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Identification of Amino Acid Sequence in the Hinge Region of Human Vitamin D Receptor That Transfers a Cytosolic Protein to the Nucleus*

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The localization of human vitamin D receptor (VDR) in the absence of its ligand 1,25-dihydroxyvitamin D_3 was investigated using chimera proteins fused to green fluorescent protein (GFP) at either the N or C terminus, and the nuclear localization signal (NLS) was identified. Plasmids carrying the fusion proteins were transiently or stably introduced into COS7 cells, and the subcellular distribution of the fusion proteins was examined. GFP-tagged wild-type VDRs were located predominantly in nuclei but with a significant cytoplasmic presence, while GFP alone was equally distributed throughout the cells. 10^{-8} M 1,25-dihydroxyvitamin D_3 promoted the nuclear import of VDR in a few hours. To identify the NLS, we constructed several mutated VDRs fused to GFP. Mutant VDRs that did not bind to DNA were also localized predominantly in nuclei, while the deletion of the hinge region resulted in the loss of preference for nucleus. A short segment of 20 amino acids in the hinge region enabled cytoplasmic GFP-tagged alkaline phosphatase to translocate to nuclei. These results indicate that 1) VDR is located predominantly in nuclei with a significant presence in cytoplasm without the ligand and 2) an NLS consisting of 20 amino acids in the hinge region facilitates the transfer of VDR to the nucleus.

The vitamin D receptor (VDR)[1] is one of the ligand-dependent transcription factors that make up the nuclear hormone receptor superfamily (1–3). To modulate the transcription of target genes in response to its cognate ligand 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), VDR must be localized in nucleus and then bind to an enhancer designated as the vitamin D-responsive element (VDRE), forming a heterodimer with retinoid X receptor (1–6). In contrast to the case for the glucocorticoid receptor (GR), which translocates from the cytoplasm to the nucleus when exposed to its ligand, VDR does not bind to heat shock protein 90, and both immunocytochemical and biochemical fractionation studies suggested the nuclear localization of VDR even in the absence of $1,25(OH)_2D_3$ (7–10).

Several reports, however, demonstrated that VDR was located in cytoplasm in the absence of ligand and transported to nucleus in response to $1,25(OH)_2D_3$ (11–13). Although the reason for conflicting results as to the distribution of VDR is not clear, the fixation and cell permeabilization procedures in immunostaining might influence the subcellular distribution of the subject protein. Consistent with this explanation, Barsony *et al.* (11), by the fixation of cells using a microwave, revealed the cytoplasmic localization of VDR in contrast to the nuclear localization detected by a conventional fixation method utilizing the same antibody against VDR.

To avoid the fixation and cell permeabilization steps required in the immunostaining procedure, in the present study we have taken advantage of fusion with green fluorescent protein (GFP), which has been proven to be a useful tag for monitoring the subcellular distribution and trafficking of various proteins in living cells (14). In the other nuclear receptors, the localization of GR and mineralocorticoid receptor was examined utilizing proteins fused with GFP (15–17), and nuclear translocation was observed in response to the ligands. However, there have been no reports about the subcellular distribution of VDR in living cells using GFP fusion protein to date.

Nuclear translocation of large size proteins involves active transport across the nuclear pore complexes and requires energy and specific supportive proteins (18–22). The amino acid sequences that confer the nuclear import ability are termed nuclear localization signal (NLSs), and have been identified in several nuclear proteins (20–24). Although NLSs do not show highly conserved amino acid sequences, the basic residues are believed to play important roles in nuclear trafficking. Simian virus 40 (SV40) large T antigen contains the classical monopartite NLS, PKKKRKV, while nucleoplasmin has a bipartite NLS, KRPAATKKAGQAKKKK. In the latter, two short clusters of basic amino acids are separated by a gap of 10 amino acids. The protein carrying the NLS(s) interacts with importin α, which binds to importin β; importin α and β act as the carrier proteins for the cargo (NLS protein), and translocate through nuclear pore complexes before releasing the cargo in the nucleus (25, 26). The interaction of importin α and the proteins carrying the NLS has been recently investigated by x-ray crystallography (27).

A previous study on progesterone receptor (PR) revealed an NLS in the hinge region (or D domain), whose sequence resembles the classical monopartite NLS of SV40 T antigen (28). The sequence of this NLS in PR is conserved in the nuclear receptor superfamily to some extent and appears to work as an NLS in the other members including GR, androgen receptor, and thy-

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[1] The abbreviations used are: VDR, vitamin D receptor; wtVDR, wild-type VDR; NLS, nuclear localization signal; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDRE, vitamin D-responsive element; GFP, green fluorescent protein; GR, glucocorticoid receptor; PR, progesterone receptor; ALP, alkaline phosphatase.

roid hormone receptor α as well. However, it is still unclear whether the same region acts as NLS in all of the nuclear receptors (29–32). In some studies on NLSs in nuclear receptors, the DNA-binding domain is also reported to contain NLSs. In the case of VDR, there is a report about an NLS between amino acids 49 and 55 in the DNA-binding domain (33). In another report, a peptide representing the amino acids 76–102 immediately C-terminal of the second zinc finger of VDR targeted fluorescein isothiocyanate-conjugated IgG to the nuclei (34). However, the nuclear accumulation was not complete, and the authors admitted the possibility that other NLS(s) exist. In addition, it is rather difficult to distinguish between the nuclear translocation and the nuclear retention, especially when an NLS is located in the DNA-binding domain, because the binding to DNA or nuclear matrix helps to maintain the protein in the nucleus after the nuclear translocation.

In this study, we investigated the subcellular distribution of VDR in living cells utilizing the protein fused with GFP in the absence and presence of $1,25(OH)_2D_3$. Subsequently, the analysis of the subcellular distribution of the various GFP-tagged deletion mutants of VDR indicated that the hinge region plays an important role to the nuclear localization of VDR. Finally, we obtained evidence that the sequence of amino acids in this region enabled a cytoplasmic protein to translocate into the nucleus.

MATERIALS AND METHODS

Plasmid Constructions of VDR-GFP Fusion Proteins—The expression vector of the human VDR cDNA, pSG5-hVDR was kindly provided by Dr. M. R. Haussler (University of Arizona) (35). Although the amino acid number is indicated according to the original paper concerning the human VDR cDNA (GenBank™ accession number J03258), the expression vector pSG5-hVDR itself contains the VDR cDNA, which starts from the second translation start point due to the existence of polymorphism, resulting in the 424 amino acids (hVDR amino acids 4–427). GFP fusion vector pGreen Lantern was purchased from Life Technologies, Inc. To construct the fusion protein in which wild type VDR was fused to the C terminus of GFP (designated as GFP-wtVDR), the termination codon of GFP cDNA in pGreen Lantern was mutated to generate a new *Hin*dIII site (designated as pGreen Lantern stop(−)), and then the full-length human VDR cDNA obtained from pSG5-hVDR was inserted into the *Hin*dIII site to yield a fusion protein in frame, resulting in pGreen Lantern-wtVDR. To construct another fusion protein in which wild type VDR was fused to N terminus of GFP (designated as wtVDR-GFP), we first removed the VDR stop codon from pSG5-hVDR by *Bgl*II digestion. There are two *Bgl*II sites in pSG5-hVDR; one of them is located immediately upstream of the VDR stop codon, and the other is located in the 3′-untranslated region of the VDR. We partially digested the plasmid with *Bgl*II to obtain the fragment that was cut only at the recognition site immediately upstream the VDR stop codon. Then the following oligomers were annealed, phosphorylated at the 5′-ends by kination, and inserted to the opened *Bgl*II site: sense, 5′-GATCAGTGCGGCCGCA-3′; antisense, 5′-GATCTGCGGCCGCACT-3′. Since the annealed oligomer was designed to have a unique *Not*I site, the plasmid was next opened by *Not*I digestion, and cDNA encoding GFP that had been excised from pGreen Lantern by *Not*I digestion was inserted to generate an in frame fusion protein, resulting in pSG5-wtVDR-GFP. This construct consists of human VDR (amino acids 4–427), 5-amino acid linker (AAAAT), GFP (amino acids 1–238), and the 3′-untranslated region of the VDR, and the fusion protein terminates using the stop codon of GFP. In the process of constructing the GFP-tagged truncated VDR mutants, we also modified pGreen Lantern vector to possess a multiple cloning site at the position of the original termination codon of GFP cDNA and designated this plasmid pGreen Lantern-MCS. The various truncated VDRs (Δ4–176, Δ178–427, and Δ80–427; the numbers indicate deleted amino acids) were obtained by digestion with appropriate restriction enzymes and some modification and cloned into the cloning site of pGreen Lantern-MCS. To generate the [Δ4–153,Δ174–427] mutant tagged with GFP, the annealed oligomers for bipartite NLS described below were inserted into the *Hin*dIII site in pGreen Lantern stop(−). For the analysis with less deletion, various deletion mutants of VDR (Δ4–88, Δ78–233, Δ78–114, Δ181–230, and Δ117–173; the numbers indicate deleted amino acids) were also generated by digestion with appropriate restriction enzymes, fused to GFP at their C termini, and cloned into pSG5 vector except for the Δ4–88 mutant, which was cloned into pSVL expression vector (Amersham Pharmacia Biotech, Tokyo, Japan). To generate C79S point mutant of VDR, polymerase chain reaction-based mutagenesis was performed, and the mutant VDRs were also fused to GFP and cloned into pSG5 vector. All plasmids were examined for the introduced mutation and desired fusion in frame using an ABI 377A model DNA sequencer (Perkin-Elmer).

Plasmid Constructions of GFP-Alkaline Phosphatase (GFP-ALP) Fusion Proteins Carrying Putative NLSs of VDR—To examine whether the putative NLS of VDR enables the cytoplasmic proteins to translocate from cytoplasm to nuclei, we constructed several plasmids of GFP-tagged ALP carrying the putative NLS sequences of VDR. Here we utilized human tissue-nonspecific ALP, whose nascent enzyme consisted of 507 amino acids. Human tissue-nonspecific ALP has a signal sequence at the N terminus as well as a hydrophobic domain at the C terminus, the latter of which is involved in glycosylphosphatidylinositol anchoring (36). We have previously reported that GFP-tagged ALP was exclusively localized to cytoplasm and cell membrane (37).

To construct the plasmid encoding GFP fused to ALP, GFP cDNA was excised from pGreen Lantern stop(−) by *Not*I digestion and fused to N terminus of mature ALP obtained from ALP expression vector pSV2Aalp (a gift from Dr. P. S. Henthorn, University of Pennsylvania) and cloned into pcDNA3.1 vector (Invitrogen, NV Leek, The Netherlands), which was named pcDNA-GFP-ALP (Fig. 6*A*). The mature ALP described above is a fragment lacking the N-terminal 17 amino acids corresponding to the secretion signal peptide of ALP. To insert the putative NLSs of VDR into GFP-ALP fusion protein, the following oligomers were annealed after the phosphorylation of 5′-ends with polynucleotide kinase and ATP, and the generated fragments were cloned into pcDNA-GFP-ALP between GFP and ALP to generate the in frame fusion proteins: bipartite NLS-sense, 5′-AGCTTCGGCCTCCAGTTCGTGTGAATGATGGTGGAGGGAGCCATCCTTCCAGGCCCAACTCCAGAA-3′; bipartite NLS-antisense, 5′-AGCTTTCTGGAGTTGGGCCTGGAAGGATGGCTCCCTCCACCATCATTCACACGAACTGGAGGCCGA-3′; RPPVR NLS-sense, 5′-AGCTTCGGCCTCCAGTTCGTTAAC-3′; RPPVR NLS-antisense, 5′-AGCTGTTAACGAACTGGAGGCCGA-3′; RKREMILKRK-sense, 5′-AGCTTAGGAAGCGGGAGATGATCCTGAAGCGGAAAG-3′; RKREMILKRK-antisense, 5′-AGCTCTTTCCGCTTCAGGATCATCTCCCGCTTCCTA-3′. All of the hybrid constructs were examined for the desired in frame fusion by sequencing as described above.

Cell Culture and Transfection—Monkey kidney epithelial cell line COS7 and CV-1 cells were cultured at 37 °C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium and Eagle's minimum essential medium, respectively (Nikken Bio-Medical Laboratory, Kyoto, Japan), supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (Life Technologies, Inc.), which had been stripped with dextran-coated charcoal to remove endogenous steroids. Human osteoblastic cell line MG63 was maintained in Dulbecco's modified Eagle's medium in the same condition.

Transient transfections to COS7 and MG63 were performed using TransFast™ reagent (Promega, Madison, WI) according to the manufacturer's instructions. CV-1 cells were transfected using Lipofectamine™ (Life Technologies, Inc.). For the microscopy, a total of 5 μg of DNA was used to transfect cells in each 6-cm dish.

Generation of Stable Transfectants—To generate the stable transfectants of wtVDR-GFP, the fragment containing the cDNA of the fusion protein was excised by *Eco*RI digestion from pSG5-wtVDR-GFP and cloned into another expression vector pcDNA3 (Invitrogen), which has a neomycin resistance cassette, resulting in pcDNA-wtVDR-GFP. The fusion plasmid was introduced into COS7 cells using TransFast™, and the stably transfected cells were selected using G418 (Geneticin; Life Technologies, Inc.). Several clonal cell lines were expanded from single foci and were screened by GFP fluorescence and Western blotting for expression of GFP-tagged VDR. COS7 cells are seldom used to make stable transfectants because of the constitutive expression of SV40 T antigen, which supports transfected plasmids in the episomal state. However, we utilized this cell line because a previous study of the intracellular trafficking of GFP-tagged GR had been performed using COS1 cells (16), and there was a report where the authors succeeded in generation of stable COS7 transfectants (38). The expression levels of wtVDR-GFP in our stable transfectants appeared to be lower than those of the transiently transfected cells, which were estimated by GFP fluorescence and Western blotting (data not shown).

Detection of GFP by Fluorescence Microscopy—Microscopy was performed to detect GFP fluorescence 24–48 h after transient transfection on an BH-2 Olympus microscope with epifluorescence illumination

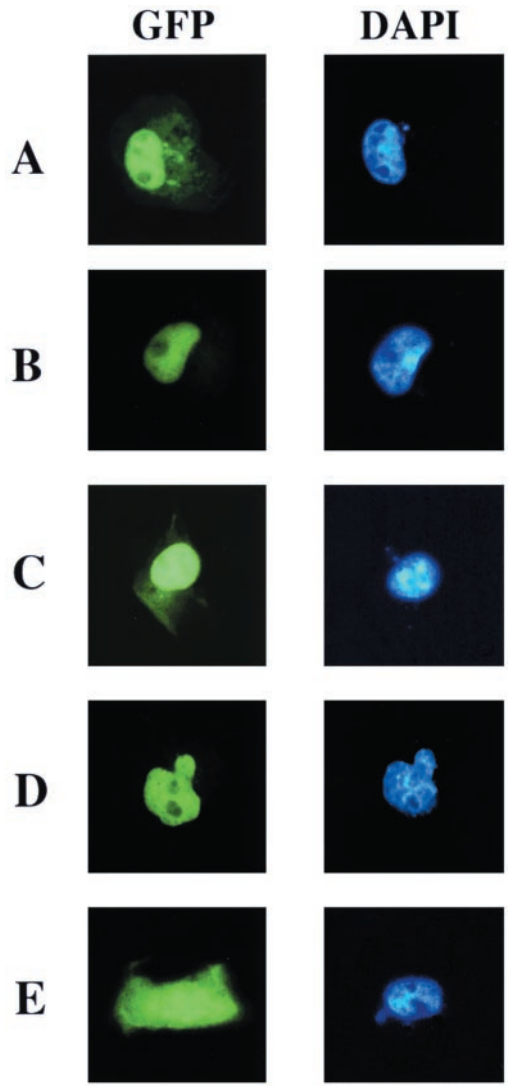

FIG. 1. **Subcellular distribution of GFP-wtVDR in transient transfections to COS7 cells in the presence or absence of ligand.** COS7 cells were transfected with GFP-wtVDR (*A* and *B*), wtVDR-GFP (*C* and *D*), or GFP alone (*E*). In order to examine the effect of the ligand, 10^{-8} M $1,25(OH)_2D_3$ (*B* and *D*) or vehicle (*A* and *C*) was added to the transfected cells 48 h after the transfection and cultured for an additional 3 h. GFP alone was distributed throughout the cells (*E*). On the other hand, even in the absence of the ligand, both GFP-wtVDR and wtVDR-GFP were predominantly localized to nuclei with significant presence in the cytoplasm (*A* and *C*). In response to the administration of $1,25(OH)_2D_3$, most VDRs were accumulated in the nuclei (*B*, *D*). Staining with 4′,6-diamidine-2′-phenylindole dihydrochloride (*DAPI*) demonstrates the intact nuclei.

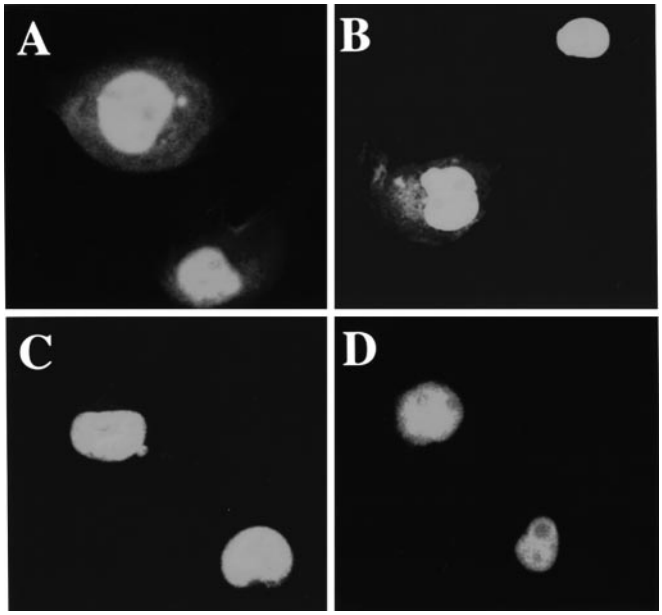

FIG. 2. **Time-dependent translocation of wtVDR-GFP induced by $1,25(OH)_2D_3$ in stably transfected COS7 cells.** COS7 cells were transfected with DNA encoding wtVDR-GFP, and stable transfectants were selected as described under "Materials and Methods." In the presence of 10% charcoal-stripped fetal calf serum, the transfectants were treated with 10^{-8} M $1,25(OH)_2D_3$ for 0 h (*A*), 1 h (*B*), 3 h (*C*), or 8 h (*D*). In the absence of $1,25(OH)_2D_3$, VDR was predominantly localized to nuclei with significant presence in the cytoplasm (*A*). An hour after the addition of $1,25(OH)_2D_3$, significant cytoplasmic VDR was still observed (*B*). Treatment with $1,25(OH)_2D_3$ for 3 h or longer resulted in more accumulation of VDR in the nuclei (*C* and *D*).

(Olympus, Tokyo, Japan). GFP fluorescence was observed in the living cells with a fluorescein isothiocyanate filter. In some experiments, the transfected cells were fixed in 4% paraformaldehyde solution (Muto Pure Chemicals, Tokyo, Japan) and stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (Roche Molecular Biochemicals, Mannheim, Germany) to confirm that the nuclei were intact.

Effect of $1,25(OH)_2D_3$ on the Subcellular Distribution of VDR—To examine the effect of the ligand on the subcellular distribution of VDR, a 10^{-8} M concentration of $1,25(OH)_2D_3$ (Wako, Tokyo, Japan) or vehicle was added to COS7 and MG63 cells that had been transiently transfected with GFP-wtVDR or wtVDR-GFP at a point 48 h after the transfection. Three hours later, the cells were subjected to microscopy. In order to examine the time-dependent translocation of the fusion protein, similar experiments were performed using COS7 cells stably transfected with wtVDR-GFP, which were generated as described above. The stable transfectants were incubated with 10^{-8} M of $1,25(OH)_2D_3$ for 0, 1, 3, or 8 h, and the subcellular distribution of the fusion protein was examined. More than 200 of the cells were subjected to microscopy, and classified into four categories according to the pattern of the subcellular distribution of the fusion proteins: N > C, stronger intensity in nucleus than in cytoplasm; N, almost exclusive presence in nucleus; N = C, homogeneous presence throughout the cells; and N < C, stronger intensity in cytoplasm. In these experiments and other experiments where the cells were needed to be classified according to the subcellular distribution of GFP-tagged mutant VDRs or GFP-ALP fusion proteins, the transfected cells were also counted by an observer from the Department of Pathology, Osaka Medical Center and Research Institute for Maternal and Child Health, who was blinded to the identity of our experimental group to ensure the reproducibility of the scoring system. There were not significant differences in the results between the observers.

Analysis of Chimera Proteins by Western Blotting—Whole cell extracts were harvested in radioimmune precipitation buffer (1% Triton, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 5 mM EDTA, protease inhibitor mixture (Complete; Roche Molecular Biochemicals)) from the transiently transfected COS7 cells 48 h after the transfection. In some experiments, cytoplasmic extracts were harvested by Dounce homogenization of the cells in swelling buffer (0.1 M Tris-Cl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 5 mM dithiothreitol) followed by centrifugation to obtain the supernatant. The extracts containing 10 μg of each protein were then subjected to 7.5% SDS-polyacrylamide gel electrophoresis. After the separation by gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad). After blocking with Block Ace reagent (Dainippon Pharmaceuticals, Osaka, Japan), the membranes were incubated with the indicated first antibodies, the monoclonal anti-GFP antibody (Roche Molecular Biochemicals) or the monoclonal anti-VDR antibody (9A7γ; Affinity Bioreagents, Inc., Golden, CO). After incubation with the corresponding second antibodies, the proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Transcription Activation Assay—The promoter region of the rat 24-hydroxylase gene (−291/+9), which contains two VDREs (a gift from Dr. Y. Ohyama, Hiroshima University, Japan) (39), was cloned into a luciferase reporter vector pGV-B2 (Toyo Ink, Tokyo, Japan) and named pGV-B2 24-hydroxylase. In the transient transfection experiments, the expression vectors of GFP-tagged wild type or mutated VDRs were introduced into CV-1 cells with pGV-B2 24-hydroxylase using Lipofectamine™ (Life Technologies, Inc.). Twenty-four hours after the

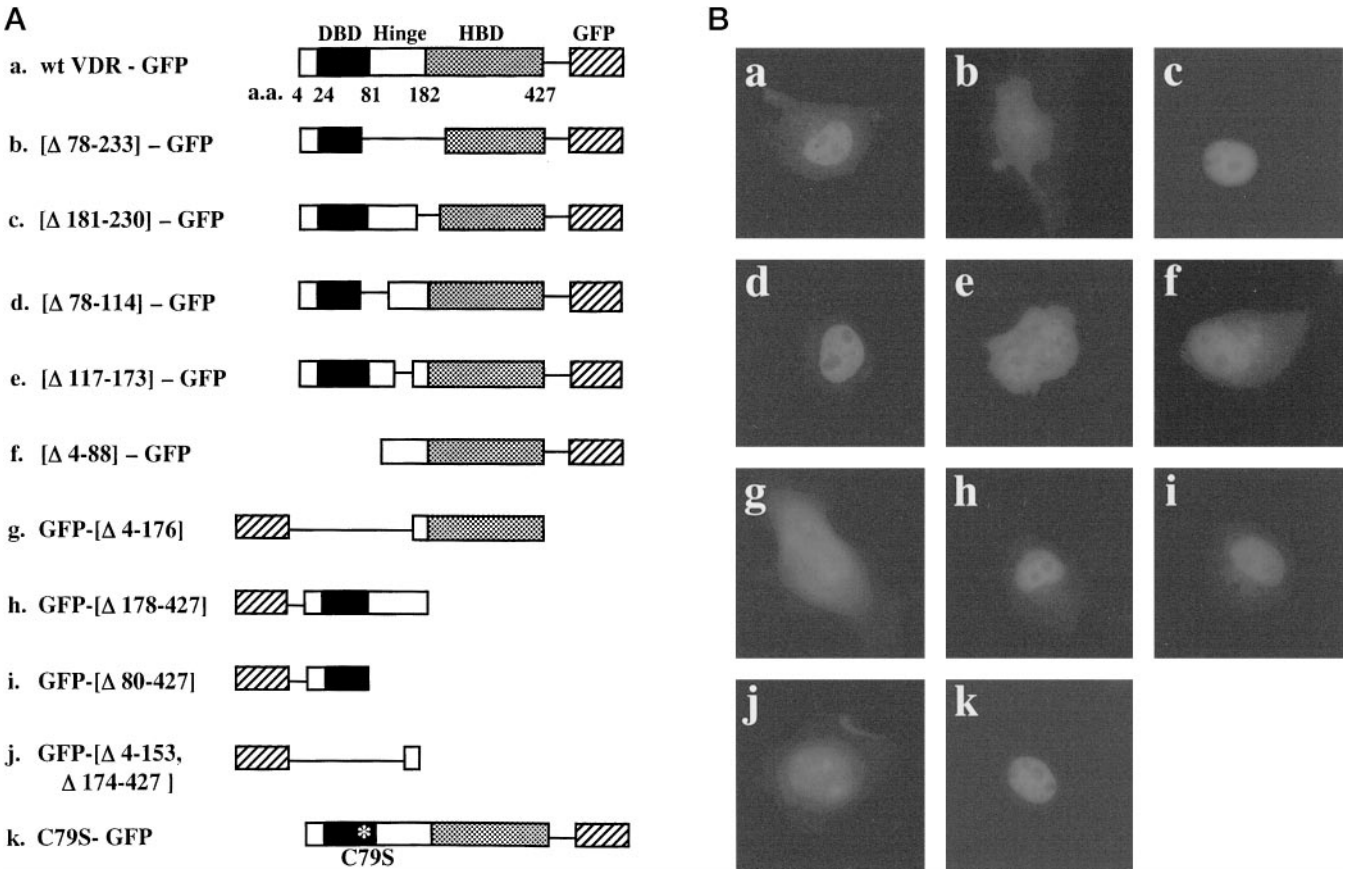

FIG. 3. **Subcellular distribution of various VDR deletion mutants tagged with GFP in the absence of ligand in transfected COS7 cells.** *A*, the constructs used in the experiments. The numbers after Δ indicate the deleted amino acids. The wild-type VDR (wtVDR) contains amino acids 4–427. Features depicted are as follows. *DBD*, the DNA-binding domain; *Hinge*, the hinge region; *HBD*, the hormone-binding domain. The *asterisk* indicates the position of the substitution of Cys^{79} for Ser. The length of each domain in the diagram is arbitrary. *B*, the fluorescent images of the COS7 cells transfected with each construct shown in *A*.

transfection, 10^{-8} M $1,25(OH)_2D_3$ or vehicle was added, and the cells were retrieved 48 h after the addition. The luciferase activities of the cell lysates were measured with luciferase assay kit (Toyo Ink) according to the manufacturer's manual. Transactivation measured as the luciferase activity was standardized by the galactosidase activities of the same lysates determined by a β-galactosidase enzyme assay system (Promega), and then the transactivation function of the GFP-tagged wild type and mutated VDRs was evaluated as the -fold induction of promoter activity by $1,25(OH)_2D_3$.

Electrophoretic Mobility Shift Assay—The electrophoretic mobility shift assay was basically performed as described previously (40). To prepare the probe, the following oligomers, which represent the VDRE of the human osteocalcin gene, were annealed to generate a double-stranded oligonucleotide with overhangs at both ends and then labeled with [α-^{32}P]dCTP (NEN Life Science Products) by a fill-in reaction using Klenow fragment of *Escherichia coli* DNA polymerase I: sense, 5′-CTAGCTTGGTGACTCACCGGGTGAACGGGGGCATTG-3′; antisense, 5′-CTAGCAATGCCCCCGTTCACCCGGTGAGTCACCAAG-3′. Whole cell extracts were harvested from the COS7 cells transfected with pSG5-VDR or pSG5-C79S VDR. The reaction mixture of the probe and the whole cell extracts was electrophoresed on a 5% polyacrylamide gel and visualized by BAS2000 (Fujix, Tokyo, Japan).

RESULTS

Nuclear Localization of GFP-tagged Wild-type VDR—In the transient transfections to COS7 cells, GFP alone was distributed throughout the cells (Fig. 1*E*). In contrast, the fusion proteins were predominantly located in nuclei with a significant presence in cytoplasm even in the absence of ligand both when wild type VDR was fused to the C terminus (GFP-wtVDR) and to the N terminus of GFP (wtVDR-GFP) (Fig. 1, *A* and *C*). In the transfectants with lower expression, where the intensity of fluorescence was relatively weak, the cytoplasmic presence of the fusion proteins was still observed. The fusion proteins were not located in the nucleoli. When the transfected cells were treated with 10^{-8} M $1,25(OH)_2D_3$, more accumulation of VDR in the nuclei was observed (Fig. 1, *B* and *D*). In the transient transfections to CV-1 and MG63 cells as well, both GFP-wtVDR and wtVDR-GFP were predominantly localized to nuclei with some cytoplasmic presence in the absence of ligand and greater accumulation in nuclei in response to 10^{-8} M $1,25(OH)_2D_3$ (data not shown).

Time-dependent Translocation of wtVDR-GFP Induced by $1,25(OH)_2D_3$ in Stable Transfectants—To examine the time-dependent effect of ligand on VDR distribution, we generated stable transfectants of COS7 cells with pcDNA-wtVDR-GFP. Similar to the observation in the transient transfection experiments, the fusion protein was predominantly localized to nuclei with some cytoplasmic presence even in the absence of ligand (Fig. 2*A*), and 96.4% of the cells were classified into group N > C (predominantly nuclear with some cytoplasmic presence), and 3.6% were N (exclusively nuclear). Western blotting using anti-VDR antibody 9A7γ and the cytoplasmic fraction obtained from the stable transfectants also revealed the cytoplasmic presence of the fusion protein (data not shown). When the cells were treated with 10^{-8} M of $1,25(OH)_2D_3$ for 1 h, most of them still exhibited cytoplasmic VDR with predominant nuclear VDR (Fig. 2*B*); 85.3 and 14.7% of the cells were classified into N > C and N, respectively. On the other hand, when the cells were treated with $1,25(OH)_2D_3$ for 3 h, more VDR came to accumulate to the nucleus (Fig. 2*C*), and 51.5% of the cells exhibited exclusive nuclear localization. Eight hours after the addition, this ligand-dependent nuclear accumulation of VDR was still observed (Fig. 2*D*), and 56.0 and 44.0% of the

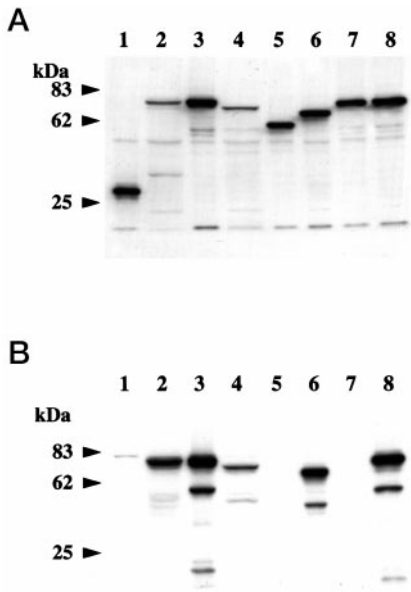

FIG. 4. **Western blot analysis of GFP-tagged wild-type and various mutant VDRs in transfected COS7 cells.** 10 μg/lane of COS7 whole cell extracts, except for *lanes 2* and *4* to which 30 μg of extracts were applied, were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membranes were probed with monoclonal anti-GFP antibody (*A*) or monoclonal anti-VDR antibody, 9A7γ (*B*). *Lane 1*, GFP alone; *lane 2*, GFP-wtVDR; *lane 3*, wtVDR-GFP; *lane 4*, [Δ4–88]-GFP; *lane 5*, [Δ78–233]-GFP; *lane 6*, [Δ181–230]-GFP; *lane 7*, [Δ78–114]-GFP; *lane 8*, [Δ117–173]-GFP. Molecular weight markers are shown on the *left*. Both antibodies detected the fusion proteins at the expected sizes, except for the Δ78–233 and Δ78–114 mutants lacking the antigenic site for 9A7γ.

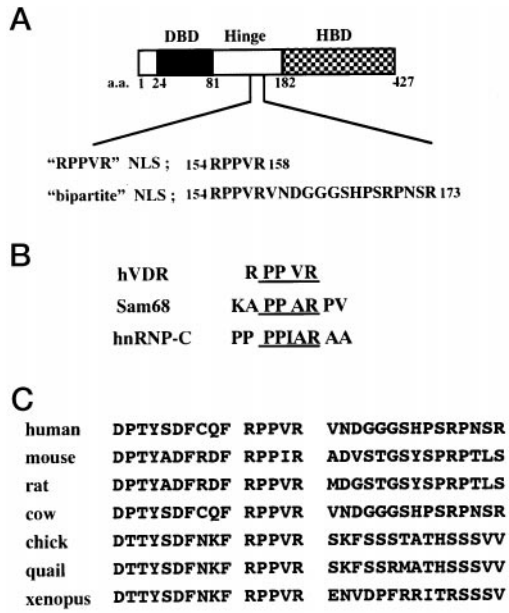

FIG. 5. **Functional domains of human VDR and the composition of the putative NLSs.** *A*, the structural features of human VDR and the amino acid sequences of the putative NLSs are represented schematically. Features depicted are as follows. *DBD*, the DNA-binding domain; *Hinge*, the hinge region; *HBD*, the hormone-binding domain. RPPVR NLS consists of the five amino acids 154–158. Bipartite NLS consists of the 20 amino acids 154–173. The length of each domain in the diagram is arbitrary. *B*, PP*X*R motif in NLSs found in other proteins. The conserved motifs are *underlined*. *C*, the conservation of the RPP*X*R motif in VDR among the species. Conserved amino acids are shown at the *center*.

cells were classified into N > C and N, respectively.

Subcellular Distribution of Deleted Mutants of VDR Tagged with GFP—In an attempt to identify the NLSs in VDR, we first examined the subcellular distribution of various deletion mutants of GFP-tagged VDR without its ligand in the transiently transfected COS7 cells (Fig. 3). Fig. 3*B* shows the representative cells transfected with each plasmid. In each transfection, although there was a variation of cells in the expression level of the protein, the subcellular distribution did not appear to be altered according to the expression level. As described above, the wtVDR-GFP was predominantly localized in nuclei with some cytoplasmic presence in the absence of ligand (Fig. 3*a*). The Δ78–233 mutant, which lacked the whole hinge region and a part of the hormone-binding domain, was distributed equally both to nuclei and cytoplasm in all of the transfected cells (Fig. 3*b*). Δ181–230 and Δ78–114 mutants exhibited predominant nuclear localization like wtVDR (Fig. 3, *c* and *d*), while the Δ117–173 mutant showed no nuclear accumulation (Fig. 3*e*), suggesting the existence of NLS(s) between amino acids 117 and 173 in the hinge region. Δ4–88 mutant lacking DNA-binding domain exhibited decreased nuclear localization but showed some nuclear preference compared with the Δ78–233 mutant (Fig. 3, *f* and *b*, respectively). The Δ4–176 mutant, which has only the hormone-binding domain, was distributed throughout the cells (Fig. 3*g*), while Δ178–427 mutant, which possesses both the DNA-binding domain and the hinge region, was strictly localized to nuclei (Fig. 3*h*). The Δ80–427 mutant also exhibited predominant nuclear localization (Fig. 3*i*), which was consistent with the incomplete loss of nuclear localization of Δ4–88 mutant. The [Δ4–153,Δ174–427] mutant, which has only the short segment (amino acids 154–173) of the hinge region, exhibited predominant nuclear localization (Fig. 3*j*).

The appropriate full-length expression of the GFP-tagged wild type and mutant VDRs was confirmed by Western blotting using anti-GFP antibody (Fig. 4*A*) and anti-VDR antibody (9A7γ, Fig. 4*B*). Both when GFP (~27 kDa) was fused to the N terminus and C terminus of wtVDR (~50 kDa), the appropriate sized fusion protein (~77 kDa) was recognized by both anti-GFP antibody and 9A7γ. Both antibodies also detected the deletion mutants as the predicted sizes except for the Δ78–233 and Δ78–114 mutants, which lack the antigenic site for 9A7γ (amino acids 89–105).

Subcellular Distribution of C79S Mutant Lacking DNA Binding—Since the Δ4–88 mutant lacking the complete DNA-binding domain still retained the nuclear preference to some extent, it was suggested that the functional domains for the DNA binding and the nuclear localization might be distinct. We therefore studied the subcellular distribution of C79S mutant VDR, which has previously been proved to lack DNA binding capability (41, 42).

In C79S mutant, cysteine in the second zinc finger of the DNA-binding domain was changed to serine. Electrophoretic mobility shift assay using the whole cell extract obtained from the COS7 cells transfected with wtVDR revealed a retarded band corresponding to the VDR·VDRE complex. On the other hand, the extract from C79S transfectant failed to form the VDR·VDRE complex, suggesting that C79S mutant VDR lacked DNA binding capability (data not shown).

Despite this lack of DNA binding capability, the C79S mutant VDR was predominantly localized to nuclei as well as the wtVDR (Fig. 3*k*).

Transactivation Function of the GFP-tagged VDRs—Transactivation function of the GFP-tagged wild type and mutated VDRs was evaluated as the -fold induction of promoter activity by $1,25(OH)_2D_3$. When the transactivation activity of the wtVDR without GFP-tag (pSG5-hVDR) was taken as 100%, that of wtVDR-GFP (pSG5-wtVDR-GFP) was 122.9%. The result suggests no major hindrance of the fusion protein by GFP

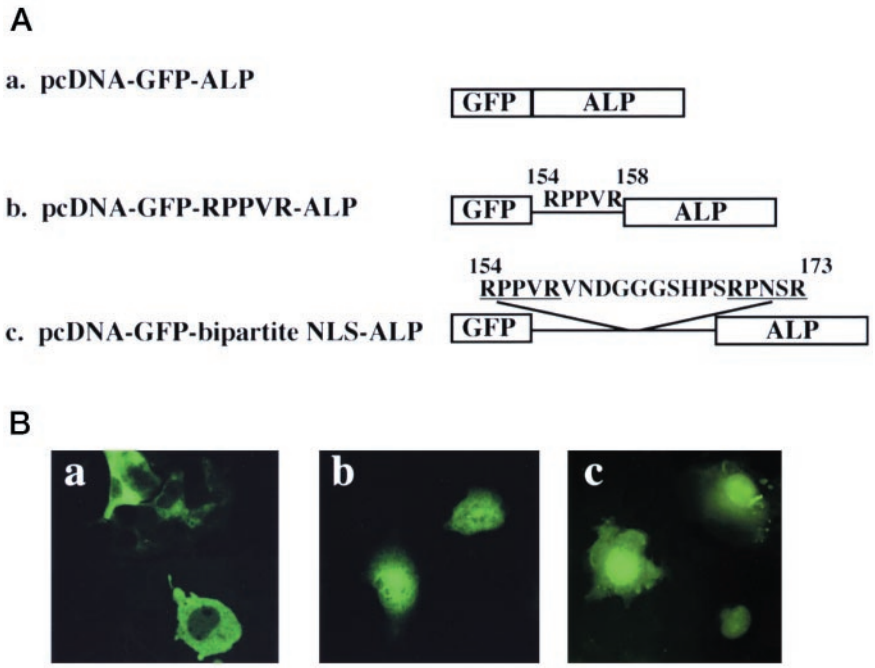

FIG. 6. **Subcellular distribution of GFP-tagged ALP carrying the putative NLSs of VDR.** *A*, the constructs of GFP-tagged ALP with or without putative NLSs of VDR. *B*, fluorescent images of the COS7 cells transfected with each construct shown in *A*. In the absence of NLS, GFP-tagged ALP was exclusively localized in cytoplasm (*a*). When RPPVR NLS was inserted (pcDNA-GFP-RPPVR-ALP), the fusion protein came to distribute both in nuclei and cytoplasm (*b*). When the putative bipartite NLS was inserted (pcDNA-GFP-bipartite NLS-ALP), the fusion protein was predominantly localized in nuclei in most of the cells (*c*).

in terms of the function of transcription regulation. The GFP-tagged deletion mutant VDRs and the C79S mutant exhibited less than 5% of the transactivation activity.

Putative NLSs of VDR—The deletion analysis suggested that the amino acids between 117 and 173 contain the NLS sequence. The sequences corresponding to the NLS identified in previous studies in VDR (33, 34) and the sequence ^{102}RKREMILKRK^{111} resembling NLS found in PR ^{637}RKFKKFNK^{644} (28, 43) was not present in this region, and they could be deleted without the significant impairment of nuclear localization in our deletion analysis. In the region between amino acids 117 and 173, we assumed that the short sequence between 154 and 158 (RPPVR) might be involved in the nuclear localization, based on the resemblance to the PP*X*R motif, which has been revealed to be a NLS in other proteins (Fig. 5, *A* and *B*) (44, 45). The longer sequence between amino acids 154 and 173 that contains RPPVR might act as a bipartite type NLS (Fig. 5*A*). The five amino acids RPPVR were highly conserved in VDRs among the species (Fig. 5*C*).

Putative Bipartite PPXR-type NLS of VDR Enables ALP to Translocate from Cytoplasm to Nuclei—To examine whether the putative NLS(s) of VDR enables a cytoplasmic protein ALP to translocate to nuclei, we constructed plasmids encoding GFP-ALP fusion proteins to which the RPPVR NLS or the putative "bipartite" NLS of VDR were inserted as described under "Materials and Methods" and named pcDNA-GFP-RPPVR-ALP and pcDNA-GFP-bipartite NLS-ALP, respectively. Each plasmid was introduced into COS7 cells, and 48 h later, the transfected cells were subjected to microscopy. More than 800 cells were observed and classified into three categories according to the subcellular distribution of the fusion proteins: N < C, N = C, and N>C.

In the absence of NLS sequence, GFP-tagged ALP exhibited exclusive cytoplasmic localization in COS7 cells, reflecting the nature of ALP as a cytoplasmic protein (Fig. 6*a*, Table I). In contrast, GFP-ALP fusion protein carrying the putative "bipartite" NLS of VDR showed predominant localization in nuclei in 54.5% of the transfectants (Fig. 6*c*, Table I). When only the first 5 amino acids, RPPVR, were inserted to GFP-ALP, the fusion protein came to equally distribute both in nucleus and cytoplasm in 36.2% of the transfected cells (Fig. 6*b*, Table I). The appropriate full-length expression of each protein was confirmed by Western blotting using anti-GFP antibody (data not shown). The data suggest that the 5 amino acids RPPVR may act as a weak NLS and that the longer sequence of amino acids 154–173 works as a stronger one.

DISCUSSION

The localization of VDR in the absence of its ligand is still to be ascertained; nuclear localization was shown by immunostaining with conventional fixation, while cytoplasmic location was detected with microwave fixation despite utilization of the same antibody (11). Despite this controversy, many researchers have suggested the exclusive nuclear localization of VDR even in the absence of ligand. However, if this is the case, how is $1,25(OH)_2D_3$ transported to the cytoplasm? To date no carrier protein has been identified that binds to $1,25(OH)_2D_3$ in cytoplasm, in contrast to the cases of thyroid hormone and retinoic acid, where specific binding proteins bring the ligands from the cell surface to the cytoplasm (46, 47). In our present study using GFP-tagged VDRs, VDR was located both in nucleus and cytoplasm in living cells even in the absence of its ligand. Treatment with $1,25(OH)_2D_3$ induced the accumulation of more VDR into nucleus, suggesting that VDR is at least one of the carrier proteins that facilitate the nuclear transport of $1,25(OH)_2D_3$ from outside of the cells into nuclei, in parallel with a previous report (12).

The authors who reported the cytoplasmic localization of VDR in the absence of ligand also observed the rapid translocation of VDR in response to the ligand in a few minutes (11, 13). In contrast, the translocation was not so rapid in our study as reported before, and it appeared to take at least 3 h for VDR to accumulate to the nucleus. In addition, the ligand-dependent translocation of VDR was not complete; some VDRs were still observed in cytoplasm even in the presence of ligand. This cytoplasmic presence of VDR does not seem to be an artifact of the overexpression of the VDR in a transient expression system using heterologous promoter, because the cytosolic distribution was also observed in the stable transfectants where the expression level of VDR analyzed by Western blotting was not so high as the transient expression level (data not shown). In addition, this cytoplasmic presence of VDR was also demonstrated in a

TABLE I
Distribution of GFP-tagged ALP carrying the putative NLSs of VDR

Transfection of each plasmid to COS7 cells was performed six times, and more than 800 cells in total transfected with each plasmid were subjected to microscopy. The cells were classified into three categories according to the pattern of distribution in GFP fluorescence, and the percentage of each category was calculated.

Plasmids	N > C	N = C	N < C
	%	%	%
pcDNA-GFP-ALP	0	5.8	94.2
pcDNA-GFP-RPPVR-ALP	6.8	36.2	57.0
pcDNA-GFP-bipartite NLS-ALP	54.5	43.8	1.7

previous report where a fractionation study combined with Western blotting was performed (33).

GFP is a useful tag with which to examine the subcellular distribution and trafficking of various proteins, because it has autofluorescence, enabling visualization of the fused proteins in living cells (14). GFP itself is distributed equally both in nucleus and cytoplasm, since it is small enough to pass through the nuclear pore complexes by passive diffusion without the requirement of NLS. However, when fused to other larger proteins, the fused protein requires active transport system and NLS. To identify the NLS of VDR, we have performed deletion analysis using GFP-tagged mutant VDRs. The analysis revealed that the amino acids 117–173 in the hinge region may be involved in the nuclear localization. To analyze the function of the putative NLSs in this region, we constructed GFP-tagged mature ALP in which the N-terminal secretion signal of ALP was deleted and observed its exclusive cytoplasmic localization. When the putative NLS of VDR was inserted, the distribution of the GFP-tagged mature ALP was remarkably altered to nucleus from in the cytoplasm. These results strongly suggest that amino acids 154–173 located in the hinge region of human VDR confer the ability to localize in nucleus to other proteins.

The NLS of VDR identified in the present study might be a member of a rather small group of NLS sequences which have a PP*X*R motif. The PP*X*R motif in NLSs was previously reported in nuclear RNA-binding proteins (ribonucleoprotein), such as Sam68 and heterogeneous nuclear ribonucleoprotein C1 (44, 45). The putative NLS of VDR identified in the present study contains the amino acid sequence RPPVR, which is homologous to the PP*X*R motif. This class of NLS looks rather unique and has not been well characterized yet. In the nuclear receptor superfamily, human VDR is the first member that has been identified to possess this type of NLS. Interestingly, despite the poor conservation in the amino acid sequence of the entire hinge region, the RPP*X*R motif is completely conserved among VDRs of all the reported species, suggesting the importance of this amino acid sequence.

The bipartite structure has not been reported in the NLS with the motif PP*X*R. However, it is likely that human VDR has the bipartite type of this motif, because the amino acid sequence RPPVRVNDGGGSHPSRPNSR was more efficient than RPPVR in transferring GFP-ALP to the nucleus. So far, the protein that interacts with the PP*X*R motif has not been identified, while both classical monopartite and bipartite NLSs have been revealed to interact with a cargo protein, importin α. Although a structural study has revealed the crucial role of amino acids, lysine and arginine in the interaction of NLS-bearing protein and importin, the precise role of the proline residue, which is also found in many NLSs, has not been elucidated (27, 48). The interaction of VDR with cargo proteins including importin α is the next issue to be addressed.

Among the nuclear hormone receptors, NLS sequence that resembles the classical NLS found in SV40 large T antigen has been identified in the hinge region of PR (28, 43). However, the NLS-like motif corresponding to the NLS sequence of PR was not thought to be responsible for the nuclear localization of VDR in our deletion analysis. In other words, the amino acid sequence, ^{102}RKREMILKRK^{111} in the human VDR, which is similar to NLS of PR ^{637}RKFKKFNK^{644}, resides in a region that can be deleted without significant impairment of the nuclear localization. Consistent with these results, the amino acids, RKREMILKRK, did not alter the cytoplasmic localization of GFP-tagged ALP (data not shown).

In some members of nuclear receptors, such as GR and PR, more than one NLS was identified (28, 43). Our results also did not exclude the possibilities of the existence of the NLS in VDR other than RPPVR and also that of nuclear export signals (49). More accurately, the data rather suggest that the DNA-binding domain is also important for VDR to be located in nucleus, because the deletion mutant having only the DNA-binding domain exhibited predominant nuclear accumulation, and the mutant that lacked the DNA-binding domain showed decreased nuclear preference compared with wild type VDR. Barsony *et al.* (11) demonstrated that nuclear localization of VDR mutated in the DNA-binding domain was not observed using fibroblasts obtained from a patient with vitamin D dependence type II. Hsieh *et al.* (33) reported an NLS in the DNA-binding domain. These reports also suggest the importance of DNA-binding domain for efficient nuclear accumulation of VDR. However, we prefer to emphasize that the nuclear localization and DNA binding are likely to be functionally distinct, since C79S mutant lacking DNA binding still retained the nuclear localization, and an NLS was identified in the hinge region of VDR.

In conclusion, we obtained results that suggest that the element, RPPVRVNDGGGSHPSRPNSR, works as an NLS in the homologous protein human VDR and also in a heterologous protein ALP when fused to the protein.

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REFERENCES

1. Haussler, M. R., Whitfield, G. K., Haussler, C. A., Hsieh, J.-C., Thompson, P. D., Selznick, S. H., Dominguez, C. E., and Jurutka, P. W. (1998) *J. Bone Miner. Res.* **13,** 325–349
2. Pike, J. W. (1997) *Vitamin D*, pp. 105–125, Academic Press, Inc., San Diego
3. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83,** 835–839
4. Lemon, B. D., Fondell, J. D., and Freedman, L. P. (1997) *Mol. Cell. Biol.* **17,** 1923–1937
5. Mangelsdorf, D. J., and Evans, R. M. (1995) *Cell* **83,** 841–850
6. Kliewer, S. A., Umesono, K., Mangelsdorf, D. J., and Evans, R. M. (1992) *Nature* **355,** 446–449
7. Walters, M. R., Hunziker, W., and Norman, A. W. (1980) *J. Biol. Chem.* **255,** 6799–6805
8. Clemens, T. L., Garrett, K. P., Zhou, X.-Y., Pike, J. W., Haussler, M. R., and Dempster, D. W. (1988) *Endocrinology* **122,** 1224–1230
9. Milde, P., Merke, J., Ritz, E., Haussler, M. R., and Rauterberg, E. W. (1989) *J. Histochem. Cytochem.* **37,** 1609–1617
10. Bidwell, J. P., van Wijnen, A. J., Fey, E. G, Merriman, H., Penman, S., Stein, J. L., Stein, G. S., and Lian, J. B. (1994) *J. Cell. Biochem.* **54,** 494–500
11. Barsony, J., Pike, J. W., DeLuca, H. F., and Marx, S. J. (1990) *J. Cell Biol.* **111,** 2385–2395
12. Kamimura, S., Gallieni, M., Zhong, M., Beron, W., Slatopolsky, E., and Dusso, A. (1995) *J. Biol. Chem.* **270,** 22160–22166
13. Barsony, J., Renyi, I., and McKoy, W. (1997) *J. Biol. Chem.* **272,** 5774–5782
14. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) *Science* **263,** 802–805
15. Htun, H., Barsony, J., Renyi, I., Gould, D. L, and Hager, G. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4845–4850
16. Ogawa, H., Inouye, S., Tsuji, F. I., Yasuda, K., and Umesono, K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11899–11903
17. Fejes-Tóth, G., Pearce, D., and Náray-Fejes-Tóth, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2973–2978
18. Nigg, E. A. (1997) *Nature* **386,** 779–787
19. Davis, L. I. (1995) *Annu. Rev. Biochem.* **64,** 865–896
20. Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) *Nature* **311,** 33–38

21. Dingwall, C., Sharnick, S. V., and Laskey, R. A. (1982) *Cell* **30,** 449–458
22. Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S., and Laskey, R. A. (1987) *EMBO J.* **6,** 69–74
23. Shields, J. M., and Yang, V. W. (1997) *J. Biol. Chem.* **272,** 18504–18507
24. Goldfarb, D. S., Gariépy, J., Schoolnik, G., and Kornberg, R. D. (1986) *Nature* **322,** 641–644
25. Pollard, V. W., Michael, W. M., Nakielny, S., Siomi, M. C., Wang, F., and Dreyfuss, G. (1996) *Cell* **86,** 985–994
26. Görlich, D., Vogel, F., Mills, A. D., Hartmann, E., and Laskey, R. A. (1995) *Nature* **377,** 246–248
27. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) *Cell* **94,** 193–204
28. Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., and Milgrom, E. (1989) *Cell* **57,** 1147–1154
29. Simental, J. A., Sar, M., Lane, M. V., French, F. S., and Wilson, E. M. (1991) *J. Biol. Chem.* **266,** 510–518
30. Robertson, N. M., Schulman, G., Karnik, S., Alnemri, E., and Litwack, G. (1993) *Mol. Endocrinol.* **7,** 1226–1239
31. Sackey, F. N. A., Haché, R. J. G., Reich, T., Kwast-Welfeld, J., and Lefebvre, Y. A. (1996) *Mol. Endocrinol.* **10,** 1191–1205
32. Lee, Y., and Mahdavi, V. (1993) *J. Biol. Chem.* **268,** 2021–2028
33. Hsieh, J.-C., Shimizu, Y., Minoshima, S., Shimizu, N., Haussler, C. A., Jurutka, P. W., and Haussler, M. R. (1998) *J. Cell. Biochem.* **70,** 94–109
34. Luo, Z., Rouvinen, J., and Mäenpää, P. H. (1994) *Eur. J. Biochem.* **223,** 381–387
35. Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., and O'Malley, B. W. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3294–3298
36. White, M. P. (1994) *Endocr. Rev.* **15,** 439–461
37. Cai, G., Michigami, T., Yamamoto, T., Yasui, N., Satomura, K., Yamagata, M., Shima, M., Nakajima, S., Mushiake, S., Okada, S., and Ozono, K. (1998) *J. Clin. Endocrinol. Metab.* **83,** 3936–3942
38. Danila, D. C., Schally, A. V., Nagy, A., and Alexander, J. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 669–673
39. Ohyama, Y., Ozono, K., Uchida, M., Yoshimura, M., Shinki, T., Suda, T., and Yamamoto, O. (1996) *J. Biol. Chem.* **271,** 30381–30385
40. Ozono, K., Liao, J., Kerner, S. A., Scott, R. A., and Pike, J. W. (1990) *J. Biol. Chem.* **265,** 21881–21888
41. Sone, T., Kerner, S. A., and Pike, J. W. (1991) *J. Biol. Chem.* **266,** 23296–23305
42. Nakajima, S., Yamagata, M., Sakai, N., and Ozono, K. (1998) *Mol. Cell. Endocrinol.* **139,** 15–24
43. Guiochon-Mantel, A., Delabre, K., Lescop, P., and Milgrom, E. (1996) *J. Steroid Biochem. Mol. Biol.* **56,** 3–9
44. Ishidate, T., Yoshihara, S., Kawasaki, Y., Roy, B. C., Toyoshima, K., and Akiyama, T. (1997) *FEBS Lett.* **409,** 237–241
45. Nakielny, S., and Dreyfuss, G. (1996) *J. Cell Biol.* **134,** 1365–1373
46. Ichikawa, K., and Hashizume, K. (1991) *Life Sci.* **49,** 1513–1522
47. Ross, A. C. (1993) *FASEB J.* **7,** 317–327
48. Lyons, R. H., Ferguson, B. Q., and Rosenberg, M. (1987) *Mol. Cell. Biol.* **7,** 2451–2457
49. Tyagi, R. K., Amazit, L., Lescop, P., Milgrom, E., and Guiochon-Mantel, A. (1998) *Mol. Endocrinol.* **12,** 1684–1695