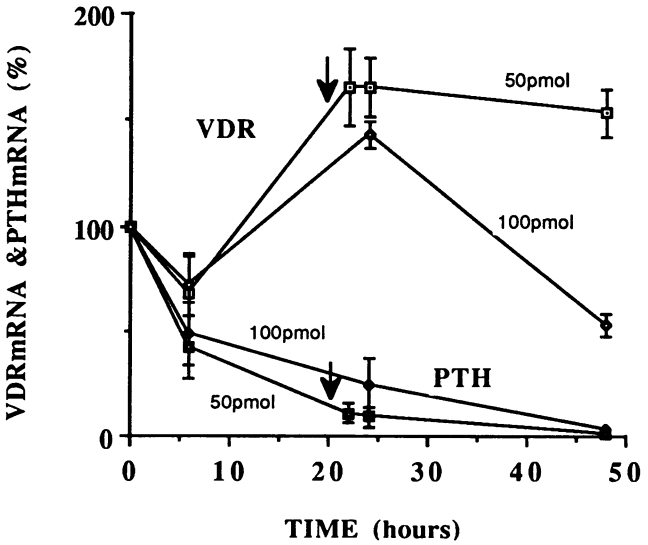

Figure 1. Time course of the effect of $1,25(OH)_2D_3$ on mRNA levels for parathyroid hormone (PTH) and the $1,25(OH)_2D_3$ receptor (VDR) in rat parathyroid-thyroid tissue. Rats were injected with either a single dose of 100 pmol $1,25(OH)_2D_3$, or 50 pmol $1,25(OH)_2D_3$ at 0 and 24 h. The arrow represents the second injection of 50 pmol $1,25(OH)_2D_3$. Levels of mRNA for PTH (♦, ■) and VDR (◇, □) were determined by sequential hybridizations. The data for PTHmRNA has been published previously (4). The results of each point are the mean±SE for four rats and are expressed as a percentage of basal.

1.7-fold increase in VDRmRNA at both doses. Rats given a single dose of 100 pmol $1,25(OH)_2D_3$ had a decrease in VDRmRNA at 48 h, whereas those given 50 pmol $1,25(OH)_2D_3$ had received a second 50-pmol dose at 24 h and they maintained their elevated VDRmRNA levels for 48 h. Actin mRNA was not affected by $1,25(OH)_2D_3$ (data not shown). This time course of $1,25(OH)_2D_3$ on VDRmRNA indicates an effect after 6 h, evident at 24 h but not at 48 h after a single dose.

A $1,25(OH)_2D_3$ dose response study at 24 h had shown a decrease in PTHmRNA levels after as little as 12.5 pmol with little change from 50 to 200 pmol (4). Actin mRNA was not affected. This data is shown in Fig. 2 together with results after rehybridization with VDR riboprobe to measure VDRmRNA levels. VDRmRNA levels were increased after 12.5 pmol peaking at 25 pmol with a twofold increase. These doses of $1,25(OH)_2D_3$ had not caused hypercalcemia.

A Northern blot of parathyroid-thyroid tissue from rats injected with $1,25(OH)_2D_3$ (100 pmol at 24 h) demonstrated a reduction in PTHmRNA which ran as a single band at 833 bp (Fig. 3). Hybridization for VDRmRNA showed 2 bands at 2.2 and 4.4 kb in the control rats, and both bands were increased by a single injection of $1,25(OH)_2D_3$ (100 pmol at 24 h). RNA extracts of duodenum were run on agarose gels and Northern blots for VDRmRNA showed a single band at 4.4 kb for a control rat. After $1,25(OH)_2D_3$ (100 pmol at 24 h) there was an increase in intensity of this single band (Fig. 4).

In vivo in the rat a low calcium increased PTHmRNA levels at 1–6 h (22). Rehybridization of those filters showed no effect of serum calcium on VDRmRNA levels (Fig. 5).

Chronic changes in dietary calcium and vitamin D regulate PTHmRNA levels in the rat. We therefore studied rats who had been maintained for 3 wk after weaning on different diets. The diets used were either vitamin D normal (ND) or deficient (−D), together with different calcium concentrations: 0.02% (−Ca), 0.4% (NCa), 2% (+Ca).

The mean serum calciums in the different groups of rats were NDNCa, 10.6 mg/dl; ND−Ca, 6.3 mg/dl; ND+Ca, 12.2 mg/dl; −DNCa, 10.6 mg/dl; −D−Ca, 5.1 mg/dl; −D+Ca, 11.2 mg/dl. We had shown in previous studies that the vitamin D–deficient diet given for 3 wk to weanling rats did not result in low serum $1,25(OH)_2D$ levels in −DNCa rats, but only in −D−Ca rats, whereas ND−Ca rats had markedly increased levels of serum $1,25(OH)_2D$ (27).

Northern blot hybridization showed that PTHmRNA ran as a single band (833 bp) which was increased by calcium deficiency (ND−Ca) even more so when combined with vitamin D deficiency (−D−Ca) (Fig. 6). A high calcium (−D+Ca) did not affect PTHmRNA mobility or decrease the intensity of the transcript. After extensive washing the Northern blot was rehybridized for VDRmRNA. The VDRmRNA ran as two bands at 2.2 and 4.4 kb (Fig. 6). In control rats (NDNCa) and calcium-deficient rats (ND−Ca) the predominant band was at 4.4 kb (Fig. 6). Similar results were seen in ND+Ca rats (not shown). In contrast in rats after 3 wk of a vitamin D–deficient diet, regardless of the calcium content of the diet (NCa, −Ca, +Ca), the predominant band was at 2.2 kb.

We have previously shown that separation of parathyroid-thyroid cells by enzymatic digestion and passage through a flow cytometer (FACS) separates parathyroid from thyroid cells (27). A parathyroid-thyroid cell suspension was passed through a FACS and the smaller parathyroid cells separated and collected separately from the larger thyroid cells (Fig. 7). The RNA extracted from cells from each peak were hybridized sequentially with PTHcDNA, thyroglobulin cDNA, calcitonin cDNA, and VDR riboprobe (Fig. 7). PTHmRNA was only present in the first peak (Fig. 7) and thyroglobulin mRNA in the second peak (27). Moreover calcitonin mRNA was only present in the second peak, whereas VDRmRNA was almost all in the first peak with a weak message in the second peak (Fig. 7). These results show that the VDRmRNA in the parathyroid-thyroid tissue extracts was mainly derived from parathyroid tissue.

We performed in situ hybridization with the VDR ribo-

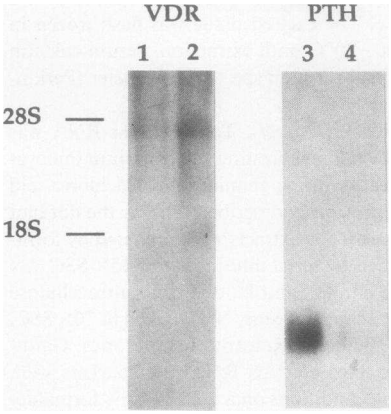

Figure 3. Gel blot analysis of total RNA from parathyroid-thyroid tissue. Filters were hybridized sequentially for PTHmRNA and VDRmRNA. Lanes: *1* and *3*, parathyroid-thyroid tissue of one control rat; *2* and *4*, 1 rat 24 h after 100 pmol $1,25(OH)_2D_3$. RNA size is shown for ribosomal 18S and 28S RNA's, indicating the approximate positions of the 2.2 and 4.4 kb transcript, respectively.

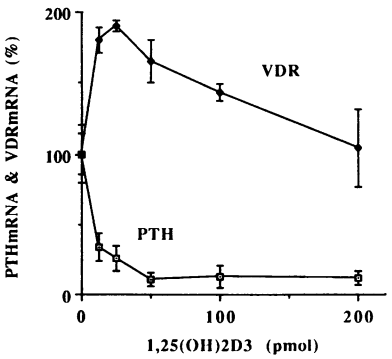

Figure 2. $1,25(OH)_2D_3$ dose-response effect on mRNA levels for parathyroid hormone (PTH) (□) and the $1,25(OH)_2D_3$ receptor (VDR) (◇), in rat parathyroid-thyroid tissue 24 h after administration of $1,25(OH)_2D_3$ ip. The data for PTHmRNA has been published previously (4). The results represent mean±SEM for four rats.

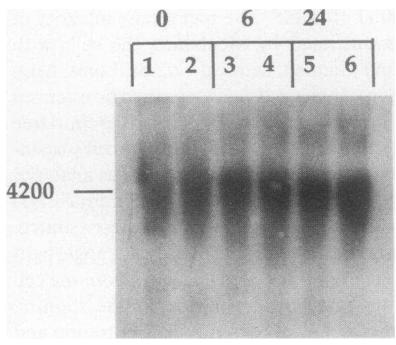

Figure 4. Gel blot analysis of total RNA from duodenum hybridized for VDRmRNA. Lanes: *1* and *2*, control; *3* and *4*, 6 h after $1,25(OH)_2D_3$ (100 pmol); *5* and *6*, 24 h after $1,25(OH)_2D_3$ (100 pmol). RNA size is shown in nucleotide base pairs.

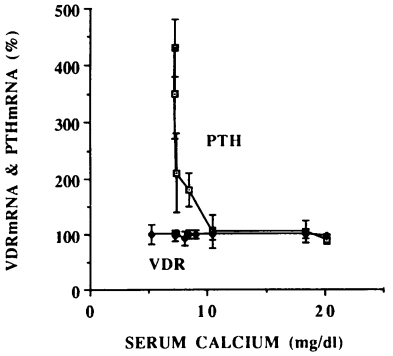

Figure 5. The effect of changes in serum calcium from control rats at 10.4 mg/dl on mRNA levels for PTH (□) and VDR (♦). The results represent mean±SEM for four rats as a percentage of basal. The data for PTHmRNA has been published previously (22).

probe. ^{35}S-labeled antisense RNA was synthesized in vitro and used as an hybridization probe. 10-μm-thick frozen sections of parathyroid-thyroid tissue and proximal small intestine were prepared from a control rat and a 1,25$(OH)_2D_3$ (100 pmol at 24 h)–treated rat.

Bright field illumination of parathyroid-thyroid tissue (Fig. 8 *A*) from a control rat (Fig. 8 *A1*) and a rat injected with 1,25$(OH)_2D_3$ (100 pmol at 24 h) (Fig. 8 *A2*) demonstrated the parathyroid glands (*white arrows*) embedded in the thyroid tissue. Duodenum from a 1,25$(OH)_2D_3$-treated rat is demonstrated on the same preparation (Fig. 8 *A3*). A higher-power magnification (Fig. 8 *B*) demonstrates the parathyroid gland (*p*) and thyroid follicles (*t*).

Sections were hybridized under conditions that favor formation of RNA–RNA hybrids (28, 29). VDRmRNA in both control and 1,25$(OH)_2D_3$-treated parathyroid-thyroid tissue was localized to the parathyroid glands (Fig. 8 *C*). Background activity was present on the thyroid follicles with a small increase in the parafollicular cells. VDRmRNA was also present in the rat proximal small intestine mucosal cells, both crypt and villi cells with none in the muscularis and serosa. The amount of VDRmRNA in the parathyroids was similar to that in the small intestine crypts (Fig. 8 *C*). In a rat injected with 1,25$(OH)_2D_3$ (100 pmol at 24 h) there was an apparent increase in VDRmRNA in the parathyroids (Fig. 8) consistant with the 1.7-fold increase shown in Fig. 1, but the in situ data was not quantified. A higher power view of the parathyroid-thyroid tissue shown in Fig. 8 *B*, revealed the marked concentration of the VDRmRNA in the parathyroid gland, as compared to the thyroid tissue (Fig. 9 *A*), and in the intestinal villus crypt cells (*cr*), and not in the muscularis (*m*) and serosal (*s*) layers (Fig. 9, *B* and *D*).

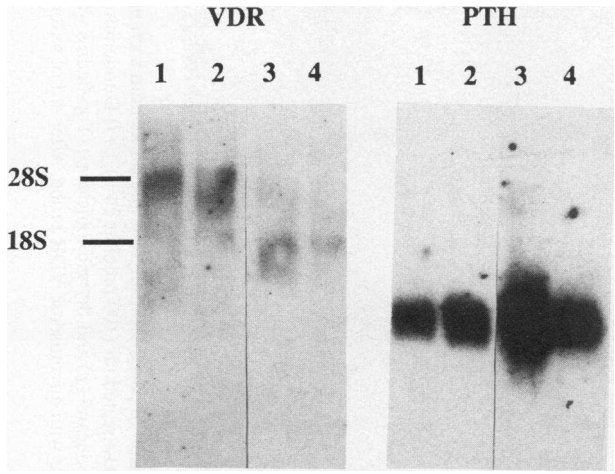

Figure 6. Gel blot analysis of total RNA from parathyroid-thyroid tissue of rats maintained for 3 wk after weaning on diets containing normal vitamin D (ND) (lanes *1* and *2*) or no vitamin D (−D) (lanes *3* and *4*), together with 0.4% calcium (NCa) (lanes *1* and *4*), or 0.02% calcium (−Ca) (lanes *2* and *3*). Lane *1*, NDNCa; lane *2*, ND-Ca; lane *3*, −D−Ca; lane *4*, −DNCa. The filters were hybridized sequentially for PTHmRNA and VDRmRNA. RNA size is shown for ribosomal 18S and 28S RNAs, indicating the positions of the 2.2 and 4.4 kb transcripts, respectively.

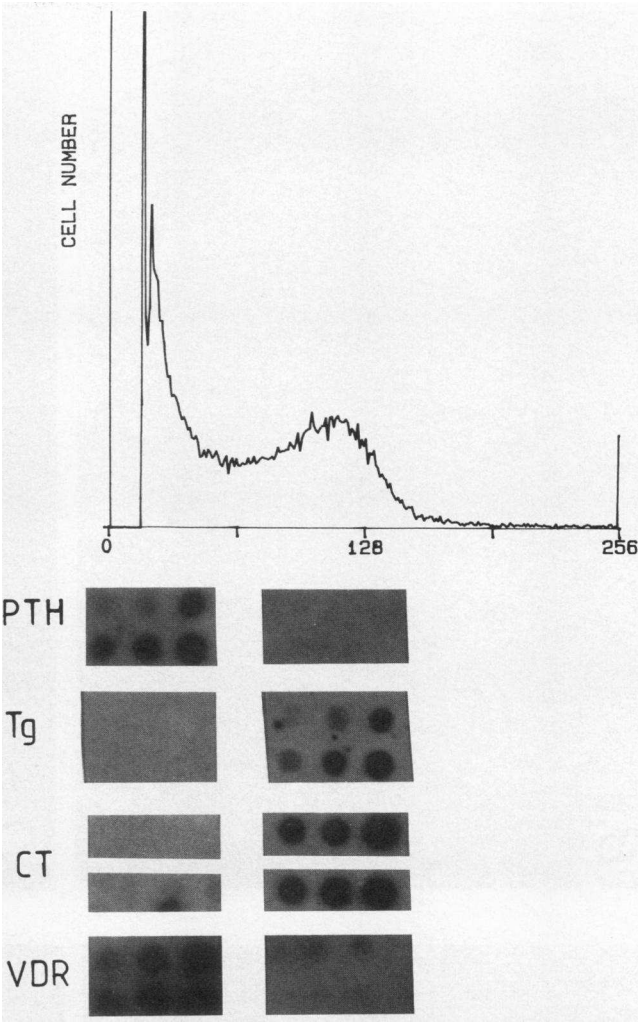

Figure 7. Flow cytometry analysis of a dispersed rat parathyroid-thyroid cell suspension. The cells were analyzed by size and the cells from the two peaks (0–110 nm and 115–200 nm) were collected separately, the RNA extracted and then hybridized sequentially with cDNA[5] for PTH, thyroglobulin (Tg), and calcitonin, and the 1,25$(OH)_2D_3$ receptor (VDR) riboprobe. The three dots in each row are from increasing volumes of total RNA extracted from a collected cell population. The second row is a duplicate.

Discussion

1,25$(OH)_2D_3$ is a potent regulator of PTH gene transcription, leading to a large decrease in PTH gene transcription and PTHmRNA levels in vivo and in vitro (3–5). We have now

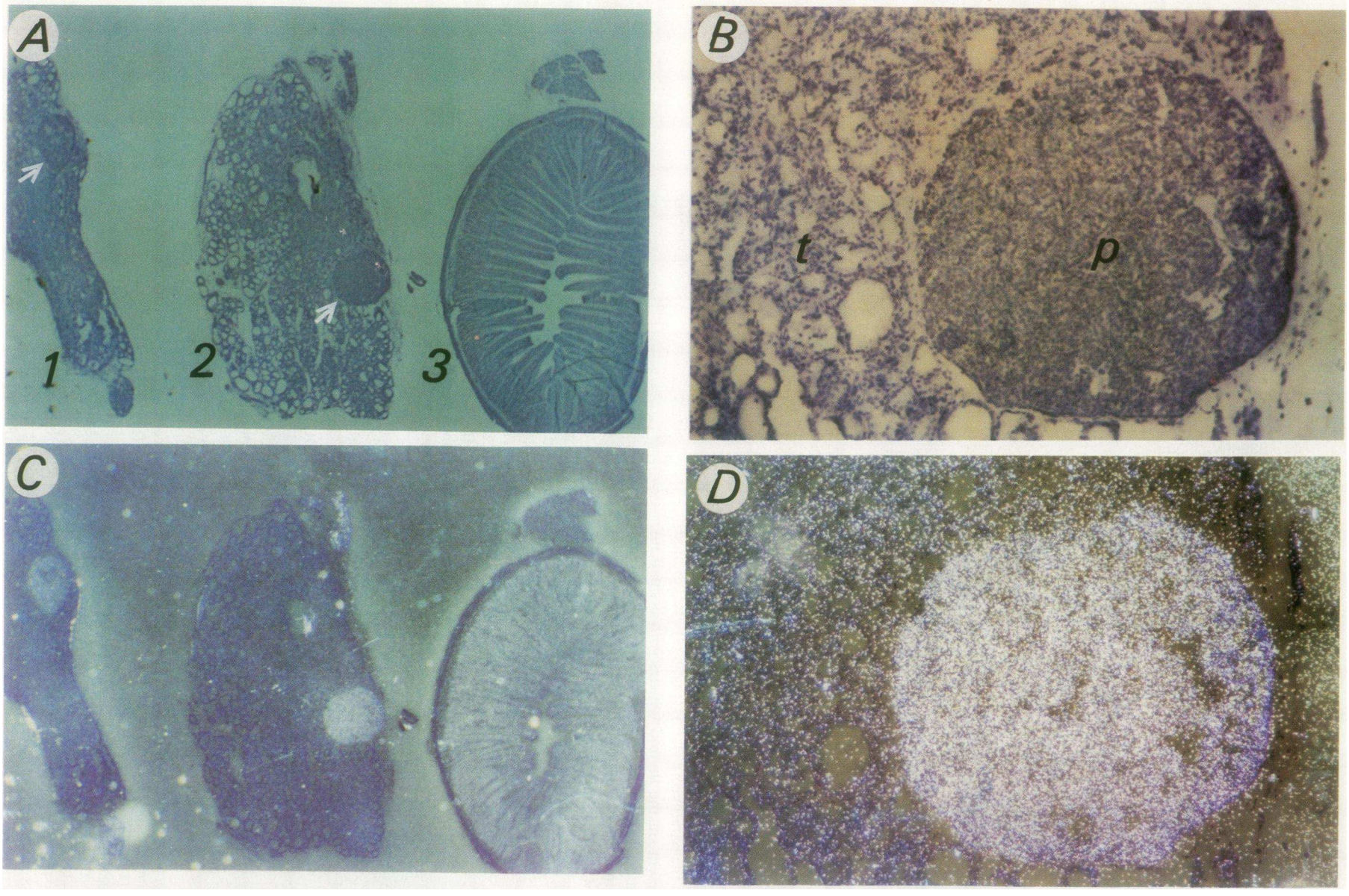

Figure 8. In situ hybridization of parathyroid-thyroid and duodenum sections with $1,25(OH)_2D_3$ receptor (VDR) probe. (*A1*) Parathyroid-thyroid tissue from a control rat. (*A2*) Parathyroid-thyroid from a $1,25(OH)_2D_3$-treated rat (100 pmol at 24 h). (*A3*) Duodenum from the $1,25(OH)_2D_3$-treated rat. The white arrows point at the parathyroid glands. (*B*) A higher power view of A2 showing the parathyroid gland (*p*) and thyroid follicles (*t*). Top figures were photographed under bright-field illumination, whereas bottom figures show dark-field illumination of the same sections. Hybridization was with an antisense VDR probe. After 4 d of autoradiographic exposure, sections were stained with Giemsa stain and photographed. Magnification in *B* is sevenfold the magnification of *A*.

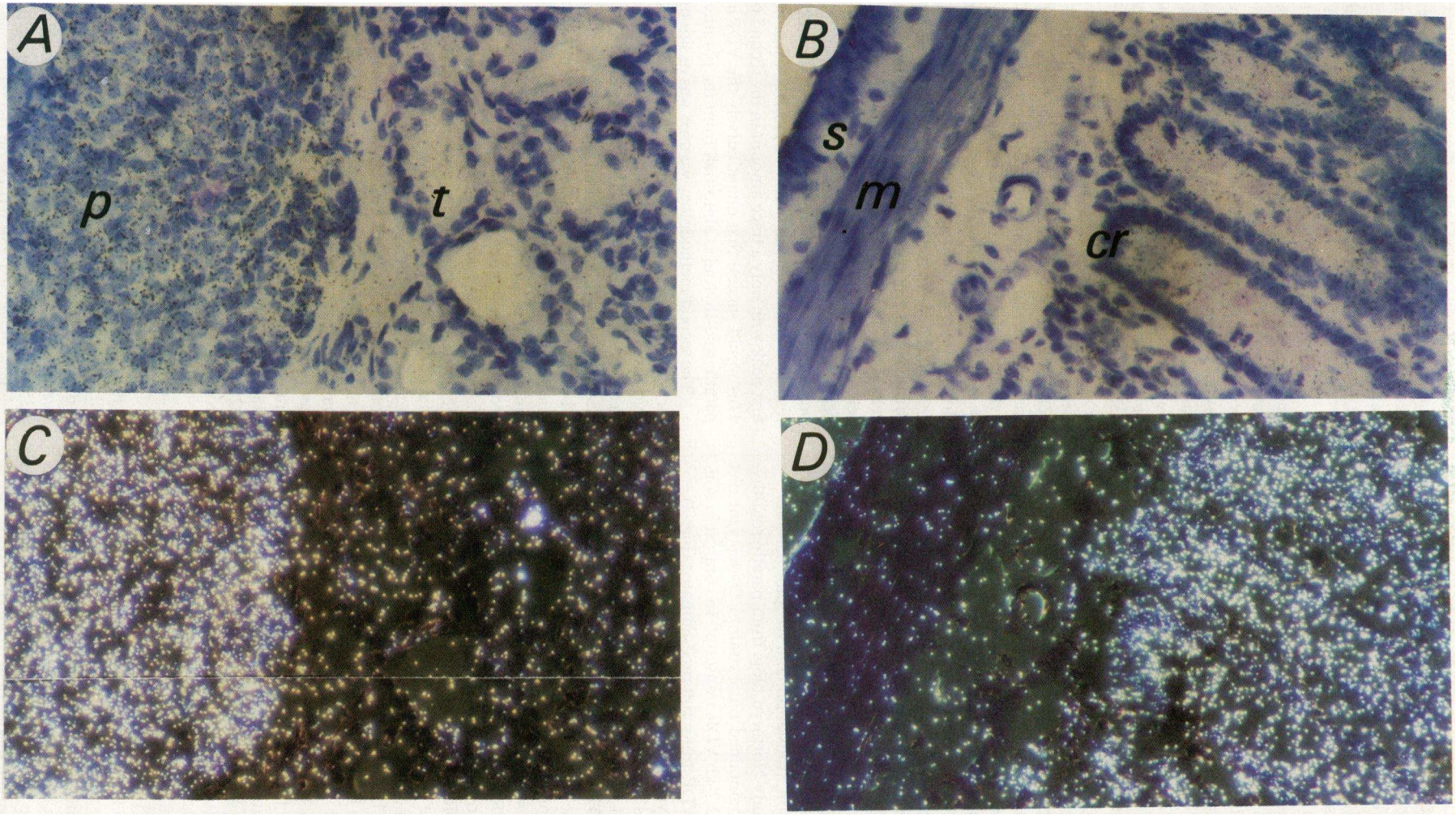

Figure 9. In situ hybridization of parathyroid-thyroid and duodenum sections with VDR probe. The sections from Fig. 8 are shown at a higher magnification. A parathyroid (*p*) and thyroid (*t*). (*B*) Duodenum; (*cr*) crypt cells; (*m*) muscularis layer; (*s*) serosa after 4 d of autoradiographic exposure. Sections were stained with Giemsa stain and photographed (at 360 magnification) under bright-field (top figures) and dark-field (bottom figures) illumination.

shown that $1,25(OH)_2D_3$ leads to a dose-dependent increase in VDRmRNA levels in the parathyroid. The increase in VDRmRNA is evident at 24 h but not at 6 h (Figs. 1 and 2).

A similar time course for VDRmRNA upregulation and downregulation by $1,25(OH)_2D_3$ has been demonstrated in intestine (18), whereas in human pomyelocytic leukemia cells (HL60) $1,25(OH)_2D_3$ has been shown to upregulate the VDR measured with a monoclonal antibody at 12 h, and downregulate the VDR at 48 and 72 h (20). Ligand binding studies have determined that $1,25(OH)_2D_3$ increases VDR number in a number of cell systems (19, 20). Huang et al. (17) studied duodenal VDRmRNA of vitamin D–deficient rats with and without $1,25(OH)_2D_3$ treatment (25 ng/day for 7 d). They found no change in VDRmRNA as measured by slot blots. The differences in the effect of $1,25(OH)_2D_3$ on duodenal VDRmRNA when given as a single injection by Strom et al. (18) and in the present study, as compared to chronic treatment (17), remain to be clarified.

Parathyroid VDR protein levels and $1,25(OH)_2D_3$ binding capacity were not determined in the present study. However, Strom et al. (18) studied rat intestine VDR, and showed that $1,25(OH)_2D_3$ lead to an increase in both VDRmRNA and VDR protein. Moreover, Costa and Feldman (16) had shown that $1,25(OH)_2D_3$ treatment in vivo lead to an increase in VDR binding. Additionally, a prolongation in VDR half-life was shown to be a major factor in the VDR upregulation by vitamin D metabolites in vitro studies (30). Therefore $1,25(OH)_2D_3$ upregulates its receptor at a number of levels, including transcription, translation, and receptor half-life.

In the present study Northern blots from parathyroid-thyroid tissue of rats maintained on a vitamin D–deficient diet had a predominant 2.2-kb VDR transcript. In contrast in control rats the 4.4-kb VDR transcript predominated (Fig. 3).

The total parathyroid VDRmRNA was not very different between control rats and rats which had been on a vitamin D–deficient diet, but rather the distribution between 2.2- and 4.4-kb transcripts. The interpretation of this finding is not clear, but it might be speculated that the larger transcript is a more stable transcript.

We showed that serum calcium had no effect on parathyroid-thyroid VDRmRNA levels. Favus et al. (31) showed that a low calcium did not affect duodenal VDRmRNA levels despite increasing VDR number, suggesting a posttranslational effect. The effects of a low calcium diet on intestinal VDR number might be due to the effect of low calcium itself or of the increased serum $1,25(OH)_2D_3$ levels in these rats (31). Rats with normal vitamin D and low calcium (ND-Ca) did not have higher parathyroid VDRmRNA levels, despite increased serum $1,25(OH)_2D_3$ levels. This might imply that a normal calcium is necessary for $1,25(OH)_2D_3$ treatment to upregulate the VDR gene's transcription. In contrast a rat duodenum extract had a single VDRmRNA band at 4.4 kb, which was increased after $1,25(OH)_2D_3$ (Fig. 4), similar to published results. VDRmRNA has been shown to run as more than one band in mouse 3T6 fibroblasts (8), chicken intestine and brain (8), and rat kidney (17) and as a single band in chicken brain (8) and rat intestine (17). The significance of this heterogeneity is not clear.

In the dietary model of secondary hyperparathyroidism due to a low calcium and deficient vitamin D intake there was a marked increase in PTHmRNA but no increase in VDRmRNA. In another model of secondary hyperparathyroidism, chronic renal failure due to 5/6 nephrectomy, there was also no increase in VDRmRNA (32). These results indicate that secondary hyperparathyroidism does not result in parathyroid cells which are stimulated to synthesize all their products at an increased level, but rather synthesize more PTHmRNA than other cellular products such as VDRmRNA.

The rat's two parathyroids are embedded in thyroid tissue and it is therefore not possible to accurately microdissect the parathyroids from the thyroids without significant contamination with thyroid tissue. For this reason the experiments reported here were all performed on rat parathyroid-thyroid tissue, which is irrelevant when measuring total rat PTHmRNA, but is relevant when presenting results for VDRmRNA. This is because the thyroid parafollicular or C cells which synthesize calcitonin also have VDR[s] (33), and calcitonin gene transcription is also regulated by $1,25(OH)_2D_3$ (6). It was therefore necessary to determine the relative contribution of parathyroid cell VDRmRNA to C-cell VDRmRNA. We used two experimental procedures, separation of the cell populations by flow cytometer (FACS) and in situ hybridization.

For flow cytometry studies a cell suspension from rat parathyroid-thyroid was separated by size into two peaks which were collected separately, and as we have reported previously this separates the parathyroid cells from the thyroid follicular cells (Fig. 7) (27). RNA extracts from the first peak of smaller cells had PTHmRNA, which was not present in the second peak of larger cells, whereas thyroglobulin mRNA was only present in the second peak (Fig. 7). Calcitonin mRNA was also restricted to the second peak. VDRmRNA was present in the first peak with only a trace amount in the second peak (Fig. 7). This would suggest that quantitatively the VDRmRNA from parathyroid-thyroid is almost all from the parathyroids.

The second methodology we used was in situ hybridization for VDRmRNA (Figs. 8 and 9). Sections from rat parathyroid-thyroid tissue and duodenum were processed and hybridized under conditions that favor formation of RNA–RNA hybrids (28, 29). Rat from parathyroid-thyroid tissue from one lobe of a control rat, together with parathyroid-thyroid and duodenum from a rat which had received $1,25(OH)_2D_3$ (100 pmol at 24 h) were fixed to a microscope slide together, in order to allow comparison among the sections. VDRmRNA was markedly concentrated to the parathyroids as compared to the thyroids, as well as to the duodenal mucosa (Figs. 8 and 9). The intensity of VDRmRNA was greater in the $1,25(OH)_2D_3$ treated rat's parathyroid than in the control. The results of the dot blots and Northern blots presented here therefore represent VDRmRNA derived from parathyroid tissue, much more than that from the parafollicular cells. Moreover, using primary cultures of bovine parathyroid cells we have demonstrated a similar upregulation of VDRmRNA by $1,25(OH)_2D_3$ (data not shown). It remains to be studied whether $1,25(OH)_2D_3$ also regulates VDR gene expression in calcitonin producing cell lines.

$1,25(OH)_2D_3$ is a potent regulator of PTH gene transcription, decreasing PTH gene transcription in vivo by > 90% after a single injection in the rat (4). We have now demonstrated that it also increases VDR gene expression in the parathyroid cell, which might result in increased VDR protein synthesis and increased binding of $1,25(OH)_2D_3$. This ligand dependent receptor upregulation would lead to an amplified effect of $1,25(OH)_2D_3$ on the PTH gene, and might help explain the dramatic effect of $1,25(OH)_2D_3$ on the PTH gene.

Acknowledgments

We thank Dr. Howard Cedar for helpful discussions, Dr. H. Mayer for the PT43 plasmid, Dr. M. J. Rosenfeld for the rat calcitonin gene, Dr. Vassart for the thyroglobulin gene, Drs. Kaiser and Eischer of Hoffman-La Roche Inc., Basel, Switzerland, for the $1,25(OH)_2D_3$, Ms. M. Ofner and A. Itin for technical assistance; and Dr. H. Gilo and Ms. G. Neiman for help with the FACS.

This work was supported by grants from the National Institutes of Health (grant DK 38696), the United States-Israel Binational Science Foundation (BSF), the German-Israel Foundation for Research and Development (GIF), and the Israel Academy of Sciences and Humanities.

References

1. Haussler, M. R., D. J. Mangelsdorf, B. S. Komm, C. M. Terpening, K. Yamaoka, E. A. Allegretto, A. R. Baker, J. Shine, D. P. McDonnell, M. Hughes, N. L. Weigel, B. W. O'Malley, and J. W. Pike. 1988. Molecular biology of the vitamin D hormone. *Recent Prog. Horm. Res.* 44:263–305.

2. Minghetti, P. P., and A. W. Norman. 1988. $1,25(OH)_2$-Vitamin D_3 receptors: gene regulation and genetic circuitry. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:3043–3053.

3. Silver, J., J. Russell, and L. M. Sherwood. 1985. Regulation by vitamin D metabolites of messenger ribonucleic acid for preproparathyroid hormone in isolated bovine parathyroid cells. *Proc. Natl. Acad. Sci. USA.* 82:4270–4273.

4. Silver, J., T. Naveh-Many, H. Mayer, H. J. Schmeizer, and M. M. Popovtzer. 1986. Regulation by vitamin D metabolites of parathyroid hormone gene transcription in vivo in the rat. *J. Clin. Invest.* 78:1296–1301.

5. Okazaki, T., T. Igarashi, and H. M. Kronenberg. 1988. 5'-Flanking region of the parathyroid hormone gene mediates negative regulation by $1,25(OH)_2$ vitamin D_3. *J. Biol. Chem.* 263:2203–2206.

6. Naveh-Many, T., and J. Silver. 1988. Regulation of calcitonin gene transcription by vitamin D metabolites in vivo in the rat. *J. Clin. Invest.* 81:270–273.

7. Cote, G. J., D. G. Rogers, E. S. C. Huang, and R. F. Gagel. 1987. The effect of 1,25-dihydroxyvitamin D_3 treatment on calcitonin and calcitonin gene-related peptide mRNA levels in cultured human thyroid C-cells. *Biochem. Biophys. Res. Commun.* 149:239–243.

8. 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.

9. Kerner, S. A., R. A. Scott, and J. W. Pike. 1989. Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D_3. *Proc. Natl. Acad. Sci. USA.* 86:4455–4459.

10. Demay, M. B., J. M. Gerardi, H. F. DeLuca, and H. M. Kronenberg. 1990. DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 87:369–373.

11. Markose, E. R., J. L. Stein, G. S. Stein, and J. B. Lian. 1990. Vitamin D-mediated modifications in protein-DNA interactions at two promoter elements of the osteocalcin gene. *Proc. Natl. Acad. Sci. USA.* 87:1701–1705.

12. Yamamoto, K. R. 1985. Steroid receptor regulated transcription of specific genes and gene networks. *Annu. Rev. Genet.* 19:209–252.

13. Hughes, M. R., P. J. Malloy, D. G. Kieback, R. A. Kesterson, J. W. Pike, D. Feldman, and B. W. O'Malley. 1988. Point mutations in the human vitamin D receptor gene associated with hypocalcemic rickets. *Science (Wash. DC).* 242:1702–1705.

14. Ritchie, H. H., M. R. Hughes, E. T. Thompson, P. J. Malloy, Z. Hochberg, D. Feldman, J. W. Pike, and B. W. O'Malley. 1989. An ochre mutation in the vitamin D receptor gene causes hereditary 1,25-dihydroxyvitamin D_3-resistant rickets in three families. *Proc. Natl. Acad. Sci. USA.* 86:9783–9787.

15. Costa, E. M., M. A. Hirst, and D. Feldman. 1985. Regulation of 1,25-dihydroxyvitamin D_3 receptors by vitamin D analogs in cultured mammalian cells. *Endocrinology.* 117:2203–2210.

16. Costa, E. M., and D. Feldman. 1986. Homologous up-regulation of the $1,25(OH)_2$ vitamin D_3 receptor in rats. *Biochem. Biophys. Res. Commun.* 137:742–747.

17. Huang, Y. C., S. Lee, R. Stolz, C. Gabrielides, A. Pansini-Porta, M. E. Bruns, D. E. Bruns, T. E. Miffins, J. W. Pike, and S. Christakos. 1989. Effect of hormones and development on the expression of the rat 1,25-dihydroxyvitamin D_3 receptor gene. *J. Biol. Chem.* 264:17454–17461.

18. 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.

19. Mahonen, A., A. Pirskanen, R. Keinanen, and P. H. Maenpaa. 1990. Effect of $1,25(OH)_2D_3$ on its receptor mRNA levels and osteocalcin synthesis in human osteosarcoma cells. *Biochim. Biophys. Acta.* 1048:30–37.

20. Lee, Y., M. Inaba, H. F. DeLuca, and W. S. Mellon. 1989. Immunological identification of 1,25-dihydroxyvitamin D_3 receptors in human promyelocytic leukemic cells (HL-60) during homologous regulation. *J. Biol. Chem.* 264:13701–13705.

21. Pan, P., B. R. Kaladhar, G. Studzinski, J. W. Pike, and S. Christakos. 1989. Regulation of 1,25 dihydroxyvitamin D_3 receptor mRNA in human leukemia cells (HL-60). *J. Bone Miner. Res.* 4:5291. (Abstr.)

22. Naveh-Many, T., M. M. Friedlaender, H. Mayer, and J. Silver. 1989. Calcium regulates parathyroid hormone messenger ribonucleic acid (mRNA), but not calcitonin mRNA in vivo in the rat. Dominant role of 1,25-dihydroxyvitamin D. *Endocrinology.* 125:275–280.

23. Baker, A. R., D. P. McDonnel, M. Hughes, T. M. Crisp, D. J. Mangelsdorf, M. R. Haussler, J. W. Pike, J. Shine, and B. W. O'Malley. 1988. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. USA.* 85:3294–3298.

24. Krieg, P. A., and D. A. Melton. 1987. In vitro RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* 155:397–415.

25. Brunius, G. 1980. Technical aspects of the use of 3',6'-diacetyl fluorescein for vital fluorescent staining of bacteria. *Curr. Microbiol.* 4:321–323.

26. Resnick, M., S. Schuldiner, and H. Bercovier. 1985. Bacterial membrane potential analyzed by spectrofluorocytometry. *Curr. Microbiol.* 12:183–186.

27. Naveh-Many, T., and J. Silver. 1990. Regulation of parathyroid hormone gene expression by hypocalcemia, hypercalcemia and vitamin D in the rat. *J. Clin. Invest.* 86:1313–1319.

28. Hogan, B., F. Constantini, and E. Lacy. 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

29. Keshet, E., R. D. Polakiewicz, A. Itin, A. Ornoy, and H. Rosen. 1989. Proenkephalin A is expressed in mesodermal lineages during organogenesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2917–2923.

30. Costa, E. M., and D. Feldman. 1987. Measurement of 1,25-dihydroxyvitamin D_3 receptor turnover by dense amino acid labeling: changes during receptor up-regulation by vitamin D metabolites. *Endocrinology.* 120:1173–1178.

31. 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.

32. Shvil, Y., T. Naveh-Many, P. Barach, and J. Silver. 1990. Regulation of parathyroid cell gene expression in experimental uremia. *J. Am. Soc. Nephrol.* 1:99–104.

33. Freake, H. C., and I. MacIntyre. 1982. Specific binding of 1,25-dihydroxycholecalciferol in human medullary thyroid carcinoma. *Biochem. J.* 206:181–184.