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Subnuclear Distribution of the Vitamin D Receptor

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Abstract The subnuclear distribution of the vitamin D receptor was investigated to begin addressing the contribution of nuclear architecture to vitamin D–responsive control of gene expression in ROS 17/2.8 rat osteosarcoma cells. The nuclear matrix is an anastomosing network of filaments that is functionally associated with DNA replication, transcription, and RNA processing. The representation of vitamin D receptor in the nuclear matrix and nonmatrix nuclear fractions was determined by the combined application of 1) sequence-specific interactions with the vitamin D receptor binding element of the rat bone-specific osteocalcin gene promoter and 2) Western blot analysis. Both methods confirmed the presence of vitamin D receptor in the nonmatrix nuclear fraction and the absence of detectable vitamin D receptors associated with the nuclear matrix. In contrast, these same nuclear matrix proteins preparations exhibited association with the general transcription factor AP-1 and a bone tissue-specific promoter binding factor NMP2. NMP-2 exhibits recognition for a promoter domain contiguous to the vitamin D-responsive element of the osteocalcin gene, although the vitamin D receptor does not appear to be a component of the nuclear matrix proteins. Interrelationships between nuclear matrix proteins and nonmatrix nuclear proteins, in mediating steroid hormone responsiveness of a vitamin D–regulated promoter, are therefore suggested.

Key words: vitamin D, nuclear matrix, protein, AP-1, NMP2

Vitamin D plays a principal role in calcium homeostasis and skeletal metabolism [reviewed by Darwish and DeLuca, 1988]. Additionally, this steroid hormone influences expression of a broad spectrum of genes in a developmental stage-specific manner, both in vivo and in vitro, contributing to regulation of gene expression functionally related to establishment and maintenance of the bone cell phenotype [reviewed by Lian and Stein, 1993; Stein and Lian, 1993; Owen et al., 1991; Pan and Price, 1987].

Consistent with molecular mechanisms mediating the action of steroid hormones in general, the vitamin D–vitamin D receptor complex, together with RXR and other accessory proteins, exhibits sequence-specific recognition for vitamin D–responsive elements in promoters of several skeletal-related genes [Lian et al., 1989; Demay et al., 1990; Markose et al., 1990; Owen et al., 1993; Sone et al., 1991; Bortell et al., 1992, 1993; Morrison et al., 1989; Terpening et al., 1991; MacDonald et al., 1993]. The promoters of the bone-specific osteocalcin gene [Lian et al., 1989; Demay et al., 1990], the collagen gene [Lichtler et al., 1989], and the osteopontin gene [Noda et al., 1990] are examples of 5′ regulatory sequences where physiological responsiveness, associated with both developmental and homeostatic control, involves modifications in competency of a steroid hormone responsive element for occupancy by the vitamin D–vitamin D receptor complex.

The subcellular distribution of the vitamin D receptor in the osteoblast, and particularly changes that occur in association with vitamin D–related modifications in gene expression, can contribute to an understanding of the molecular mechanisms associated with actions of vitamin D on bone. Immunocytochemical localization reveals that vitamin D receptors are present in both the cytoplasm and nucleus and that the unoccupied receptor as well as the activated receptor-hormone complex predominantly re-

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side in the nucleus of osteoblasts [Barsony et al., 1990; Clemens et al., 1988]. A similar cellular distribution of other steroid receptors has been observed [Getzenberg et al., 1990].

The vitamin D receptor functions as a trans-acting factor responsive to an integrated series of cellular signalling mechanisms which determine vitamin D–mediated modifications in gene expression [Darwish and DeLuca, 1993; Norman and Hurwitz, 1993]. It is therefore important to further define mechanisms associated with nuclear vitamin D receptor concentrations, accessibility to factors that modify the structure and/or activities of the vitamin D receptor within the nucleus, and the proximity of vitamin D receptor to cognate promoter elements. In this context the involvement of nuclear architecture is an important consideration.

The nuclear matrix is a primary structural and functional component of nuclear architecture which has been associated with DNA replication and the regulation of gene expression at multiple levels [Berezney, 1991; Nickerson and Penman, 1992; Cockerill and Garrard, 1986; Berezney, 1991; Berezney and Coffey, 1975]. The relation of nuclear matrix composition to cell, tissue type, developmental stages during differentiation, and specific tumor phenotypes suggests nuclear matrix participation in expression of genes [Dworetzky et al., 1990; Bidwell et al., 1993; Pienta et al., 1991; Penman, 1991; Getzenberg et al., 1991; Fey and Penman, 1988; Stuurman et al., 1989]. Other results supporting involvement of the nuclear matrix in the regulation of gene expression include preferential association of actively transcribed genes as well as the localization of RNA synthesis and pre-mRNA splicing to this substructure [Pienta et al., 1991; Xing et al., 1993; Carter et al., 1993; Spector et al., 1991; Zeitlin et al., 1987]. Nuclear matrix involvement in transcriptional control is also suggested by recent demonstrations that transcription factors which interact with a broad spectrum of gene promoter elements [van Wijnen et al., 1993] and transcription factors which bind in a sequence-specific manner to a cell growth regulated gene promoter [Dworetzky et al., 1992] and the bone-specific osteocalcin gene promoter [Bidwell et al., 1993] are nuclear matrix components. Tissue-specific factors that exhibit selective partitioning between the nuclear matrix and the nonmatrix nuclear fractions provide additional possibilities for involvement of nuclear architecture in transcriptional regulation that is responsive to regulatory signals. The presence of several steroid hormone receptors with the nuclear matrix that include the estrogen receptor, the progesterone receptor, and an androgen receptor provides a basis for postulating a role for the nuclear matrix in steroid hormone-mediated control of transcription [Getzenberg et al., 1990; Pienta et al., 1991; Schuchard et al., 1991; van Steensel et al., 1991; Ciejek et al., 1983].

In this study we addressed contributions of nuclear architecture to vitamin D–mediated transcriptional control by determining the extent to which the vitamin D receptor is nuclear matrix associated in ROS 17/2.8 osteosarcoma cells. Our results indicate that, in contrast to other liganded steroid hormone receptor complexes that are nuclear matrix associated, the vitamin D receptor is principally found in the nonmatrix nuclear fraction. These findings are discussed in relation to activities of bone tissue-specific promoter binding factors which recognize sequences contiguous to the vitamin D receptor binding domain of the bone-specific osteocalcin gene.

MATERIALS AND METHODS

Cell Culture

The rat osteosarcoma cells, ROS 17/2.8 (a gift from Dr. S. Rodan and Dr. G. Rodan), were grown in Hams F12 medium supplemented with L-glutamine, pen/strep, and 5% horse serum, 2% fetal calf serum at 37°C under 95% air/5% CO_2. For the experiments, cells were treated as follows or as specified in the figure legends. Cells were seeded in 100 × 20 mm plastic dishes at 5×10^5 cells/plate in Hams F12 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and 5% charcoal-stripped fetal bovine calf (FBS) serum. These cells were fed fresh medium 48 h and 96 h after seeding. At the 96 h feeding, 1,25-dihydroxyvitamin D_3 (kindly provided by Dr. M. Uskokovic, Hoffmann-La Roche Inc.), (10 nM), or an equal volume of ethanol, was added to the medium. Cells were harvested 20 h later.

Cell Fractionation Protocols

Nuclear matrix proteins were obtained from the ROS 17/2.8 cells by a sequential extraction protocol [Fey et al., 1984]. Briefly, membrane and soluble proteins were extracted from the adherent cells with a Triton X-100 based buffer

followed by removal of the cytoskeletal proteins with a buffer containing Tween-40 and sodium deoxycholate. Chromatin proteins were extracted with ammonium sulfate after digestion with nuclease. The nuclear matrix–intermediate filament (NM-IF) pellet was either disassembled in 8 M urea, 20 mM MES (pH 6.6), 1 mM EGTA, 0.1 mM $MgCl_2$, 1.2 mM PMSF, and 1% 2-beta-mercaptoethanol and used for 1-D SDS/polyacrylamide gel (SDS/PAGE) analysis or, for Western analysis and gel mobility shift assays, was further processed by reassembly of the intermediate filaments through dialysis and their separation from the solubilized nuclear matrix proteins by ultracentrifugation [Bidwell et al., 1993]. Nonmatrix nuclear proteins were prepared by extraction of nuclei with 0.42 M KCl as described previously [van Wijnen et al., 1993].

Protein Analysis

Western analysis was used to evaluate the cellular compartmentalization of the vitamin D receptor [Ausubel et al., 1989]. The monoclonal antibody for this receptor, IVG8C11 (a gift from Dr. H. DeLuca) binds to the mammalian and porcine receptor [Dame et al., 1986]. Changes in nuclear matrix protein synthesis were characterized by 1-D PAGE analysis (10% acrylamide) [Laemmli, 1970] of ^{35}S-methionine–labelled NM-IF cellular fractions.

Gel Mobility Shift Assays

Gel mobility shift assays were used to characterize sequence-specific DNA-binding activity of nuclear matrix (NM) and nonmatrix nuclear (NE) fractions. Binding reactions, 20 μl in volume, consisted of 1 μg of total nuclear protein, 200–500 ng of poly (dI-dC) *poly (dI-dC) DNA, 1.9 mm dithiothreitol, 15% glycerol, 75 mM KCl, 16 mM Hepes, 0.15 mM EDTA, 10 fmole probe DNA, pH 7.5. Reactions were electrophoresed in 5% (80:1) PAGE gels in TGE buffer [Ausubel et al., 1989] followed by autoradiographic exposure. Oligonucleotide probes were prepared by 5′ end-labelling with T4 polynucleotide kinase.

RESULTS AND DISCUSSION

Restricted Representation of the Rat Vitamin D Receptor in the Nonmatrix Nuclear Fraction

To directly address the extent to which the vitamin D receptor is associated with the nuclear matrix in ROS 17/2.8 osteosarcoma cells, we examined the representation of vitamin D receptor binding activity by gel mobility shift analysis using the rat osteocalcin gene vitamin D responsive element as a probe. Sequence-specific binding activity to the osteocalcin gene vitamin D responsive element (VDRE) was assayed in nuclear matrix–associated proteins and nonmatrix nuclear proteins. The probe detects formation of one primary protein/DNA complex which includes the vitamin D receptor as established by competition and methylation interference analysis as well as by immunoreactivity [Owen et al., 1993]. The vitamin D receptor protein/DNA complex represents a heterodimer of the liganded vitamin D receptor and RXR-β [Bortell et al., 1993; Carlberg et al., 1993; MacDonald et al., 1993]. As indicated in Figure 1, the vitamin D receptor complex is present only at very low levels in the nonmatrix nuclear protein fraction of cells cultured in 5% serum but is highly abundant in the nonmatrix nuclear protein fraction following vitamin D treatment. In contrast, the vitamin D receptor complex is below the threshold for detection in the nuclear matrix fraction prior to and following vitamin D treatment of ROS 17/2.8 osteosarcoma cells. These results suggest that the vitamin D receptor is not associated with the nuclear matrix in cells that exhibit pronounced vitamin D–responsive transcriptional upregulation.

By direct comparison, levels of the bone-specific nuclear matrix protein NMP2 [Bidwell et al., 1993] are high in nuclear matrix preparations of both control and vitamin D–treated ROS 17/2.8 cells (Fig. 2). This protein binds with sequence specificity to sites flanking the VDRE, but its concentration is not modulated by vitamin D. In addition, we observe a series of C/EBP-related nuclear proteins that are unique to the nonmatrix nuclear fraction, and these are also not vitamin D inducible. The extent to which the vitamin D receptor resides solely in the nonmatrix nuclear fraction was confirmed by western blot analysis (Fig. 3). We detected vitamin D–dependent upregulation of the ~50 kD rat vitamin D receptor protein only in the nonmatrix nuclear fraction.

Selective Partitioning of General and Tissue-Specific Transcription Factors Between the Nuclear Matrix and Nonmatrix Nuclear Fractions

To further examine the vitamin D–dependent modifications in partitioning of transcription factors between nuclear compartments, we monitored AP1 binding activity (Fig. 4). Consistent

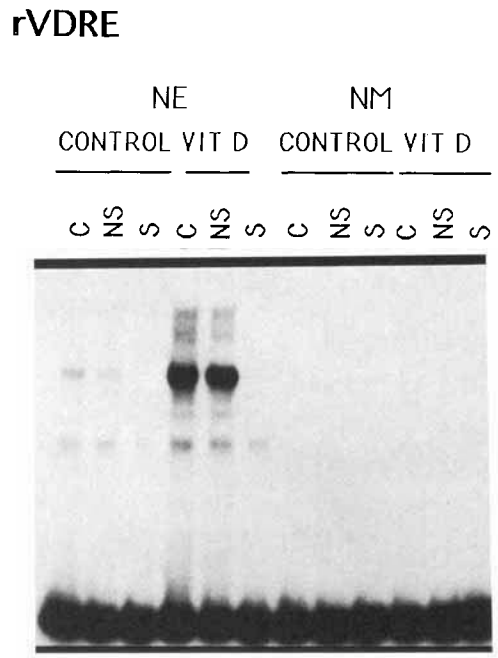

Fig. 1. DNA-binding activity of nonmatrix nuclear proteins (NE) and nuclear matrix (NM) proteins to the vitamin D receptor binding element of the osteocalcin gene promoter. Gel mobility shift assays compare DNA-binding activities of NM and NE proteins from ROS 17/2.8 cells that had been treated with vitamin D (10 nM) or carrier (an equal volume of ethanol) for 20 h as detailed in Materials and Methods. Nuclear matrix proteins were extracted as described in Materials and Methods. Binding reactions, 20 μl volume, contained 10 ng/μl poly(dI-dC)*poly(dI-dC), 15% glycerol, 75 mM KCl, 16 mM Hepes, pH 7.5, 0.15 mM EDTA, 10 fmol of probe DNA, and 1 μg of nuclear protein from the matrix (NM) or nonmatrix (NE) fractions. The oligonucleotide containing the rat vitamin D responsive element (rVDRE), 5'-CTGCACTGGGTGAATGAGGACATTACTGA-3', was end-labelled with T4 polynucleotide kinase and used as the DNA probe. Binding reactions were electrophoresed in 5% (80:1) polyacrylamide gels in TGE buffer [Ausubel et al., 1989], followed by exposure to preflashed X-ray film for autoradiographic analysis. NE extracts from treated ROS 17/2.8 cells exhibit a dramatic upregulation in DNA-binding activity as compared to controls. No DNA-binding activity using the rVDRE oligonucleotide as a probe was observed with NM extracts from control or treated cells. Binding reactions were performed in absence (C; control lane) and presence of 100-fold of unlabelled specific DNA competitor (S lane) or identical amount of nonspecific (NS) competitor DNA.

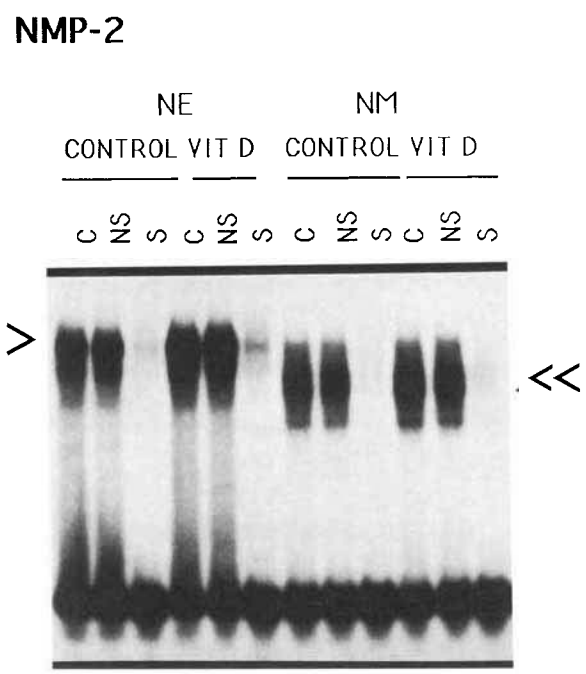

Fig. 2. Abundant NMP2 activity is observed in NM extracts from ROS 17/2.8 cells treated with vitamin D demonstrating the integrity of this nuclear subfraction. Cells were cultured and treated and the nuclear matrix proteins extracted as described in Fig. 1. Conditions for the gel mobility shift assay were identical to those described in Fig. 1. The oligonucleotide used for the probe DNA, 5'-GATCCCGAAAAACCACTAAAGCA-3', contains the consensus sequence for NMP2. This protein (double arrowhead) is specific to the nuclear matrix in osteoblasts [Bidwell et al., 1993]. NMP2 binding activity was observed in the NM fractions of both control and vitamin D–treated cells. The single arrowhead indicates DNA binding activity in ROS 17/2.8 cell nuclear extracts having a mobility distinct from NMP2. Binding reactions were performed in absence (C; control lane) and presence of 100-fold of unlabelled specific DNA competitor (S lane) or identical amount of nonspecific (NS) competitor DNA.

with previous observations [Bortell et al., 1993], we observe that AP-1 activity is upregulated upon vitamin D treatment. In addition, AP-1 activity is present in both the nuclear matrix and nonmatrix nuclear fractions. We note that in these proliferating cells, representation of AP-1 activity is higher in the nuclear extract compared to a previous report where AP-1 activity distributed to the nuclear matrix in postconfluent ROS 17/2.8 cells [van Wijnen et al., 1993]. This is consistent with cell growth-dependent partitioning of this transcription factor complex. Taken together the findings presented in Figures 1–4 suggest that transcription factors can selectively partition between the nuclear matrix and nonmatrix nuclear fractions. Moreover the AP-1 activity (Fig. 4) observed in the nuclear matrix fraction is vitamin D dependent, whereas the representation of NMP2 in the nuclear matrix is independent of vitamin D (Fig. 2).

We have observed modifications in the representation of specific nuclear matrix proteins as a function of osteoblast differentiation [Dworetzky et al., 1990] and in osteoblastic cells following treatment with PTH [Bidwell et al., 1994]. These results support involvement of the nuclear matrix in the integration and/or amplification of signal transduction pathways and are consistent

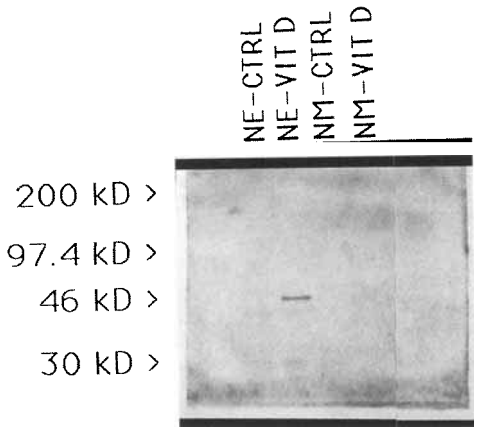

Fig. 3. Vitamin D receptor protein (VDR) levels are upregulated and localized in the NE fraction of the ROS 17/2.8 cells following hormone treatment. Western analysis [Ausubel et al., 1989] was employed to characterize the cellular distribution of the vitamin D receptor protein in both control and vitamin D–treated ROS 17/2.8 cells. Nuclear protein fractions (27.5 μg) were ethanol precipitated at −70°C overnight. These proteins were then pelleted and the supernatant removed and air dried. Samples were dissolved in SDS sample buffer, boiled, and loaded onto an 8% SDS-polyacrylamide gel. After electrophoretic fractionation the proteins were transferred to a PVDF Immobilon-P membrane (Millipore, Bedford MA), blocked, and probed with an antibody to the vitamin D receptor. The monoclonal antibody (a gift from Dr. H.F. DeLuca, Madison, WI) was raised against the porcine intestinal vitamin D receptor and cross-reacts with the rat vitamin D receptor (antibody IVG8C11). The blot was developed using chemiluminescence (BioRad, Richmond, CA). A band is evident at 55 kD in the NE fraction from treated cells which corresponds with the established electrophoretic mobility of the rat vitamin D receptor. The vitamin D receptor was not observed in the NM fractions of control or treated cells.

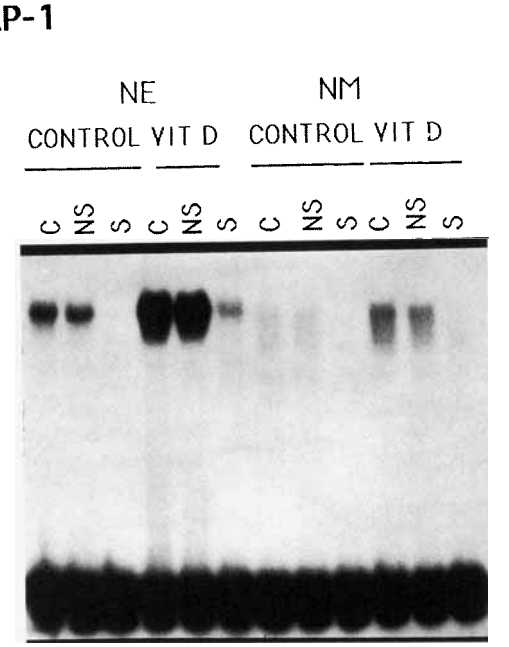

Fig. 4. AP-1 DNA-binding activity is exhibited by NM and NE proteins of both control and vitamin D–treated ROS 17/2.8 cells. Cells were cultured and treated and the nuclear matrix proteins extracted as described in Fig. 1. Conditions for the gel mobility shift assay were identical to those described in Fig. 1A. The oligonucleotide used for the probe DNA, 5'-CGTGACT-CAGCGCGCG-3', contains an AP-1 binding site. AP-1 activity was upregulated in both the NE and NM fractions following vitamin D treatment. The NE fraction exhibited significantly more AP-1 DNA-binding activity than the NE and NM fractions of confluent ROS 17/2.8 cells [van Wijnen et al., 1993]. AP-1 activity was observed to localize in the NM fraction in confluent ROS 17/2.8 cells. In the present study the cells were harvested before reaching confluency which is consistent with the reciprocal distribution of this trans-acting complex between these nuclear compartments.

with hormone-mediated changes in the nuclear matrix that may facilitate phenotypic responsiveness of bone cells. However, it would be naive to postulate a single regulatory mechanism. While vitamin D influences the representation of early response gene-encoded transcription factors (e.g., Fos and Jun complexes that bind to AP1 sites [Candeliere et al., 1991; Owen et al., 1993]), this steroid hormone does not affect the partitioning of the vitamin D receptor between the nuclear matrix and nonmatrix nuclear fractions, and only subtle modifications in the synthesis of nuclear matrix proteins are observed from 8–72 hours following addition of vitamin D (Fig. 5).

Potential Contributions of Nuclear Architecture to Transcriptional Control by Integrating Regulatory Activities of Steroid Hormones and Other Transcription Factors

Modifications in nuclear matrix composition may support integration of regulatory signals that determine the extent to which hormone responsiveness controls gene transcription. To date several classes of DNA binding proteins that influence transcription have been identified as components of the nuclear matrix. These include general transactivation factors that interact with a broad spectrum of gene promoters (e.g., ATF-NMP1, SP1, and AP1) [van Wijnen et al., 1993], steroid hormone receptors (estrogen receptor, androgen receptor, and glucocorticoid receptor) [Getzenberg et al., 1990; Pienta et al., 1991; Schuchard et al., 1991; van Steensel et al., 1991; Ciejek et al., 1983], and tissue-specific promoter binding factors (e.g., NMP2) [Bidwell et al., 1993]. The association of these modulators of transcription with the nuclear matrix implies a contribution of nuclear architecture to regulation of gene expression by facilitating concentration and targeting of transcription fac-

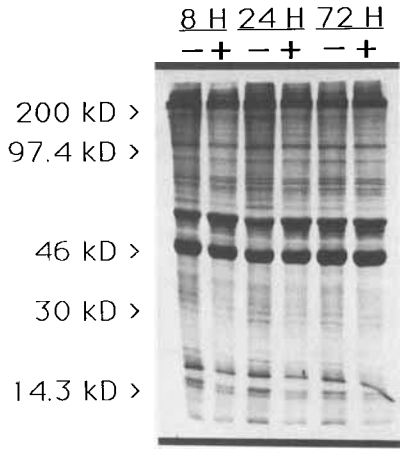

Fig. 5. Nuclear matrix–intermediate filament protein synthesis in ROS 17/2.8 cells following exposure to vitamin D. SDS-PAGE analysis of NM-IF proteins obtained from ^{35}S-methionine–labelled ROS 17/2.8 cells treated with vitamin D. NM-IF extracts were derived from cells exposed to vitamin D (10 nM) or an equal volume of vehicle (ethanol) for 8, 24, and 72 h as described in the text. Upon nearing confluency, ROS 17/2.8 cells (day 8, postseeding) were switched to Hams F12 medium supplemented with 5% stripped serum (FBS). After 24 h, cells were replenished with fresh medium containing vitamin D (10 nM) or an equal volume of ethanol. Cultures were fed daily, and exposure to hormone was staggered to permit harvesting of all cultures on day 12 postseeding. One hour prior to harvest, cells were exposed to ^{35}S-methionine, 22–30 μCi/ml (Amersham >1000 Ci/mmol) in MEM, without methionine, supplemented with hormone/carrier and 10% fetal calf serum (dialyzed against phosphate buffered saline).

tors. Variations in the partitioning of transcription factors between the nuclear matrix and nonmatrix subnuclear compartments may further provide a basis for determining accessibility of transcription factors to their cognate promoter regulatory elements. However, the present findings indicate that all steroid hormone receptors that influence transcription may not be nuclear matrix associated. It therefore appears that steroid hormone-mediated cellular signaling mechanisms which determine transcriptional activity may be mediated through the integrated activities of DNA binding proteins which reside in both the nuclear matrix and nonmatrix nuclear fractions.

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