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Identification of a Response Element for Vitamin D3 and Retinoic Acid in the Promoter Region of the Human Fructose-1,6-bisphosphatase Gene[1]

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Fructose-1,6-bisphosphatase (FBPase) is a key gluconeogenic enzyme. The data herein show that both the enzyme activity and mRNA level of the human FBPase gene are enhanced by 9-*cis* retinoic acid (9cRA) and all-*trans* retinoic acid (atRA) as well as by 1,25-dihydroxyvitamin D3 (VD3) in human promyelocytic HL60 cells and normal monocytes in peripheral blood, which were used as an alternative source to liver for the DNA diagnosis of FBPase deficiency. To understand the molecular mechanism of this enhancing action, the 2.4 kb 5′-regulatory region of the human FBPase gene was isolated and sequenced. Using luciferase reporter gene assays, a 0.5 kb FBPase basal promoter fragment was found to confer induction by VD3, 9cRA, and atRA that was mediated by the vitamin D3 receptor (VDR), retinoid X receptor (RXR), and retinoic acid receptor (RAR). Within this region, a direct repeat sequence, 5′-TAACCTttcTGAACT-3′ (–340 to –326), which functions as a common response element for VD3, 9cRA, and atRA, was identified. The results of electrophoretic mobility shift assays indicated that VDR-RXR and RAR-RXR heterodimers bind this response element. Collectively, these observations indicate that VD3 and RA are important modulators of the expression of the human FBPase gene in monocytic cells.

Key words: fructose-1,6-bisphosphatase, gluconeogenesis, nuclear receptor, RARE, VDRE.

Fructose-1,6-bisphosphatase (FBPase; D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11), a key gluconeogenetic enzyme, catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate. Human FBPase mRNA was first isolated from a promyelocytic cell line, HL60 cells, that had been treated with 1,25-dihydroxyvitamin D3 (VD3) (*1*). FBPase is expressed in various tissues, and the enzymes expressed in liver and kidney are probably identical. This conclusion is supported by the observations that FBPase cDNAs isolated from human liver, kidney, and monocyte(s) were found to be indistinguishable (*2*). Although liver FBPase is subject to hormonal control (*3*, *4*), the transcriptional regulation of the enzyme in each tissue is not clear. Congenital FBPase deficiency (MIM 229700) is characterized by hypoglycemia and severe metabolic acidosis as a result of fasting at birth, and is associated with sudden infant death (*5*). Therefore, a simple and reliable diagnosis of this disease is very important. We established a diagnostic procedure of FBPase deficiency using peripheral blood monocytes cultured in the presence of VD3, for both enzyme activity and cDNA, as an alternative source to liver biopsy samples (*2*). Using this procedure, we were able to identify the common mutation among Japanese patients with FBPase deficiency (*6*, *7*). However, we were unable to detect FBPase mRNA in some European patients for whom the impaired expression of the FBPase gene may also cause enzyme deficiency. These findings emphasize the need to understand the regulatory mechanisms and factors that modulate FBPase expression in monocytes. No studies to characterize the *trans*-acting factors that interact with their *cis*-elements in the promoter region of the FBPase gene have yet been performed. The DNA sequences of the 5′-noncoding regions of the FBPase gene of rats and humans are completely divergent,

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Abbreviations: atRA, all-*trans* retinoic acid; bp, base pairs; β-gal, β-galactosidase; 9cRA, 9-*cis* retinoic acid; DR, direct repeat; EMSA, electrophoretic mobility shift assay; kbp, kilobase pairs; Luc, luciferase; FBS, fetal bovine serum; FBPase, fructose-1,6-bisphosphatase; HSV, herpes simplex virus; TBE, Tris-borate-EDTA; TGE, Tris-glycine-EDTA; TK, thymidine kinase; VDR, vitamin D3 receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; VD3, 1,25-dihydroxyvitamin D3; VDRE, vitamin D3 response element.

despite conservation in the coding regions (*4*, *8*). These facts led us to initiate an independent study on the regulation of human FBPase gene expression. Since another lipophilic vitamin, retinoic acid (RA), is a known differentiation factor of HL60 cells as well as VD3 (*1*, *9*), we focus on how VD3 and RA affect FBPase gene transcription.

VD3 and RA modulate numerous genes by acting through their cognate nuclear receptors, the vitamin D3 receptor (VDR), retinoic acid receptor (RAR) and retinoid X receptor (RXR) (*10–12*). The receptors exert their actions by binding, as homodimers or heterodimers, to specific DNA sequences located within or near the promoter region of the target genes, and stimulate transcription upon binding to their ligands. These nuclear receptor homodimers or heterodimers bind preferentially to direct repeat arrangements of a core binding consensus (AGGTCA) sequence, a half site motif, with spacing (*13*, *14*). The spacing between the half sites is an important determinant of binding specificity for the nuclear receptors. Typically, the VDR-RXR heterodimer binds to direct repeat (DR) half sites separated by 3 bp (designated DR3), termed the vitamin D3 response element (VDRE). RAR-RXR heterodimers are less discriminating, since DR5, DR2, and DR1 can function as retinoic acid response elements (RARE).

In this study, we first demonstrate the induction of FBPase by 9-*cis* retinoic acid (9cRA) and all-*trans* retinoic acid (atRA), as well as VD3, in human leukemia HL60 cells and normal monocytes in peripheral blood. In subsequent experiments, we show that the human FBPase promoter region is capable of mediating responses to both VD3 and RA for transcriptional upregulation. In addition, a single response element was found to function as both a VDRE and RARE by transfection assay and electrophoretic mobility shift assay (EMSA).

EXPERIMENTAL PROCEDURES

Cell Culture and Monocyte Preparation—HL60 cells, grown in RPMI1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), were inoculated at a density of 1×10^6 cells/ml and cultured in the presence or absence of 9cRA and atRA at concentrations ranging from 1 nM to 10 μM. After incubation at 37°C for up to 72 h under 5% CO_2, cells were collected for enzyme activity assay and northern blot analysis. Human mononuclear cells were separated from a fresh blood sample by Leucoprep (Becton and Dickinson, Franklin Lake, NJ) and cultured in RPMI1640 with 10% FBS. The medium was changed after 3 h, and cells that had attached to flasks were prepared as monocytes. The monocytes were further incubated for 72 h with or without ligands and were scraped for enzyme activity and northern blotting analysis. Under these conditions, 40 μg total RNA was obtained from 4×10^6 mononuclear cells from 200 ml of fresh whole blood provided by a single donor and used for 4 reactions (10 μg total RNA/reaction).

The vitamin A profile in the medium was checked by liquid chromatography. The medium was confirmed to contain no detectable retinoid, which could have been carried over by FBS.

Assay of FBPase Activity—Cells, suspended in phosphate buffered saline (PBS) and supplemented with protease inhibitors, were lysed by freezing and thawing. After centrifugation, the clear supernatant was assayed for enzyme activity. FBPase activity was determined spectrophotometrically by measuring the rate of $NADP^+$ reduction at 340 nm in an assay system containing fructose-1,6-bisphosphate, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase (*15*). Protein concentration was determined by dye-binding assay (Bio-Rad, Richmond, CA) using bovine serum albumin as the standard.

RNA Isolation and Northern Blot Analyses—Total cellular RNA was isolated from duplicate cultures by the guanidium thiocyanate/phenol method (*16*). Northern blotting was carried out as described previously (*17*). ^{32}P-labeled FBPase-cDNA was used for hybridization (*2*). RNA recovery from the cells was monitored by the amount of β-actin mRNA determined by stripping the same membrane and rehybridizing with labeled β-actin cDNA. RT-PCR was performed in order to confirm FBPase transcripts in RA-treated monocytes as described previously (*2*).

Isolation of the 5′-Flanking Region of the FBPase Promoter and DNA Sequencing—The 5′-flanking region of the promoter was screened from a human placental genomic DNA library in EMBL3 (Clontech, Palo Alto, CA) by hybridization with the ^{32}P-labeled oligonucleotide (5′-ACAGTGCGGGTGGAGGGCAC-3′, positions –243 to –224) in the promoter region (*8*). The isolated clone contains about 4 kilobase pairs (kbp), consisting of a 5′-regulatory region, exon 1, and the 5′-site of intron 1. After subcloning the appropriate fragments into pUC118 plasmid, the nucleotide sequences of both strands were determined using a DNA sequencer and the Dye Terminator Cycle Sequencing FS kit (Applied Biosystems, Foster City, CA).

Construction of a Reporter Gene—The subcloned 5'-flanking region DNA of the FBPase promoter was completely or partially digested with the restriction endonucleases shown in Fig. 4. After electrophoretic separation, the appropriate DNA fragments were cloned into the corresponding restriction site(s) of the herpes simplex virus thymidine kinase promoter/luciferase reporter (pTK-Luc) vector (*18*). Typically, for pFI71, pFI75, and pFI72, the *Pst*I fragment (–415 to +150 of the FBPase gene), the *Bam*HI-*Pst*I fragment (–143 to +150), and the *Pst*I–*Bam*HI fragment (–415 to –143) were ligated to the pTK-Luc reporter plasmid. The synthesized oligonucleotides (5′-agct tact TAACCT TTC TGAACT tccg agct-3′ and its complementary sequence), which are recognized as a VDRE-like sequence and their mutant derivatives (shown in Fig. 4) were also cloned into pTK-Luc to construct pFI80, pFI84, pFI86, and pFI87. To clarify the enhancer activity, a triplet derivative consisting of three copies of the VDRE-like sequence was constructed as pFI85. The structure of each construct was confirmed by DNA sequencing. DNAs for the following transfection experiments were prepared with a plasmid Maxi-kit (Qiagen, Hilden, Germany).

DNA Transfection and Reporter Assay—CV-1, a monkey kidney cell line, was selected for these experiments because CV-1 cells lack VDR and contains negligible RARs and RXRs. In addition, it is necessary to exclude possible interactions with transcription factors that exist endogenously in liver cells and that could affect the FBPase gene promoter. Small changes in the combination of *trans*-acting factors might result in profound alterations in gene expression. A synergistic interaction between different classes of transcription factors bound to distinct sites within a pro-

moter or enhancer region represents one pathway by which this might occur. CV-1 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% FBS at 37°C in 5% CO_2. At 8 h before transfection, 5×10^6 cells/well were inoculated into 24-well plates in fresh medium and incubated. Subsequently, the medium was replaced with fresh medium 1 h before transfection. DNA transfection was carried out using the calcium phosphate precipitation method (*19*). The amounts of plasmids used in each transfection were as follows: FBPase-TK-Luc reporter (200 ng), CMX-β-galactosidase reporter (pCMX-β-gal, 350 ng) (*14*), pCMX-receptor expression vector (50 ng), and carrier pUC119 (a total of 750 ng DNA). After 8 h of transfection, the cells were rinsed and fresh medium containing 1 μM of 9cRA, atRA, or VD3 was added. After additional incubation for 32 h, the cells were rinsed twice with ice-cold PBS and lysed with lysis buffer (Promega, Madison, WI) for β-galactosidase and luciferase assay. At least 15 transfection reactions per assay were carried out.

Luciferase activity was measured using an LB9501 luminometer (Berthold, Wildbad/Schwarzwald, Germany) and luciferase assay kit (Promega). The enzyme activity was then normalized for the efficiency of transfection on the basis of β-galactosidase activity.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed under the previously described conditions (*20, 21*) with the following modifications. Synthetic oligonucleotides of VDRE/RARE and their mutant derivatives (shown in Fig. 4) were end-labeled with [γ-^{32}P]ATP (111 Tbq/mmol) using T4 polynucleotide kinase (about 10^8 cpm/μg DNA). Human nuclear receptor proteins were synthesized *in vitro* by pCMX-VDR, pCMX-RARα, and pCMX-RXRα DNA (*14*) using a coupled T7 Quick transcription/translation system (Promega). Two microliters each of the *in vitro* translated proteins containing RXR and VDR or RAR were preincubated at 23°C for 20 min in binding buffer (*21*) with 1 μM ligand. In the next step, 1 μl of DNA probe (10^6 cpm/reaction) was added to this reaction mixture. For competition assays, oligonucleotides containing artificial VDRE, RXRE, and RARE were used as competitors and named DR3, DR1, DR5, and VDRE/RARE itself, respectively (*14*). A 10-, 30-, or 100-fold molar excess of competitor was added along with the probe DNA. After incubation for 20 min at 23°C, the reaction mixtures were analyzed by 5% polyacrylamide gel electrophoresis in 0.5 × TBE (Tris-borate-EDTA) buffer for VDRE or 0.5 × TGE (Tris-glycine-EDTA) buffer for RARE assay. Finally, the gels were dried and visualized by autoradiography.

RESULTS

Induction of FBPase in Human Leukemia HL60 Cells and Monocytes by 9-cis Retinoic Acid and All-trans Retinoic Acid—We first examined whether retinoic acid, another representative lipophilic vitamin and a typical differentiation-inducing agent, is capable of inducing FBPase activity in human leukemia HL60 cells and monocytes. HL60 cells were initially used to characterize the effect of RA on monocytic cells. As shown in Fig. 1A, 9cRA increased FBPase enzyme activity in a dose-dependent manner with maximal induction occurring at a concentration of 1 μM. However, at concentrations higher than 2 μM, 9cRA caused a reduction of FBPase enzyme activity due to a significant reduction in cell viability. AtRA was also effective, although the maximal level of induction was slightly less than that elicited by 9cRA (Fig. 1B). FBPase activity increased progressively with increasing levels of atRA. Under the same conditions, 1 μM of VD3 induced FBPase to 34.2 ± 2.1 nmol/min/mg protein.

Northern blot analysis allowed the detection of FBPase mRNA in HL60 cells treated with concentrations of 9cRA and atRA higher than 0.1 μM for 72 h (Fig. 1, C and D). The expression of FBPase mRNA (1.7 kb in size) was in agreement with FBPase enzyme activities at the corresponding concentrations of 9cRA. Furthermore, 9cRA was found to induce FBPase mRNA more efficiently than atRA. Weak mRNA signals at 10 μM 9cRA and atRA may reflect the different stabilities of the mRNA and FBPase enzyme (*22*) under conditions that produce a significant reduction in cell viability. In order to clarify whether the FBPase induction is the primary effect of these vitamins or not, mRNAs were prepared from HL60 cells and treated with each vitamin in the presence of cycloheximide, a protein synthesis inhibitor (*23*). FBPase mRNA was detected for

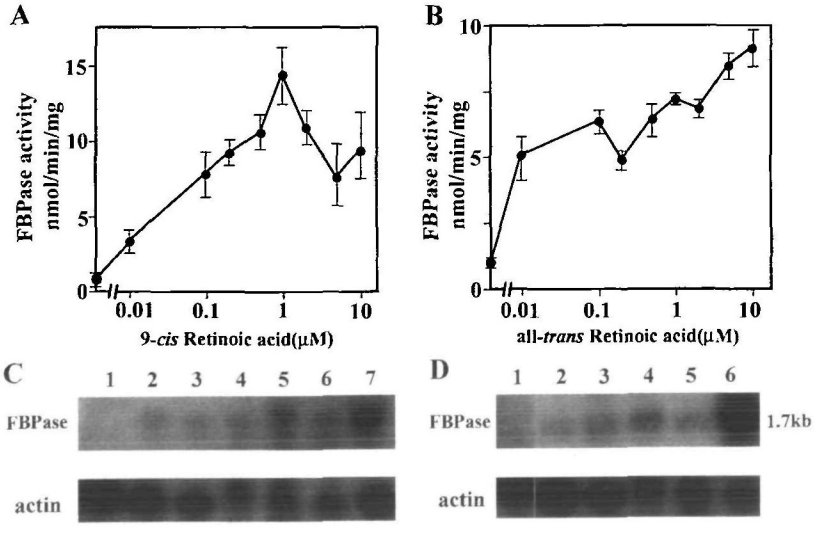

Fig. 1. **Effect of 9cRA and atRA levels on the induction of FBPase and FBPase mRNA abundance in HL60 cells.** HL60 cells were cultured for 72 h at the indicated concentrations of RA and analyzed as described in "EXPERIMENTAL PROCEDURES." (A) and (B) FBPase activity induced by 9cRA and atRA, respectively. Values represent mean ± SEM of at least 15 experiments; (C) FBPase mRNA and β-actin mRNA after induction with various concentrations of 9cRA: lane 1, 0 M; lane 2, 1 nM; lane 3, 10 nM; lane 4, 100 nM; lane 5, 1 μM; lane 6, 10 μM. Lane 7 contained 1 μM of VD3 as a positive control. (D) FBPase mRNA and β-actin mRNA after induction with various concentrations of atRA: lane 1, 0 M; lane 2, 10 nM; lane 3, 100 nM; lane 4, 1 μM; lane 5, 10 μM. Lane 6 contained 1 μM of VD3.

periods of up to 4 h of treatment, even in the presence of cycloheximide, suggesting that *de novo* protein synthesis is not required for induction (Fig. 2). These data also suggest that 9cRA, atRA, and VD3 activate the transcription of the FBPase gene through endogenous RAR, RXR, and VDR in HL60 cells.

In human monocytes, 9cRA and atRA induce the expression of the FBPase gene. Treatment with 1 μM 9cRA or atRA for 72 h induced FBPase to 13.5 ± 1.5 or 10.1 ± 1.3 nmol/min/mg protein, respectively, whereas FBPase activity with 1 μM VD3 was 23.1 ± 2.2 and, in the absence of ligand, the level was 1.4 ± 0.3 nmol/min/mg, as shown in Fig. 3A. RT-PCR amplified a 1,099 bp DNA corresponding to the coding region of FBPase plus linkers (*2*) from both 9cRA- and atRA-induced monocytes (Fig. 3B). FBPase mRNA was detected by northern blot in VD3-treated monocytes, although the cells appeared very faint due to the limitation of monocytes prepared from a single donor (data not shown).

Isolation and Sequencing of the Transcriptional Regulatory Region of the Human FBPase Gene—To investigate the mechanisms involved in the transcriptional regulation of FBPase gene expression by RA and VD3, we isolated and sequenced the 5′-flanking region (2.4 kb) of the human FBPase gene (Fig. 4). The transcriptional start site was assigned based on previous data as reported by El-Maghrabi *et al.* (*8*) who determined an approximately 600 bp sequence in this region. Compared with the sequences described in their study, our results differ with regard to the insertion of AATT at –210 and the transition of A to G at –432 within the 600 bp.

Localization of a Positive Regulatory Enhancer Domain for RA and VD3 in the FBPase Promoter Region—In order to identify possible regulatory element(s) for RA and VD3 within the FBPase promoter, overlapping DNA fragments of the 5′-flanking region were ligated to a luciferase reporter plasmid (pTK-Luc), resulting in 9 constructs. pTK-Luc was selected as a parental reporter, since the inclusion of the TK basal promoter made it possible to test the enhancer/response element activities of various DNA fragments isolated from the FBPase 5′-flanking region, because of the weak promoter activity of the FBPase gene itself under the following experimental conditions. Each of the 9 reporter constructs was then transfected into CV-1 cells, along with the receptor expression vector(s) and pCMX-β-*gal* as an internal control. Subsequently, the cells were incubated with or without 1 μM RA or VD3. Analysis of luciferase activity among the 9 constructs showed that multiple regions responded to vitamin ligands (data not shown).

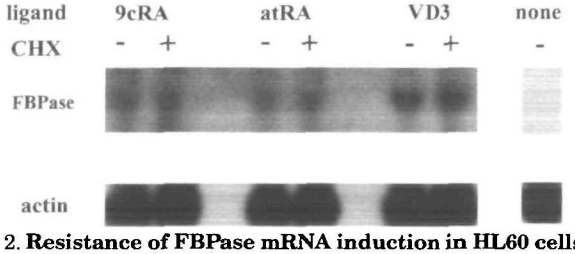

Fig. 2. **Resistance of FBPase mRNA induction in HL60 cells to cycloheximide.** Total RNA was isolated from cells treated in the presence (+) or absence (–) of 10 μg/ml of cycloheximide (CHX) with 1 μM of 9cRA, atRA, or VD3. Cells were incubated for 4 h and analyzed for FBPase mRNA by northern blotting.

The focus of this study was to characterize the proximal regulatory region. The reporter plasmid pFI71 encoding the *Pst*I fragment conferred 1.5 ± 0.1-, 4.2 ± 0.5- and 2.8 ± 0.3-fold induction with VD3, 9cRA, and atRA, respectively, in the presence of their cognate receptor expression plasmids (Fig. 4B). In the absence of these receptor expression plasmids, 9cRA and atRA activated luciferase activity by 2.7 ± 0.2- and 1.7 ± 0.2-fold, due to endogenous receptors present in CV-1 cells. Deletion of the *Pst*I–*Bam*HI fragment, located between –415 and –143 (pFI75), resulted in a complete loss of the response to VD3 and a significantly reduced response to RA ligands in comparison with pFI71 (Fig. 4, B and C). However, the atRA-response on pFI75 cannot completely exclude the possibility of another RARE within the region of –143 to +150, although there is no consensus DNA sequence. To confirm that the *Pst*I–*Bam*HI fragment includes response element, we prepared pFI72, which the *Pst*I–*Bam*HI fragment ligated to the TK promoter. This reporter plasmid conferred 1.66 ± 0.18, 3.38 ± 0.26, and 2.98 ± 0.38-fold induction with VD3, 9cRA, and atRA, respectively, in the presence of receptor expression plasmid (Fig. 4D). The pTK-Luc vector alone showed no responsiveness to VD3 or RA ligands or their receptors at all (1.05 ± 0.08-fold activation). These results indicate that the *Pst*I–*Bam*HI fragment (272 bp) encodes a major response element(s).

Identification of a Response Element to VD3 and RA—Inspection of the nucleotide sequence within this region revealed the presence of TAACCTTTCTGAACTT, positioned at –340 to –325, which is closely related to the DR3 type element (Fig. 4A). To systematically examine the potential interactions of nuclear receptors and to exclude

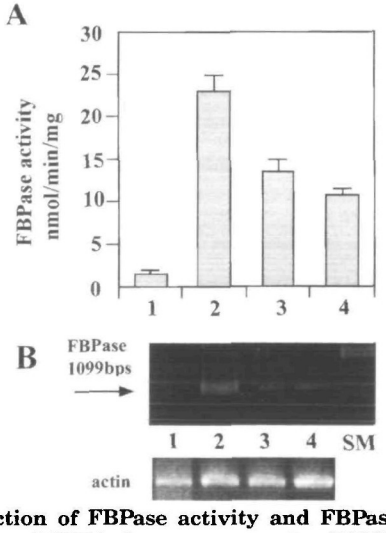

Fig. 3. **Induction of FBPase activity and FBPase-mRNA by 9cRA, atRA, and VD3 in human monocytes.** (A) FBPase activity in monocytes after induction for 72 h with no ligand (lane 1), or 1 μM of VD3 (lane 2), 9cRA (lane 3), or atRA (lane 4). Values represent mean ± SEM of at least 10 experiments. (B) Detection of FBPase mRNA in monocytes induced as in (A). FBPase and β-actin cDNA were synthesized by reversetranscriptase and amplified by 25 and 20 cycles of PCR respectively (*4*), followed by electrophoresis in a 1.2% agarose gel. SM is *Hin*dIII-λ DNA fragments indicating 2,322 and 2,027 bp. The indicated band in lane 2 seems smeared; however, it composed of a single band as indicated in lanes 3 and 4.

the effect of other transcription factors, we constructed a series of reporter plasmids containing the putative responsive element. Oligonucleotides encoding the sequence between positions –344 and –322 (termed VDRE1/RARE1) were synthesized and a single copy of this VDRE1/RARE1 was inserted into pTK-Luc to prepare reporters pFI80 (forward orientation) and pFI84 (reverse orientation) (Fig. 5, B and C). Exposure to 9cRA or atRA at 1 μM caused a 4- to 5-fold increase in luciferase activity in cells transfected with both forward and reverse constructs. Interestingly, VD3 induction was more effective with the reverse-oriented construct (3.7-fold *versus* 1.7-fold for the forward-oriented construct).

To test the sequence specificity of VDRE1/RARE1, four bases were substituted within the putative core sequence (Fig. 5A) and pTK-Luc plasmids containing the mutant element, pFI86 (forward) and pFI87 (reverse), were prepared and assayed. Neither of these reporters responded to ligand treatment in comparison with the corresponding pFI80 and 84, except for the weak residual activity of atRA on pFI86 (Fig. 5, B and C). Deletion of 4 bp, which flank both ends of the putative 15 bp-core sequence from pFI80 or pFI84 had no effect on luciferase induction, suggesting the importance of the core nucleotide sequence (data not shown).

In addition, we also tested whether multimerization of the response elements would enhance the sensitivity of the ligand response. Three copies of VDRE1/RARE1 were inserted to the pTK-Luc reporter plasmid in the forward direction (pFI85). As shown in Fig. 6, pFI85 showed an increased level of induction by VD3 and RA, *i.e.*, about a 5-fold induction for VD3 and about a 25-fold activation for 9cRA and atRA. Collectively, these observations demonstrate that the 15 bp direct repeat located at –340 to –326 in the FBPase promoter region is capable of functioning as a common response element for both VD3 and RA in CV-1 cells. Accordingly, we refer to this sequence as *fbp*-VDRE/RARE.

It is interesting that the increase in activation cotransfected with VDR and RXR in pFI80 and pFI85 was lower than that of VDR alone. This tendency was also observed in

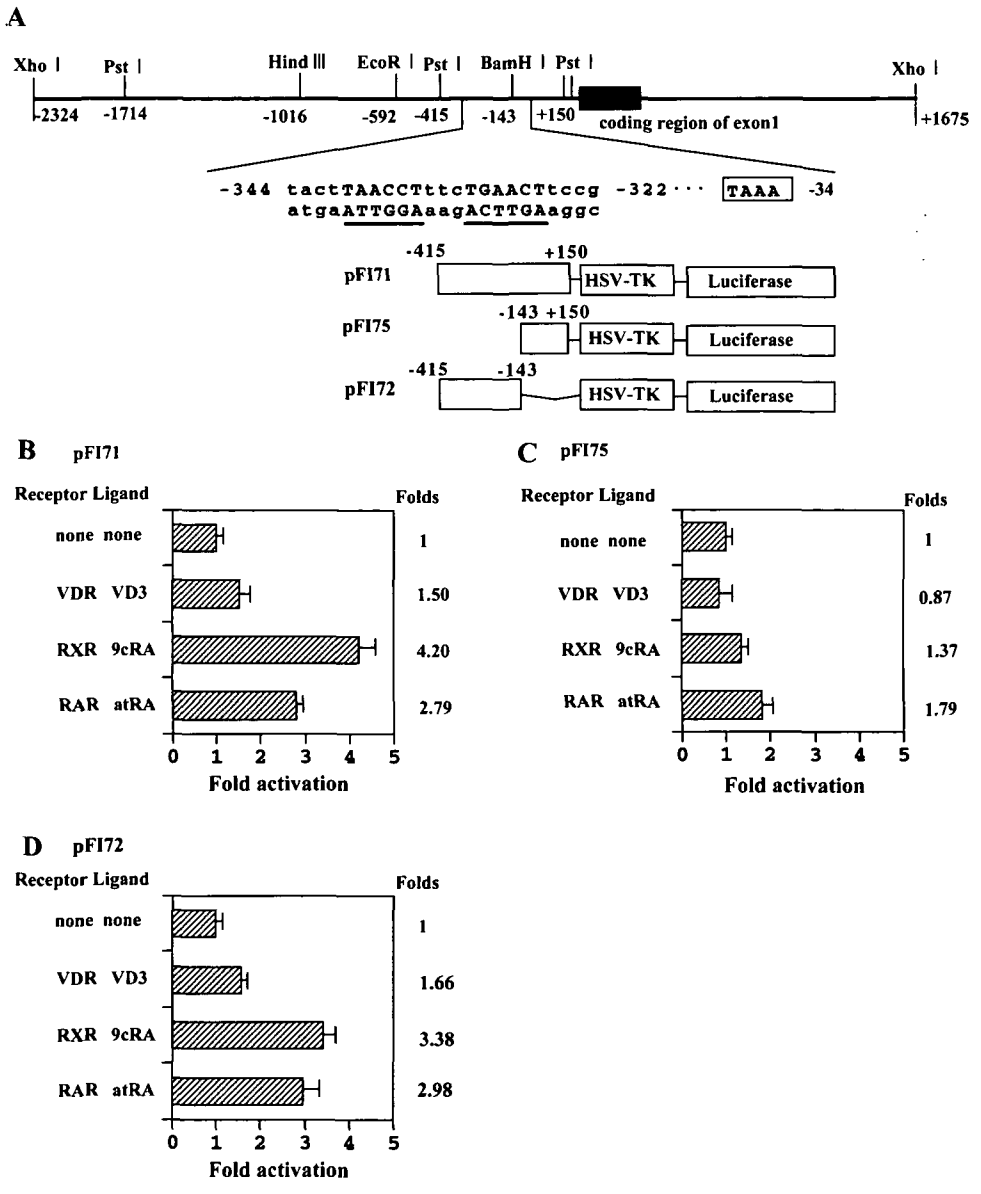

Fig. 4. **Identification of potential VDRE and RARE regions within the transcriptional regulatory sequence of the human FBPase gene.** (A) Organization of the regulatory region and scheme of typical reporter constructs. The transcription start site is at position 1, as determined by El-Maghrabi *et al.* (*8*). Direct repeated sequences at –340/–325 are underlined. Reporter plasmids of pFI71 (B), pFI75 (C), and pFI72 (D) were cotransfected into CV-1 cells with expression vectors for the indicated receptors as described in "EXPERIMENTAL PROCEDURES." Values are mean fold induction (± SEM) of activity of cultures (n = 15) treated with 1 μM of the indicated ligand compared with a control culture as shown. All values in (B), (C), and (D) are significant at p = 0.05.

pFI84. The reason for this is not clear but we speculate that the expressed RXR dimerized with another endogenous receptor bound to this element without a ligand, and this suppressed the binding of VDR-RXR heterodimer. In the case of cotransfection with RXR and RAR, the increase in activation is slightly decreased compared to RXR or RAR alone in each reporter gene. The reason for this also remains unclear.

Binding of VDR-RXR and RXR-RAR to FBPase-VDRE/RARE—*Fbp*-VDRE/RARE was examined *in vitro* by EMSA to determine whether it acts as a specific binding site for VDR, RXR, and RAR proteins in either the homo- or heterodimeric form. Human VDR, RXRα, and RARα proteins were synthesized *in vitro* in a rabbit reticulocyte system. Using labeled oligonucleotides encoding the *fbp*-VDRE/RARE, VDR alone, with or without VD3, and VDR with RXR in the absence of VD3 did not reveal any DNA-protein complexes (Fig. 7). A specific retarded band was detected only in the presence of VD3, VDR, and RXR together, indicating that binding of the VDR-RXR heterodimers to this sequence is ligand-dependent. The inclusion of a 10 to 100-fold molar excess of non-radiolabeled competitor DNA containing a synthetic DR3 element markedly reduced the intensity of the retarded bands. On the other hand, the mutated *fbp*-VDRE/RARE oligonucleotides shown in Fig. 5A, were incapable of acting as a binding site. Thus, this complex formation was VD3-dependent, proper to VDR-RXR and nucleotide sequence–specific.

A similar approach was undertaken to test RARE properties (Fig. 8A). Labeled *fbp*-VDRE/RARE, RAR, and RXR formed a complex without any ligand under the condition of EMSA with VDRE (data not shown). However, it was interesting that the *fbp*-VDRE/RARE and RAR-RXR heterodimer formed a complex only in the presence of either 9cRA or atRA in 0.5 × TGE buffer. The presence of a 30- or 100-fold molar excess of unlabeled competitor DNAs encoding synthetic DR1 and DR5 elements competed for this binding. In addition, complex formation was greatly diminished by the presence of a 10-fold molar excess of the unlabeled *fbp*-element itself. The unlabeled synthetic DR3 element was also used as competiter DNA (Fig. 8B). The shifted band did not disappear, even in the presence of a 200-fold molar excess of the DR3 element. The mutated VDRE/RARE oligonucleotides failed to support complex for-

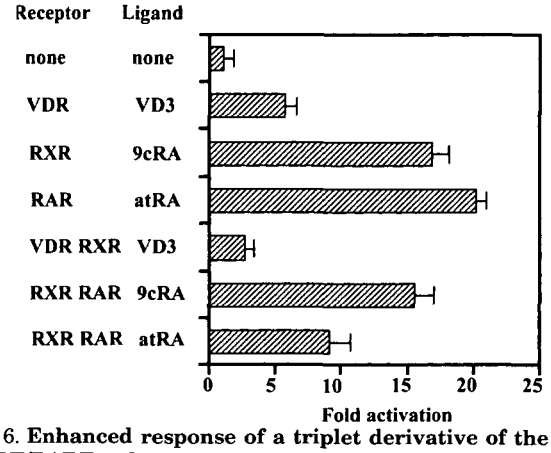

Fig. 6. **Enhanced response of a triplet derivative of the fbp-VDRE/RARE to VD3, 9cRA, and atRA.** To enhance the gene expression respondent to VDR and VD3, three copies of the oligonucleotides were inserted into pTK-Luc in the normal orientation (forward direction) for pFI85. Reporter assays (at least $n = 15$) were performed under the indicated conditions.

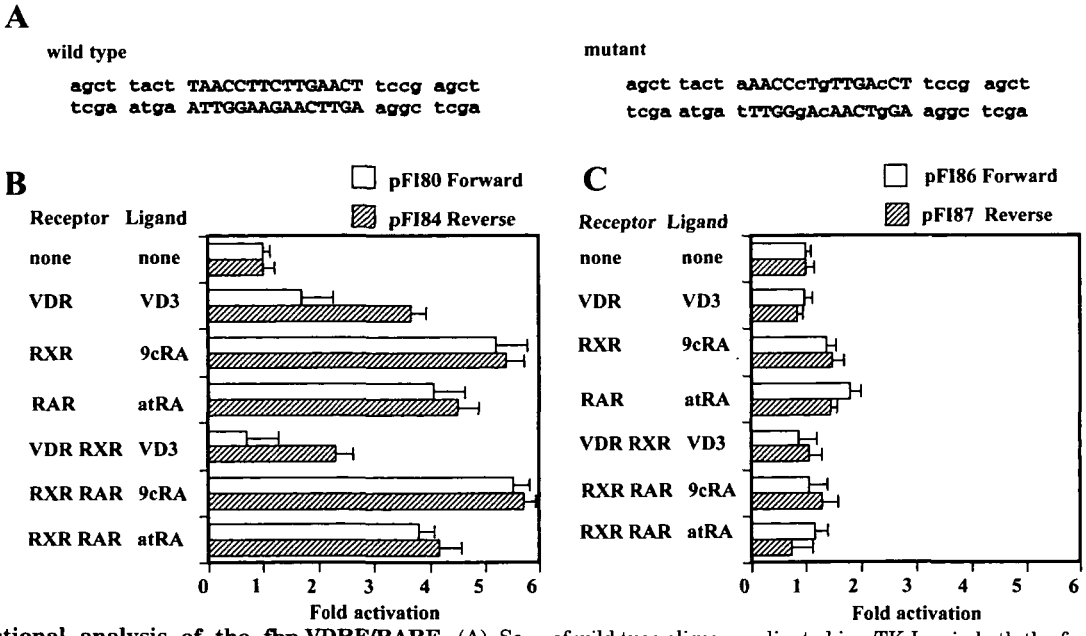

Fig. 5. **Functional analysis of the fbp-VDRE/RARE.** (A) Sequences of wild type and mutant oligonucleotides of the element corresponding the FBPase promoter sequence at –344/–321 with a 4 bp linker at both ends. Capital letters represent the direct repeat with a 3 bp spacer at –340/–325. In the mutant oligos, 4 bp were base-substituted and shown in small letters within the core motif. (B) One copy of wild type oligos was ligated in pTK-Luc in both the forward and reverse directions for pFI80 and pFI84, respectively. Reporter assays (at least $n = 15$) were carried out as shown in Fig. 4(C). The transverse-mutated oligos were used to construct pFI86 and pFI87, and the resultant constructs were assayed for enhancer activity.

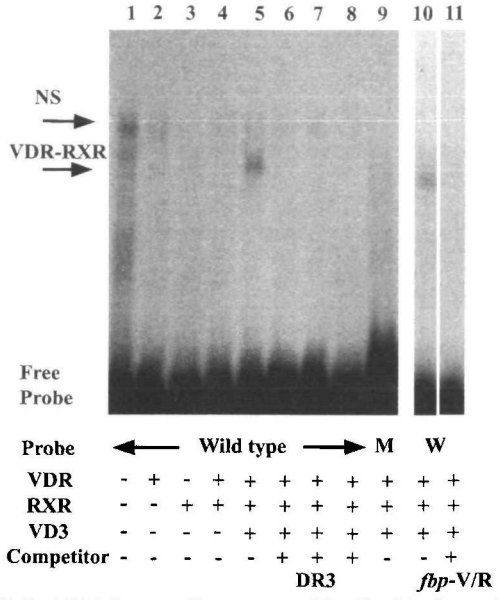

Fig. 7. **VDR-RXR heterodimers specifically bind to the fbp-VDRE/RARE oligonucleotides in the presence of cognate ligands.** ^{32}P-labeled oligonucleotides, wild type and mutant (M) fbp-VDRE/RARE, shown in Fig. 5A were used as the probe DNA in gel EMSA. (A) Binding of VDR-RXR. VDR, RXR proteins, and 1 μM VD3 were added as indicated. Lane 1, a TNT Quick reaction mixture was added as a negative control lacking any receptor protein. No shifted bands were observed even in the presence of 1 μM of VD3 (data not shown). In lanes 6 to 8, as a competitor, 10-, 30-, and 100-fold molar excesses of DR3 oligonucleotides were added to the reaction mixtures. In lane 11, a 10-fold molar excess of nonlabeled fbp-VDRE/RARE was used as a competitor. NS, nonspecific; VDR-RXR, VDR-RXR heterodimer–DNA complex.

mation, even in the coexistence of receptors and ligands. To test whether the smeared bands (indicated as NS in Fig. 6) above the complexes are non-specific, we used ^{35}S-labeled receptor proteins instead of the oligonucleotide probes and confirmed that the signals co-migrated with those discussed above (data not shown). Thus, complex formation of this element is RAR-RXR dependent and DNA sequence–specific. To compare the binding strength of fbp-VDRE/RARE with DR3 and DR5 to VDR-RXR or RAR-RXR heterodimers, labeled DR3 and DR5 were also used as probes (Fig. 9). The binding strength of fbp-VDRE/RARE was weaker than DR3 as VDRE, however it was stronger than DR5 under our customized conditions of EMSA with RARE. DR3 also could bind weakly to RAR-RXR hetero-

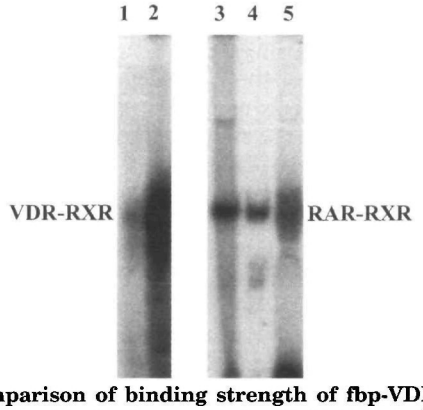

Fig. 9. **Comparison of binding strength of fbp-VDRE/RARE, DR3, and DR5.** The binding strength of VDR-RXR and RAR-RXR heterodimers to fbp-VDRE/RARE, DR3, and DR5 is indicated. Lane 1, fbp-VDRE/RARE; lane 2, DR3; lane 3, fbp-VDRE/RARE; lane 4, DR5; lane 5, DR3.

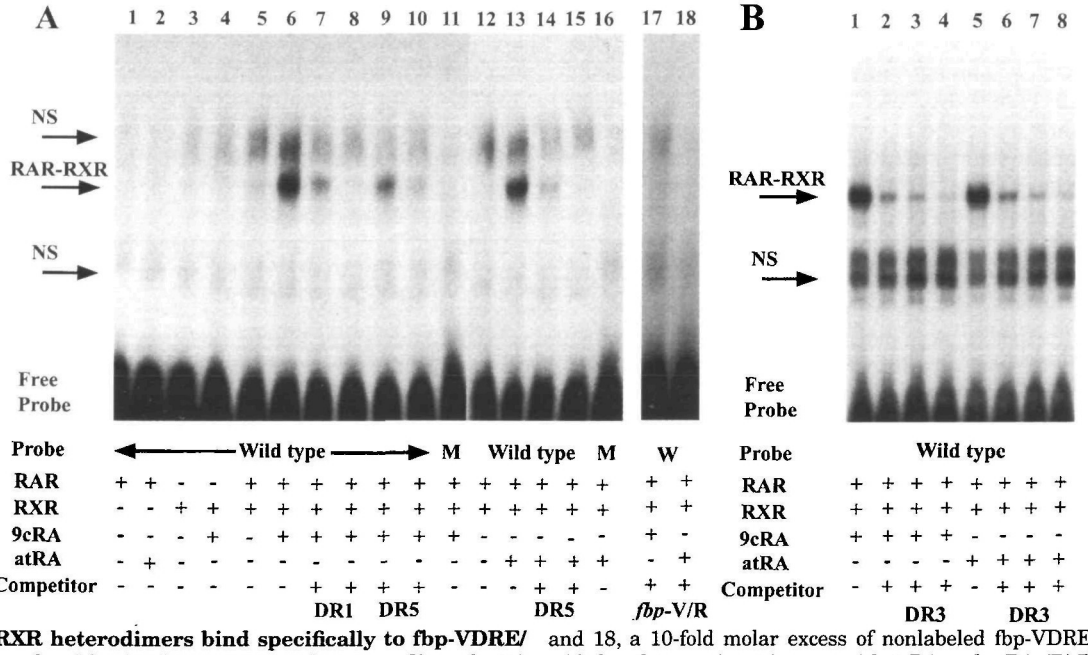

Fig. 8. **RAR-RXR heterodimers bind specifically to fbp-VDRE/RARE oligonucleotides in the presence of cognate ligands.** (A) Binding of RAR-RXR, RAR, and RXR proteins. 9cRA and atRA were added as indicated. The additional effects of DR1 and DR5 competitor oligonucleotides were tested at 30- and 100-fold molar excess of probe in lanes 7 and 8, 9, and 10, and 14 and 15, respectively. In lanes 17 and 18, a 10-fold molar excess of nonlabeled fbp-VDRE/RARE was added to the reaction mixtures with 9cRA and atRA. (B) DR3 oligonucleotides were used as competitors at 50-, 100-, and 200-fold molar excess of probe in lanes 2 and 6, 3 and 7, and 4 and 8. RAR-RXR, RAR-RXR heterodimer–DNA complex.

dimers, and their formed complex band showed the band representing the complex formed appears smeared under these conditions.

DISCUSSION

The induction of human FBPase gene expression by RA and VD3 and the molecular mechanisms underlying this induction were examined. We found that both 9cRA and atRA induce FBPase enzyme activity in a manner similar to that seen with VD3, in human leukemia HL60 cells and normal monocytes (Figs. 1 and 2). Although Mizunuma and Tashima reported the induction of FBPase by atRA in HL60 cells, the mechanism was not examined (*24*). It is noteworthy that active forms of lipophilic vitamins, VD3, 9cRA, and atRA, are capable of inducing the key enzyme of gluconeogenesis in monocytic cells. The physiological role of FBPase expression in these cells remains unknown. However, the data point to the role of gluconeogenesis during cell differentiation, because RA and VD3 differentiate HL60 cells to granulocytes and monocytes/macrophages (*1*, *9*). The present observations suggest that glucose production through gluconeogenesis might be involved in phagocytosis by these differentiated blood cells.

Using cotransfection assays through reporter constructs in CV-1 cells, we identified a DR3-like sequence, TAACCTttcTGAACT, *fbp*-VDRE/RARE, in the FBPase promoter region, which could function as a common response element for VD3, 9cRA, and atRA *via* their receptors. The DNA sequence–specificity of this element with regard to the response to these vitamins was also functionally and physically demonstrated by the mutated elements (Figs. 3–6). VDR-RXR heterodimers bind to this element sequence specifically and competitive inhibition was observed with the authentic DR3 oligonucleotide (Fig. 6A). RAR-RXR heterodimers also interact with *fbp*-RARE/VDRE in EMSA. Complex formation was competitively inhibited by authentic DR1 and DR5 oligos (Fig. 6B). These results further indicate that this element functions as both VDRE and RARE. To our knowledge, this is a unique case where a VDRE sequence in a gene is involved in sugar metabolism and also functions as a RARE. Dual VDRE/RAREs are known to exist in the *pit-1* gene and osteocalcin gene, although they have been detected only in transfection systems (*25*, *26*). We observed different responsiveness to ligands between the induction of FBPase in HL60 cells and monocytes and luciferase activities in CV-1 cells (Figs. 1 and 3–5). VD3 action in CV-1 cells was considerably lower than in HL60 cells. The binding strength of fbp-VDRE/RARE to VDR-RXR was weaker than to RAR-RXR heterodimers (Figs. 7 and 8A). The reason for the dissociation between binding ability of fbp-VDRE/RARE to nuclear receptors and FBPase induction in HL60 cells and monocytes is not clear. These unexpected results suggest an association of active coregulator(s) (*27*) in FBPase induction by VD3, in addition to dependence on different amounts of expression of the RXR and VDR or RAR in these cells (*28*). The detected mRNA of FBPase treated with cycloheximide for 4 h indicated the dominance of VD3 over RA in the stimulation of transcription (Fig. 2). This suggests the lack of any influence of differentiation on the induction of FBPase in HL60 cells and monocytes.

For the VDRE sequence, Ohyama *et al.* (*20*) reported that sequences in the upstream half-site are more conserved than those downstream. In this regard, in the complementary sequence of *fbp*-VDRE/RARE (AGTTCAgaaAGGTTA), the upstream half-site is more conserved than the downstream site, and VD3 induction is more effective in a reverse-oriented construct than the forward-oriented construct (Figs. 4 and 5), adding further support for this sequence property being VDRE. In various RA-inducible genes, RAREs with 5 bp spacing (DR5) are commonly found, such as RARβ (*29*, *30*), mRARα (*31*), and RARγ (*32*). However, RAR-RXR heterodimers are thought to be less discriminating as to their target, since DR2 and DR1 can function as a RARE (*33–36*). DR2 type elements are reported to be activated by both atRA and 9cRA *via* their target receptors, RAR and RXR (*37*, *38*). FBPase-RARE could be a DR2-type variant, since both 9cRA and atRA enhance transcription through this element. However, it seems unlikely that the *fbp*-element is a DR2 variant because of the large difference in the sequence of the downstream half-site (TAACCTttCTGAAC or AGTTCAgaAAGGTT) compared to the consensus half-site. To examine the property of each half-site sequence, we prepared mutant reporters in which two more base pairs were inserted into the spacer between the half sites, resulting in a DR-5 type element. These reporter constructs induced luciferase activity by retinoic acid about three times more efficiently than the wild *fbp*-RARE/VDRE construct (unpublished data). These results suggest that the original *fbp*-RARE functions as a DR3. The *fbp*-RARE consisting of 15 bp enhances transcription in the presence of 9cRA and atRA and has a high affinity for RXR-RAR heterodimers. The binding strength of fbp-VDRE/RARE is weaker than that of DR3 as a VDRE, however it is stronger than DR5 under our conditions of EMSA with RARE. DR3 binds to RAR-RXR heterodimer weakly and the band for the complex formed is smeared under these conditions. Umesomo previously described a clear activity of DR3 for RA while DR5 confers the best RA response using a transfection system (*14*). It is very interesting that the fbp-VDRE/RARE shows different activities for VD3 and RA compared with DR3. This might depend on the difference of the 3 bps spacer sequence in addition to the 6 bps repeated half site motif.

DNA sequences of the 5'-noncoding regions of this gene between rats and humans are completely divergent (*4*, *8*), and we did not find any sequences that were homologous to the human fbp-VDRE/RARE in the 5'-flanking sequence of the rat FBPase gene (*4*). This suggests that a different family of transcription factors might interact in the expression of the FBPase gene in rats and humans. Another key gluconeogenic enzyme, phosphoenoylpyruvate carboxykinase, has two RAREs on its promoters and is also induced by retinoic acid in rat hepatoma cells (*39*, *40*). The 5'-flanking sequences of the human and rat genes are highly conserved (*41*). Recently, we confirmed that hepatic FBPase is also induced by 9cRA and atRA in HepG2 cells, a human hepatoma cell line (Fujisawa *et al.*, manuscript in preparation). RA may represent the common inducer between liver and monocytes/HL60 cells in humans. We are also interested in the possibility that RA, a derivative of vitamin A, when fed as a nutrient, serves as a common inducer of the above two key enzymes, FBPase and phosphoenoylpyruvate carboxykinase in human liver cells. It is noteworthy that the regulation of gluconeogenesis is under the control

of nutritional conditions in addition to rapid hormonal regulation. We were not able to detect FBPase activity or mRNA in HepG2 cells treated with VD3 (data not shown), because of the limited expression of VDR in the liver (*42*). In this respect, VD3 can not mediate FBPase expression in hepatic cells, and VDR mediation in transcription may be a specific regulation in monocytic cells. FBPase and its isoenzymes are also expressed in lung (*43*) and muscle (*44*) in humans. Its expression in each tissue would be under the control of a tissue-specific enhancer.

In conclusion, we used transfection and EMSA to demonstrate that a single element placed on the human FBPase promoter at –340/–326 is capable of mediating responses to both VD3 and RA for the transcriptional upregulation through ligand-dependent interactions with VDR-RXR and RAR-RXR heterodimers. These findings are consistent with our observations that both enzyme activity and transcription of the human FBPase gene are enhanced by 9cRA and atRA as well as VD3 in HL60 cells and normal monocytes. Further studies are underway in our laboratory to elucidate the regulatory mechanisms of human FBPase gene expression. Data from these studies will help in determining the cause of the genetic defects in FBPase deficiency that are suspected to be due to impaired expression.

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