Immunocytology with Microwave-fixed Fibroblasts Shows 1α,25-Dihydroxyvitamin D_3-dependent Rapid and Estrogen-dependent Slow Reorganization of Vitamin D Receptors

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Abstract. Prior studies have given no evidence for regulation of vitamin D receptor (VDR) compartmentalization or subcellular organization. Microwave fixation (9–15 s) and an indirect immunodetection system of avidin-biotin enhancement and phycoerythrin fluorophore resulted in sufficient spatial and temporal resolution to allow analysis of these processes. We studied cultured fibroblasts from normals or from patients with four different types of hereditary defect compromising VDR function (mutant cells).

Compartmentalization of VDRs in the absence of 1,25-dihydroxyvitamin D_3 (calcitriol) was regulated by serum or estrogen. VDRs were mainly cytoplasmic in cells cultured without serum and phenol red, but VDRs were mainly intranuclear after addition of serum or an estrogen to cells for at least 18 h (slow regulation).

Calcitriol initiated a rapid and multistep process (rapid regulation) of reorganization in a portion of VDRs: clumping within 15–45 s, alignment of clumps along fibrils within 30–45 s, perinuclear accumulation of clumps within 45–90 s, and intranuclear accumulation of clumps within 1–3 min. We found similar rapid effects of calcitriol on VDRs in various other types of cultured cells. These sequential VDR pattern changes showed calcitriol dose dependency and calcitriol analogue specificity characteristic for the VDR. In mutant fibroblasts VDR pattern changes after calcitriol were absent or severely disturbed at selected steps. Treatment of normal cells with wheat germ agglutinin, which blocks protein transport through nuclear pores, also blocked calcitriol-dependent translocation of VDRs.

We conclude that immunocytology after microwave fixation provides evidence for regulation of VDR organization and localization.

HORMONE binding to the vitamin D receptor (VDR)[1] and other steroid receptor-related proteins initiates a process of receptor activation that allows the receptor to become competent to regulate transcription (7, 27). Steroid-related receptors may also mediate certain rapid (so-called nongenomic) effects of their hormonal agonists (4, 17, 34, 68, 70). Initiation of these genomic and nongenomic effects may be similar among steroid-related receptors, through biochemical and physical changes in the receptors, such as changes in phosphorylation (46, 52) and/or changes in binding to other cell components (i.e., identical proteins or other proteins [8, 22, 36]).

Despite many studies, there is controversy concerning receptor compartmentalization and possible subcellular changes in receptor distribution patterns after binding between a steroid hormone and its receptor (44, 73). Studies of steroid-related receptors in the 1960s and 1970s, principally with radioactive hormones, suggested that unstimulated receptors were mainly in the cytoplasm. Parallel studies suggested that steroid hormone binding initiated receptor translocation into the nucleus within minutes (76) or, in most studies, later than 0.5–4 h after hormone exposure (73). Subsequently, immunocytology and cell fractionation suggested that even nonactivated steroid receptors are predominantly intranuclear, and the evidence for receptor translocation was widely criticized as an artifact (20, 21, 33, 24, 44, 73). However, further evidence has accumulated recently that the hormone-free glucocorticoid receptor, in particular, resides in the cytoplasm, complexed to the 90-kD heat shock protein (hsp90) (56) and translocates into the nucleus after agonist binding (1, 25, 61, 64).

Methodologic limitations (antigen diffusion or modification during fixation, poor specimen permeability to antibodies, loss of antigen during tissue permeabilization, poor spatial resolution, poor temporal resolution, suboptimal antibody affinity or specificity, and other causes for inadequate signal to noise ratios) may have precluded visualization of

1. *Abbreviations used in this paper*: VDR, vitamin D receptor; WGA, wheat germ agglutinin.

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changes in receptor distribution, particularly during the first minutes of the receptor activation process. Recent developments such as microwave energy fixation of tissues (5, 37, 42), high efficiency immunodetection systems (23), and computer-aided image analysis and enhancement (31) suggested the possibility of sufficient temporal and spatial resolution to allow evaluation of compartmentalization and organization of VDRs and the rapid effects of agonist binding. VDRs may be better suited for this evaluation than some other steroid-related receptors (29) because VDRs seem to be synthesized as a single species of protein (27; Pike, J. W., unpublished data; DeLuca, H. F., unpublished data).

Materials and Methods

Reagents

1α,25-$(OH)_2D_3$ was from M. Uskokovic (Hoffman-LaRoche, Nutley, NJ); 1β,25-$(OH)_2D_3$ was from M. Holick and Rahul Ray (Boston University Medical School, Boston, MA). Immunocytology reagents were from Vector Laboratories, Inc. (Burlingame, CA), Sigma Chemical Co. (St. Louis, MO), and Zymed Lab. Inc. (San Francisco, CA). Rat monoclonal antibody (9A7) against chicken VDR was previously described (45, 57); mouse monoclonal antibodies (IVG8C11 and VD2F12) against porcine VDR were also described previously (16, 56). Cholecalci-ferol, 17β-estradiol, 17α-estradiol, diethylstilbestrol, androstenediol, and progesterone were from Sigma Chemical Co. Wheat germ agglutinin (WGA) was from Vector Laboratories, Inc.

Cell Sources

Normal dermal fibroblasts were from three subjects (6). Dermal fibroblasts from seven patients with hereditary resistance to calcitriol (mutant fibroblasts) had VDR functional defects characterized previously (6, 19, 39, 40, 59, 74).

Other cells and their sources were as follows: CV-1 monkey fibroblasts, CL9 rat liver cells, and LLC-PK_1 porcine kidney epithelial cells (American Type Culture Collection, Rockville, MD); MCF-7 human breast cancer cells (Dr. Marc Lippman, Georgetown University, Washington, DC); ROS 17/2.8 rat osteosarcoma cells (Dr. Gideon Rodan, Merck Sharp & Dohme, West Point, PA); and UMR-106 rat osteosarcoma cells (Dr. N. Partridge, Washington University, St. Louis, MO).

Cell Culture and Incubation with Steroids

Cells were grown in Dulbecco's minimal essential medium (from Biofluids, Rockville, MD) supplemented with 0.2 mM glutamine, 80 mg/liter gentamycin (both from Gibco Laboratories, Grand Island, NY), and with 0.1 μM insulin (regular Iletin I, beef-pork; Eli Lilly & Co., Indianapolis, IN), and with 10% defined fetal bovine serum (from HyClone Laboratories, Logan, UT). 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 negative results. For all experiments, cells were subcultured (10,000 per well) into two-chamber tissue culture glass slides (Lab-Tek, Naperville, IL), precoated with human fibronectin (10 μg/well; Collaborative Research Inc., Bedford, MA). Cells were used for experiments at 70% confluency.

For certain experiments, the monolayers were preincubated for 2–8 d before the experiments in phenol red-free (11) and serum-free medium (Gibco Laboratories) supplemented by 1% ITS-Premix additive (Collaborative Research Inc.). Such cells express a normal effect of 1,25-$(OH)_2D_3$ on DNA synthesis or on 250HD_3 24-hydroxylase activity (6). For one subgroup of these experiments, the cells were exposed for 30 min to WGA (2 mg/ml with detergent [5 μg/ml; Alconox Inc., New York]) in Eagle's minimal essential medium without phenol red (Gibco Laboratories), with 0.3 g/liter bovine serum albumin (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.5 mM calcium, 0.5 mM magnesium, 25 mM Hepes, pH 7.4 (medium A). Pilot studies (not shown) with fluorescein isothiocyanate-labeled WGA (Vector Laboratories, Inc.) indicated that with these conditions WGA accumulated in a perinuclear distribution and along the nuclear envelope.

For analysis of rapid regulation of VDRs, we added steroid analogues to cells in medium A. We dissolved the analogues in ethanol, and final ethanol concentration in control and in hormone-supplemented media was 0.1%. We found that 0.1% ethanol did not influence VDR compartmentalization but much higher concentrations (10–70%) did (data not shown).

Cell Fixation

After brief incubation with a steroid, cell monolayers were fixed immediately with brief microwave irradiation as recently described in detail (5). We used a commercial microwave oven (model No. RCS 700; 650 watt maximum output, 2.45 GHerz; Amana Refrigeration Inc., Amana, IA), with a continuous mode, making brief energy exposure possible. We fixed only a single slide at a time, without presence of an added water load in the oven. This was possible because of a built-in ceramic shelf and magnetron protection in this mode (50). Uniformity of heating was further improved by a model stirrer. Before placing cells in the oven, we turned on the power for 1 min. Cell monolayers with a small covering layer of medium (total volume of cells plus medium was 200 μl/chamber) were exposed for 9–15 s to maximal power. Final temperature of the cells monitored with a needle microprobe thermometer (Sensortek, Clifton, NJ) was 50 ± 6°C. Optimal slide position in the oven and optimal fixation time were established by monitoring homogeneity of temperature changes on a chamber slide covered with a temperature indicator (Omegalaq; Omega Engineering Inc., Stamford, CT), integrity of cell structure by light microscopy after conventional staining, and signal to background ratio by immunocytology. Fixation for shorter time prevented development of specific VDR immunoreactivity due to poor antibody accessibility, while excessive microwave exposure caused structural damage to cells. Optimal fixation time was 10 s for fibroblasts, 9 s for MCF7 cells, 12 s for ROS 17/2.8 cells, and 15 s for LLC-PK_1 cells. Dehydration was minimized by use of a lid to cover each slide chamber. Immediately after microwave exposure, 1 ml PBS was added to each chamber.

For comparison, some samples were fixed with five different conventional methods: cryofixation (12), 3.5% fresh paraformaldehyde (48), methanol-acetone at −20°C (10), ethanol-based fixative (Cytoprep per manufacturer's instructions; Fisher Scientific Co., Silver Spring, MD), or microwave fixation of specimen in aldehyde solution (43).

Immunostaining

The upper chamber of the slide unit was removed, and cell monolayers on the lower glass slide were incubated 15 min each with avidin solution and then with biotin solution (Blocking Kit; Vector Laboratories, Inc.) and then for 30 min with blocking serum (normal serum [5% in PBS] from species of second antibody). Primary anti-VDR antibody (9A7 at 1:1,000 dilution, VD2F12 at 1:2,000 dilution, or IVG8C11 at 1:1,500 dilution) was diluted in 4% human serum and then incubated with specimens for 1 h.

Affinity-purified biotinylated second antibody was selected for each primary antibody to give the highest signal-to-noise ratio. The 9A7 primary antibody was characterized for binding to a panel of potential second antibodies. For 9A7 we selected either the anti-mouse IgG from Vector Laboratories, Inc. (this has high cross-reaction with rat IgG) or the anti-rat IgG from Zymed; the anti-mouse IgG gave the best signal-to-background ratio with 9A7 primary antibody. For VD2F12 and for IVG8C11 we used anti-mouse IgG from Vector Laboratories, Inc. or anti-mouse IgG from Accurate Chemical & Scientific Corp. (Westbury, NY).

Biotinylated second antibodies were diluted according to manufacturer's recommendation and were applied for 30 min, followed by addition of avidin/biotinylated phycoerythrin (Vector Laboratories, Inc.) for 30 min. Each incubation step was followed by three 5-min rinses with PBS. After the final rinse cells were mounted in aqua-polymount medium (Polysciences, Inc., Warrington, PA). All staining procedures were at room temperature in a humidified box, shielded from light, and continuously tilted. Every cell line with each experimental condition was analyzed at least three times. Additional experiments were done with conventional stains to evaluate related cell structures; we used azure II (0.05%/methylene blue (0.02%)/sodium borate or methyl green or acridine orange stains (72).

Photomicroscopy and Image Processing

We analyzed slides with a Zeiss Photomicroscope III equipped for epifluorescence analysis. Phycoerythrin gives a signal with epifluorescent illumination with or without an associated phase-contrast image. Images were photographed directly from the microscope with a built-in camera; alternatively, images were captured with video cameras, digitized, then

Table I. Comparison of Vitamin D Receptor (VDR) Content by Immunocytology with 9A7 Antibody and by Calcitriol High Affinity Binding Capacity from Competitive Binding Studies with [^{3}H]Calcitriol in Various Cell Lines

Cells	VDR amount by calcitriol high affinity binding capacity*	VDR amount by immunocytology
	sites/cell	
MCF-7	15,000–18,000	++++++
ROS 17/2.8	15,000–18,000	++++++
UMR-106	14,000	+++++
Human fibroblasts (normals)	3,000–10,000	+++
LLC-PK$_1$	5,000–7,000	+++
CL9	<800	+
CV1	<500	Very low
Mutant fibroblasts (patient No. 11 with calcitriol-binding defect of VDRs)	Undetectable	Very low

* Calcitriol binding capacities from prior publications; references available by request to J. Barsony.

processed and analyzed with computer assistance, and then photographed from the computer screen. Paired control and experimental fluorescent images were photographed with the shutter opened for the same duration, or with camera controller set for the same gain and offset. Fluorescent images were captured with a Dage/MTI CCD-72 Solid State Camera equipped with an Opelco intensifier KS3-CD. Color images were captured with a Sony XC-007 precision Tricolor Camera. Images were processed and analyzed using a system from G. W. Hannaway and Associates (Boulder, CO). Receptor immunoreactivity was expressed as fluorescent intensity or color signal and analyzed by computer for intensity, size, and distribution of that signal. Each type of analysis was internally consistent with regard to immunocytology method, magnification, and instrument settings. Variability is expressed as mean and 1 SD.

Results

Validation of Immunocytology Method

Immunocytology of VDRs using microwave fixation, the 9A7 primary antibody, and the immunostaining system based on the phycoerythrin fluorophore produced an intense and relatively uniform signal over large areas of the slide (not shown). Cell morphology was at least as good as from that after fixation with other conventional methods except that after microwave fixation there were occasional burning artefacts evidenced by structural damage (vacuolization or reticular pattern in cytoplasm, or disruption of plasma membrane or nuclear membrane) in small regions close to the edges of the slides.

We found variability in the amount of VDR per cell within each sample; a similar variability has been reported with other steroid receptors after conventional fixation (62). Staining heterogeneity may reflect differences in physiological state among members of the cell population (such as asynchrony in the cell cycle).

Since we found strong signals from and novel distributions of VDR (see below), we did detailed analyses to rule out the possibility that much of the signal might be nonspecific. (*a*) With anti-VDR antibody omitted or replaced with a nonimmune rat ascites fluid, we did not detect a phycoerythrin signal (not shown). Similar controls have been used with 9A7 antibody and aldehyde fixatives (9, 12, 15, 48). (*b*) The amount of VDR by immunocytology in different cell lines correlated with the amount of receptor predicted from biochemical studies, including virtual absence of VDR signal in CV1 cells and in one mutant cell line with undetectable high affinity binding of calcitriol (Table I). (*c*) When 9A7 primary antibody was replaced by either of two different primary antibodies against VDRs and with appropriate second antibody, we found the same amount and distribution of VDRs as with the 9A7 anti-VDR antibody (not shown).

Slow Effect of Serum or Estrogen on Intranuclear Accumulation of Vitamin D Receptors

When normal human fibroblasts were cultured with medium containing serum and phenol red (final concentration of calcitriol was 0.53 pg/ml, total estrogen was 9.5 pg/ml; both analyzed by Nichols Institute Lab., San Juan Capistrano, CA), at least 50% of VDRs were in the nucleus (Fig. 1 *a*). Less than 5% of VDRs in the nucleus and in the cytoplasm were in the form of clumps (defined as VDR signal area >10 pixels), and VDRs showed no alignment along fibrils before calcitriol addition.

When we cultured fibroblasts without serum and phenol red for 48 h, the VDRs were dispersed as fine granules principally in the thick perinuclear zone of the cytoplasm with fewer VDRs inside the nucleus (Fig. 1 *B*). We saw no association of VDRs with plasma membrane or nuclear membrane. Longer periods of culture without serum and phenol red caused VDRs to become exclusively cytoplasmic; VDRs were much more peripherally distributed in the cytoplasm after 7 d than after 2 d of culture in this medium (Fig. 1 *C*).

We cultured cells without serum and phenol red after 48 h and then added back serum for 24 h; VDRs were then predominantly intranuclear. If we added for 24 h only phenol red (11) or graded doses of a series of analogues of 17β-estradiol, we noted effects similar to those from serum. This slow effect of a steroid on VDR distribution showed the analogue specificity of an action mediated by the estrogen receptor. Similar effects were induced by 17β-estradiol at 10^{-9} M and higher and by 17α-estradiol or diethylstilbestrol at 10^{-7} M and higher; androstenediol or progesterone were ineffective at concentrations up to 10^{-6} M. We also evaluated the time course of this process. After culturing fibroblasts 48 h without serum or phenol red, we added 17β-estradiol (10^{-7} M) for varying times (including 0.5, 3, 12, and 18 h and at 1-d increments up to 8 d). VDR intranuclear accumulation became detectable after 18 h and was maximal (80%) after 5 d. We termed these effects of estrogen or serum "slow" to contrast them with the more rapid effects of calcitriol (see below).

Rapid Effects of Calcitriol on Vitamin D Receptor Organization and Compartmentalization

Cells cultured for 48 h without serum and phenol red were treated briefly with calcitriol (10^{-8} M) to study rapid time-dependent changes of VDR organization and compartmentalization. Within 15 s (the earliest time point evaluated) after calcitriol addition, the VDR pattern changed. Analysis

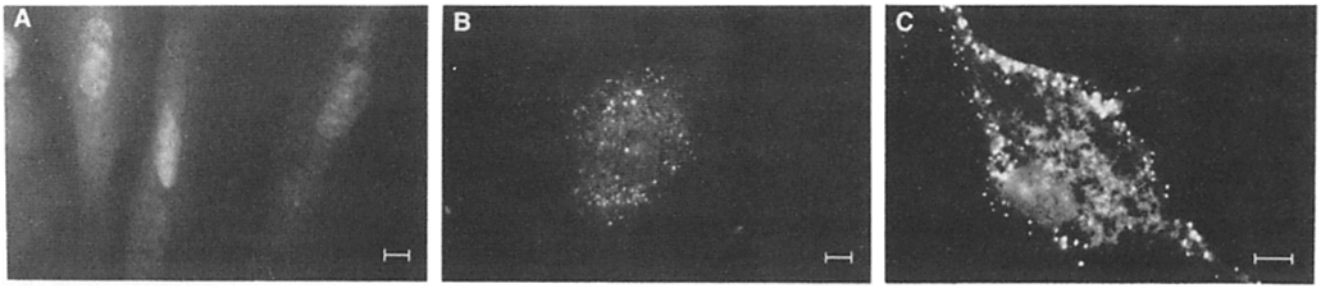

Figure 1. Subcellular localization of VDRs in normal human dermal fibroblasts (*A*) cultured with serum and phenol red, (*B*) cultured without serum and phenol red for the final 2 d, or (*C*) cultured without serum and phenol red for the final 7 d. Fluorescence images. Bar, 10 μm.

of 100 cells showed that before calcitriol addition 8.5 ± 3% of VDR immunoreactivity was accounted for by clumps (defined as signal area >10 pixels); by 15 s after calcitriol the percent of VDRs in clumps was 26 ± 11 ($P < 0.001$). Larger clumps (VDR signal area >20 pixels) were a smaller fraction; at 0, 15, and 45 s the fractions of VDRs in larger clumps were 0, 3 ± 1, and 16 ± 4 ($P < 0.001$ for all comparisons) (Fig. 2; Fig. 3 *A* vs. Fig. 3 *B*).

By 30 s after calcitriol addition ~40% of all VDRs appeared in clumps; by this time the clumps were mainly aligned along fibrils, radiating from the nucleus to the periphery (Fig. 3 *B*). By 45 s, the VDR clumps were distributed closer to the nucleus. By 1 min, the clumps were mostly along the nuclear envelope, and some of them were already inside the nucleus and nucleoli (Fig. 3 *C*). By 3 min most of the VDRs were within the nucleus with a small proportion still detectable near the nuclear membrane; about 30% of all VDRs remained dispersed in the cytoplasm (Fig. 3 *D*). Thereafter, the pattern of VDRs was essentially unchanged during 3–30 min of exposure to calcitriol. We observed the same calcitriol-induced changes in VDR patterns with any of the three monoclonal antibodies against VDRs.

About 30% of the total cell population seemed to react to calcitriol more slowly, being at various stages of this process between 3 and 30 min after hormone addition.

Lower calcitriol doses caused similar stepwise reorganization of VDRs with threshold dose of 10^{-11} M, but the reorganization was slower with lower doses (i.e., VDR clumping started after 1 min at 10^{-11} M but after 30 s with 10^{-9} M calcitriol). Incubation for 0.25–10 min with 17β-estradiol (10^{-7} M), dexamethasone (10^{-7} M), retinoic acid (10^{-7} M), 1β-dihydroxycholecalciferol (10^{-8} M), and cholecalciferol (10^{-7} M) did not change VDR organization or localization.

When fibroblasts were cultured with serum and phenol red, calcitriol (10^{-7} M) also caused rapid changes in VDR organization. VDRs in cytoplasm and nucleus were mainly dispersed before calcitriol addition (Fig. 4 *A*). During the brief incubation with calcitriol, nuclear clumping of VDRs occurred immediately in 20% of all cells (at 15 s: the earliest time point evaluated), quickly involving 40% of all cells (within 30 s). Many VDRs aligned along intranuclear fibrils (beginning at 30 s) and then accumulated in the nucleoli (Fig. 4 *C*) and close to the nuclear membrane (beginning at 60 s and reaching maximal level by 3 min). Simultaneously a portion of cytoplasmic VDRs became aligned along fibrils (Fig. 4 *B*). Intranuclear translocation of VDRs was detectable though usually subtle. Computerized analysis of single cells in one experiment showed a ratio of average nuclear/average whole cell VDR fluorescence in 30 cells before and after calcitriol of 1.08 ± 0.13 vs. 2.1 ± 1.0 ($P < 0.001$). Both cytoplasmic and nuclear reorganization of VDRs were complete within 3 min, involving 60–70% of all the VDRs. Calcitriol caused identical time-dependent reorganization of VDRs in MCF-7 breast cancer cells, in LLC-PK_1 kidney epithelial

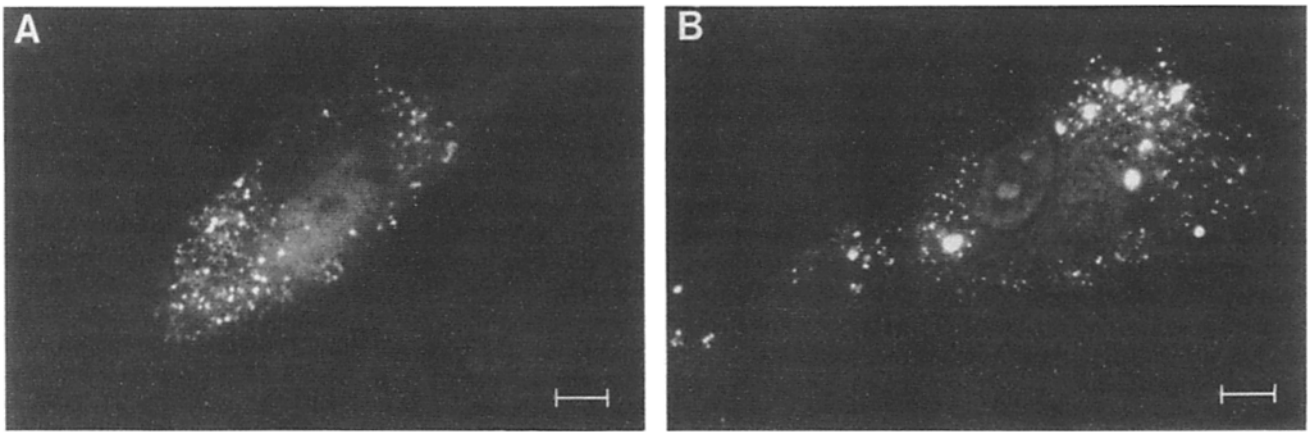

Figure 2. Rapid clumping of VDRs after calcitriol (10^{-8} M) addition. Fibroblasts were cultured for final 2 d without serum or phenol red. (*A*) Control fibroblasts without calcitriol; (*B*) fibroblasts with calcitriol for 30 s. Fluorescence images. Bar, 10 μm.

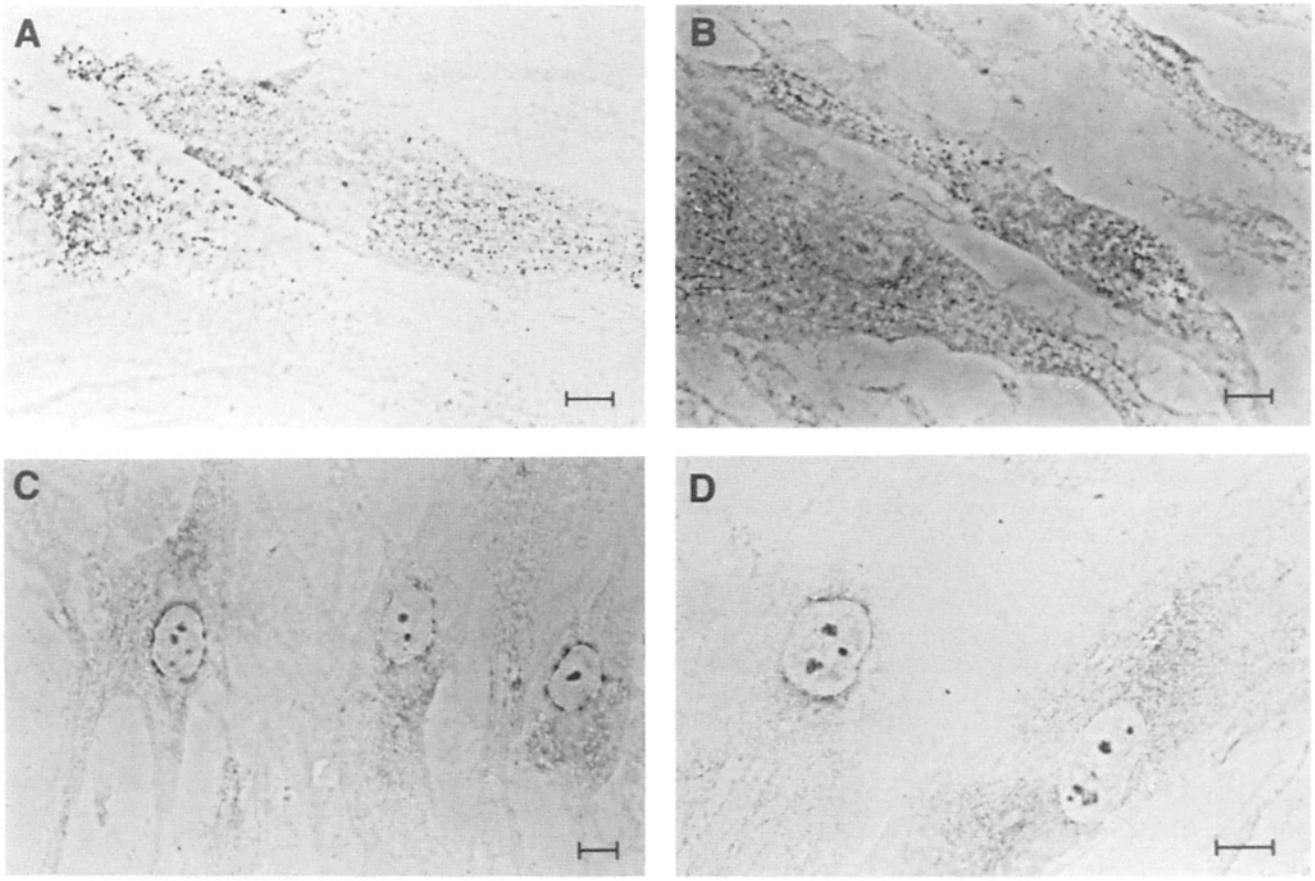

Figure 3. Time course of rapid effects of calcitriol (10^{-8} M) on VDR organization and compartmentalization in fibroblasts. Fibroblasts were cultured without serum and phenol red for 2 d: (*A*) no added calcitriol, (*B*) with calcitriol for 30 s (clumping of VDRs and alignment of VDR clumps along fibrils, directed radially towards the nucleus), (*C*) with calcitriol for 1 min (perinuclear and intranuclear accumulation of clumped VDRs), (*D*) with calcitriol for 3 min (VDR accumulation within nucleus and nucleolus). Each panel shows VDR fluorescence combined with simultaneous phase-contrast image. Bar, 10 μm.

cells, in ROS 17/2.8 osteosarcoma cells, CL9 rat liver cells, and in normal human dermal fibroblasts.

When we fixed fibroblasts with any of five standard fixation protocols, (see Materials and Methods) the sensitivity of VDR staining was lower, almost all detectable VDRs were inside the nucleus, and no VDR reorganization after brief calcitriol addition or chronic estrogen addition could be detected.

Rapid Effects of Calcitriol in Fibroblasts Pretreated with Wheat Germ Agglutinin (WGA)

We added WGA because of its potential to block protein transport through nuclear pores (51, 77). These studies were done with cells cultured for 48 h without serum and phenol red.

WGA preincubation did not have an effect on VDR distri-

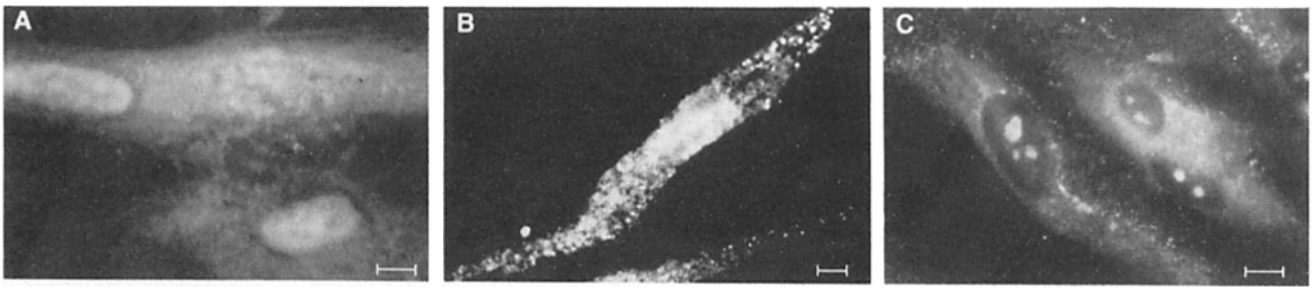

Figure 4. Time course of rapid effects of calcitriol (10^{-7} M) on VDR organization in fibroblasts. Fibroblasts were grown only in complete medium, which included serum and phenol red, and then tested: (*A*) no added calcitriol, (*B*) calcitriol added for 30 s (VDRs in cytoplasm and nucleus have become clumped), (*C*) calcitriol added for 3 min (clumped VDRs have accumulated in nucleolus). Fluorescence images. Bar, 10 μm.

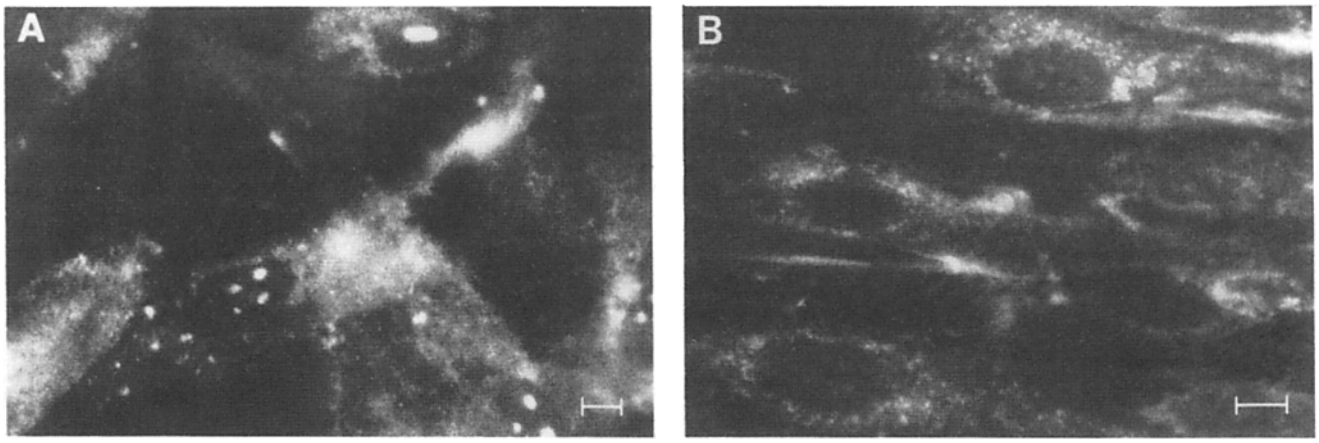

Figure 5. Effect of brief preincubation with WGA on the subsequent rapid change of VDR compartmentalization after calcitriol (10^{-8} M) for 5 min) addition. (*A*) Control cells with calcitriol but without WGA preincubation (most VDRs are inside the nucleus, compare with Fig. 2 *A*); (*B*) cells preincubated for 30 min with WGA before calcitriol addition (most VDRs are outside the nuclear envelope). Fluorescence images. Bar, 10 μm.

bution in unstimulated cells, nor did it influence VDR clumping and alignment after calcitriol addition. However, WGA prevented intranuclear accumulation of VDR 3–30 min after addition of calcitriol and stabilized a greater than normal perinuclear accumulation of VDRs (Fig. 5).

Rapid Effects of Calcitriol in Fibroblasts from Patients with Defective Vitamin D Receptor Function

We analyzed cells from seven patients with defects of VDR function (mutant cells) (Table II). We tested the mutant cells after 48 hours culture without serum and phenol red.

VDR-binding to calcitriol was undetectable in cells from three patients (No. 8, No. 10, and No. 11). Two mutant cell lines (No. 8 and No. 10) showed variably decreased immunoreactivity with different anti-VDR antibodies, and the signal was undetectable in a variable number of cells. Most importantly, no reorganization of the signal was seen in cells exposed to calcitriol.

In the other of these three (No. 11) the amount of VDR was <10% of normal (Table I) (compare Fig. 3 *A* with Fig. 6 *A*) with all three primary antibodies. After addition of 10^{-7} M calcitriol, we observed a rapid clumping, alignment, and intranuclear accumulation of VDRs. Although the number of VDR clumps was strikingly decreased, the entire time course of VDR reorganization was normal (Fig. 6, *A–C*). The particularly low amount of refractory VDR in cytoplasm of this cell line beyond 1 min after calcitriol addition suggested that much of the refractory cytoplasmic signal in the normal cell lines was specific for VDR.

In two mutant cell lines (No. 1a and No. 2a) with normal VDR binding to calcitriol and normal VDR binding to nonspecific DNA but with defective calcitriol uptake into the nucleus the amount of VDR by immunocytology was normal. Within 30 s after calcitriol addition, VDRs became clumped; then these clumps aligned along cytoplasmic fibrillar elements with abnormal, whorl-like orientations (Fig. 7). After 1 min with calcitriol almost all VDRs were close to the nucleus. We could not detect intranuclear accumulation of VDRs between 1 and 30 min.

In two mutant cell lines (No. 3 and No. 7) with decreased VDR binding to DNA, the distribution of VDRs in unstimu-

Table II. Relations Between Hereditary Defects in Vitamin D Receptor (VDR) Function and Rapid Changes of Organization and Compartmentalization of VDR After Calcitriol (10^{-8} M)

Source of fibroblasts*	Biochemical classification of VDR function*	Calcitriol-induced rapid change in VDR organization and compartmentalization
Normals (n = 3)	Normal	Initially dispersed in cytoplasm, then rapid clumping, then radial alignment along fibrils, then perinuclear accumulation, then accumulation in nucleus (especially in nucleoli)
Patient 11	VDR calcitriol-binding defect	Very low amount of VDR; all VDR changes normal after calcitriol
Patients 8 and 10	VDR calcitriol-binding defect	Variable amount of VDR; no VDR changes after calcitriol
Patients 1a and 2a	Defective uptake of calcitriol into the nucleus	Clumping, abnormal whorl-like alignment, exaggerated perinuclear accumulation; no intranuclear accumulation of clumped VDRs
Patients 3 and 7	VDR DNA-binding defect	Normal cytoplasmic clumping, alignment, and perinuclear accumulation; no intranuclear accumulation or clumping of VDRs

* The VDR functional categories were established in prior reports (6, 19, 39, 40, 59, 74). Patients and their cells are assigned the same identification symbols as in those reports. Cells from patients No. 3 and No. 7 have an identical homozygous point mutation in the VDR DNA-binding zinc finger domain, although the two patients are not known to have common ancestors (69). Cells from patient No. 8 have a homozygous point mutation causing early termination to transcription of the VDR mRNA (Pike, J. W., unpublished data). This mutation is identical to a recently reported mutation in several members of an extended kindred (63) though no common ancestry is known between patient No. 8 and the other kindred.

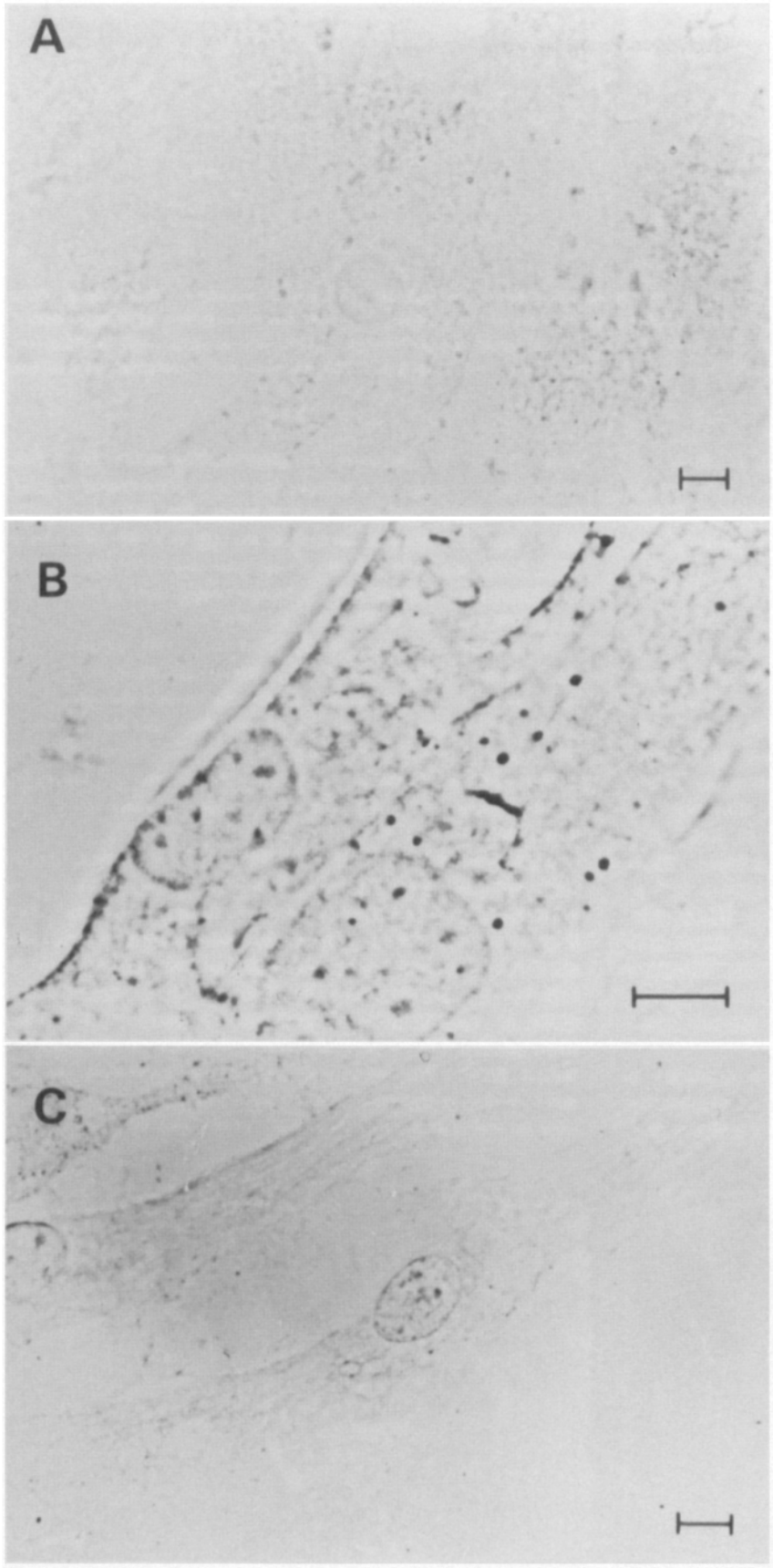

Figure 6. Rapid effect of calcitriol (10^{-8} M) on VDR distribution in dermal fibroblasts from a patient with an hereditary defect in VDR binding to calcitriol (mutant fibroblasts; No. 11): (*A*) no calcitriol added to cells (very low numbers of dispersed VDRs); (*B*) 30 s after calcitriol added to cells (VDRs clumped, but fewer VDR clumps than normal); (*C*) 3 min after calcitriol added to cells (clumped VDRs in nucleoli, but fewer clumps than normal). All panels show simultaneous VDR fluorescence combined with phase-contrast image. Bar, 10 μm.

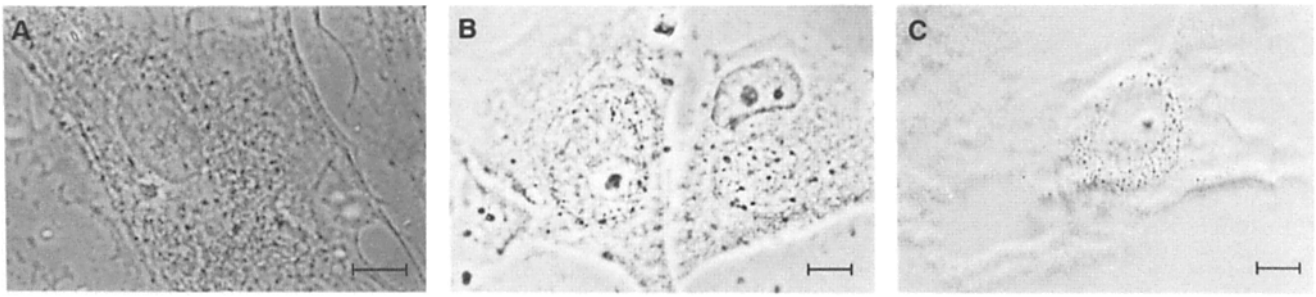

Figure 7. Rapid calcitriol (10^{-8} M) effect on VDR distribution in mutant fibroblasts from patient No. 2a with hereditary defect in calcitriol uptake into the nucleus. Mutant cells (*A*) without calcitriol (VDRs in normal pattern), (*B*) after 30 s with calcitriol added (abnormal whorl-like orientation of aligned VDR clumps in cytoplasm), (*C*) after 3 min with calcitriol added (clumped VDRs near nucleus with circumferential alignment; VDR clumps fail to accumulate inside nucleus). All panels show simultaneous VDR fluorescence combined with phase-contrast image. Bar, 10 μm.

lated cells was normal. After addition of calcitriol, there was apparently normal clumping and alignment of VDRs within 1 min, but there was no reorganization of intranuclear VDRs and no accumulation of VDRs in the nucleus for up to 30 min (Fig. 8).

Discussion

We selected microwave fixation because it could have advantages over other fixation methods (5, 37, 42). First, the rapidity of fixation allows excellent time resolution (5, 50). Although rapid freezing can also give excellent time resolution, this generally requires subsequent specimen incubation in a fixative solution (71), and the fixation step may change retention, organization, or compartmentalization of many proteins. Second, microwave fixation with or without added chemical fixatives can preserve antigenic epitopes as well as or even better than other methods (5, 30, 32, 38). Third, antibodies efficiently can reach intracellular sites in intact cells after microwave fixation without further permeabilization steps (5). Permeabilization steps, such as with detergents, can change compartmentalization and even remove some proteins.

We considered whether some of our findings might be, in part, an artifact of microwave fixation. But since microwave fixation does not cause reorganization of most cellular proteins (5, 32, 38; Barsony, J., unpublished data), the VDRs would have to acquire a unique lability to microwave irradiation after brief exposure to calcitriol. We believe it more likely that our findings including the rapid VDR subcellular reorganization after calcitriol exposure are valid and may be morphologic counterparts to changes suggested in biochemical analyses with VDRs (13, 28, 60).

We established the specificity of the VDR signal in this study with multiple standard criteria (see Results). Three types of experimental result supported further the specificity of our VDR signal: (*a*) the calcitriol dose and analogue dependency of VDR reorganization, (*b*) the extreme rapidity of the VDR reorganization after calcitriol, and (*c*) the types of disruptions of VDR cytologic patterns associated with hereditary defects in VDRs.

Approximately 30% of the VDR signal was refractory to reorganization after calcitriol addition in normal cells, and all of the signal was refractory in two mutant cell lines (No. 8 and No. 10). (VDR protein was undetectable by two-site immunoradiometric assay in fibroblasts from patients No. 10 and No. 11 [see reference 66; DeLuca, H. F., unpublished data] and undetectable by immunoblot assay in fibroblasts from patient No. 8 [see reference 58; Pike, J. W., unpublished data].) The refractory signal was randomly distributed in the cytoplasm and nucleus. This refractory signal may be from proteins other than VDRs and/or from incom-

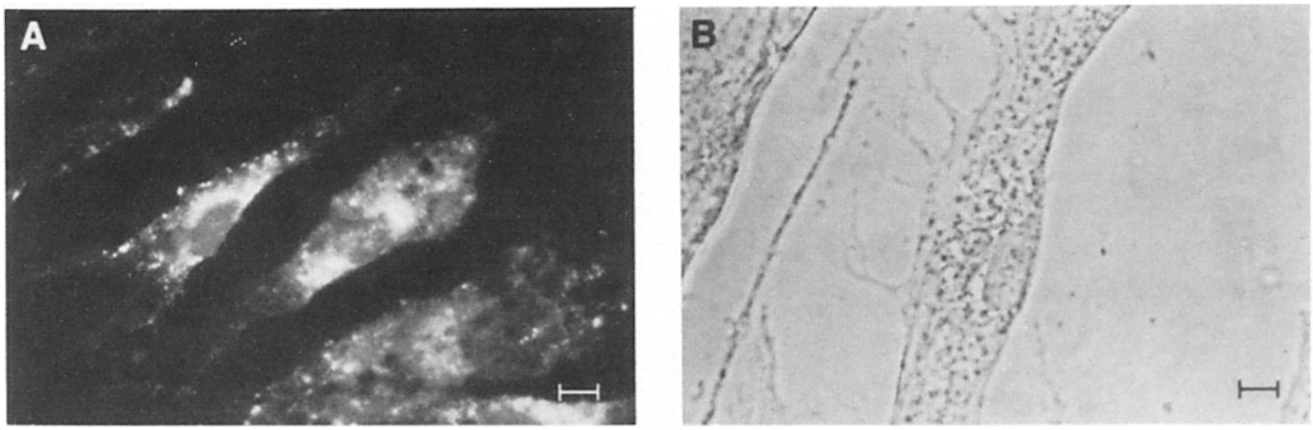

Figure 8. Maximal effect of calcitriol (10^{-8} M for 3 min) on VDR distribution in mutant fibroblasts from patient No. 7 with hereditary defect in VDR binding to DNA (VDRs are clumped in cytoplasm, but clumped VDRs are not present in the nucleus). (*A*) Fluorescence image; (*B*) Simultaneous VDR fluorescence combined with phase-contrast image. Bar, 10 μm.

pletely functional VDRs such as immature VDRs or degraded VDRs (49). The three monoclonal primary antibodies are likely to react principally with differing epitopes on the VDR, and each may cross-react with other proteins. 9A7, the principal anti-VDR antibody that we used, is a monoclonal IgG recognizing one VDR epitope common to birds and mammals (45, 59). We do not presently know if the 9A7 antibody shares a portion of the epitope recognized by the VD2F12 antibody (11–20% overlap between 9A7 and VD2F12 antibodies in an assay based on competition of antibodies for partially purified VDRs) (Barsony, J., and R. F. Schuman, unpublished data [Antibody Resources Inc., Rockville, MD]). However, the VD2F12 and the IVG8C11 monoclonal antibodies bind to two distinct VDR epitopes (16, 56).

We found with microwave fixation that a variable fraction of VDRs was cytoplasmic. When cells were cultured without serum and phenol red, most VDRs were dispersed in the cytoplasm. But even with culture in standard media and brief addition of calcitriol, we still found some VDRs in the cytoplasm. Differences in cell culture, fixation methods, and detection methods account for our differences from previous reports, in which immunocytology with 9A7 antibody detected VDRs only inside the nucleus (8, 12, 15, 48). And we verified that the VDR distributions with these prior methods were different than with our method. A feature common to all of those prior cytologic studies was slow fixation in aldehyde-based solutions. We suggest that rapid interactions of aldehydes or other fixatives with a mobile receptor protein or other cellular components could largely account for the dependency of VDR compartmentalization on fixation method. Furthermore, prior studies of VDRs used detergents or other solutions that could have selectively depleted VDRs from the cytoplasm (75).

We studied VDR subcellular compartmentalization and organization and recognized a slow regulation by estrogen and a rapid regulation by calcitriol. After long-term culture without serum and without phenol red, the subsequent addition of serum or an estrogen was sufficient to induce predominant intranuclear localization of VDRs. Culture components such as serum and phenol red can influence immunocytochemical localization of the glucocorticoid receptor (54); slow effects of estrogen on the compartmentalization of the estrogen receptor are controversial (53, 55). Phenol red preparations are mixtures that include estrogenic agonists (11), and we found estrogen analogue specificity of the slow effect of phenol red preparations or steroids on VDRs, suggesting mediation through estrogen receptors. The long time lag (18 h) necessary for development of the estrogen effect on VDR redistribution between cytoplasm and nucleus suggests that it involves new protein synthesis.

Since there were traces of estrogen and calcitriol in the serum we used, either or both might contribute to slow redistribution of VDRs caused by serum. There may be serum components other than estrogen, which can induce this slow effect. The slow effects of estrogen and/or serum may be physiologically important and warrant further studies.

In the intact organism estrogen, calcitriol, or other modulators may indirectly cause 70% or more of VDRs to reside within the nucleus, but the extranuclear portion of receptors could also have important functions. For example, physiologic evidence for VDR-mediated rapid calcitriol actions at the plasma membrane or in cytoplasm (14, 18, 41, 67) suggests that some extranuclear VDRs are functional.

Unlike the slow effect of estrogen or serum on VDR distribution, the calcitriol effects on VDRs were rapid. It was easier to visualize this rapid VDR reorganization in cells cultured without serum and phenol red. However, we found similar rapid calcitriol-induced VDR reorganization steps in the cytoplasm and nucleus also with fibroblasts cultured with serum and phenol red.

The earliest VDR change we observed after calcitriol addition was clumping of VDRs. This could be an anatomic counterpart for the steroid receptor homodimerization process (36). The next rapid step we observed was alignment of VDR clumps along cytoplasmic fibrils, directed radially toward the nucleus. Receptor clumps moved closer to the nucleus at 30–45 s than at 15 s after calcitriol. Cytoskeleton elements may be involved in the activation process of other steroid receptors (65).

After 1 min with calcitriol, clumped VDRs were principally around the nuclear envelope. This pattern suggested that reorganizing VDRs do not move unimpeded across the nuclear envelope. The rapid, calcitriol-dependent translocation of VDRs into the nucleus could be disrupted by WGA. WGA binds to nuclear pore proteins and thereby can stop the translocation of many large molecules, which would normally be actively transported through those pores (51, 77). Accentuated perinuclear accumulation and lack of intranuclear accumulation of VDRs after calcitriol was seen after WGA pretreatment and also in two mutant cell lines, suggesting disruption at similar steps.

Calcitriol caused within 1–5 min not only intranuclear VDR accumulation but also a reorganization of the intranuclear VDR pattern. Calcitriol caused VDRs to accumulate preferentially in the nucleoli, as well as in other intranuclear aggregates and close to the nuclear envelope. These distribution patterns were similar to those reported for at least two other proteins that could interact with VDRs: estrogen receptors (22, 62) and the 70-kD heat shock protein (47), which has been reported to form complexes with receptors for one or more steroid hormones (35). Some fibrillar elements were visible in the nucleus also, with VDR clumps aligned along them. This rapid rearrangement of VDRs in the nucleus may be an extension of previous biochemical studies showing interaction of steroid receptors with the nucleus (2, 3).

One class of mutant cells may have a defect in these intranuclear steps as there was lack of intranuclear accumulation and of intranuclear reorganization but no accentuated perinuclear accumulation of VDRs. This was caused by a homozygous point mutation near the base of the second zinc finger (69) in a region without recognized nuclear localization signal. A prior study also implicated the zinc finger region as contributing to the nuclear localization process for the progesterone receptor (26). Our study illustrates that mutagenesis will be suitable to define further the VDR domains determining components of the reorganization processes that we have imaged.

We conclude that immunocytology with microwave fixation is suitable for the study of VDR organization and its regulation by steroids and possibly other factors. The reorganization and, in particular, the cytoplasm-to-nucleus translocation of VDRs after calcitriol were more rapid than the translocation suggested by most prior studies of steroid-related receptors (55, 73). The rapidity of the changes after calcitriol is sufficient for these changes to be involved in

one or more physiologic processes. These simultaneous processes include rapid nongenomic effects of calcitriol, calcitriol transport into the nucleus, VDR maturation or recycling, and VDR activation for transcriptional competence.

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