Selective Expression of a Normal Action of the 1,25-Dihydroxyvitamin D_3 Receptor in Human Skin Fibroblasts with Hereditary Severe Defects in Multiple Actions of that Receptor

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Abstract

We evaluated three actions of 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] in human skin fibroblasts to test for heterogeneity in hormone-response coupling. In fibroblasts from normal subjects the 1,25-$(OH)_2D_3$ concentrations for half-maximal effect (EC_{50}) were: for mitogenic effect 0.0001–0.0005 nM, for antimitogenic effect 1 nM, and for induction of 25-OHD_3 24-hydroxylase (24-OHase) 5 nM. To evaluate the effects of mutations presumed to be in the gene for the 1,25-$(OH)_2D_3$ receptor we examined cell lines representing four kindreds with hereditary resistance to 1,25-$(OH)_2D_3$ ("mutant" cell lines). In one mutant cell line all three 1,25-$(OH)_2D_3$ actions were severely abnormal. In one mutant cell line 24-OHase induction and mitogenic action were undetectable, but EC_{50} and maximal effect were normal for antimitogenic action of 1,25-$(OH)_2D_3$. In two mutant cell lines 24-OHase induction and antimitogenic actions were undetectable or severely impaired but mitogenic action was normal in EC_{50} and normal or increased in maximal effect. The mitogenic and antimitogenic actions in normal cells showed a similar profile of potency ratios for 1,25-$(OH)_2D_3$ and six analogues. Whenever a mutant cell showed a normal or even an abnormal mitogenic or antimitogenic effect of 1,25-$(OH)_2D_3$, these effects showed potency ratios similar to wild type, suggesting mediation by a similar 1,25-$(OH)_2D_3$ receptor.

We conclude that three 1,25-$(OH)_2D_3$ actions show important differences in hormone response coupling indicated by differences in EC_{50} for 1,25-$(OH)_2D_3$ and by different consequences of receptor mutations.

Introduction

It has been generally believed that the induction mechanism of all 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)[1] receptor actions is the same, and that a defect in one 1,25-$(OH)_2D_3$ receptor action implies similar defects in all 1,25-$(OH)_2D_3$ receptor actions. However, certain actions of 1,25-$(OH)_2D_3$ in a homogeneous cell population differ in 1,25-$(OH)_2D_3$ concentration for half-maximal effect (EC_{50}) (1), suggesting differences in the process of hormone-response coupling. Moreover, recent data suggest diverse mechanisms of action for other v-erb-A related receptor molecules (2–7). To characterize possible differences in 1,25-$(OH)_2D_3$ coupling to three presumably nuclear actions (mitogenic action, antimitogenic action, and induction of 25-OH-D_3-24-hydroxylase [24-OHase]) we studied 1,25-$(OH)_2D_3$ analogue specificity, EC_{50}, and effect of 1,25-$(OH)_2D_3$ receptor mutations.

Methods

Materials. Cell culture media were from Gibco Laboratories (Grand Island, NY); defined fetal bovine serum was from HyClone Laboratories (Logan, UT); 1α,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol (24,25-$(OH)_2D_3$), 25-hydroxycholecalciferol (25-OH-D_3), 1α,25-$(OH)_2$-24,24F_2D_3 (RO-22-9343), and 1α,25-dihydroxy-Δ16-23yne-cholecalciferol (RO23-7553) were gifts from M. Uskokovic (Hoffmann-LaRoche, Nutley, NJ); 1β,25-dihydroxycholecalciferol (1β,25-$(OH)_2D_3$) was a gift from M. Holick and Rahul Ray (Boston University Medical School). Cholecalciferol and thymidine were from Sigma Chemical Co. (St. Louis, MO). Insulin (regular iletin I, beef-pork) was from Eli Lilly & Co. (Indianapolis, IN). Hoechst dye 33342 was from Polysciences, Inc. (Warrington, PA). [*Methyl*-1′,2′-^{3}H]Thymidine (100–130 mCi/mmol sp act) and 1α,25-dihydroxy-[26,27-*methyl*-^{3}H]-cholecalciferol (sp act 176 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). All other reagents were of the highest possible purity.

Calciferol analogues were always tested for purity on reverse-phase high performance liquid chromatography within 2 wk before use. Calciferols were dissolved in ethanol. Final concentration of ethanol in media for experiments was 0.1%, either with or without calciferol. 1,25-$(OH)_2D_3$ content of untreated serum was 39 pg/ml, and after charcoal treatment it was below the detection limit (5 pg/ml).

Cell culture. Two normal skin fibroblast lines were obtained from the American Type Culture Collection (Rockville, MD) and two additional lines were established from skin biopsies in our laboratory (8).

Three of four cell lines from patients with hereditary resistance to 1,25-$(OH)_2D_3$ ("mutant cell lines")[2] had been tested previously to characterize their defects in 1,25-$(OH)_2D_3$ receptors (8–11) (Table I). The fourth cell line from a patient with hereditary resistance to 1,25-$(OH)_2D_3$ was kindly provided by Dr. K. Kruse (12), and we characterized it using the same methods (Table I).

Cells were grown in Dulbecco's minimal essential medium/10% fetal bovine serum/0.2 mM glutamine/80 mg per liter gentamycin/0.1 μM insulin. Cells were maintained in 150-cm^2 plastic tissue culture flasks (Costar, Cambridge, MA), at 37°C in an atmosphere of 5% CO_2/95% air. Cells from passages 12–25 were used. All cell lines were tested for mycoplasma (American Type Culture Collection) with nega-

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1. *Abbreviations used in this paper:* 24,25-$(OH)_2D_3$, 24,25-dihydroxycholecalciferol; 25-OH-D_3, 25-hydroxycholecalciferol.

2. The term "mutant" cell line refers to a cell line from a subject with hereditary severe resistance to 1,25-$(OH)_2D_3$.

Table I. Characterization of Receptor Defects in Skin Fibroblasts from Subjects with Hereditary Resistance to 1,25-$(OH)_2D_3$

Cell line	1,25-$(OH)_2D_3$ binding to receptor*	1,25-$(OH)_2D_3$ receptor uptake by nuclei‡	1,25-$(OH)_2D_3$ receptor binding to DNA§	Classification of 1,25-$(OH)_2D_3$ receptor defect
10	Undetectable	NA	NA	Hormone-binding defect
11	Undetectable	Undetectable	NA	Hormone-binding defect
2	Normal	Undetectable	Normal	Cytosol-to-nucleus translocation defect
7	Normal	Mild decrease	Abnormal	DNA-binding defect

* Measured as [^{3}H]1,25-$(OH)_2D_3$-binding at 4°C to extracts solubilized from cells with high KCl concentration (11). ‡ Measured as [^{3}H]1,25-$(OH)_2D_3$ uptake into nuclei of intact cells at 37°C (11). § Measured as the elution position of [^{3}H]1,25-$(OH)_2D_3$ in a KCl gradient applied to a DNA-cellulose column containing adsorbed receptors (previously bound to [^{3}H]1,25-$(OH)_2D_3$) (8). NA, not analyzed.

tive results. Cells were incubated in serum-free medium for 24 h before experiments.

Induction of 24-OHase. All 24-OHase assays on normal or mutant cells were done as previously described (8), with minor modifications. These modifications include increase in cell number/tube (3 million/tube), and filtration of extracts prior to chromatography on a microfilter (Millipore HV 0.45 μm; Nichon Millipore, Kogyo, Japan) to prevent loss of materials on the guard column. The three mutant cell lines that had been tested previously (8, 10) were reanalyzed in one experiment. The fourth mutant cell line (12) and the normals were analyzed in two separate experiments in triplicate. All 1,25-$(OH)_2D_3$ was added in serum-free media, for 24 h.

Measurement of DNA synthesis by [^{3}H]thymidine incorporation. Cells were subcultured to 24-vial plates (Costar). Experimental conditions were then optimized for testing either antimitogenic effect (A) or mitogenic effect (B) of calciferols. Each cell line was tested with both protocols at least four times.

For protocol A cells were used within 1 d after reaching confluency. They were placed into serum-free media for 24 h; then different concentrations of 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogue were added in serum-free medium for another 24 h.

For protocol B cells were used at half confluency and also maintained in serum-free medium for 24 h before testing; then 1,25-$(OH)_2D_3$ analogues were added in a medium supplemented with 0.2 μM insulin and with 10% calciferol-free bovine serum for another 24 h.

After 24 h the media were replaced with fresh aliquots of the same media (without or with calciferols) with [^{3}H]thymidine at a final concentration of 1 μCi/ml, for another 24 h. The reaction was stopped with removal of medium, and cell monolayers were washed with ice-cold PBS containing 2 mM thymidine. Cell associated acid insoluble materials were precipitated by addition of 500 μl 5% trichloroacetic acid solution to the monolayers and incubation for 2 h at 4°C. Then the dried precipitate was solubilized with 1 N sodium chloride. Radioactivity was counted in a liquid scintillation counter, and data were expressed as percentage of counts in control wells without hormone added (mean±1 SE). Control values for different experiments were 5,700±300 counts/min n = 504 for protocol A, and 3,000±200 cpm n = 336 for protocol B. Normal cells and mutant cells always were included in the same assay, and their control values did not differ significantly.

Measurement of DNA content with microfluorimetry. Effects of 1,25-$(OH)_2D_3$ analogues on cell growth were monitored by serial measurement of DNA content with a rapid staining technique (13). For each experiment, cells were subcultured to six 96-well microtiter plates (USA Scientific Plastics, Ocala, FL) at 1,500 cells/well. Cells in microtiter plates were grown to confluency before testing (for protocol A), or to 75% confluency (for protocol B). Each cell line was tested under both conditions. Before calciferol exposure cells were washed and maintained in serum-free medium for 24 h. For antimitogenic effect (A) or mitogenic effect (B) cells were further cultured either in serum-free medium (A) or in medium supplemented with insulin and 10% calciferol-free fetal bovine serum (B) without and with varying calciferol concentrations (between 10^{-14} and 10^{-6} M). Each plate included 16 replicates without calciferol added and 8 replicates at each calciferol concentration. Cells were incubated with test substances for 7 d, with media and test substances replaced every other day. After 24 h one plate was assayed for DNA content, and after 2, 3, 5, 6, 7 d further plates were taken for DNA measurement. During this 7-d period cell number in control wells (without hormone added) increased in protocol A to 145±22% and in protocol B to 206±64% of the cell number on day 0 (3,200±1,800 n = 464 for protocol A, and 1,800±700 n = 432 for protocol B).

DNA content was measured as follows: media were removed, then cells were washed with serum-free assay media (Eagle's No. 2. media with 50 mM tricine pH 7.4), and Hoechst 33342 stain solution (50 ng/ml in assay medium, 150 μl/well) was added. Cells were incubated with the staining solution for 60 min at 37°C. After removal of staining solution microfluorometric analysis was carried out using a MicroFLUOR reader (Dynatech Laboratories Inc., Chantilly, VA; excitation filter-365 nm, emission filters: 450 nm narrow band, UV blocking nonfluorescent filter 400 nm). Background was subtracted by measuring fluorescence in wells without cells in each experiment. In preliminary experiments a relationship between cell number in wells and fluorescence units was established. Using this linear relationship, we pooled data by converting fluorescence units to percent of cell number in control wells. In each experiment normal cells and cells with 1,25-$(OH)_2D_3$ receptor defects were studied together. We report response amplitude on the day of maximal amplitude. There were no significant variations in half maximally effective hormone dose between days 1 and 7.

Statistics. Data were evaluated by analysis of variance, Student t test, and Scheffe's test for comparison of multiple means. Data are expressed as mean±1 SD, except where indicated. EC_{50}s were estimated by visual interpolation from curves of pooled data.

Results

Effects of 1,25-$(OH)_2D_3$ on normal human skin fibroblasts

1,25-$(OH)_2D_3$ effect on 24-OHase activity. 1,25-$(OH)_2D_3$ stimulated 24-OHase with a maximum stimulation (25–40-fold above baseline) at 10^{-7} M 1,25-$(OH)_2D_3$. Half maximal effect occurred at 5 nM 1,25-$(OH)_2D_3$ (Fig. 1).

Antimitogenic effect of 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ inhibited [^{3}H]thymidine incorporation in normal skin fibroblasts. Under serum-free condition (Fig. 2, *left*) the maximal inhibitory effect reached 18±4% of control, with an EC_{50} at 1 nM 1,25-$(OH)_2D_3$. In the presence of serum (Fig. 2, *right*) the maximal inhibitory effect was similar (21±3), but the EC_{50} was at 10-fold higher 1,25-$(OH)_2D_3$ concentration (10 nM).

Using microfluorimetry we also found an inhibitory effect of 1,25-$(OH)_2D_3$ on cell growth (Fig. 3, *left*). This effect of

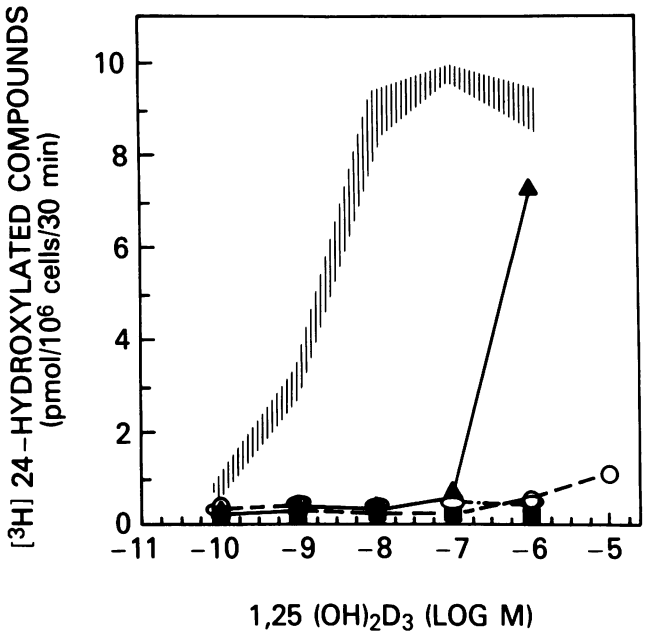

Figure 1. Induction of 25-OHD$_3$-24-hydroxylase activity by 1,25-(OH)$_2$-D$_3$ in normal and in mutant skin fibroblasts (normal: *striped zone;* line 11:○; line 7: ■; line 2: ▲; line 10: ○). Means±SD were pooled from previous data (8, 10) and from additional recent data. Standard errors are equal to or less than 8% of mean.

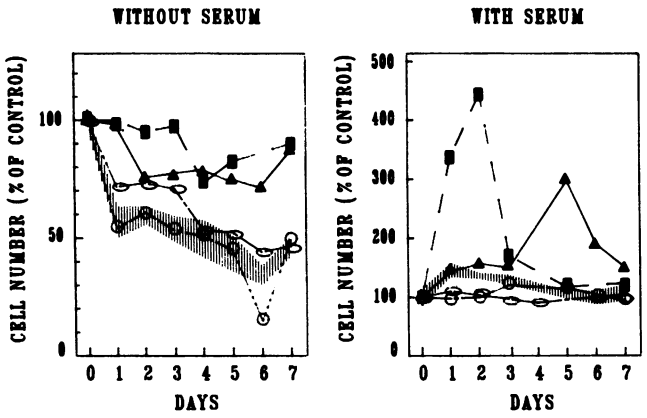

Figure 3. Effect of 1,25-(OH)$_2$D$_3$ on total DNA synthesis, measured by microfluorimetry, in normal human skin fibroblasts (*striped area*), and in fibroblasts from patients with hereditary resistance to 1,25-(OH)$_2$D$_3$ (line 11,○; line 7, ■; line 2, ▲; line 10, ○). Data (mean±1 SE) represent percent of cell number at each day compared to cell number without hormone on the same day of incubation. Each line was tested in at least three experiments. Error ranges are given only for normal cells; in all other lines errors are equal to or less than 9% of the mean. (*Left*) Maximal antimitogenic effect of 1,25-(OH)$_2$D$_3$ (10^{-7} M for normal cells and for mutant cell line subject 11, and 10^{-6} M for mutant cell lines 10, 2, and 7). (*Right*) Maximal mitogenic effect of 1,25-(OH)$_2$D$_3$ (at 10^{-11} M for normal fibroblasts and for mutant cell lines 7, and 2, and at 10^{-10} M for mutant cell lines 10 and 11).

1,25-(OH)$_2$D$_3$ was greater ($P < 0.01$) without (39±10%) than with serum (65±7%) (at 10^{-7} M 1,25-(OH)$_2$D$_3$ at the sixth day of a 7-d incubation). Half maximal inhibition on days 1–7 was found at 1 nM 1,25-(OH)$_2$D$_3$ in serum-free, and 30 nM 1,25-(OH)$_2$D$_3$ in serum supplemented media.

Mitogenic effect of 1,25-(OH)$_2$D$_3$. Stimulatory effect of 1,25-(OH)$_2$D$_3$ on [^{3}H]thymidine incorporation in normal fibroblasts was reproducible only after we optimized the incubation conditions. Without serum, with serum from another source, or without insulin this effect was not detectable in normal fibroblasts (for example, see Fig. 2, *left*). Under our optimized conditions (Fig. 2, *right*) [^{3}H]thymidine incorporation was stimulated by 1,25-(OH)$_2$D$_3$ maximally (148±3% of control) at 0.01 nM 1,25-(OH)$_2$D$_3$. EC$_{50}$ for mitogenic action was 0.0005 nM 1,25-(OH)$_2$D$_3$.

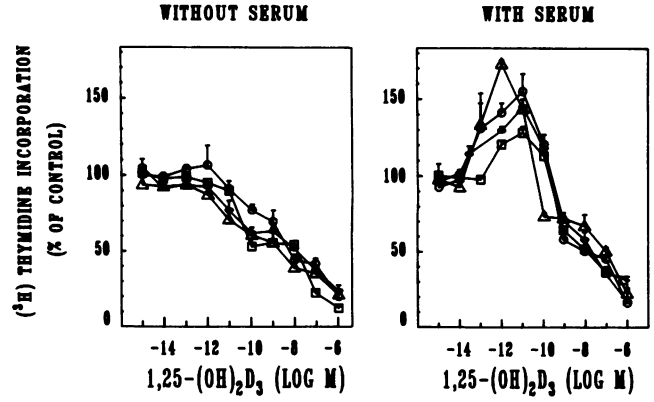

Figure 2. Effect of 1,25-(OH)$_2$D$_3$ on DNA synthesis in normal skin fibroblasts. Data are mean±1 SE from 22 experiments in triplicate, on four different cell lines. (*Left*) Without serum. (*Right*) With serum.

Using microfluorometric measurement of DNA content, we were also unable to detect a mitogenic action of 1,25-(OH)$_2$D$_3$ in the absence of serum and insulin. In the presence of serum and insulin, however, mitogenic action occurred after 24 h incubation with hormone (Fig. 3, *right*). This action decreased with longer 1,25-(OH)$_2$D$_3$ exposure, and became undetectable after 5 d. Maximal stimulation (146±23%) occurred after 24 h at 0.005 nM 1,25-(OH)$_2$D$_3$ with an EC$_{50}$ of 0.0001 nM 1,25-(OH)$_2$D$_3$.

1,25-(OH)$_2$D$_3$ actions in cell line 10 from subject with hereditary resistance to 1,25-(OH)$_2$D$_3$

This mutant cell line was classified as having defective hormone-binding to receptors (Table I).

1,25-(OH)$_2$D$_3$ effect on 24-OHase activity. Stimulatory effect of 1,25-(OH)$_2$D$_3$ on 24-OHase activity in cell line 10 was highly abnormal (Fig. 1). There was no significant increase between 10^{-11} and 10^{-7} M 1,25-(OH)$_2$D$_3$; 10^{-6} M 1,25-(OH)$_2$D$_3$ caused only 2.3-fold and 10^{-5} M 1,25-(OH)$_2$D$_3$ a 4.6-fold increase in 24-OHase activity. In the same experiments 10^{-7} M 1,25-(OH)$_2$D$_3$ caused a 40-fold increase in normal cells.

Antimitogenic effect of 1,25-(OH)$_2$D$_3$. Under serum-free conditions (Fig. 4, *left*) the maximal inhibitory effect (16±4%) of 10^{-6} M 1,25-(OH)$_2$D$_3$ was within the normal range, but EC$_{50}$ (10 nM) was 10-fold greater than normal. In the presence of serum (Fig. 4, *right*) the maximal inhibitory effect (63±4% of baseline) was less ($P < 0.001$) than in normal cells (21±4%), and the EC$_{50}$ for 1,25-(OH)$_2$D$_3$ (100 nM) was 100-fold greater than normal.

Using microfluorometric detection of DNA content, maximal inhibitory action of 1,25-(OH)$_2$D$_3$ with serum-free media

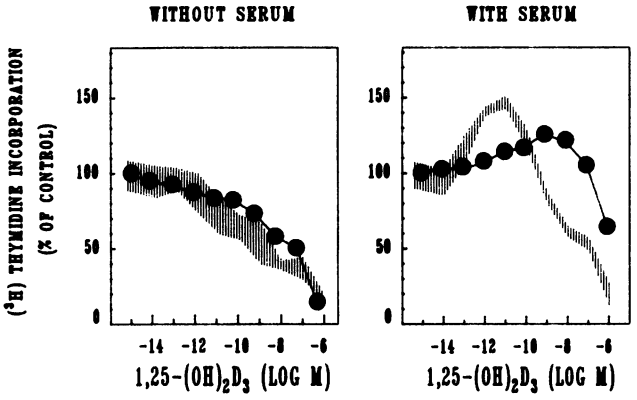

Figure 4. Effect of 1,25-$(OH)_2D_3$ on DNA synthesis in a skin fibroblast line derived from patient 10 with hereditary resistance to 1,25-$(OH)_2D_3$. Data are mean±1 SE of 10 experiments (*left*) or of 5 experiments (*right*). Symbols accommodate errors. Normal range is given as a striped zone, based on pooled data from Fig. 2.

was within the normal range, reaching maximum on the 6th day (17±8% of control), and EC_{50} was increased 10-fold to 10 nM. In the presence of serum the inhibitory action was less (maximum inhibition 68%±5% of control on the 7th day) than in normal cells ($P < 0.05$), and EC_{50} was 100 nM.

Mitogenic effect of 1,25-$(OH)_2D_3$. The stimulatory effect of 1,25-$(OH)_2D_3$ on [^{3}H]thymidine incorporation was detectable in serum-supplemented media (maximum 128±6%, and EC_{50} 100-fold greater than normal) (Fig. 4, *right*). The maximal increase in DNA content (123±2% of control by microfluorimetry) occurred after 3 d of hormone exposure, with an EC_{50} of 0.1 nM.

1,25-$(OH)_2D_3$ actions in cell line 11 from subject with hereditary resistance to 1,25-$(OH)_2D_3$

This mutant cell line was classified as having calcitriol receptors with hormone-binding defect (Table I).

1,25-$(OH)_2D_3$ effect on 24-OHase activity. 24-OHase in cell line 11 was not induced by any 1,25-$(OH)_2D_3$ dose up to 10^{-5} M (Fig. 1).

Antimitogenic effect of 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ inhibited [^{3}H]thymidine incorporation in cell line 11 (8 experiments) (Table II) exactly as in normal cell lines. Under serum-free conditions (Fig. 5, *left*) maximal inhibitory effect reached 14±3% of control with an EC_{50} at 0.5 nM 1,25-$(OH)_2D_3$. In the presence of serum (Fig. 5, *right*) the maximal inhibitory effect was 45±4% of control, and the EC_{50} for 1,25-$(OH)_2D_3$ was the same as in the normal cells in four experiments.

Cell line 11 (tested with or without serum) also showed normal inhibitory action of 1,25-$(OH)_2D_3$ on cell growth measured by microfluorimetry. Maximum inhibition was reached on day six (55±5% with and 45±3% without serum at 10^{-7} M 1,25-$(OH)_2D_3$). The concentration for half maximal inhibition was also normal, 0.5 nM 1,25-$(OH)_2D_3$ in serum-free media and 10 nM in serum-supplemented media.

Mitogenic effect of 1,25-$(OH)_2D_3$. The stimulatory effect of 1,25-$(OH)_2D_3$ on [^{3}H]thymidine incorporation was not detectable in four experiments (Fig. 5, *right*). We were unable to find stimulatory effect also with microfluorimetry.

1,25-$(OH)_2D_3$ actions in cell line 7 from subject with hereditary resistance to 1,25-$(OH)_2D_3$

This mutant cell line was classified as showing 1,25-$(OH)_2D_3$ receptors with a DNA-binding defect (Table I).

1,25-$(OH)_2D_3$ effect on 24-OHase activity. There was no 24-OHase induction detected even with the highest hormone concentrations (7) (Fig. 1).

Antimitogenic effect of 1,25-$(OH)_2D_3$. In these cells 1,25-$(OH)_2D_3$ inhibited [^{3}H]thymidine incorporation only at very high dose in the presence or absence of serum. With serum, maximal inhibition was 35±11% of control (Fig. 6, *right*). Without serum, inhibition of [^{3}H]thymidine incorporation was 50±2% of control. EC_{50} was 1,000-fold greater than normal (Fig. 6, *left*).

DNA quantitation by microfluorimetry revealed a similar defect in this 1,25-$(OH)_2D_3$ action. There was only a slight inhibitory action (74±2% of control) after 4 d with 10^{-6} M 1,25-$(OH)_2D_3$.

Mitogenic effect of 1,25-$(OH)_2D_3$. Mutant cell line 7 retained a normal or even exaggerated stimulatory action of 1,25-$(OH)_2D_3$ on DNA synthesis. Stimulatory effect on [^{3}H]-thymidine incorporation in the presence of serum at 10^{-13} and 10^{-12} M 1,25-$(OH)_2D_3$ was the same as in normal cells (Fig. 6, *right*). The maximal stimulatory effect (245±9%) at 10^{-11} M 1,25-$(OH)_2D_3$ was greater than the normal maximal stimulatory effect ($P < 0.001$) in nine experiments.

Microfluorometric measurement of DNA content revealed similarly accentuated stimulatory effect reaching a maximum (444±6%) after 48 h hormone exposure at 10^{-12} M 1,25-$(OH)_2D_3$, in the presence of serum (Fig. 3).

1,25-$(OH)_2D_3$ effects in cell line 2 from subject with hereditary resistance to 1,25-$(OH)_2D_3$

This mutant cell line was classified as having 1,25-$(OH)_2D_3$ receptors with cytosol-to-nucleus translocation defect (Table I).

Table II. Summary of Three 1,25-$(OH)_2D_3$ Actions in Four Cell Lines from Subjects with Hereditary Resistance to 1,25-$(OH)_2D_3$

Cell line	Classification of 1,25-$(OH)_2D_3$ receptor defect	1,25-$(OH)_2D_3$ action		
		24OHase induction	Mitogenic action	Antimitogenic action
10	Hormone-binding defect	Abnormal*	Abnormal	Abnormal
11	Hormone-binding defect	Undetectable	Undetectable	Normal
7	DNA-binding defect	Undetectable	Normal or increased	Undetectable
2	Cytosol-to-nucleus translocation defect	Abnormal	Normal or increased	Abnormal

* Abnormal implies detectable action (with normal or deficient amplitude) but abnormally increased EC_{50} for 1,25-$(OH)_2D_3$.

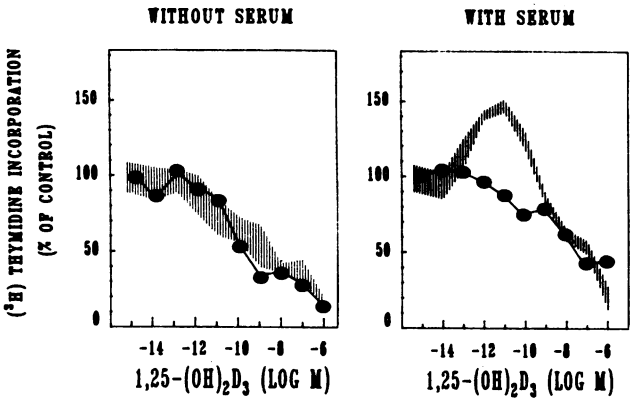

Figure 5. Effect of 1,25-$(OH)_2D_3$ on DNA synthesis in a skin fibroblast line from patient 11 with hereditary resistance to 1,25-$(OH)_2D_3$. Data are mean±1 SE of eight (*left*) or four (*right*) experiments. Symbols accommodate errors. Normal range is given as a striped zone based on pooled data from Fig. 2.

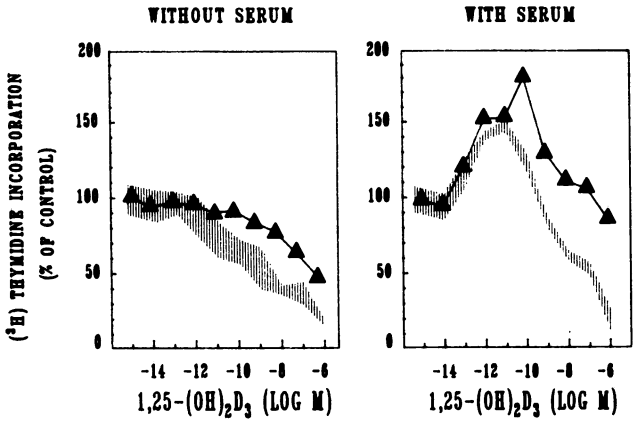

Figure 7. Effect of 1,25-$(OH)_2D_3$ on DNA synthesis in a skin fibroblast line derived from patient 2 with hereditary resistance to 1,25-$(OH)_2D_3$. Data are mean±1 SE of six experiments (*left*) or of eight experiments (*right*). Symbols accommodate errors. Normal range is given as striped zone based on pooled data from Fig. 2.

1,25-$(OH)_2D_3$ effect on 24-OHase activity. There was no increase in 24-OHase activity up to 10^{-7} M 1,25-$(OH)_2D_3$, but at very high 1,25-$(OH)_2D_3$ concentrations 24-OHase was induced to a normal maximal activity (7) (Fig. 1).

Antimitogenic effect of 1,25-$(OH)_2D_3$. In cell line 2 inhibitory effect of 1,25-$(OH)_2D_3$ on DNA synthesis was highly abnormal. In the presence of serum (Fig. 7, *right*) we could not detect inhibition of [^{3}H]thymidine incorporation at any 1,25-$(OH)_2D_3$ dose. In the absence of serum (Fig. 7, *left*) inhibitory action of 1,25-$(OH)_2D_3$ on [^{3}H]thymidine incorporation was attenuated: maximal inhibitory effect was 45±8% of control, the EC_{50} for 1,25-$(OH)_2D_3$ was increased 1,000-fold above normal.

Using microfluorimetry we found inhibitory effect on cell growth only with 10^{-6} M 1,25-$(OH)_2D_3$ under serum-free conditions.

Mitogenic effect of 1,25-$(OH)_2D_3$. In cell line 2 the mitogenic effect of 1,25-$(OH)_2D_3$ was retained in approximately normal fashion. Stimulatory effect of 1,25-$(OH)_2D_3$ on [^{3}H]-thymidine incorporation started with the same hormone concentrations as in normal cells, but it increased to a greater maximum (185±7%; $P < 0.01$ compared to normal maximum) at 10^{-10} M 1,25-$(OH)_2D_3$ (Fig. 7, *right*).

Using microfluorimetry we found a time-dependent increase in mitogenic effect with a maximum of 300% increase on the 5th day at 10^{-12} M 1,25-$(OH)_2D_3$, in the presence of serum (Fig. 3).

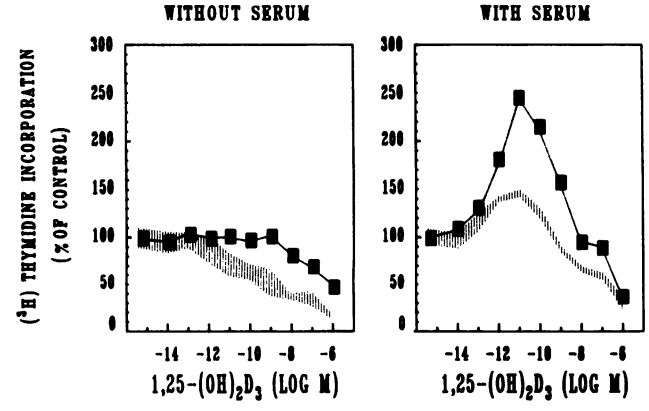

Figure 6. Effect of 1,25-$(OH)_2D_3$ on DNA synthesis in a skin fibroblast line derived from patient 7 with hereditary resistance to 1,25-$(OH)_2D_3$. Data are mean±1 SE of five experiments (*left*) or of nine experiments (*right*). Symbols accommodate errors. Normal range is given as striped zone, based on pooled data from Fig. 2.

1,25-$(OH)_2D_3$ analogue specificity for mitogenic or antimitogenic actions in normal cells

The selective retention of normal 1,25-$(OH)_2D_3$ stimulatory action on DNA synthesis in some mutant cells (lines 2 and 7), and of normal 1,25-$(OH)_2D_3$ inhibitory action in another (line 11) with severe defect in 1,25-$(OH)_2D_3$ receptor actions raised the question whether these effects in normal cell lines or in mutant cell lines are 1,25-$(OH)_2D_3$ receptor mediated. To address this we studied 1,25-$(OH)_2D_3$ analogue specificity of these actions in normal cells and in mutant cells.

1,25-$(OH)_2D_3$ and six analogues showed in normal cells indistinguishable order of potency for mitogenic and antimitogenic actions (though the EC_{50}s for the two actions differed strikingly) (Table III). The relative unresponsiveness of normal cells to both actions of 1β,25-$(OH)_2D_3$ established stereospecificity for these actions at the 1-hydroxyl residue.

1,25-$(OH)_2D_3$ analogue specificity for mitogenic or antimitogenic actions in mutant cells

In similar experiments, each response to 1,25-$(OH)_2D_3$ that was expressed in a normal manner by a mutant cell line showed analogue potency ratios indistinguishable from normal (compare the ratios for mutant cell line 11 in Table IV or mutant cell lines 7 and 2 in Table V to normal in Table III).

Responses that were defective (in 1,25-$(OH)_2D_3$ maximal effect or EC_{50}) but measurable in mutant cells also showed normal analogue potency ratios (mutant cell lines 2 and 10 in Table IV or line 10 in Table V compared to normal in Table III).

Discussion

Our results indicate important differences in induction mechanisms for three 1,25-$(OH)_2D_3$ actions. We have now found

*Table III. EC_{50}s of 1,25-$(OH)_2D_3$ and Its Analogues for Mitogenic and Antimitogenic Action in Normal Skin Fibroblasts**

Vitamin D_3 analogue	EC_{50} for mitogenic action		EC_{50} for antimitogenic action	
	nM	*Ratio‡*	*nM*	*Ratio‡*
1α,25-$(OH)_2$-Δ16-23yne-D_3	0.0001	5	0.1	10
1α,25-$(OH)_2$-24,24F_2D_3	0.0005	1	0.5	2
1α,25-$(OH)_2D_3$	0.0005	1	1	1
24,25-$(OH)_2D_3$	0.001	0.5	10	0.1
25-OHD_3	0.1	0.005	200	0.005
1β,25-$(OH)_2D_3$	0.5	0.001	100	0.01
D_3	50	0.00001	500	0.002

* All data are means from at least two experiments in triplicate. These EC_{50}s were derived from cell proliferation assays, based on [^{3}H]thymidine incorporation. Mitogenic assays used a serum and insulin supplemented medium, and antimitogenic assays used a medium without serum or insulin. Calciferol analogues were present for 48 h, with [^{3}H]thymidine added for the last 24 h.
‡ Ratio between EC_{50} for 1α,25-$(OH)_2D_3$ and EC_{50} for a vitamin D_3 analogue.

that some mutant cell lines can selectively retain at least one 1,25-$(OH)_2D$ action (mitogenic or antimitogenic action) in an approximately normal fashion while 24-OHase and some other actions are severely abnormal.

We have established incubation conditions in human skin fibroblasts to optimize mitogenic and antimitogenic effects of 1,25-$(OH)_2D_3$. Our observations with different media suggest that the mitogenic effect of 1,25-$(OH)_2D_3$ depends upon the presence of serum (i.e., presumably growth factors in serum) and insulin (presumably IGF-1/somatomedin-C since insulin acts on the latter's receptor at the high concentrations we employed [14]). Prior reports have also suggested that the mitogenic action of 1,25-$(OH)_2D_3$ may involve serum or growth factors (15), such as PDGF (15), interleukins (16), or epidermal growth factor (16). We also found that confluency status had to be optimized, supporting prior studies on the importance of confluency (17) or differentiation (18).

We observed biphasic effects of 1,25-$(OH)_2D_3$ on growth of normal skin fibroblasts, using a short-term assay based on [^{3}H]thymidine incorporation or using a long-term assay based on total DNA content. Biphasic 1,25-$(OH)_2D_3$ effects with stimulation of DNA synthesis at low doses and inhibition at higher doses have been observed previously in normal or malignant cells (19–21).

Antimitogenic effects of 1,25-$(OH)_2D_3$ on DNA synthesis have been observed in many cell systems, often accompanied by induction of cell differentiation (20, 22).

Mitogenic effects of 1,25-$(OH)_2D_3$ on DNA synthesis have been described in embryonic fibroblasts (22), in cartilage (17), in T lymphocytes (23), and in monocytes (24). While mitogenic effects of 1,25-$(OH)_2D_3$ have been found in only few systems and have been of modest magnitude, the strikingly lower EC_{50} for mitogenic than for antimitogenic effect in our studies and in prior studies (19–22) suggests that the mitogenic effect is physiologically important.

In mutant cell lines 2 and 7 with the most severe deficiency of antimitogenic effect, we noted an exaggerated amplitude of the mitogenic effect. Apparently the biphasic 1,25-$(OH)_2D_3$ dose-response relations result from interactions of two opposing effects; loss of one 1,25-$(OH)_2D_3$ effect (by selective assay conditions or by receptor mutation) allows the opposing one to be seen more clearly. The exaggerated mitogenic effects of 1,25-$(OH)_2D_3$ in cell lines 2 and 7 showed time courses different from normal and from each other (Fig. 3, *right*); the determinants of these time courses are not known.

We found EC_{50} differences by as much as 10,000-fold among three 1,25-$(OH)_2D_3$ actions in normal cells. EC_{50}s for 1,25-$(OH)_2D_3$ actions were: 0.0001–0.0005 nM for mitogenic effect, 1 nM for antimitogenic effect (without serum or insulin), and 5 nM for 24-OHase induction. Differences in EC_{50} among several 1,25-$(OH)_2D_3$ actions were also shown previously (1). Heterogeneity in EC_{50} among multiple actions of other steroids have also been documented (25), but the molecular basis for this form of heterogeneity in action of 1,25-$(OH)_2D_3$ or other steroids has not been determined.

The absolute value of an EC_{50}, per se, must be interpreted with caution; we and others (24) noted a 10-fold difference in the EC_{50} for antimitogenic action of 1,25-$(OH)_2D_3$ depending on serum addition, and EC_{50} for mitogenic actions were gener-

*Table IV. EC_{50}s for Antimitogenic Effect of 1,25-$(OH)_2D_3$ and Its Analogues in Cell Lines 11, 10, and 2 from Subjects with Hereditary Resistance to 1,25-$(OH)_2D_3$**

Vitamin D_3 analogue	EC_{50} for antimitogenic action by cell line					
	Cell line 11		Cell line 10		Cell line 2	
	nM	*Ratio‡*	*nM*	*Ratio*	*nM*	*Ratio*
1α,25-$(OH)_2$-Δ16-23yne-D_3	0.5	1	1	10	50	10
1α,25-$(OH)_2$-24,24F_2D_3	NA		NA		500	1
1α,25-$(OH)_2D_3$	0.5	1	10	1	500	1
24,25-$(OH)_2D_3$	1	0.5	20	0.5	1,000	0.5
25-OHD_3	50	0.01	100	0.1	1,000	0.5
1β,25-$(OH)_2D_3$	100	0.005	500	0.02	No effect	
D_3	500	0.001	1,000	0.01	No effect	

* All data are means from at least three experiments. These EC_{50}s were derived from cell proliferation assays, based on [^{3}H]thymidine incorporation. Antimitogenic assays used a medium without serum, with calciferol analogue for 48 h, and with [^{3}H]thymidine for the last 24 h.
‡ Ratio between EC_{50} for 1α,25-$(OH)_2D_3$ in that cell line and EC_{50} for a vitamin D_3 analogue. NA, not analyzed.

*Table V. EC_{50}s for Mitogenic Actions of 1,25-$(OH)_2D_3$ and Its Analogues in Cell Lines 7, 10, and 2 from Subjects with Hereditary Resistance to 1,25-$(OH)_2D_3$**

Vitamin D_3 analogue	EC_{50} for mitogenic action by cell line					
	Cell line 7		Cell line 10		Cell line 2	
	nM	*Ratio*‡	*nM*	*Ratio*	*nM*	*Ratio*
1α,25-$(OH)_2$-Δ16-23yne-D_3	NA		0.1	1	0.0001	10
1α,25-$(OH)_2$-24,24F_2D_3	0.0002	8	0.1	1	0.001	1
1α,25-$(OH)_2D_3$	0.001	1	0.1	1	0.001	1
24,25-$(OH)_2D_3$	0.001	1	1	0.1	0.001	1
25-OHD_3	0.07	0.14	10	0.01	10	0.0001
1β,25-$(OH)_2D_3$	1	0.001	5	0.02	100	0.00001
D_3	20	0.00005	20	0.005	50	0.00002

* All data are means from at least three experiments. These EC_{50}s were derived from cell proliferation assays, based on [^{3}H]thymidine incorporation. Mitogenic assays used a serum and insulin supplemented medium with calciferol analogues for 48 h, and with [^{3}H]thymidine for the last 24 h. NA, not analyzed. ‡ Ratio between EC_{50} for 1α,25-$(OH)_2D_3$ in that cell line and EC_{50} for a vitamin D_3 analogue.

ally lower for long-term cell growth (microfluorimetry) than for the short-term [^{3}H]thymidine incorporation) assay. The EC_{50} for antimitogenic effect of 1,25-$(OH)_2D_3$ varied more than 10-fold among a spectrum of myeloid cell lines grown under identical conditions (18). These limitations do not apply to studies, such as ours or others (1), in which markedly differing EC_{50}s are manifested as biphasic curves in cells tested under a single condition.

Our studies of fibroblasts with hereditary defects in the 1,25-$(OH)_2D_3$ receptor revealed evidence for heterogeneity in mechanisms of coupling between hormone and response; certain mutations impaired several 1,25-$(OH)_2D_3$ actions but allowed another action to remain normal. Among the four lines we tested from subjects with hereditary resistance to 1,25-$(OH)_2D_3$ one line showed selective retention of a normal antimitogenic effect of 1,25-$(OH)_2D_3$, two showed selective retention of a normal mitogenic effect of 1,25-$(OH)_2D_3$, and one showed abnormalities in all 1,25-$(OH)_2D_3$ effects. Since the molecular sites of mutations (most or all mutations are presumed to be in the 1,25-$(OH)_2D_3$ receptor gene) in these cells are not yet known, and since we were not able to study larger numbers of mutant cell lines, we cannot directly relate the retained 1,25-$(OH)_2D_3$ actions to any characteristic alterations of receptor structure. In fact, in other skin fibroblast lines or peripheral mononuclear cells from subjects with hereditary resistance to 1,25-$(OH)_2D_3$ and undetectable hormone-binding to receptors, there was no retention of antimitogenic effect of 1,25-$(OH)_2D_3$ (26, 27). On the other hand, peripheral mononuclear cells showing a DNA-binding defect seemed to retain a subtle mitogenic effect of 1,25-$(OH)_2D_3$ (that was not commented upon) (27). Retention of the normal inhibitory effect of 1,25-$(OH)_2D_3$ on c-*myc* mRNA synthesis in a 1,25-$(OH)_2D_3$ resistant HL-60 cell line was also reported recently (28).

The selective retention of normal mitogenic or antimitogenic responses to 1,25-$(OH)_2D_3$ in some lines from subjects with hereditary resistance to 1,25-$(OH)_2D_3$ raised the question whether all these actions are mediated by the same receptor. To address this question we tested 1,25-$(OH)_2D_3$ analogue specificity of these actions in normal or in mutant cell lines. In normal cells the mitogenic and antimitogenic actions showed similar relative potencies among 1,25-$(OH)_2D_3$ and six analogues, suggestive of mediation by the same receptor. Others also reported similar potency ratios for mitogenic and antimitogenic actions of 1,25-$(OH)_2D_3$ and several analogues (19, 21, 22, 29). Retained responses in mutant cell lines also showed analogue potency ratios similar to normal, suggesting mediation by a 1,25-$(OH)_2D_3$ receptor. Our data indicate that three actions of 1,25-$(OH)_2D_3$, differing by EC_{50} and affected differently by receptor mutations, are mediated by receptors with shared hormone-binding characteristics.

Our data suggest that a mutation compromising several actions of the 1,25-$(OH)_2D_3$ receptor may leave other 1,25-$(OH)_2D_3$ receptor functions intact. These phenomena might have clinical importance. One of the patients (subject 11), whose cells displayed abnormal antimitogenic effect, responded to high doses of vitamin D_3 with suppression of PTH secretion but without an intestinal response[3] (12). Since the 1,25-$(OH)_2D_3$ receptor action in the parathyroid might involve suppression of gene expression (30) while that in the intestine may involve stimulation of gene expression, differences in coupling mechanisms for these two effects are possible. Prior studies showed that 1,25-$(OH)_2D_3$ induction of 24-hydroxylase correlated well with calcemic response to 1,25-$(OH)_2D_3$.[3] Our current findings show that mitogenic or antimitogenic response to 1,25-$(OH)_2D_3$ predict calcemic responsivity less well.

Studies of the v-erbA related family of receptors have suggested several potential mechanisms for heterogeneity of receptor coupling to responses. Heterogeneity could reflect differing effects from multiple forms of hormone receptor. These receptor subspecies can be encoded by one gene (31) or by several genes (32). To date there is no evidence for multiple genes or for alternate transcripts from one gene for the 1,25-$(OH)_2D_3$ receptor. Alternately, one receptor could couple differently to distinct responses; in particular, various functional domains of the same receptor can mediate coupling to

3. The calcemic response to calciferols, which was actually measured, undoubtedly involves multiple components, but the principal 1,25-$(OH)_2D_3$ action in raising serum calcium is at the level of calcium transport across the intestinal mucosa.

different target genes (5). The amino terminus of the progesterone receptor has been shown to help select the target gene, and a synthetic receptor construct was shown to retain selectively one but not another effect (33). Some steroid receptor target genes have variable numbers of receptor recognition elements, thereby presenting opportunities for varying cooperative effects in receptor actions (2, 4, 6). Heterogeneous coupling mechanisms are also possible through receptor interactions with an increasing spectrum of transcription factors (34, 35). For example, in certain target genes, no receptor recognition element may be present, and the receptor may modulate the gene by interacting with another DNA-binding protein (5, 36). In addition to transcriptional actions, posttranscriptional effects have been suggested for 1,25-$(OH)_2D_3$ (37), and other steroids (7, 38).

While we do not know if our molecular classification of receptor defects will predict similar patterns of response retention in other mutant cell lines, we present below several speculations to illustrate how the observed response patterns might arise.

Cell line 11 with a defect in 1,25-$(OH)_2D_3$-binding to receptor retained a normal antimitogenic effect of 1,25-$(OH)_2D_3$. Since the antimitogenic effect showed the analogue specificity characteristic of a 1,25-$(OH)_2D_3$ receptor, we postulate mediation by receptors present in such small numbers or with such low 1,25-$(OH)_2D_3$ affinity that they are beneath the detection limit of the assay of [^{3}H]-1,25-$(OH)_2D_3$-binding to soluble extract. This could be analogous to a glucocorticoid resistant lymphoma cell line, which retained one glucocorticoid receptor function normally in the face of reduced numbers of glucocorticoid receptors (3). Such a postulated receptor subpopulation, arising within a presumably homogeneous cell line, must also have features that target it selectively to one or a limited number among target elements.

Cell line 7 with a defect in receptor-binding to DNA retained a normal (or even exaggerated) mitogenic effect of 1,25-$(OH)_2D_3$. We speculate that the 1,25-$(OH)_2D_3$ receptor may interact indirectly with its target for this effect by interacting directly with another protein. Such a 1,25-$(OH)_2D_3$ action need not require an intact DNA-binding domain of the 1,25-$(OH)_2D_3$ receptor (5, 36). In fact, the retained mitogenic effect in mutant lines 2 and 7 with defective nuclear interactions of receptor suggests this effect could be at a cytoplasmic target of the receptor.

We have shown that 1,25-$(OH)_2D_3$ coupling to three different actions differs strikingly in EC_{50} and in dependency on receptor functions that are compromised by certain mutations. This heterogeneity implies that full information on the normal mechanism of 1,25-$(OH)_2D_3$ receptor actions will require detailed studies of multiple responses. 1,25-$(OH)_2D_3$ effects on DNA synthesis seem to provide indices for two distinct receptor functions, while induction of 24-hydroxylase activity measures a third that correlates best of these three with the calcemic effect[3] of 1,25-$(OH)_2D_3$.

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