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Novel Nuclear Localization Signal Between the Two DNA-Binding Zinc Fingers in the Human Vitamin D Receptor

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Abstract The human vitamin D receptor (hVDR) possesses a unique array of five basic amino acids positioned between the two DNA-binding zinc fingers that is similar to well-characterized nuclear localization sequences in other proteins. When residues within this region are mutated to nonbasic amino acids, or when this domain is deleted, the receptor is still well expressed, but it no longer associates with the vitamin D-responsive element in DNA, in vitro, and hVDR-mediated transcriptional activation is abolished in transfected cells. Concomitantly, the mutated hVDRs exhibit a significant shift in hVDR cellular distribution favoring cytoplasmic over nuclear retention as assessed by subcellular fractionation and immunoblotting. Independent immunocytochemical studies employing a VDR-specific monoclonal antibody demonstrate that mutation or deletion of this basic domain dramatically attenuates hVDR nuclear localization in transfected COS-7 cells. Although wild-type hVDR is partitioned predominantly to the nucleus in the absence of the 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) hormone, treatment with ligand further enhances nuclear translocation, as it does to some degree in receptors with the basic region altered. The role of 1,25$(OH)_2D_3$ may be to facilitate hVDR heterodimerization with retinoid X receptors, stimulating subsequent DNA binding and ultimately enhancing nuclear retention. Taken together, these data reveal that the region of hVDR between Arg-49 and Lys-55 contains a novel constitutive nuclear localization signal, RRSMKRK. J. Cell. Biochem. 70:94-109, 1998.

Key words: steroid hormone receptor; 1,25-dihydroxyvitamin D_3; nuclear retention; DNA-binding; transcriptional activation

The vitamin D receptor (VDR) is classified as a member of the steroid/retinoid/thyroid hormone receptor superfamily of proteins by virtue of amino acid homologies within two separate domains and by similar biochemical functions among this family of nuclear regulatory macromolecules [Beato, 1989; Evans, 1988; Haussler et al., 1988]. The N-terminal domain of VDR is configured into two zinc-coordinated fingers responsible for DNA recognition and binding, whereas the C-terminal domain binds the 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) hormone [Haussler et al., 1995]. VDR also possesses a protein kinase C (PKC) phosphorylation site located between the two zinc fingers, namely, serine-51 (Fig. 1), that is proposed to modulate DNA binding depending on its phosphorylation state [Hsieh et al., 1993]. Analogous to other steroid/retinoid/thyroid hormone receptors and their cognate ligands, the intranuclear VDR mediates the biological effects of 1,25$(OH)_2D_3$ on target cells. Ligand-occupied VDR regulates specific gene transcription by binding to a DNA enhancer sequence, termed the vitamin D-responsive element (VDRE), within the promoter region of controlled genes [Noda et al., 1990; Ozono et al., 1990; Terpening et al., 1991]. Finally, VDR alone does not bind efficiently to the VDRE. It requires another protein(s) such as retinoid X receptor (RXR) for optimal binding to the VDRE as a heterodimer [Liao et al., 1990; MacDonald et al., 1991, 1993; Ross et al., 1992], rendering VDR most similar to the thy-

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roid hormone receptor (TR) and retinoic acid receptor (RAR), which also heterodimerize with RXR on DNA [Mangelsdorf and Evans, 1995].

Biochemical fractionation studies [Walters et al., 1980] suggest that even predominantly unoccupied $1,25(OH)_2D_3$ receptors are localized (>60%) to nuclear preparations from various vitamin D target tissues, including chick intestinal mucosa, parathyroid, kidney, testes, and pancreas, rat intestinal mucosa, kidney, and testes, as well as a mouse osteoblast-like bone cell line. These experiments also revealed that the apparent nuclear/cytosol distribution depends upon the ionic strength of the extraction buffer used in the subcellular fractionation procedure [Walters et al., 1980]. Nevertheless, immunocytochemical localization studies confirm that, in situ, VDR is primarily a nuclear protein even in the ligand unoccupied state [Berger et al., 1988; Clemens et al., 1988; Milde et al., 1989; Zanello et al., 1997] .

In spite of this evidence that VDR is partitioned predominately in the nucleus [reviewed in Haussler et al., 1997], little is known about the nature of nuclear localization signals (NLSs) within the VDR molecule or the process by which VDR is transferred into the nucleus. A number of NLSs in nuclear proteins have been identified, and most contain a short stretch of basic amino acids, lys-lys/arg-x-lys/arg (x=lys, arg, pro, val, ala) [Chelsky et al., 1989]. One of the best characterized signals is PKKKRKV of the SV40 T-antigen [Kalderon et al., 1984; Lanford and Butel, 1984], and this NLS sequence may represent a prototype for similar sequences in other nuclear proteins. Such a short sequence of basic amino acids that apparently

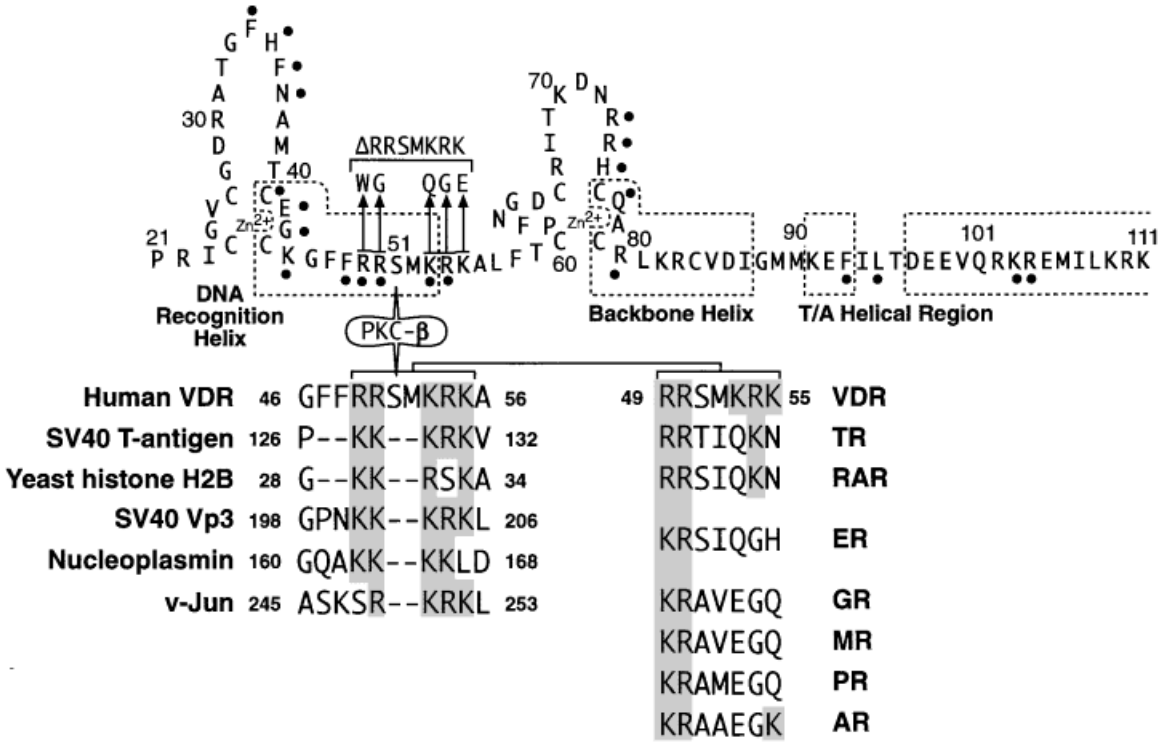

Fig. 1. Comparison of the proposed nuclear localization signal in hVDR with known or putative signaling domains identified in other nuclear proteins. Amino acids within the DNA binding domain (DBD) in hVDR are indicated by the one-letter code and a residue number above the sequence (top half of figure). Boxed with dashes are putative helical regions based on the crystal structure of the highly homologous TR DBD [Rastinejad et al., 1995], and amino acids proposed to participate in DNA contacts on the VDRE are indicated by the closed circles. Depicted above the DBD (arrows) are one double (RR) and one triple (KRK) mutant, along with a deletant (ΔRRSMKRK) in the putative nuclear localization signal (residues 49-55), evaluated in the present study. Serine-51, a protein kinase C phosphorylation site, also resides in this domain. Shown below the DBD (lower left) is a sequence alignment of hVDR residues 46-56 and the nuclear proteins, SV40 T-antigen [Kalderon et al., 1984], yeast histone H2B [Moreland et al., 1987], SV40 Vp3 [Clever and Kasamatsu, 1991], *Xenopus laevis* nucleoplasmin [Bürglin and De Robertis, 1987], and v-Jun [Chida and Vogt, 1992]. Additional sequence homology is shown between hVDR residues 49-55 and other members of the nuclear receptor superfamily (lower right). MR is the mineralocorticoid receptor. Conserved (and conservatively replaced) residues are shaded in the lower portion of the figure.

facilitates transfer to the nucleus has been identified in several of the steroid hormone receptors, including the glucocorticoid receptor (GR) [Picard and Yamamoto, 1987], progesterone receptor (PR) [Guiochon-Mantel et al., 1989], and androgen receptor (AR) [Simental et al., 1991], as well as in a number of nuclear protein kinases and cyclins [Boulikas, 1996].

Figure 1 illustrates the deduced amino acid sequence of the human VDR (hVDR) N-terminal region that contains a number of clusters of basic residues and is responsible for DNA binding [Baker et al., 1988]. This domain possesses two zinc fingers that, by analogy to the reported structure of TRβ cocrystallized with RXR on DNA [Rastinejad et al., 1995], presumably participate in VDRE binding via a DNA-recognition helix and a phosphate backbone-associating helix situated on the C-terminal side of the first and second fingers, respectively. Putative DNA-binding amino acids are designated with closed circles in Figure 1 for the residues in hVDR corresponding to those in TR that actually contact DNA elements [Rastinejad et al., 1995]. Two additional helical regions positionally equivalent to the T- and A-boxes of nuclear receptors [Wilson et al., 1992] are also present in hVDR (Fig. 1) and apparently participate in both DNA binding and heterodimerization with the RXR partner on the nucleic acid scaffold [Hsieh et al., 1995; Lee et al., 1993; Rastinejad et al., 1995]. In analyzing basic amino acid stretches present in hVDR, a region between residues 70 and 111 was selected [Luo et al., 1994] to probe for the existence of a NLS capable of conferring nuclear transfer. Three peptides, hVDR(79-105), hVDR(70-83), and hVDR(100-111), were conjugated to fluorescein-labeled IgG and the chimeras were then microinjected into the cytoplasm of human osteosarcoma MG-63 cells. The hVDR(79-105) construct was able to enter the nucleus as did an SV40 T-peptide, but the hVDR(70-83) and hVDR(100-111) conjugates, each of which contains only one of the basic clusters in hVDR(79-105), remained in the cytoplasm. These results [Luo et al., 1994] suggest that the basic residues at both ends of hVDR(79-105) are equally necessary for nuclear accumulation. However, further characterization of this bipartite region is required to determine which individual basic residues actually constitute a VDR NLS(s).

By site-directed mutagenesis, we now report the identification of a distinct, more N-terminal basic sequence between the two zinc fingers of hVDR that represents a second NLS region. When positively charged residues between arg-49 and lys-55, a seven residue stretch containing five basic amino acids (Fig. 1), are mutated to nonbasic residues or deleted, a significant shift in VDR distribution results, favoring cytoplasmic retention as analyzed by cell fractionation/Western blotting and immunocytochemistry.

MATERIALS AND METHODS

Site-Directed Mutagenesis

Specific deletion or alteration of residues by site-directed mutagenesis of codons in the hVDR cDNA [Hsieh et al., 1991] was performed by a method described in detail elsewhere [Kunkel et al., 1987] using the Muta-Gene kit (Bio-Rad Laboratories, Richmond, CA). Single-stranded phagemid containing the hVDR cDNA was produced and annealed to oligonucleotides complementary to the region of interest in the hVDR sequence. These oligonucleotides contained bases in the central portion of their sequence that mismatched with, or resulted in the deletion of, the natural hVDR sequences, creating altered hVDRs (see top portion of Fig. 1). After annealing of these mutagenic oligonucleotides, the second strand of the pSG5hVDR phagemid was completed using T4 DNA polymerase in an in vitro reaction. Double-stranded phagemid was then propagated in a bacterial host and isolated colonies were screened for the presence of pSG5hVDR phagemids containing the desired mutation by DNA sequencing.

Cell Culture, Transfections, and Transcription Assay

COS-7, an SV40-transformed African Green monkey kidney cell line, was obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts, Calabasas, CA). Cells were transfected with the appropriate expression and/or reporter plasmid for 18 h using the calcium phosphate-DNA coprecipitation technique without glycerol shock [Kingston, 1990]. After transfection, cells were washed twice with DMEM and incubated in either the presence of 10 nM $1,25(OH)_2D_3$ hormone or ethanol vehicle for an additional time

period dictated by the type of experiment (transcription assay or cell fractionation). Transcriptional activity of hVDR mutants was measured in COS-7 cells cotransfected with the appropriate pSG5hVDR expression plasmid (7.5 µg) and the reporter plasmid $(CT4)_4$TKGH (5 µg) containing four copies of the rat osteocalcin vitamin D responsive element (VDRE) [Terpening et al., 1991] inserted upstream of the viral thymidine kinase promoter-growth hormone reporter gene (Nichols Institute, San Juan Capistrano, CA). The media from these cells were assayed for the expression of human growth hormone by radioimmunoassay using a commercial kit (Nichols Institute Diagnostics) 48 h after completion of the transfection, and cells were harvested at that time for immunoblot analysis.

Immunoblotting

Transfected COS-7 cells were lysed directly in 2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, and 20% glycerol, and 40 µg of cellular protein were run on 5-20% gradient SDS/polyacrylamide gels. After electrophoretic fractionation, proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Transblot apparatus (Bio-Rad Laboratories) in 25 mM Tris-HCl, pH 7.4, 192 mM glycine, 0.01% SDS, and 20% methanol. Immunodetection of bound hVDR proteins was then performed using the 9A7γ monoclonal anti-VDR antibody [Pike et al., 1983]. After the first antibody treatment, the Immobilon-P membrane was washed and treated at room temperature for 3 h with goat anti-rat IgG conjugated to biotin. After four 15-min washes, the blot was incubated with avidin-alkaline phosphatase for 1 h and then was washed four more times, followed by a fifth wash with biotin blot buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100). Finally, the blot was exposed to color reagent containing 50 µg/ml of 5-bromo-4-chloro-3-indolyl-phosphate and 100 µg/ml of 4-nitro blue tetrazolium chloride. The color reaction was stopped by washing with distilled water.

Cell Fractionation

After allowing for maximal receptor expression in the absence of $1,25(OH)_2D_3$, 48 h following the completion of transfection, COS-7 cells expressing wild-type or mutant hVDRs (2×10^7 cells/150 mm plate) were washed with DMEM and treated for 2 h at 37°C with ethanol vehicle alone, or with a final concentration of 10 nM $1,25(OH)_2D_3$, followed by harvesting and resuspension in ice-cold Dulbecco's phosphate buffered saline (PBS). The washed cell pellet was suspended in 1.5 ml of swelling buffer (0.1 M Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 5 mM dithiothreitol), incubated on ice for 10 min, and then disrupted by Dounce homogenization with a tight fitting pestle (type B). The homogenate was subjected to centrifugation at 1,100 x *g* for 15 min and the resultant supernatant was referred to as "cytosol extract." The crude nuclear pellet was suspended in 0.5 ml of KETD-0.3 (0.3 M KCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol) and extracted for 30 min. Soluble nuclear proteins, referred to as "nuclear extract," were obtained by centrifugation of the nuclear suspension at 27,000 x *g* for 10 min. Immunodetection of hVDRs in cytosolic and nuclear extracts was performed as described above.

Immunofluorescent Staining of Human Vitamin D Receptor

COS-7 cells (3×10^5) were plated on sterile #1 cover glasses and grown in a 3 cm dish with 2 ml DMEM containing 10% FBS in a CO_2 incubator. The next day, cells were rinsed twice with serum-free DMEM and incubated for 4 h at 37°C with a 1 ml mixture of wild-type or mutant pSG5hVDR cDNA (1 µg) and lipofectin (10 µl, GIBCO), followed by addition of 1 ml of DMEM containing 20% FBS. Cells were incubated at 37°C for 24 h, the medium was changed to fresh DMEM containing 10% FBS, and cells were grown for an additional 48 h. When testing the effect of $1,25(OH)_2D_3$ (10 nM), the ligand was present for 2 h at 37°C prior to fixing the cells. The cells were then washed twice with PBS, fixed with 3% formaldehyde in PBS for 10 min at room temperature, and washed three times with PBS followed by permeabilization with 0.2% Triton X-100 in PBS for 2 min. Next, the cells were rinsed four times with PBS and incubated with 10 mg/ml of rat anti-human VDR monoclonal antibody (9A7γ) or rabbit anti-sheep IgG in PBS containing 3% BSA for 1 h at room temperature and then washed three times with PBS. The cover glass was subsequently treated with biotinylated goat anti-rat IgG (10 mg/ml) for 30 min at room temperature. Cells were washed three times with PBS and stained

with FITC-avidin (5 mg/ml) for 15 min at room temperature. The cover glass was rinsed three times with PBS and mounted on a slide with PBS containing 10% glycerol. Cells were observed by confocal laser scanning microscopy (BIO-RAD MRC600 image analyzer). The subcellular distribution of hVDR in cytosol and nuclei was quantitated using a laser microscopy program.

RESULTS

Conserved Basic Amino Acid Sequences Among the Nuclear Proteins

The molecular action of the $1,25(OH)_2D_3$ ligand is predominantly in the nucleus [Haussler et al., 1995; Stumpf, 1995], and because VDR has been found to occur as a nuclear protein via immunocytochemistry [Berger et al., 1988; Bidwell et al., 1994; Clemens et al., 1988; Colston et al., 1994; Milde et al., 1989; Tuohimaa et al., 1992], it is apparent that VDR contains one or more nuclear localization signals. NLSs often are comprised of basic amino acids and, in addition to a series of small segments of basic amino acids distributed between residues 70 and 111 of hVDR [Luo et al., 1994], there exists an array of five basic amino acids between residues 49 and 55 (Fig. 1). As shown in Figure 1 (lower right), the ensemble of these five positively charged groups is unique to VDR, with the C-terminal portion not well conserved in other members of the superfamily of nuclear receptors. In contrast, this stretch of amino acids is absolutely conserved in all species of VDR sequenced to date [Li et al., 1997], suggesting that it may perform important and perhaps novel functions. One such role illustrated in Figure 1 is to establish apparent base contact sites for VDRE binding [Hsieh et al., 1993]. A second function of these positively charged residues is to provide for substrate recognition by PKC, which catalyzes the phosphorylation of ser-51 [Hsieh et al., 1993]. Such phosphorylation of ser-51 (Fig. 1), which is imbedded in the basic stretch, negatively regulates DNA binding and may also affect nuclear retention [Hsieh et al., 1993]. Precedents exist for the presence of kinase phosphorylation sites within bipartite nuclear localization signals [Moll et al., 1991], with these and other phosphorylation/dephosphorylation reactions modulating nuclear translocation [Hennekes et al., 1993; Jans, 1995; Ruff and Leach, 1995; Vancurova et al., 1995]. Comparison of the amino acid sequence within this cluster of basic residues (49-55) in hVDR with those of well-characterized basic NLSs in nuclear proteins such as SV40 T-antigen [Kalderon et al., 1984], yeast histone H2B [Moreland et al., 1987], SV40 Vp3 [Clever and Kasamatsu, 1991], *Xenopus laevis* nucleoplasmin [Bürglin and De Robertis, 1987], and Jun protein [Chida and Vogt, 1992] reveals some sequence similarity (Fig. 1, lower left), especially if one considers conservative replacement of positively charged groups and accommodates the insertion of a serine-methionine pair within the cluster. Taken together, this information suggests that the RRSMKRK region is a potential NLS for hVDR, a hypothesis that was tested by constructing the three mutants illustrated in Figure 1 (top) for further studies, namely, R49WR50G, K53QR54GK55E, and ΔRRSMKRK. In the ΔRRSMKRK mutant, the RRSMKRK region was completely deleted, whereas the basic residues on either side of the PKC site were replaced with other amino acids in the R49WR50G double- and K53QR54GK55E triple-mutant. The biochemical and biological properties of these VDR mutants were studied, with the goal of elucidating a possible third role for RRSMKRK involving nuclear localization.

Transcriptional Activation and VDRE-Binding Capacities of Wild-Type and Mutant hVDRs

The functional activity of mutant hVDRs lacking all or part of the putative NLS was first assessed in cotransfection assays. COS-7 cells, which express low levels of endogenous VDR that are undetectable in immunoblots (see Fig. 2B, lane 1 and Fig. 3, first two lanes of each panel), were transfected with the pSG5hVDR expression vector and a reporter plasmid possessing several copies of a VDRE [Terpening et al., 1991]. A comparison of transcriptional activity of the wild-type and mutant hVDRs is shown in Figure 2A. The results indicate that in all three of the mutants, R49WR50G, K53QR54GK55E, and ΔRRSMKRK, transcriptional activity in response to $1,25(OH)_2D_3$ is abolished. As demonstrated by immunoblot analysis (Fig. 2B), the observed transcriptional inhibition was not a consequence of a decrease in mutant hVDR expression. Thus altering the basic cluster of amino acids between the two zinc fingers of hVDR affects neither expression nor stability of the receptor, but renders it transcriptionally

inactive. This observation is not surprising given the fact that residues in a presumed α-helix crucial for DNA recognition (Fig. 1) have been disrupted, and we have previously observed that none of the three mutants in question associates with a VDRE as a heterocomplex with RXR, in vitro [Hsieh et al., 1993]. However, the integrated assay of transcriptional activation in intact cells (Fig. 2A) is likely more sensitive and relevant to biological function than is the in vitro gel mobility shift assay. Furthermore, the transcription results raise the question of whether hVDRs with mutations in the basic cluster of residues between amino acids 49 and 55 are able to access the nuclear compartment in order to gain the potential for DNA binding, in vivo.

Analysis of the Subcellular Distribution of Wild-Type and Mutant hVDRs by Western Blot

To test whether the alterations in this positively charged region of the receptor influence nuclear transfer, the subcellular partitioning of wild-type, R49WR50G, K53QR54GK55E, and ΔRRSMKRK mutant hVDRs was examined by immunoblotting after cellular fractionation of transiently transfected COS-7 cells. Nuclear extracts and cytoplasmic fractions from COS-7 cells expressing hVDRs were isolated and examined separately following incubation of intact cells in the presence or absence of $1,25(OH)_2D_3$. The results in Figure 3 reveal that wild-type hVDR is predominately localized in the nucleus, especially following exposure of cells to

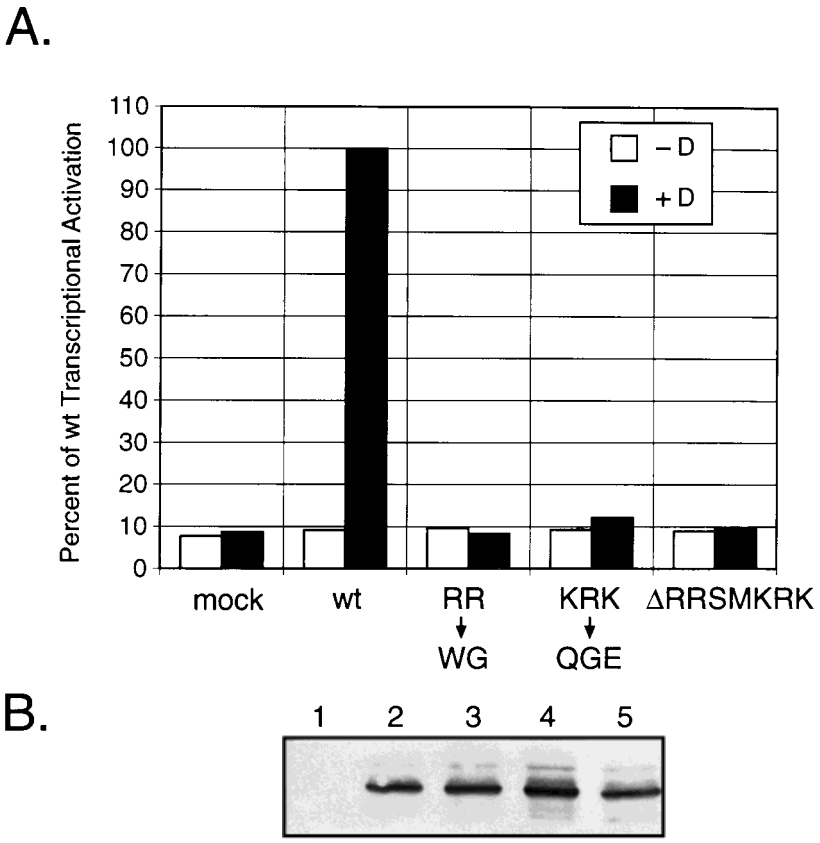

Fig. 2. Transcriptional activity and expression of the wild-type (wt) and mutant hVDRs. **A**. The ability of transfected RR, KRK, and ΔRRSMKRK mutants to activate transcription of a vitamin D-responsive human growth hormone reporter gene construct. The indicated hVDR expression vectors (7.5 μg) were cotransfected into COS-7 cells along with reporter plasmid $(CT4)_4$TKGH (5 μg) containing the VDRE. Cells were transfected by the calcium phosphate-DNA coprecipitation method for 18 h and treated for 48 h posttransfection with 10 nM $1,25(OH)_2D_3$ (abbreviated as D) or ethanol as a control. Medium was assayed for the expression of human growth hormone (an index of hVDR-mediated transactivation) by radioimmunoassay. Mock transfected cells received empty (pSG5) expression vector. **B**. Expression of wild-type and mutant hVDRs via immunoblot assessment 48 h after the completion of transfection. **Lane 1** represents extract from mock transfected cells; **lanes 2-5** are derived from the following respective transfections: wild-type hVDR, RR-, KRK-, and ΔRRSMKRK-mutant receptors.

1,25$(OH)_2D_3$, whereas the majority of R49WR50G and ΔRRSMKRK mutant hVDRs accumulated in the cytosolic compartment. A similar cytosolic localization of K53QR54GK55E was also observed (data not shown). From these preliminary data, the RRSMKRK basic region does appear to contribute significantly to the nuclear retention of hVDR and may therefore contain an important NLS. However, appreciable VDR is still able to enter the nuclear compartment in the mutants (Fig. 3), and, except in the case of wild-type receptor, it is difficult to detect an effect of 1,25$(OH)_2D_3$ ligand to enhance nuclear uptake utilizing this assay. Hormone-independent nuclear translocation of steroid hormone receptors is observed for estrogen and progesterone receptors [King and Greene, 1984; Welshons et al., 1984], but the major concern with respect to the current data is that cross-contamination or leeching may occur between cytoplasm and nuclei during fractionation procedures and extract preparations. Therefore, another strategy was employed to confirm the observations presented in Figure 3, involving the use of direct fluorescence microscopy to detect the cellular distribution of transfected hVDR in COS-7 cells.

Immunocytochemistry of Wild-Type and Mutant hVDRs

The subcellular distribution of expressed VDR in the absence of 1,25$(OH)_2D_3$, and the effect of replacement or deletion of the basic cluster of amino acids, was investigated by immunocytochemistry. Initially, control studies were performed in which COS-7 cells were examined using biotinylated goat anti-rat IgG and FITC-avidin (Fig. 4A), and rabbit anti-sheep IgG, biotinylated goat anti-rat IgG and FITC-avidin (Fig. 4B), with very minimal background staining observed in each case. With mock transfection of COS-7 cells (Fig. 4C), a small degree of endogenous VDR staining in nuclei was observed (Fig. 4C) when cells were treated with

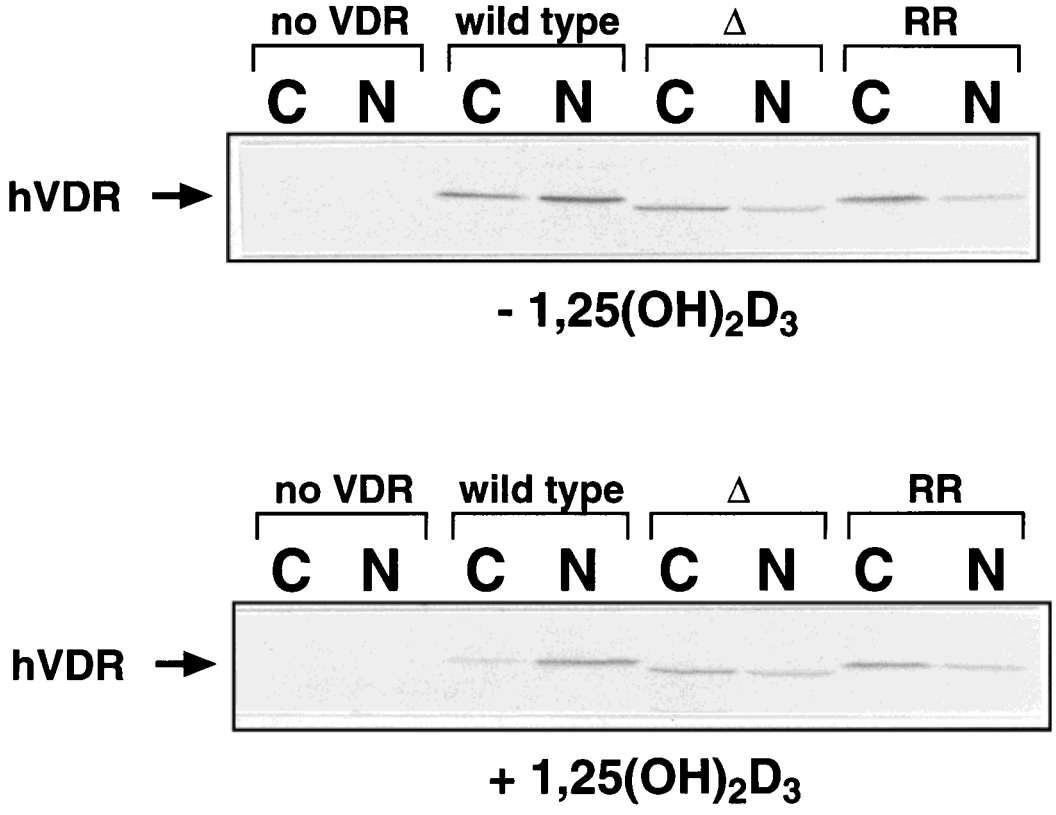

Fig. 3. Subcellular distribution of wild-type and mutant hVDRs as assessed by Western blotting of cell subfractions. Nuclear and cytoplasmic extracts from transfected COS-7 cells treated with ethanol (top panel) or 10 nM 1,25$(OH)_2D_3$ (bottom panel) were prepared as described in Materials and Methods under Cell Fractionation. Forty μg protein equivalents of nuclear (**lanes N**) or cytosolic extracts (**lanes C**) were loaded onto each lane of a 10% SDS-polyacrylamide gel followed by electrophoresis and immunoblot analysis. Mock-transfected COS-7 cells (first two lanes of each panel) posses undetectable levels of hVDR.

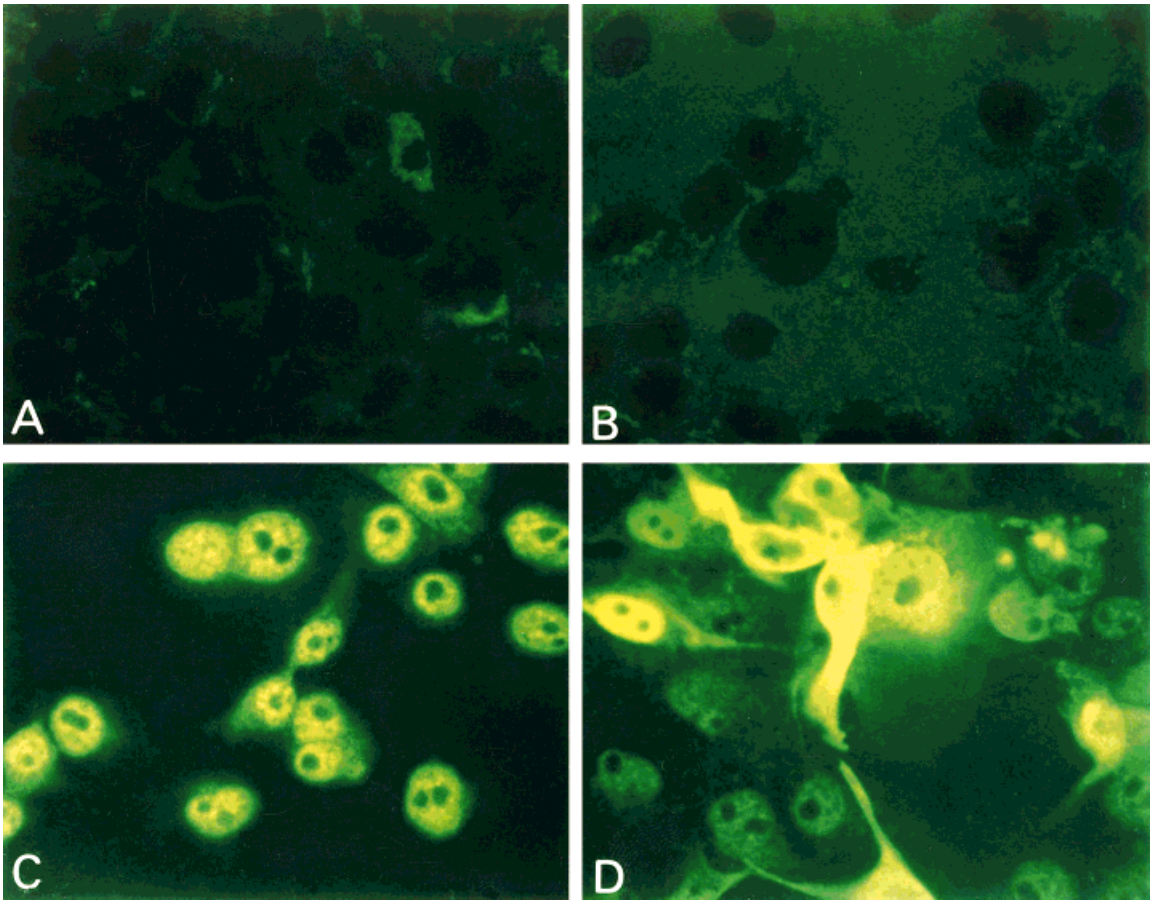

Fig. 4. Immunofluorescent staining of COS-7 cells with control and anti-VDR antibodies. Untransfected COS-7 cells were stained by incubation with (**A**) biotinylated goat anti-rat IgG and FITC-avidin (negative control I) or (**B**) rabbit anti-sheep IgG, biotinylated goat anti-rat IgG and FITC-avidin (negative control II). COS-7 cells transfected with either an empty (mock, **C**) or hVDR-containing (pSG5hVDR, **D**) expression plasmid were stained by treatment with rat anti-VDR monoclonal antibody 9A7γ, biotinylated goat anti-rat IgG, and FITC-avidin.

rat anti-hVDR, biotinylated goat anti-rat IgG and FITC-avidin. Transfected wild-type hVDR, under the same conditions in COS-7 cells (Fig. 4D), exhibits strong nuclear immunostaining in the absence of $1,25(OH)_2D_3$, with the nucleolar regions clearly excluded (Figs. 4D, 5A). Note that not all cells display intense immunostaining, likely because only a small percentage of cells actually take up the DNA plasmid during the transfection procedure. Nevertheless, these results (Figs. 4D, 5A) reveal dramatic nuclear accumulation of overexpressed hVDR, suggesting that hVDR undergoes primarily a $1,25(OH)_2D_3$-independent translocation from the cytoplasmic/perinuclear region to the nucleus.

In sharp contrast to apparent $1,25(OH)_2D_3$-independent nuclear targeting of wild-type hVDR, each of the R49WR50G, K53QR54GK55E, and ΔRRSMKRK mutants displayed predominantly cytoplasmic localization (Fig. 5B-D). Strikingly, in the stained cells pictured in Figure 5, nuclear/cytoplasmic receptor distribution was 44, 0.03, 0.04, and 0.03 for wild-type, R49WR50G, K53QR54GK55E, and ΔRRSMKRK, respectively. This near qualitative inhibition of VDR nuclear retention in basic cluster mutants may result from the elimination of a required protein-protein recognition between this putative NLS and nuclear pore associated proteins, or possibly from a conformational constraint introduced into the receptor by the mutations. The data in Figures 4D and 5 provide evidence in a small number (2-4) of efficiently transfected cells for a marked qualitative influence on VDR nuclear localization exerted by the basic cluster of amino acids located between the zinc fingers in the DNA recognition α-helix. To extend this finding to a quantitative level and to evaluate the role of $1,25(OH)_2D_3$ on VDR

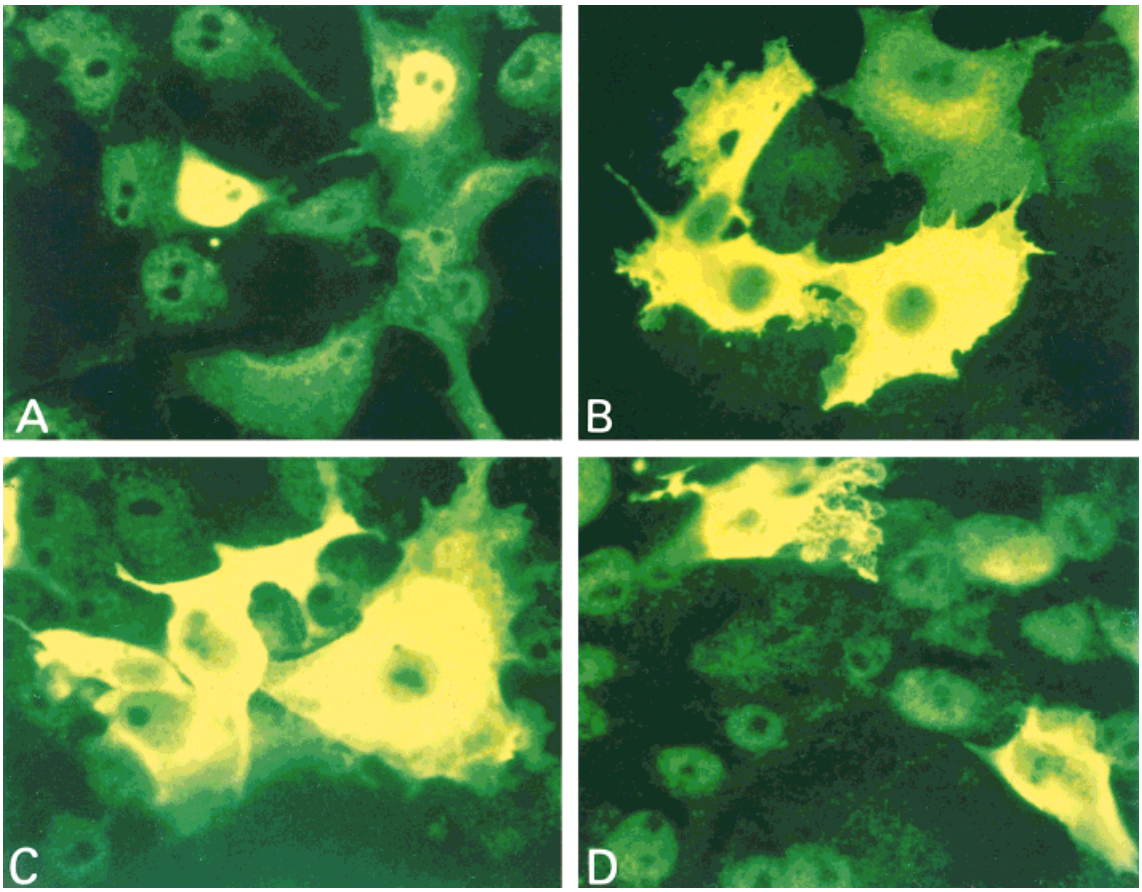

Fig. 5. Immunofluorescent detection of wild-type and mutant hVDRs in transfected cells. COS-7 cells were transiently transfected with (**A**) wild-type hVDR, (**B**) mutant R49WR50G, (**C**) mutant K53QR54GK55E, or (**D**) mutant ΔRRSMKRK, and immunostained by incubation with rat anti-VDR monoclonal antibody followed by biotinylated goat anti-rat IgG and FITC-avidin.

distribution in intact cells, we next treated COS-7 cells with 10 nM $1,25(OH)_2D_3$ for 2 h and carried out quantitative immunofluorescent staining analysis.

At least 30 (and up to 43) effectively transfected cells were observed for wild-type or receptor mutants in this experiment, and each cell was scored as to whether the localization of VDR was predominantly nuclear or cytoplasmic. The results are presented in Table I and indicate that for wild-type hVDR without $1,25(OH)_2D_3$ ligand treatment, 70% of the analyzed cells stained more intensely in the nucleus, whereas following treatment of cells with ligand, 100% of the viewed cells stained mainly in nuclei. Importantly, by such a quantitative analysis (Table I), all three mutant hVDRs are retained in the cytoplasm of the majority (58-80%) of cells viewed. These data in intact cells (Fig. 5 and Table I) strengthen the conclusion reached from Figure 3 that efficient VDR nuclear localization depends upon an intact basic cluster within the DNA recognition α-helix. Finally, in the more extensive survey summarized in Table I, it is clear that, although still suboptimal, nuclear translocation is enhanced by $1,25(OH)_2D_3$ in the case of all three mutant hVDRs. Figure 6 illustrates representative data for the RR mutant *vs.* wild-type hVDR from which the results in Table I were derived. With the RR mutant, nuclear accumulation is precluded (Fig. 6A), whereas slight but significant reversal of this impairment occurs when cells are exposed to $1,25(OH)_2D_3$ (Fig. 6B). Although more subtle because constitutive nuclear localization is already very high, the hormone (Fig. 6D) also increases nuclear translocation of wild-type hVDR over that seen in the absence of ligand (Fig. 6C). The conclusion is that the basic cluster between residues 49 and 55 of

TABLE I. Distribution of VDR in Various Transfectants

VDR plasmid	Predominant localization	+Ethanol cell number (%)	+1,25$(OH)_2D_3$ cell number (%)
Wild type	Nucleus	21 (70)	30 (100)
	Cytoplasm	9 (30)	0 (0)
RR → WG	Nucleus	12 (40)	17 (57)
	Cytoplasm	18 (60)	13 (43)
KRK → QGE	Nucleus	6 (20)	13 (37)
	Cytoplasm	24 (80)	22 (63)
ΔRRSMKRK	Nucleus	15 (42)	23 (53)
	Cytoplasm	21 (58)	20 (47)

hVDR constitutes a constitutive nuclear retention sequence, and 1,25$(OH)_2D_3$ is capable of further augmenting nuclear accumulation of hVDR by a mechanism independent of this sequence.

DISCUSSION

The major pathway for nuclear import of proteins is mediated by nuclear localization signals characterized by one or more clusters of basic amino acids lacking a strict consensus sequence [Dingwall and Laskey, 1991]. Nuclear import apparently consists of at least three major steps: (1) the binding of the NLS in the transported protein to a mobile carrier (heterodimeric importin) that associates with a recognition site on the cytoplasmic face of the nuclear pore complex, (2) GTP-dependent translocation of the complex into the nucleus, and (3) release of the imported nuclear protein followed by recycling of the carrier within the nuclear pore complex [Nigg, 1997]. The nuclear pore complex may contain fibrils that extend in both directions that could function as either static rails or molecular motors to propel proteins into the nucleus [Dingwall and Laskey, 1992]. That myosin-like cytoskeletal elements are proposed to constitute part of the translocation apparatus could be relevant to recent data on VDR obtained utilizing microwave energy to rapidly fix cells. With this technique [Barsony et al., 1997], it was observed that following 1,25$(OH)_2D_3$ treatment, cytoplasmic VDR redistributed within seconds to foci aligned along fibers oriented in a radial fashion toward the nucleus; in the first minute, VDR entered the nucleus along this microtubular-like network. Such an energy-dependent, rapid translocation of VDR may occur also with unliganded receptor, explaining the partial nuclear localization of VDR when 1,25$(OH)_2D_3$ is absent (Table I). Studies employing autoradiographic localization of tritiated VDR ligand [Stumpf, 1995] and a fluorescent analog of 1,25$(OH)_2D_3$ [Barsony et al., 1997], as well as immunochemical investigations [Bidwell et al., 1994; Colston et al., 1994; Tuohimaa et al., 1992], clearly reveal that the majority of hormone occupied VDR resides in the nucleus (Table I).

In the original model of steroid hormone action, the ligand was thought to bind to its cognate receptor in the cytoplasm of target cells, with the resulting ligand-receptor complex then entering the nucleus and recognizing a responsive element for inducing or repressing specific gene transcription [Jensen et al., 1972]. In contrast, the current data derived from biochemical and immunofluorescent staining experiments with overexpressed receptor suggest that unoccupied VDR may predominantly reside in the nucleus, which is consistent with subcellular fractionation data [Walters et al., 1980], as well as a number of immunocytochemical studies [Berger et al., 1988; Bidwell et al., 1994; Clemens et al., 1988; Colston et al., 1994; Milde et al., 1989; Tuohimaa et al., 1992], all of which probed endogenous concentrations of receptor. Such a result is not without precedent, since the unoccupied estrogen receptor [King and Greene, 1984; Welshons et al., 1984], progesterone receptor [Guiochon-Mantel et al., 1989; Perrot-Applanat et al., 1985], and thyroid hormone receptor [Samuels and Tsai, 1973] also have been reported to be nuclear in subcellular location. In the case of estrogen receptor, cytoplasts prepared by enucleating GH_3 cells contained little estrogen-binding activity, whereas most of the unoccupied estrogen receptors were present in the nuclear fraction [Welshons et al., 1984]. It is therefore concluded that a major role of steroid and thyroid hormone ligands described above is not to effect nuclear translocation, but instead may be to facilitate high affinity homodimeric or heterodimeric binding of receptors to their respective responsive elements in DNA, concomitant with conformational changes that expose transactivation surfaces. However, nuclear localization of glucocorticoid receptors is ligand-dependent [Picard and Yamamoto, 1987], indicating that nuclear concentration of unoccupied receptors is not a universal finding

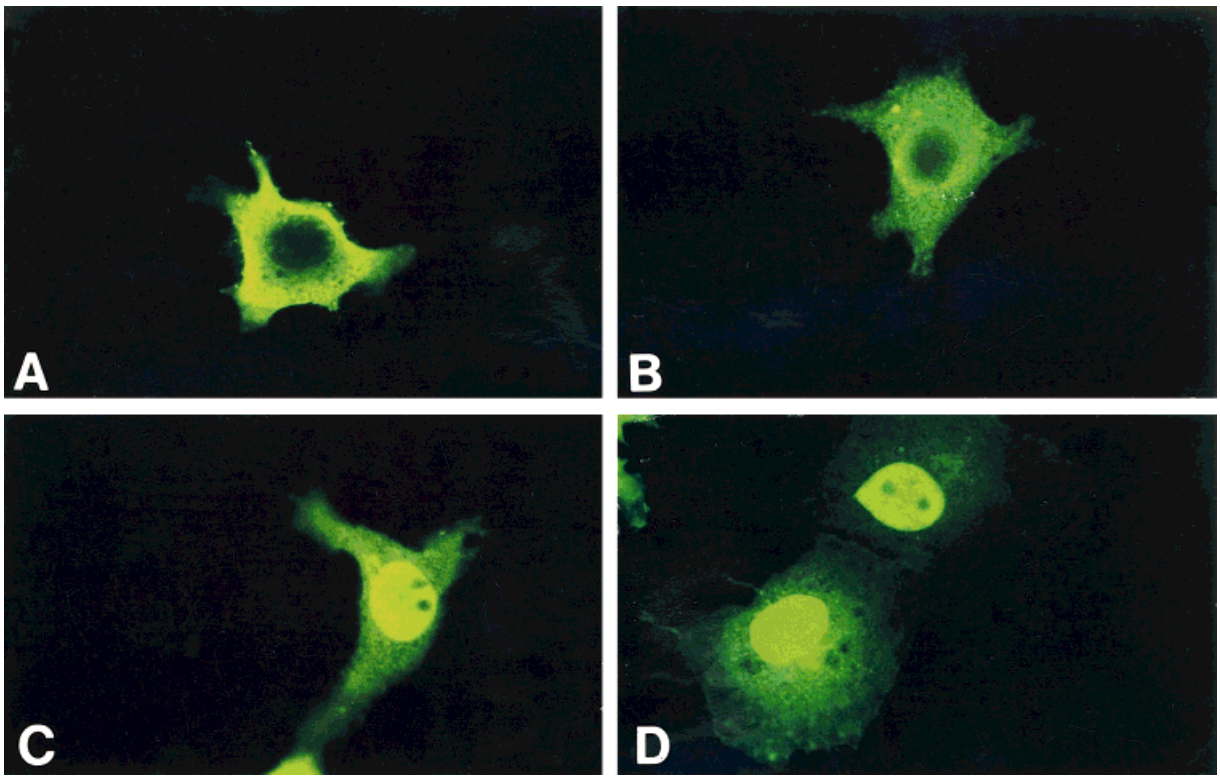

Fig. 6. Effect of $1,25(OH)_2D_3$ ligand on the distribution of hVDR as detected by immunofluorescent microscopy. COS-7 cells were transfected with the following constructs ± 10 nM $1,25(OH)_2D_3$ for 2 h and analyzed as described in Materials and Methods. **A**: R49WR50G – $1,25(OH)_2D_3$, **B**: R49WR50G + $1,25(OH)_2D_3$, **C**: wild-type hVDR – $1,25(OH)_2D_3$, and **D**: wild-type hVDR + $1,25(OH)_2D_3$.

for members of the nuclear receptor superfamily.

The NLS in VDR that we have identified in the present report, RRSMKRK, fits the general criteria for nuclear targeting sequences, namely, it is <12 amino acids in length, and it contains a high proportion of positively charged amino acids. Although the SV40 large T-antigen possesses the prototypic NLS, e.g., PKKKRKV, later studies of nuclear proteins like nucleoplasmin and N1 revealed that bipartite basic stretches, in which both clusters of positively charged residues are required for nuclear import, represent a second paradigm for the NLS [Dingwall and Laskey, 1991; Nigg, 1997]. In the bipartite motif, the spacer is usually 10 amino acids, but this intervening region ranges as high as 37 residues in the case of an adenovirus DNA-binding protein [Dingwall and Laskey, 1991]. Figure 7 illustrates schematically the position of the RRSMKRK nuclear localization signal (denoted NLS-1) in hVDR, as well as the location of an apparent bipartite nuclear targeting sequence (denoted NLS-2) reported previously [Luo et al., 1994]. A comparison of the two proposed VDR NLSs with other published nuclear translocation signals in members of the nuclear receptor superfamily is also shown in Figure 7. In rat glucocorticoid receptor (rGR), two distinct nuclear localization signals, NLS1 and NLS2, are defined. NLS1 maps to a 28 amino acid segment closely associated, but not coincident with, the DNA binding domain; NLS2 resides within a 256 amino acid region (residues 540-795 in Fig. 7) that includes most of the C-terminal hormone binding domain [Picard and Yamamoto, 1987]. In human androgen receptor (hAR), a nuclear targeting signal similar in sequence and position to that in GR (NLS1) and homologous to the SV40 T-antigen was required for androgen-induced nuclear uptake of wild-type AR [Simental et al., 1991]. In the case of rabbit progesterone receptor (rPR), deletion experiments revealed that removal of residues 638-642 (RKFKK) caused the rPR to localize entirely in the cytoplasm [Guiochon-Mantel

et al., 1989]. In the human estrogen receptor (ER), the nuclear localization domain between amino acids 256 and 303 [Picard et al., 1990] contains a basic region (residues 256-260) similar to that in the glucocorticoid receptor, as well as two other stretches of basic residues (Fig. 7). Finally, the thyroid hormone receptor (Fig. 7) possesses a pair of basic clusters positioned similarly to those in ER, with the N-terminal motif (KRVAKRK) resembling the RRSMKRK NLS-1 identified for VDR in the present communication.

Therefore, when the NLSs in VDR are compared to those in other nuclear receptors (Fig. 7), it is clear that, like signal sequences for secretion of proteins into the endoplasmic reticulum, no sequence consensus exists. Even positioning of the NLSs varies within the superfamily, yet a general theme emerges with the occurrence of at least one NLS just C-terminal of the zinc finger, DNA-binding region. Like VDR, the GR possesses 2 NLSs, although both the AR and PR appear to employ a single basic amino acid cluster for nuclear accumulation. The ER is quite similar to VDR, with three stretches of positively charged residues within the complex, 48 amino acid NLS; VDR has three such stretches within the 56 amino acids encompassing both NLSs. TR [Dingwall and Laskey, 1991] possesses a classic bipartite candidate NLS (Fig. 7) that is not conserved in its close relative, VDR. As a result, we propose that instead of a more C-terminal region being significant in nuclear translocation as it is in GR, AR, PR, ER, and TR, this functional domain in VDR is replaced in part with a unique sequence (Fig. 1, lower right) that occurs within the DNA recognition helix. This sequence,

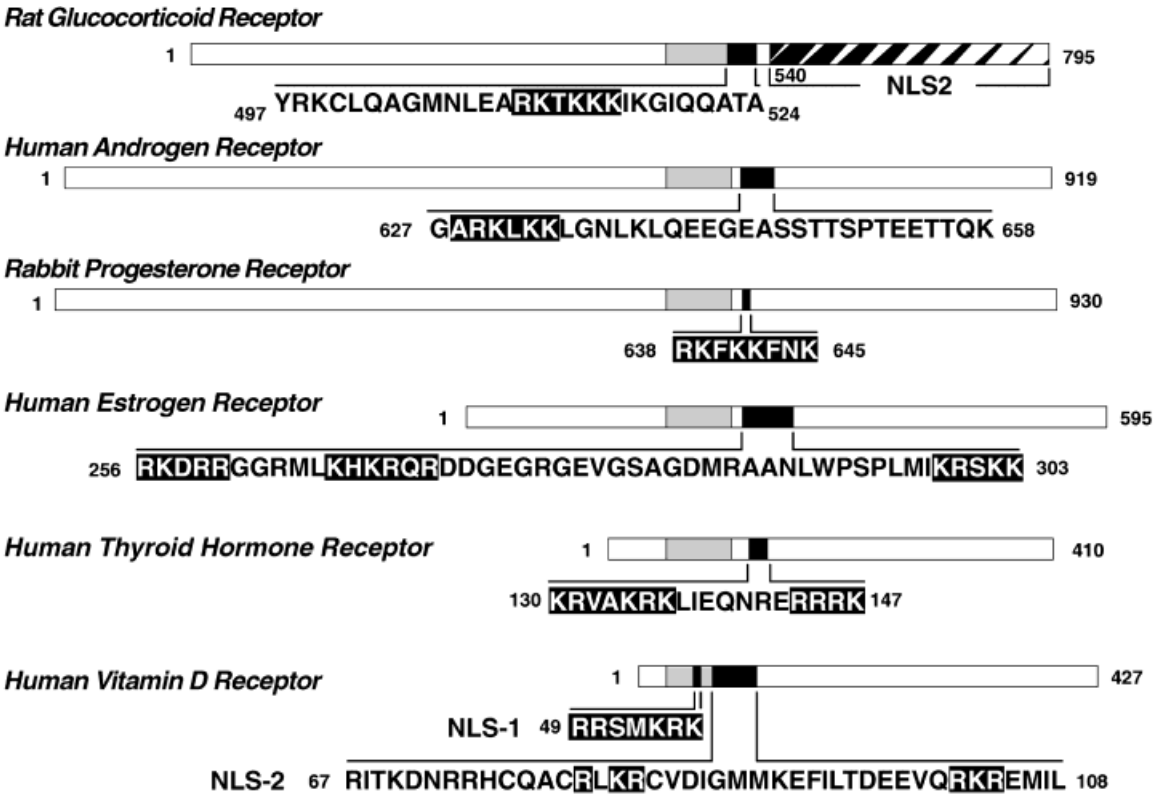

Fig. 7. Comparison of amino acid sequences identified as nuclear localization signals in various members of the nuclear receptor superfamily. The numbers refer to the amino acid position of each sequence. The DNA binding domain is indicated by a light gray box, whereas the nuclear localization region is depicted by a black box (the long, C-terminal NLS in the glucocorticoid receptor is "tiger" striped). Sequences within the nuclear localization regions are listed in black single letter code, whereas stretches of basic residues that likely mediate nuclear translocation are illustrated as black boxes with white single letters for the amino acids. Nuclear receptors shown from the top are: rat glucocorticoid receptor [Picard and Yamamoto, 1987]; human androgen receptor [Simental et al., 1991]; rabbit progesterone receptor [Guiochon-Mantel et al., 1989]; human estrogen receptor [Picard et al., 1990]; human thyroid hormone receptor α [Benbrook and Pfahl, 1987]; human vitamin D receptor [Baker et al., 1988; Luo et al., 1994].

RRSMKRK, contains the requisite number of basic amino acids, but differs from the SV40-T-antigen prototype in that it consists of a mini-bipartite motif with two residues (SM) separating the positively charged amino acids (Fig. 1, lower left). A similar sequence (KILKKRHI) was recently characterized as an NLS in cGMP-dependent protein kinase, in which nuclear translocation is triggered by the cyclic nucleotide ligand [Gudi et al., 1997].

There are numerous precedents for the occurrence of two NLSs in transcription factors, including the basic helix-loop-helix proteins MyoD [Vandromme et al., 1995] and USF2 [Luo and Sawadogo, 1996]. At present, we do not know what the functional interrelationship is between NLS-1 and NLS-2 of VDR, which are necessary for nuclear targeting of the parent protein and sufficient to direct a nonnuclear protein to the nucleus, respectively. Neither NLS-1 nor NLS-2 appears to be $1,25(OH)_2D_3$-dependent in terms of its action, and the data in Table I reveal that the sterol ligand enhances nuclear retention of NLS-1 mutant VDRs almost as efficiently as it does the wild-type receptor. Such findings are not surprising because both NLS-1 and NLS-2 reside in the DNA-binding domain of hVDR. In the case of transcription factor USF2, the basic region involved in specific DNA recognition contains an intrinsic NLS [Luo and Sawadogo, 1996], analogous to NLS-1 within the DNA recognition helix of VDR (Fig. 1). Moreover, recent results with GR imply that the entire DNA binding domain, as a functional unit, may be required for nuclear transfer and optimal retention in the nucleus [Sackey et al., 1996]. Some insight into the relative significance of NLS-1 and NLS-2 in VDR can be gleaned from the occurrence of natural point mutations in each of the putative domains that renders the patient resistant to $1,25(OH)_2D_3$ and elicits the phenotype of hereditary hypocalcemic vitamin D resistant rickets (HVDRR). In the case of NLS-1, a unique alteration of Arg-50 to glutamine (R50Q) [Saijo et al., 1991] produces an hVDR phenotype of normal cytoplasmic ligand binding and severely impaired nuclear uptake, consistent with the present conclusion that NLS-1 is crucial for the transfer of VDR into the nucleus. Similarly, mutation of Arg-80 to glutamine (R80Q) [Barsony et al., 1997] in NLS-2 of hVDR prevents nuclear accumulation of the receptor as monitored by localization of a fluorescent VDR ligand. Because of the functional impairment of nuclear translocation in naturally occurring mutants of hVDR, R50Q, and R80Q, residing within the DNA-recognition and phosphate-backbone-binding helices, respectively, we conclude that both exposed positively charged helices are required for the nuclear localization of VDR. We also argue that it is not DNA-binding, per se, that endows VDR with the property of nuclear translocatability. This latter conclusion is based upon artificial mutant S51G [Hsieh et al., 1993], which localizes normally to the nucleus, but does not bind to the VDRE in DNA. This mutant proves that it is not simply DNA attraction of the recognition helix/NLS-1 domain that causes nuclear retention, but that the basic residues flanking serine-51 actually mediate nuclear translocation as well as subsequent DNA binding.

The current studies demonstrate that the RRSMKRK sequence between the two zinc fingers of the human vitamin D receptor is important in determining its nuclear localization. Interestingly, it has been demonstrated that the serine-51 residue located in this domain is a protein kinase C phosphorylation site in vitro and in vivo [Hsieh et al., 1991]. One function of hVDR phosphorylation by PKC at serine-51 may involve an attenuation of DNA binding activity [Hsieh et al., 1993], but because this residue is also located in the presently defined nuclear retention domain, phosphorylation of serine-51 may also represent a "cross-talk" mechanism for modulating hVDR entry into the nucleus. Indeed, phosphorylation of lamin B by PKC strongly inhibits transport into the nucleus [Hennekes et al., 1993]. In the case of SV40 T antigen, there are a number of phosphorylation sites that are substrates for casein kinase II or the cell cycle-dependent cdc2 kinase. Mutation of consensus casein kinase II phosphorylatable serine residues 111 and 112 reveals that these two amino acids are required for the known enhanced rate of SV40 T antigen nuclear uptake [Hubner et al., 1997; Rihs et al., 1991]. However, phosphorylation of SV40 T antigen threonine-124, a substrate for cdc2 kinase, reduces the maximal extent of nuclear accumulation [Jans et al., 1991], a phenomenon similar to that occurring when the yeast transcription factor SWI5 is phosphorylated by cdc28 kinase [Jans, 1995]. In fact, analogous to hVDR, the nuclear localization signal in SWI5 is bipartite in nature, with an imbedded cdc28 kinase

site that mediates attenuation of nuclear import [Moll et al., 1991]. Thus the present research identifies hVDR as the first example of a nuclear receptor superfamily member that possesses a mini-bipartite NLS,which is apparently regulatable by phosphorylation/dephosphorylation. Support for this conclusion is supplied by our previous observation [Hsieh et al., 1993] that the S51D mutant hVDR, which contains a constitutive negative charge within NLS-1, neither localizes to the nucleus nor binds to DNA. The current results provide new insight into steroid/thyroid hormone action in target cells by defining the forces involved in VDR nuclear retention and may facilitate the understanding of the mechanism of ligand-inducible transcription by the vitamin D hormone.

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