Transcriptional Activity of a Fluorinated Vitamin D Analog on VDR−RXR-Mediated Gene Expression†

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ABSTRACT: The transcriptional activity of the hexafluorinated derivative of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3 [F_6-1,25-$(OH)_2D_3$], was examined in cultured cells by a transient expression assay (CAT assay) using expression vectors for the rat nuclear vitamin D_3 receptor (VDR) and the rat 9-*cis*-retinoic acid receptor (RXRβ), and a reporter plasmid containing a consensus vitamin D_3 response element (VDRE) consisting of two directly repeated AGGTCA motifs spaced by 3 bp (DR3). At physiological concentrations, the transcriptional activity of F_6-1,25-$(OH)_2D_3$ was 2−4 times more potent than that of 1,25-$(OH)_2D_3$ in both nontarget (HeLa) and target (UMR106) cells for 1,25-$(OH)_2D_3$. The transcriptional activity of F_6-1,25-$(OH)_2D_3$ was also higher when the endogenous target gene (osteopontin), which has a VDRE related to the DR3 in its promoter, was induced. A gel-shift assay using DR3 as a probe and in vitro synthesized receptors showed that the ligand-induced DNA binding of VDR required RXR to form a heterodimer. Moreover, in this assay we found that F_6-1,25-$(OH)_2D_3$ induced the receptor−DNA complex at a 10-fold lower concentration than 1,25-$(OH)_2D_3$ without influencing the dissociation kinetics. However, the binding affinity of F_6-1,25-$(OH)_2D_3$ for VDR was slightly lower than that of 1,25-$(OH)_2D_3$. The increased DNA binding of ligand-bound VDR by introducing hexafluorines into 1,25-$(OH)_2D_3$ may potentiate the transcriptional activity. Thus, the higher biological activity of F_6-1,25-$(OH)_2D_3$ may be exerted at least in part by enhanced transcriptional activity.

Hexafluorinated 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$],[1] 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3 [F_6-1,25-$(OH)_2D_3$] (Kobayashi *et al.*, 1980), is more potent than 1,25-$(OH)_2D_3$ in terms of a wide range of actions, especially in curing rickets and maintaining plasma calcium and phosphate levels (Tanaka *et al.*, 1984). Moreover, F_6-1,25-$(OH)_2D_3$ also positively influenced Interleukin 2 production in HL-60 cells as well as cytodifferentiation (Inaba *et al.*, 1987), though its binding to 1,25-$(OH)_2D_3$ nuclear receptor (VDR) was slightly less than that of 1,25-$(OH)_2D_3$ (Tanaka *et al.*, 1984; Inaba *et al.*, 1987). The discrepancy between the biological activities and the affinity for VDR is thought to be due to the different rates of their metabolic processes, as the substituted fluoro groups at the 26- and 27-carbons of 1,25-$(OH)_2D_3$ are supposed to be more resistant to metabolic inactivation (Inaba *et al.*, 1987).

It is generally accepted that most of the biological actions of vitamin D_3 and its synthetic analogs are exerted by VDR-mediated gene expression (Darwish & DeLuca, 1993). VDR is a member of a nuclear steroid/thyroid receptor superfamily, which acts as a ligand-inducible transcription factor (Green & Chambon, 1988; Evans, 1988; Beato, 1989). The nuclear receptors control transcription of their target genes by binding to the cognate *cis*-acting enhancer DNA elements referred to as ligand response elements. Synthetic and natural ligand response elements have been extensively characterized. All of these consensus ligand response elements can be divided so far from the DNA sequence into two types, consisting of a repetition of one core motif. These two types of consensus ligand response elements differ in the orientation of the two core motifs, that is, inverted and direct repeated motifs (Kato *et al.*, 1992a; Mader *et al.*, 1993a). Systematic studies using a series of synthetic target elements for nuclear receptors (Näär *et al.*, 1991; Umesono *et al.*, 1991; McDonald *et al.*, 1993) have demonstrated that VDR has a preference for two direct repeat AGGTCA motifs, spaced 3 bp apart [DR3 = consensus vitamin D response element (VDRE)], by forming a heterodimer with RXR (Yu *et al.*, 1991; Leid *et al.*, 1992a), the ligand for which has been recently identified as 9-*cis*-retinoic acid (9CRA) (Heyman *et al.*, 1992; Levin *et al.*, 1992; Leid *et al.*, 1992b). This VDR−RXR heterodimer activates transcription through the VDRE in a ligand-dependent manner (Darwish & DeLuca 1993).

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[1] Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CAT, chloramphenicol acetyltransferase; DR3, direct repeat 3; F_6-1,25-$(OH)_2D_3$, 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D_3; F_6-1,23-(*S*),25-$(OH)_3D_3$, 26,26,26,27,27,27-hexafluoro-1,23(*S*),25-trihydroxyvitamin D_3; 9CRA, 9-*cis* retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; TR, thyroid hormone receptor; VDR, vitamin D receptor; VDRE, vitamin D response element; OPN, osteopontin; HPLC, high-performance liquid chromatography.

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Inaba *et al.* (1989) have shown that, in affinity chromatography using total DNA, F_6-1,25-$(OH)_2D_3$ enhances the binding affinity of VDR to DNA more than 1,25-$(OH)_2D_3$, leading to the speculation that the higher biological activity of F_6-1,25-$(OH)_2D_3$ may be achieved by increased affinity of ligand-bound VDR for DNA. Although their observations indicated that the increased affinity to DNA may potentiate transcription, there has not been direct information about the transcriptional activity of F_6-1,25-$(OH)_2D_3$-mediated VDR upon the VDRE. Therefore, to investigate the molecular mechanism of the higher biological activity of F_6-1,25-$(OH)_2D_3$, its transcriptional activity was evaluated using a transient-expression assay (CAT assay) with expression vectors for rat VDR and rat RXRβ and a reporter plasmid containing a consensus VDRE (DR3) in cultured cells. Moreover, to understand how F_6-1,25-$(OH)_2D_3$ exerts a higher transcriptional activity at the molecular level, we used a gel shift assay with rat VDR and RXR synthesized in vitro and the VDRE (DR3). We found that F_6-1,25-$(OH)_2D_3$ and its major metabolite F_6-1,23(*S*),25-$(OH)_3D_3$ were more potent than 1,25-$(OH)_2D_3$ in terms of VDR-mediated transcription. The activity of F_6-1,25-$(OH)_2D_3$ was also higher during the induction of the endogenous target gene (osteopontin) for 1,25-$(OH)_2D_3$. The gel shift assay revealed, in agreement with the transcriptional activity, that F_6-1,25-$(OH)_2D_3$ induced the DNA binding of the VDR−RXR heterodimer to the VDRE (DR3) at 10 times lower concentration than 1,25-$(OH)_2D_3$ without influencing the dissociation kinetics, though the ligand binding potency of F_6-1,25-$(OH)_2D_3$ to VDR was slightly less than that of 1,25-$(OH)_2D_3$. Thus, these findings suggest that the introduction of fluorines into vitamin D at carbons 26 and 27 increases the binding affinity of ligand-bound VDR to DNA without affecting its affinity for VDR, thereby enhancing transcriptional activity.

MATERIALS AND METHODS

Plasmids. A reporter plasmid containing the consensus VDRE was constructed to insert a synthetic DNA(5′-AGCTTCAGGTCAAGGAGGTCAG-3′) containing AGGTCA-based direct repeats (DR3) (Umesono *et al.*, 1991), with *Hin*dIII−*Xba*I ends, into the cognate sites of pGCAT (Kato *et al.*, 1992a). To clone rat VDR, we first amplified a cDNA fragment corresponding to the region of the VDR encoding amino acids 1−68, using the synthetic primers deduced from the VDR DNA sequence (Brumer *et al.*, 1988) by PCR. Using this fragment as a probe, we screened the full-length VDR cDNA from the rat kidney cDNA library (Stratagene) and isolated two overlapping clones. The expression vector for VDR was generated from the two clones by double insertion into the *Eco*RI−*Bam*HI sites of pSG5 (Green *et al.*, 1988). A Kozak initiation sequence (5′-CCACC-3′) was then introduced in front of the translation start site (5′-ATG-3′) by subcloning the PCR product (Kamei *et al.*, 1993). The full-length of rat RXRβ cDNA was cloned from the rat liver cDNA library (Stratagene) in the same way (Mano *et al.*, 1994) and verified by dideoxy nucleotide sequencing.

Cell Transfection and CAT Assay. HeLa and UMR106 cells were maintained in Dulbecco's modified Eagle's medium without phenol red supplemented with 5% fetal calf serum stripped with dextran-coated charcoal. The cells were transfected at 40%−60% confluence in 9 cm Petri dishes with a total of 20 μg of DNA by means of calcium phosphate coprecipitation. The reporter plasmid (2 μg/dish) was cotransfected with 0.5 μg each of the expression vectors for VDR and RXRβ and 3 μg of the reference plasmid pCH110 (Pharmacia). Bluescribe M13+ (Stratagene) was used as the carrier to adjust the amount to 20 μg. A vitamin D derivative or 1,25-$(OH)_2D_3$ was added to the medium 1 h after transfection and every 8 h at each exchange of the medium. After 48 h, CAT was assayed as described (Kato *et al.*, 1992a), after normalizing the transfection efficiency with β-galactosidase activity expressed from pCH110 in HeLa cells (5 units) and in UMR106 cells (2.5 units).

RNA Isolation and Northern Blots. Total RNA was isolated by the AGPC method (Chomczynski & Sacchi, 1987). Total RNA (40 μg) was separated on a 1.1 M formaldehyde/1% agarose gel and then transferred to a nitrocellulose membrane (Schleicher & Schuell, BAS85) by capillary action in 20 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0). The membranes were cross-linked under UV light (Stratagene) and prehybridized at 42 °C in 50% formamide, 5 × SSPE (1 × SSPE = 0.1 M sodium chloride, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.0), 5 × Denhardt's solution (1 × Denhardt's = 0.02% polyvinylpyrrolidone, 0.02% BSA, 0.02% Ficoll 400), 1 mg/mL salmon-sperm DNA (ssDNA), and 0.1% SDS for 4 h. Thereafter, the membranes were hybridized at 42 °C for 18 h in 50% formamide, 5 × SSPE, 1 × Denhardt's solution, 0.2 mg/mL ssDNA, and 1 × 10^6 cpm/mL specific cDNA probe. The membranes were washed at room temperature for 15 min in 2 × SSPE, 0.03% $NaPP_i$, 0.1% SDS, then at 65 °C for 15 min in 1 × SSPE, 0.03% $NaPP_i$, 0.1% SDS, and then at 65 °C in 0.1 × SSPE, 0.03% $NaPP_i$, 1% SDS and exposed to X-ray film at −80 °C for 3 or 10 days. The membranes were dehybridized at 90 °C for 15 min in 0.1 × SSPE, 0.1% SDS and rehybridized with other specific probes (Kato *et al.*, 1992b, Mano *et al.*, 1993, 1994). The full-length cDNAs of rat VDR and rat RXRβ were cloned as described above, and mouse osteopontin cDNA was a kind gift from Dr. S. Nomura (Nomura *et al.*, 1988). The transcripts were quantified by densitometrically scanning the autoradiograms, using rat β-actin transcript as an internal control for each sample (Kato *et al.*, 1992b, Mano *et al.*, 1993, 1994).

High-Performance Liquid Chromatography. Lipid extraction of the cultured cells and medium incubated with either [1β-^{3}H]-1,25-$(OH)_2D_3$ (15.9 Ci/mmol) or [1β-^{3}H]-F_6-1,25-$(OH)_2D_3$ (18.8 Ci/mmol) was performed by the method of Bligh and Dyer (1959). The chloroform layer containing the lipid-soluble metabolites of 1,25-$(OH)_2D_3$ or F_6-1,25-$(OH)_2D_3$ (Miller *et al.*, 1990) was dried under N_2 gas at 37 °C. Samples were dissolved in absolute ethanol and used for high-performance liquid chromatography (HPLC) analysis. Analysis of the vitamin D analogs by HPLC was performed using a modular instrument composed of a Model 600 pump and a Model 440 fixed-wavelength ultraviolet (UV) detector (Waters Associates, Inc.) with a Zorbax-SIL (DuPont Instruments) 4.6 mm × 25 cm column using *n*-hexane−2-propanol (92:8), at a flow rate of 2.0 mL/min for 1,25-$(OH)_2D_3$, and *n*-hexane−dichloromethane−methanol (49:48:3), at a flow rate of 1.5 mL/min for F_6-1,25-$(OH)_2D_3$, as the solvents. Radioactivity in the fractions separated by HPLC was measured using a liquid scintillation counter.

Gel Retardation. DNA binding to ligand-bound VDR complexes was determined by the electrophoretic mobility

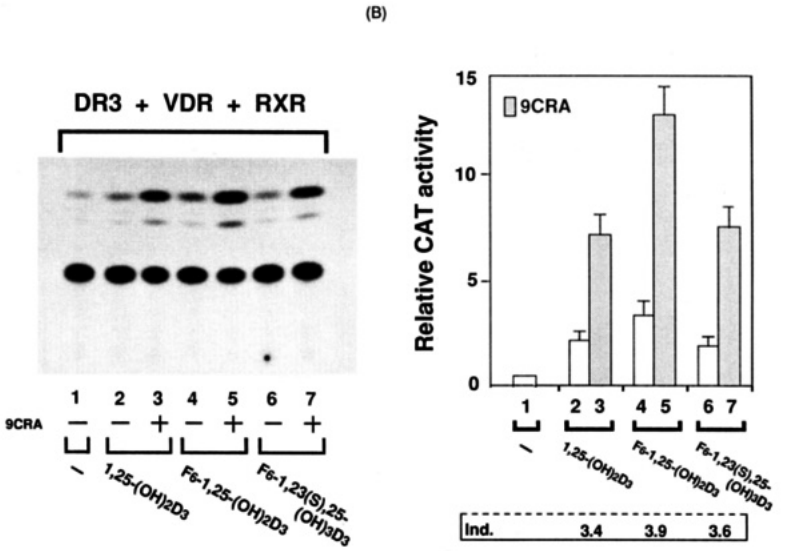

FIGURE 1: The transcriptional activity of VDR ligands is enhanced by 9-*cis*-retinoic acid in the presence of RXR. (A) A representative CAT assay is shown. UMR 106 cells were cotransfected with 2 μg of a CAT reporter plasmid containing consensus VDRE in front of the rabbit β-globin promoter and 500 ng each of the expression vectors for VDR and RXRβ, together with 3 μg of the β-galactosidase expression vector pCH110. The cells were maintained for 48 h in the absence (−) or presence (+) of the ligand and then collected and assayed for CAT with normalization of the β-galactosidase activity. The ligand was added at each exchange of the medium every 8 h. (B) Relative CAT activity. The CAT activities of the cell extracts are expressed as the means ± SD. calculated from the values of at least three independent experiments.

shift assay as described (Ozono *et al.*, 1990; Kato *et al.*, 1992a). The VDR and RXRβ proteins were synthesized by in vitro translation in the rabbit reticulocyte lysate using in vitro synthesized mRNAs from the expression vectors (pSG5). Increasing amounts of VDR ligands dissolved in ethanol were dried under N_2 gas in the bottoms of tubes, then the reticulocyte lysates in the reaction buffer [0.7 × TETGEN (1 × TETGEN = 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 7.5, 0.2% Tween 20, 15% glycerol, 0.1 M NaCl)] were added. Binding was started by adding the binding buffer (0.12 M Hepes, pH 7.9, 6 mM EDTA, pH 8.0, 30 mM $MgCl_2$, 40 mM Tris-HCl, pH 8.0, 6 mM KCl, 30 mM 2-mercaptoethanol) in the presence of poly(dIdC) (2 μg/tube). After a 20 min incubation at room temperature, 1 μL of [^{32}P]-5′-end-labeled (1 × 10^4 cpm) synthetic oligonucleotide [consensus VDRE = two directly repeated AGGTCA motifs spaced by 3 bp (DR3)] was added and the mixture incubated at room temperature for 20 min. Samples were then resolved by electrophoresis on 5% polyacrylamide gels. The dissociation kinetics were studied by means of gel retardation "off" curves (Mader et al., 1993b). Complex formation proceeded for 20 min at room temperature and a 500-fold excess of cold competitor oligonucleotide was added at 0 min. The gels were quantified by phosphoimaging using a Fuji BAS 2000.

Assay of Ligand Binding to VDR. All procedures were performed essentially as described (Tanaka *et al.*, 1984). Increasing amounts of cold VDR ligand with [^{3}H]-1,25-$(OH)_2D_3$ (13 Ci/mmol, Amersham) dissolved in ethanol were dried under N_2 gas in the bottoms of tubes, then 4 μL of the reticulocyte lysate containing VDR and 21 μL of TENM buffer [20 mM Tris-HCl (pH 7.3), 1 mM EDTA, 50 mM NaCl, 2 mM 2-mercaptoethanol] was added. After a 16 h incubation at 4 °C, 50 μL of stop solution (0.5% Norit A, 0.05% dextran T-70, 10 mM Tris-HCl (pH 7.5)] was added, then the reaction mixture was centrifuged for 5 min at 10000*g* at 4 °C. Specific binding was calculated from the radioactivity in the supernatant using a liquid scintillation counter.

RESULTS

The Transcriptional Activity of a Fluorinated Vitamin D_3 Analog on VDR−RXR-Mediated Gene Expression. To determine the transcriptional activity of a vitamin D_3 analog, we performed a transient expression study using expression vectors for VDR and RXRβ and the reporter plasmid for the chloramphenicol acetyltransferase (CAT) gene containing the consensus VDRE (DR3) driven by a rabbit β-globin short promoter (pDR3GCAT) in two cell lines. One was UMR106, which was established from osteoblasts and supposed to be the target cells for vitamin D_3. HeLa cells were used as the nontarget cells.

The cotransfection of RXRβ and the addition of its ligand, 9CRA (10^{-9} M), potentiated the transcriptional activity of the VDR ligands (10^{-8} M) 3−4-fold in target (Figure 1) and nontarget cells (data not shown), but only 9CRA showed slight inducibility (1.1−1.4-fold) on the VDRE in the presence of VDR and RXR (data not shown), in agreement with previous reports (Yu *et al.*, 1991; Carlberg *et al.*, 1993). Therefore, the expression vector for RXRβ and 9CRA were included in all assays to undergo efficient transmission of the VDR ligand signal into transcription. The transfected cells were incubated for 48 h in the absence or presence of ligands. To avoid the influence of metabolic inactivation on the ligands, the medium was changed every 8 h.

In UMR106, 1,25-$(OH)_2D_3$ transactivated the reporter genes several fold at physiological concentrations (10^{-9}−10^{-8} M). At the same concentrations, F_6-1,25-$(OH)_2D_3$ and its major metabolite, F_6-1,23(*S*),25-$(OH)_3D_3$ (Honda *et al.*, 1993), also transactivated 2−3 times more efficiently than 1,25-$(OH)_2D_3$. Moreover, even at a very low concentration (10^{-10} M), F_6-1,25-$(OH)_2D_3$ and F_6-1,23(*S*),25-$(OH)_3D_3$ clearly activated (2−4-fold) CAT activity, whereas 1,25-$(OH)_2D_3$ could not (Figure 2). The fluorinated vitamin D analogs at low concentrations also had higher activity also in HeLa cells (Figure 3). The activity of the analogs was almost double that of 1,25-$(OH)_2D_3$ at physiological con-

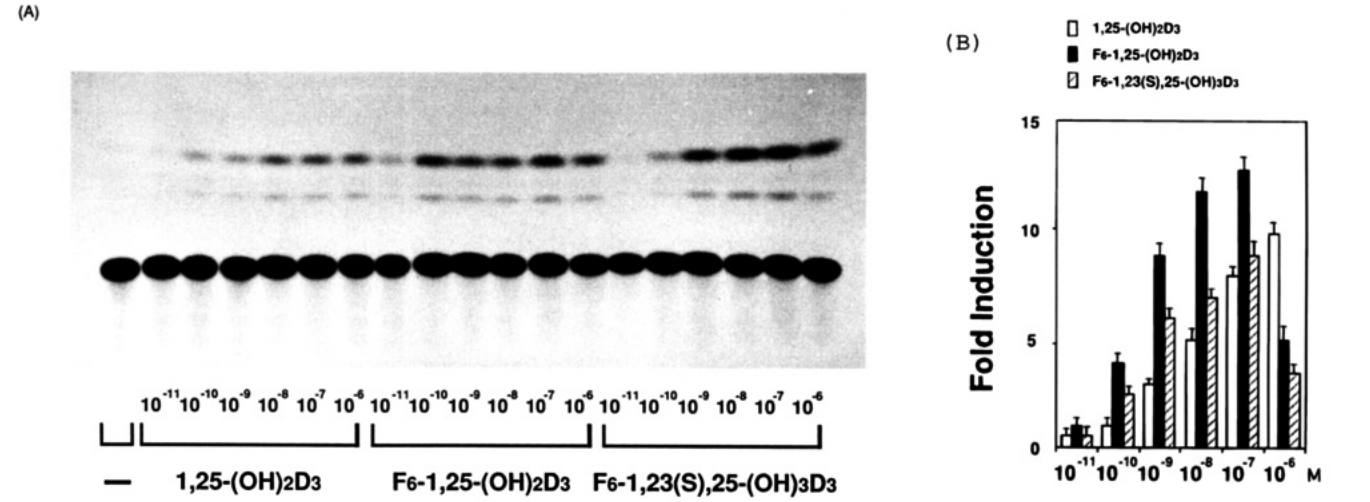

FIGURE 2: Transcriptional activities of 1,25-$(OH)_2D_3$, F_6-1,25-$(OH)_2D_3$, and F_6-1,23(*S*),25-$(OH)_3D_3$ upon the consensus VDRE in target cells for 1,25-$(OH)_2D_3$. UMR 106 cells were cotransfected with 2 μg of a CAT reporter plasmid containing consensus VDRE in front of the rabbit β-globin promoter and 500 ng each of the expression vectors for VDR and RXRβ, together with 3 μg of the β-galactosidase expression vector pCH110. The CAT assay was performed as described in the legend to Figure 1. A representative CAT assay is shown in panel A. Fold induction (panel B) was calculated by dividing the CAT activity determined in the extracts from ligand-treated cells by that in untreated control cells.

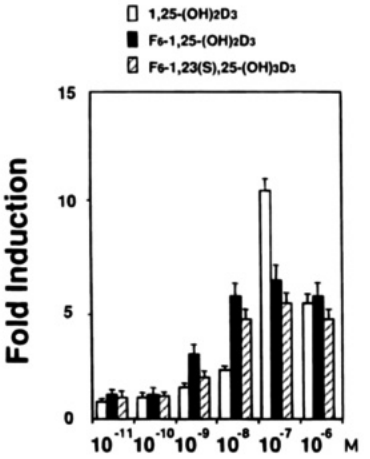

FIGURE 3: Transcriptional activities of 1,25-$(OH)_2D_3$, F_6-1,25-$(OH)_2D_3$, and F_6-1,23(*S*),25-$(OH)_3D_3$ upon the consensus VDRE in nontarget cells for 1,25-$(OH)_2D_3$. HeLa cells were cotransfected with 2 μg of a CAT reporter plasmid containing consensus VDRE in front of the rabbit β-globin promoter and 500 ng each of the expression vectors for VDR and RXRβ, together with 3 μg of the β-galactosidase expression vector pCH110. The CAT assay was performed as described in the legend to Figure 1. Fold induction is expressed as described in the legend to Figure 2.

centrations such as 10^{-9} and 10^{-8} M (Figure 3). However, at higher concentrations around 10^{-6} M in UMR106 and HeLa cells, 1,25-$(OH)_2D_3$ was equal to or more active than the vitamin D_3 analogs. Even though the activation of the VDR by these ligands was weaker in the absence of 9CRA, the fluorinated vitamin D analogs were more potent than 1,25-$(OH)_2D_3$ in both cell lines (data not shown).

To assess the transcriptional activity of the vitamin D analog on the expression of endogenous target genes for vitamin D, we selected the osteopontin (OPN) gene, which has a well-characterized VDRE related to the DR3 in its promoter (Noda *et al.*, 1990; Zhang *et al.*, 1992). When the UMR106 cells were incubated with the ligand (10^{-8} M) for 48 h, the OPN gene was induced. As shown in Figure 4, F_6-1,25-$(OH)_2D_3$ was twice as active as 1,25-$(OH)_2D_3$, which was in good agreement with the results in the transient expression assay. Note that the elevated biological activity of F_6-1,25-$(OH)_2D_3$ was not due to the increased levels of

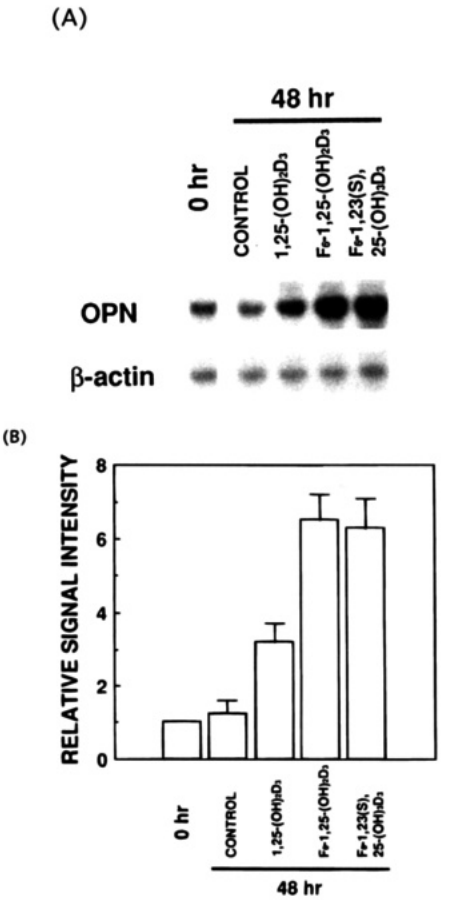

FIGURE 4: F_6-1,25-$(OH)_2D_3$ enhanced the induction of the endogenous osteopontin gene. UMR 106 cells were maintained for 48 h in the absence (−) or presence (+) of the ligand (10^{-8} M), and total RNA was isolated. The ligand was added at each exchange of the medium every 8 h. The relative abundance of OPN mRNA was calculated by densitometrically scanning Northern blots of the specific bands (a representative result is shown in panel A). The relative values in panel B were normalized to the amount of β-actin transcript and are expressed as the means ± SD of more than three samples.

VDR and RXR, since the mRNA levels of these receptors were not affected by the ligands (Figure 5).

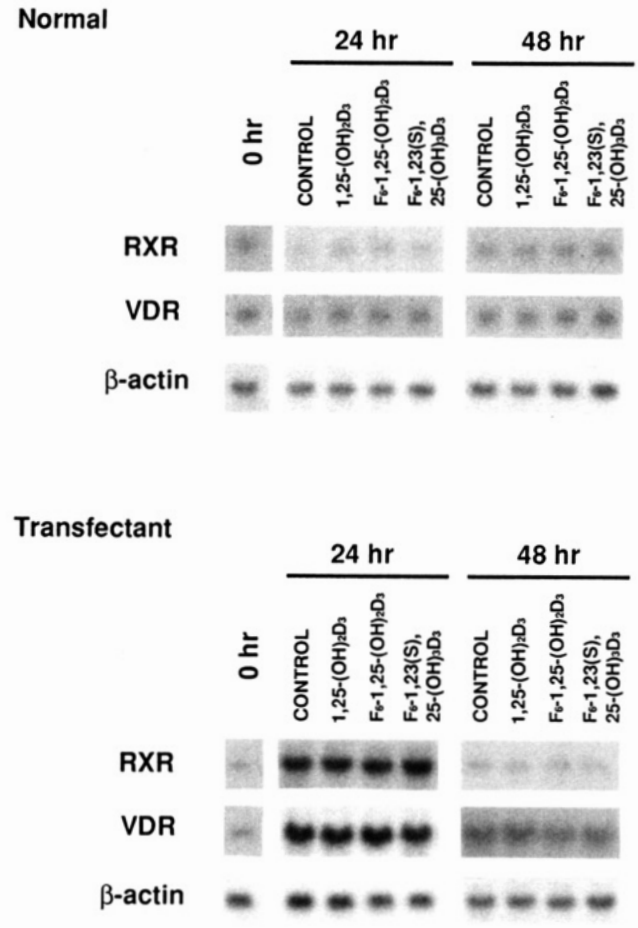

FIGURE 5: Effect of 1,25-$(OH)_2D_3$ and F_6-1,25-$(OH)_2D_3$ on the mRNA levels of VDR and RXR. UMR 106 cells were transfected with 500 ng each of the expression vectors for VDR and RXRβ as described in Figure 1. The control and transfected cells were maintained for 48 h in the absence (−) or presence (+) of the ligand. The ligand was added at each exchange of the medium every 8 h. The relative abundance of the VDR and RXR mRNAs was calculated and expressed as described in the legend to Figure 4.

Table 1: Metabolism of 1,25-$(OH)_2D_3$ and F_6-1,25-$(OH)_2D_3$ by HeLa Cells

incubn time (h)	concn (M)	% 1,25-$(OH)_2D_3$	% F_6-1,25-$(OH)_2D_3$
8	10^{-9}	91	91
	10^{-8}	94	95
	10^{-7}	100	98
24	10^{-9}	89	90
	10^{-8}	92	93
	10^{-7}	93	95

[a] HeLa cells were incubated with either [1β-^{3}H]-1,25-$(OH)_2D_3$ (15.9 Ci/mmol) of [1β-^{3}H]-F_6-1,25-$(OH)_2D_3$ (18.8 Ci/mmol) for the indicated period. Each compound was measured as described in Materials and Methods. The values are expressed as a percentage of the amount in the incubated medium without the cells.

Metabolism of 1,25-$(OH)_2D_3$ and F_6-1,25-$(OH)_2D_3$ in HeLa cells was studied by HPLC analysis (Table 1) to discover whether the higher activity of F_6-1,25-$(OH)_2D_3$ is due to the delayed metabolic inactivation. However, a significant difference between their metabolisms reflecting the activity was not observed.

Thus, these findings indicate that, at physiological concentrations, the vitamin D analogs are more potent than 1,25-$(OH)_2D_3$ in terms of VDR-mediated transactivation.

F_6-1,25-$(OH)_2D_3$ Enhanced the Binding Affinity of VDR to a Consensus Vitamin D Response Element. To investigate how the fluorinated vitamin D analogs elicit higher transactivation activity, we examined the activity of ligand-induced DNA binding of the VDR−RXR heterodimer and the structural change of ligand-bound VDR by a gel-shift assay. We used in vitro synthesized VDR and RXRβ and the same DR3 element that we used for the transactivation assay. Efficient complex formation with DR3 occurred only when both receptors and the ligand for VDR were present (Figure 6A), suggesting that ligand-induced DNA binding requires heterodimerization of VDR and RXR. The ligand for VDR drastically induced DNA binding of the VDR−RXR heterodimer to the DR3, whereas the addition of 9CRA to any reaction mixture did not significantly affect DNA binding (Figure 6A). Under these conditions, F_6-1,25-$(OH)_2D_3$ induced the DNA binding of the VDR−RXR heterodimer at 10^{-8}−10^{-9} M (Figure 6B), whereas 1,25-$(OH)_2D_3$ and F_6-1,23(*S*),25-$(OH)_3D_3$ required 10 times greater concentrations (10^{-7}−10^{-8} M). Moreover, it is notable that the dissociation kinetics between the 1,25-$(OH)_2D_3$ and F_6-1,25-$(OH)_2D_3$ bound VDR−RXR heterodimers were the same (Figure 6C,D). This higher activity of F_6-1,25-$(OH)_2D_3$ reflects its transcriptional activity, as shown in Figures 2 and 3. However, a difference in the migration of ligand-bound receptor complexes was undetectable in the gel and even in those run for longer periods, indicating that the increase in DNA binding affinity of F_6-1,25-$(OH)_2D_3$ may not be due to a drastic structural change of the ligand-bound VDR.

The Binding Affinity of F_6-1,25-$(OH)_2D_3$ to in Vitro Synthesized VDR. We determined whether or not the higher activity of F_6-1,25-$(OH)_2D_3$ in the induction of DNA binding to the VDR−RXR heterodimer is due to its increased affinity for the VDR. We investigated the binding affinity of the fluorinated vitamin D analogs to the VDR synthesized in rabbit reticulocyte lysates, by means of a competition assay using ^{3}H-labeled 1,25-$(OH)_2D_3$. The binding affinities of the fluorinated vitamin D analogs for the VDR were slightly lower than that of 1,25-$(OH)_2D_3$ (Figure 7). These results were similar to those using VDR in the crude extract of chick intestine and HL-60 cells (Tanaka *et al.*, 1984; Inaba *et al.*, 1987). Although the gel-shift assay (Figure 6) showed that RXR is required for ligand-bound VDR to form an efficient binding complex with the consensus VDRE, the presence of RXR as well as the DR3 element did not affect the binding affinity of these ligands to the VDR (data not shown).

DISCUSSION

F_6-1,25-$(OH)_2D_3$ is more potent than 1,25-$(OH)_2D_3$ in terms of the healing of rickets, the elevation of serum calcium and phosphate levels, and in the cytodifferentiation of HL-60 cells (Tanaka *et al.*, 1984; Inaba *et al.*, 1987). As the VDR is a member of a thyroid/steroid nuclear receptor superfamily, which acts as a ligand-inducible transcription factor (Green & Chambon, 1988; Evans, 1988; Beato, 1989), most of the biological actions of vitamin D_3 and its analogs are supposed to be exerted by activating transcription through the binding of the VDR complex to target enhancers (Darwish & DeLuca, 1993). Though several lines of study have indicated that introducing fluorides into vitamin D_3 potentiates transactivation (Inaba *et al.*, 1989), there has not been direct evidence that supports this notion. The results presented here show that F_6-1,25-$(OH)_2D_3$ is indeed more

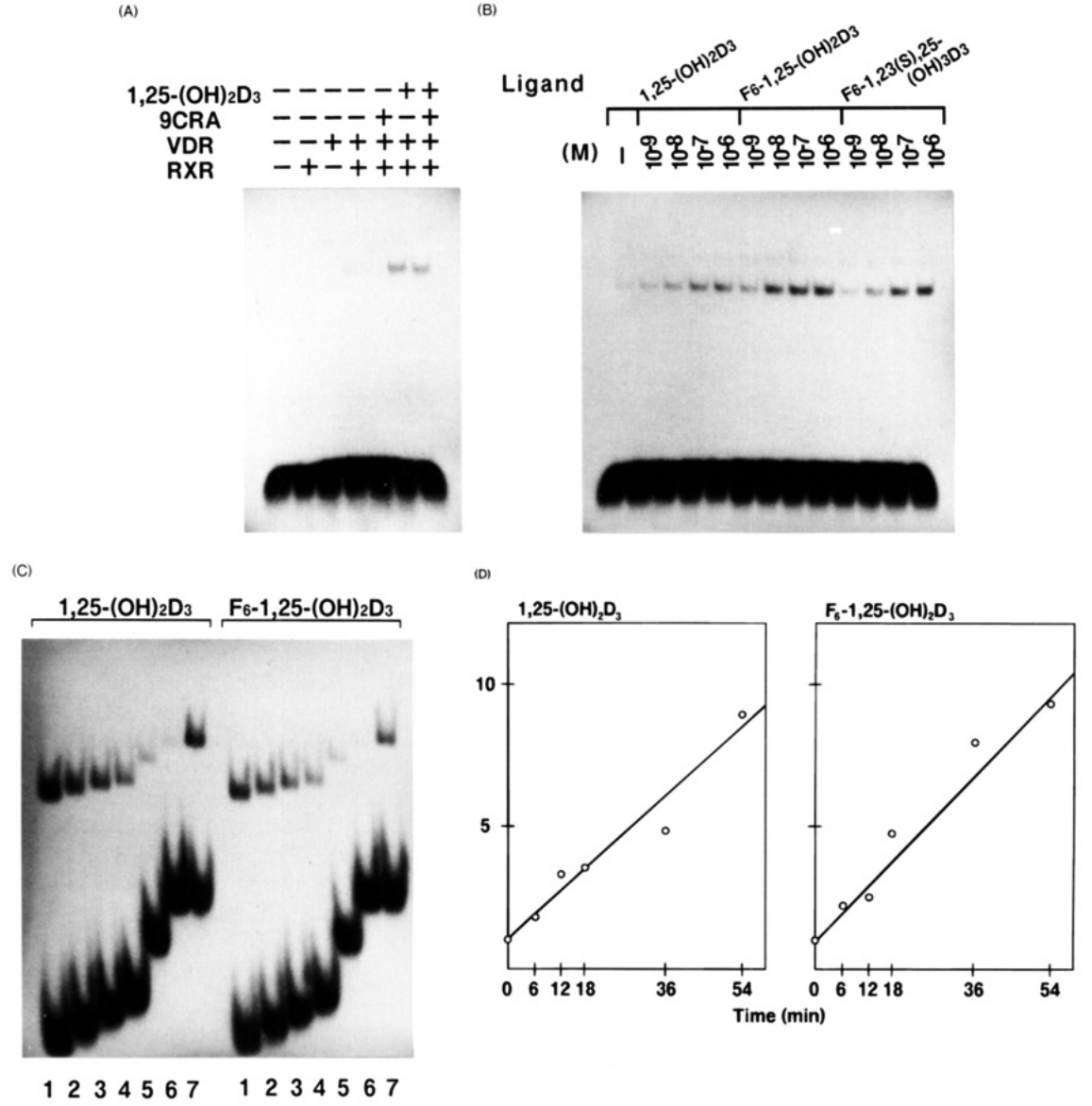

FIGURE 6: F_6-1,25-$(OH)_2D_3$ enhanced the ligand-induced DNA binding of VDR to a consensus vitamin D response element. (A) Requirement of VDR−RXR heterodimerization for ligand-induced DNA binding. (B) Ligand-induced DNA binding of a VDR−RXR heterodimer. (C and D) Dissociation kinetics of the VDR−RXR heterodimer bound to the DR3. A gel-shift assay was performed with the [^{32}P]-5′-end-labeled oligonucleotide containing DR3 and in vitro synthesized VDR and RXRβ as described in Materials and Methods. The ligand was added to the tube and dried under N_2 gas. The DNA probe and the receptors in the reaction buffer were then added. After an incubation at room temperature for 20 min, the binding reaction was started by adding the DNA probe. The dissociation kinetics of the complex between the ligand-bound VDR−RXR and DR3 were determined by adding a 500-fold excess of unlabeled DR3 competitor DNA at time point 0 (lane 1). After 6, 12, 18, 36, and 54 min at 0 °C, the reaction was loaded onto a running gel. The last lane (7) shows a gel-shift experiment performed after a 54 min incubation under identical conditions, but in the absence of competitor DNA. Graphical representations of the dissociation kinetics (panel D) are derived from quantifying the retarded complexes at various times after competition with the corresponding nonlabeled probe and show the ratio of the radioactivity associated with the retarded band at $t = 0$ min over that measured at a given time after competition.

active than 1,25-$(OH)_2D_3$ in terms of transcriptional activation.

Recent studies have demonstrated that, upon the well-characterized VDRE, efficient DNA binding of VDR requires RXR to form a heterodimer (Yu *et al.*, 1991; Leid *et al.*, 1992a; McDonald *et al.*, 1993). Moreover, RXR serves as an auxiliary protein for the retinoic acid receptor (RAR) and the thyroid receptor (TR) (Yu *et al.*, 1991; Leid *et al.*, 1992b). Though the relationship between the combination of the two ligands and the transcriptional function upon those heterodimers has not been fully established, it is likely that ligand inducibility on transactivation is positively and negatively modulated in a sequence-specific manner (Carlberg*et al.*, 1993; Hallenbeck *et al.*, 1993). In this study, we used a well-characterized VDRE, namely the two AGGTCA motifs spaced by 3 bp (DR3), which have been identified as the consensus VDRE from a comparison among the target enhancers for the other nuclear receptors (Näär *et al.*, 1991; Umesono *et al.*, 1991; Mader *et al.*, 1993a). In fact, we confirmed by a gel-shift assay that, for this VDRE (DR3), RXRβ was essential for ligand-bound VDR to form the retarded complex with DNA (Figure 4). In this respect, the expression vector for RXRβ was included in all the transfection experiments. Moreover, though 9CRA itself did not significantly affect transactivation, 9CRA (10^{-8} M) enhanced the induction by 1,25-$(OH)_2D_3$ and its fluorinated analogs

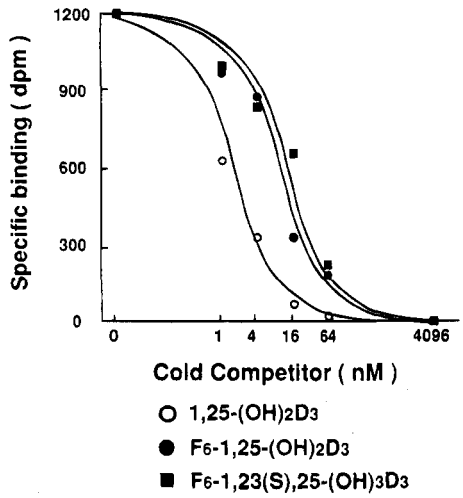

FIGURE 7: Binding affinity of F_6-1,25-$(OH)_2D_3$ for the in vitro synthesized VDR. VDR synthesized in vitro was incubated overnight with 1 nM [^{3}H]-1,25-$(OH)_2D_3$ in the absence or presence of various concentrations of nonradioactive competitors at 4 °C. Unbound vitamin D_3 analogs were removed with dextran-coated charcoal. The charcoal was removed by centrifugation and the receptor-bound radioactivity was measured using a liquid scintillation counter. Each point represents an average of duplicate samples and is expressed as a percentage of the control samples in the absence of a nonradioactive competitor. Only one representative result is shown from three independent experiments.

(Figure 1), suggesting that the VDR−RXR heterodimer also binds the DR3 in vivo, as shown by the in vitro gel-shift assay. However, the degrees of the 9CRA-enhanced transcription did not differ among the VDR ligands at any concentration of 9CRA (data not shown), and 9CRA itself could not induce DNA binding of the VDR−RXR heterodimer in vitro (Figure 4). Thus, these findings showed a distinct relationship between VDR and RXR on ligand-induced transactivation. Upon the DR3, unlike the VDR ligand, 9CRA itself cannot activate transcription, but it synergistically enhanced the effect of the VDR ligand. Thus, these observations raised the possibility that the DNA binding of the receptors may not simply reflect the transactivation function of the ligand, and rather, another mechanism may be involved. Nevertheless, in VDR-mediated transduction, especially in the intact animal, the tissue-specific generation of 9CRA should be considered when the actions of a VDR ligand are assessed (Heyman *et al.*, 1992; Levin *et al.*, 1992).

Fluoro groups at the 26- and 27-carbons of 1,25-$(OH)_2D_3$ have been introduced to prolong the half-life of the analogs by protecting against metabolic inactivation (Tanaka *et al.*, 1984; Inaba *et al.*, 1987). When this compound was administered in vivo, its biological activity lasted longer than that of 1,25-$(OH)_2D_3$. Therefore, its higher activity had been supposed to be mainly due to a delayed metabolic inactivation. To avoid the influence of metabolic inactivation, and to precisely assess the transcriptional activity of the vitamin D analogs in our transient expression assay system, the culture medium containing the ligand was exchanged every 8 h. Under these conditions, however, the contents of 1,25-$(OH)_2D_3$ in the medium were almost the same as those of the vitamin D analogs in HeLa cells after an 8 h incubation (Table 1), though the metabolism in the UMR106 cells may be different (Miller *et al.*, 1990). In addition, the biological activities of the ligands may be modulated by distinct affinities for plasma vitamin D binding protein (DBP) in the culture medium. The lower affinity of F_6-1,25-$(OH)_2D_3$ for DBP than 1,25-$(OH)_2D_3$ may contribute to the enhanced signal transmission of the fluorinated vitamin D analog into gene expression. Nevertheless, at any physiological concentration, F_6-1,25-$(OH)_2D_3$ and F_6-1,23(*S*),25-$(OH)_3D_3$ were more potent on ligand-induced transactivation on the DR3 (Figures 2 and 3). It is therefore unlikely that the higher activities of the fluorinated analogs are simply due to their delayed metabolic inactivation and cellular contents.

An in vitro DNA binding assay (gel-shift assay) revealed a possible molecular mechanism for the higher transcriptional activation of F_6-1,25-$(OH)_2D_3$. As reported (McDonald *et al.*, 1993), ligand-induced DNA binding occurred in the VDR−RXR heterodimer on the consensus VDRE (DR3) (Figure 6). In agreement with the results of transcriptional activation of the VDR ligands, at physiological concentrations the ligand induced the formation of a VDR−RXR and DR3 complex. In this assay, F_6-1,25-$(OH)_2D_3$ was 10 times more efficient than 1,25-$(OH)_2D_3$ without influencing the dissociation kinetics. However, its major metabolite, F_6-1,23(*S*),25-$(OH)_3D_3$, has almost the same activity as 1,25-$(OH)_2D_3$, suggesting that hydroxylation at the 23-carbon of F_6-1,25-$(OH)_2D_3$ neutralizes the enhanced activity of DNA binding affinity (Inaba *et al.*, 1989). The modification of some ligand analogs for the other nuclear receptors causes a structural change of the receptor by ligand binding, judging from the gel migration of the retarded complex of the ligand-bound receptor with the target DNA element (Meyer *et al.*, 1990; Fawell *et al.*, 1990a,b). However, as shown in Figure 6, there was no significant difference in the gel migration of the ligand-bound VDR complexes. The fluorides introduced into vitamin D_3 may cause a slight structural change of the VDR, which may, in turn, have a preference for the target element.

It is supposed that nuclear receptors such as other enhancer binding transcription factors activate transcription by direct and/or indirect protein−protein interactions with the basic transcription machinery (Goodrich *et al.*, 1993). Moreover, interference (squelching) studies have predicted the presence of coactivators, which are supposed to tether the nuclear receptor with the basic transcription machinery (Meyer *et al.*, 1989; Tasset *et al.*, 1990). Until now, the precise molecular mechanism of how these factors cooperatively enhance transcription has remained unknown. However, in this respect it will be valuable to understand how the enhanced DNA affinity of F_6-1,25-$(OH)_2D_3$-bound VDR for the target element potentiates transcription. A reconstitution study with purified factors associated with VDR-mediated transactivation is required.

In conclusion, these findings indicate that the introduction of fluorides at carbons 26 and 27 on 1,25-$(OH)_2D_3$ increases the binding affinity of ligand-bound VDR to target elements without changing the binding affinity of the ligand to VDR, thereby enhancing transcriptional activity. The higher biological activity of F_6-1,25-$(OH)_2D_3$ may be exerted by enhanced transcriptional activity, in addition to delayed metabolic inactivation.

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