Structural Organization of the Human Vitamin D Receptor Chromosomal Gene and Its Promoter

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The vitamin D receptor (VDR) is known to mediate the pleiotropic biological actions of 1,25-dihydroxyvitamin D_3 through its ability to modulate the expression of target genes. The regulation of this ligand-activated cellular transcription factor is reported to occur at both transcriptional and post-translational levels. To begin to address the molecular basis by which the VDR gene is regulated transcriptionally, we report here an initial characterization of the human VDR gene and its promoter. We isolated several overlapping λ-phage and cosmid clones that cover more than 100 kb of human DNA and contained the entire VDR gene. The gene is comprised of 11 exons that, together with intervening introns, span approximately 75 kb. The noncoding 5′-end of the gene includes exons 1A, 1B, and 1C. Eight additional exons (exons 2–9) encode the structural portion of the VDR gene product. While primer extension and S1 nuclease-mapping studies reveal several common transcriptional start sites, three unique mRNA species are produced as a result of the differential splicing of exons 1B and 1C. The DNA sequence lying upstream of exon 1A is GC rich and does not contain an apparent TATA box. Several potential binding sites for the transcription factor SP1 and other activators are evident. Fusion of DNA fragments containing putative promoter sequences upstream of the luciferase structural gene followed by transient transfection of these plasmids into several mammalian cell lines resulted in significant reporter activity. Due to the size and complexity of the 5′-end of the VDR gene, we examined the activity of a DNA fragment surrounding exon 1C. An intron fragment 3′ of exon 1C conferred retinoic acid responsivity when fused to a reporter gene plasmid, suggesting a molecular mechanism for the previously observed ability of retinoic acid to induce the VDR. The recovery of the gene for the human VDR will enable further studies on the transcriptional regulation of this gene. (Molecular Endocrinology 11: 1165–1179, 1997)

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INTRODUCTION

The biological actions of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] that regulate transcriptional events within the nucleus of target cells are mediated by the vitamin D receptor (VDR)(1–3). This ligand-activated transcription factor belongs to a superfamily of genes that encode receptors for the steroid, thyroid, and vitamin A (retinoic acid and 9 *cis*- retinoic acid) hormones as well as orphan receptor proteins for which ligands have not yet been identified (4–6). Most members of this receptor gene family have undergone considerable scrutiny during the past several years, and significant progress has been made in understanding their structure and function. Although genes that encode proteins involved in mineral metabolism are a principal focus of VDR action and its 1,25-$(OH)_2D_3$ ligand *in vivo* (7, 8), the receptor is also capable of influencing genes whose products regulate more fundamental processes of cellular proliferation and differentiation (9, 10). The extensive biological effects now ascribed to 1,25-$(OH)_2D_3$ can be attributed, in large part, to the nearly ubiquitous tissue distribution of the VDR as well as to the highly selective regulation of

gene expression by this hormone in specific cell types (1).

Many of the chromosomal genes for the nuclear receptor family members have been cloned and their structural organization determined (11–15). These genes are often more than 50 kb in length and are comprised of multiple protein domain-associated exons separated by exceedingly large introns. An exception to this appears to be the orphan receptors, chicken ovalbumin upstream promoter-transcription factors α and β, which are encoded by genes that span 4–6 kb of DNA, contain only two introns, and thus may represent ancestral members of this superfamily of genes (16). The promoter regions of several of the receptor genes have been characterized. They often appear to resemble housekeeping genes, and many are embedded in GC-rich islands (11, 13). The absence of a TATA box is a common feature of several of these genes as is the existence of multiple start sites for transcription. In some cases, mRNA transcripts are produced from more than one promoter (17, 18); in others, transcripts are differentially spliced to create unique mRNA species that encode functionally distinct receptor isoforms (19, 20). Thus, nuclear receptor genes can be typified by the general structural organization of the encoding exons as well as through the overall characteristics of many of their promoters.

Transcriptional regulation of nuclear receptor gene expression is clearly evident. While receptors for metabolites of vitamin D and vitamin A, as well as the glucocorticoids are widely expressed (21), receptors for estrogen, progesterone, and androgens display a more restricted tissue-specific expression profile (22). Determinants of restricted expression of these genes in tissues and cells as well as the mechanisms that lead to activation of otherwise silent receptor genes during development or during the differentiation of specific cell types are generally not known. Expressed genes are, however, regulated by a number of non-peptides hormones as well as peptide hormones, growth factors, and cytokines. Transcriptional autoregulation of receptor genes by their respective gene products through *cis* elements localized adjacent to cognate promoters is not uncommon. The β-retinoic acid receptor gene (RARβ), for example, is autoregulated through a β-retinoic acid response element located immediately adjacent to its promoter (23, 24) and accounts for substantial up-regulation of RARβ by retinoic acid both *in vivo* and *in vitro* (25, 26).

The VDR is regulated at both transcriptional and posttranslational levels. VDR gene expression is up-regulated in NIH-3T3 mouse fibroblasts through activation of the protein kinase A pathway (27) and down-regulated through activation of the protein kinase C pathway (28). Both of these signal transduction pathways mediate the actions of PTH on target cells (29) and thus may represent homeostatic mechanisms that ultimately control cellular sensitivity to 1,25-$(OH)_2D_3$. Other growth factors and cytokines are also known to regulate VDR gene expression, possibly through the above pathways. Estrogens (30), thyroid hormone (31), glucocorticoids (32), and retinoic acid (33, 34) are likewise able to alter VDR mRNA levels in what appear to be tissue-specific patterns of expression. Interestingly, both cell cycle (35) and the differentiation state of cells in culture (36) influence the extent of VDR mRNA expression. The level of the VDR under these circumstances may play a regulatory role in the control of cellular proliferation and differentiation by the vitamin D hormone in a wide variety of cell types. Finally, homologous up-regulation of VDR mRNA by 1,25-$(OH)_2D_3$ has also been demonstrated both *in vitro* and *in vivo*, again in a very tissue-specific fashion (37–39). Whether this regulatory action occurs directly as a result of VDR interaction with a *cis*-element(s) at the 5′-end of the VDR gene, analogous to the interaction of RARs with the RARβ gene promoter, or indirectly as a result of the induction or activation of other transcription factors remains to be demonstrated.

To gain a better understanding of the molecular mechanisms by which the VDR is regulated at the transcriptional level, we cloned the chromosomal gene for the human VDR. We report here on structural characterization and preliminary functional activity of its promoter. The single gene for human VDR spans more than 75 kb of genomic DNA and contains 11 exons. Three exons make up the 5′-noncoding region, and the eight additional exons encode the structural component of the VDR. The promoter is characterized by the lack of a TATA box initiator, its GC-rich nature, and the presence of putative binding sites for SP1 and a variety of transcription factors. It directs the transcription of at least three VDR mRNA transcripts in kidney that appear to arise from the differential splicing of 5′-noncoding exons. The promoter also directs the transcription of a chimeric gene when fused upstream of an expression plasmid containing the luciferase structural gene. The recovery of the human VDR gene and these initial studies provide the basis for further detailed examination of the transcriptional regulation of the VDR gene. This effort is particularly warranted in view of the central role of the VDR in 1,25-$(OH)_2D_3$ action and the potential therapeutic role of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs in blocking cellular proliferation and promoting the differentiation of tumor cells (40, 41).

RESULTS

The Structure and Sequence of hVDR mRNAs

We established first the authenticity of the 5′-end of the hVDR mRNA sequence reported initially by Baker *et al.* (42) using the 5′-RACE (rapid amplification of cDNA ends) PCR technique. Human kidney RNA was employed as template to prepare first-strand cDNA, and the latter was amplified using an anchored oligonucleotide (primer A) as well as a downstream primer (primer B2) corresponding to hVDR cDNA sequence as outlined in Fig. 1A. Primary amplification was fol-

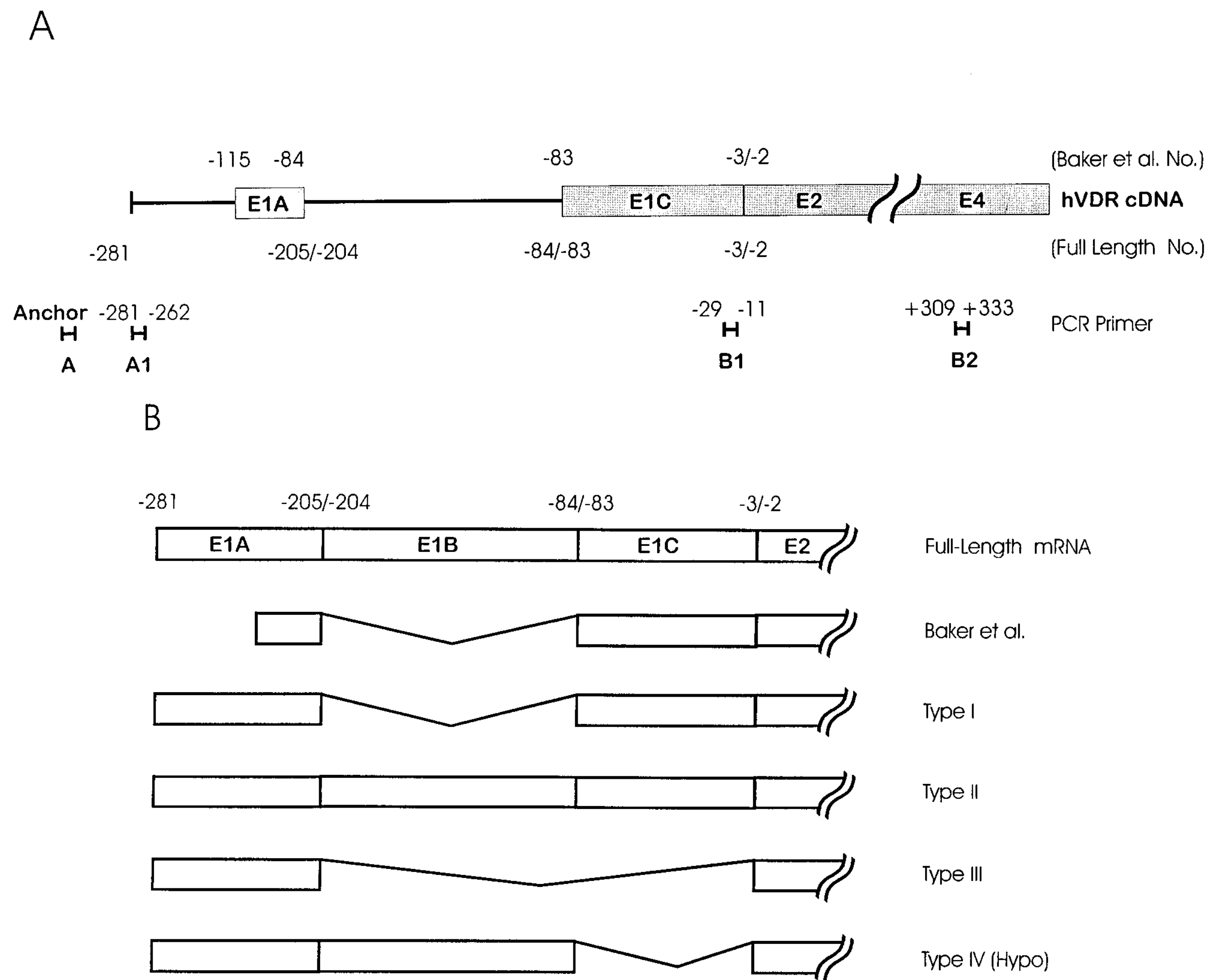

Fig. 1. Analysis of hVDR mRNA Transcripts Using 5′-RACE

A, Location of oligonucleotide primers used for DNA amplification. The *shaded rectangle* illustrates portions of the hVDR transcript originally reported by Baker *et al.* (42), whereas the *single line* indicates additional portions of the hVDR mRNA transcript revealed through 5′-RACE. The *numbering above* the diagram corresponds to that of Baker *et al.* (42) whereas the *numbering below* corresponds to the largest hVDR mRNA transcript that was identified. The location of DNA primers used to characterize the hVDR mRNA transcripts by 5′-RACE are indicated relative to the largest unspliced hVDR mRNA transcript identified. The anchor primer (corresponding to vector sequence) was obtained from Life Sciences. B, The three hVDR mRNA transcripts identified through the 5′-RACE technique are illustrated (type I, II, and III) relative to the transcript identified by Baker *et al.* (42). Type I and type III transcripts contain deletions that correspond to proposed exon 1B (type I) and exons 1B and 1C (type III), respectively. The type III transcript contains all the proposed exons: 1A, 1B, and 1C. Exon 2 (43) is common to all transcripts. The type IV transcript is hypothetical. The illustration is numbered relative to the full-length transcript (type II).

lowed by a secondary amplification as described in *Materials and Methods* using primers A and B1. The cloning and DNA sequence analysis of the resulting products revealed two individual transcripts. As observed in Table 1, the sequence of the first (type 1) was identical to that reported by Baker *et al.* (42) with the exception that the sequence of the DNA product extended some 45 bp upstream. This suggests that the original cDNA sequence reported by Baker *et al.* (42) was incomplete at the 5′-end by 45 bases and that the type 1 PCR product might represent the authentic 5′-end of the hVDR mRNA. The second transcript (type 2) was identical to the type 1 transcript (see Table 1) with the exception that a 121-bp insert was discovered lying between nucleotides −84 and −83 relative to the translation start site reported by Baker and colleagues (42). An additional amplification of kidney RNA was then carried out using primer A1 complementary to the authentic 5′-end of the mRNA established above and primer B2 as illustrated in Fig. 1A. Cloning and sequence analysis of the products of this reaction revealed the previous two mRNAs as well as a third (type 3). As documented in Table 1, type 3 was similar to type 1 with the exception that nts −83 to −3 relative to the sequence of Baker *et al.* (42) were deleted. The failure of primer B1 to hybridize to type 3 transcripts clearly prevented detection of type 3 in the original primary amplification described above. The

Table 1. Nucleotide Sequences of hVDR in RNA Transcripts Detected by 5′-RACE

Baker *et al*	5′-**GGAACAGCTT GTCCACCCGC CGGCCGGACC AGAAGCCTTT GGGTCTGAAG**	+50
	TGTCTGTGAG ACCTCACAGA AGAGCACCCC TGGGCTCCAC TTACCTGCCC	+100
	CCTGCTCCTT CAGGGATG-3′	+150
Type I	5′-CTGCTTGTCA AAAGGCGGCA GCGGAGCCGT GTGCGCCGGG AGCGC**GGAAC**	+50
	AGCTTGTCCA CCCGCCGGCC GGACCAGAAG CCTTTGGGTC TGAAGTGTCT	+100
	GTGAGACCTC ACAGAAGAGC ACCCCTGGGC TCCACTTACC TGCCCCCTGC	+150
	TCCTTCAGGG ATG-3′	+200
Type II	5′-CTGCTTGTCA AAAGGCGGCA GCGGAGCCGT GTGCGCCGGG AGCGC**GGAAC**	+50
	AGCTTGTCCA CCCGCCGGCC GGACCAGGCT CCTGAACCTA GCCCAGCTGG	+100
	ACGGAGAAAT GGACTCTAGC CTCCTCTGAT AGCCTCATGC CAGGCCCCGT	+150
	GCTCATTGCT TTGCTTGCCT CCCTCAATCC TCATAGCTTC TCTTTGGG**AA**	+200
	GCCTTTGGGT CTGAAGTGTC TGTGAGACCT CACAGAAGAG CACCCCTGGG	+250
	CTCCACTTAC CTGCCCCCTG CTCCTTCAGG GATG-3′	+300
Type III	5′-CTGCTTGTCA AAAGGCGGCA GCGGAGCCGT GTGCGCCGGG AGCGC**GGAAC**	+50
	AGCTTGTCCA CCCGCCGGCC GGACCAGGGA TG-3′	+100

The preexisting sequences documented by Baker *et al.* (42) are indicated in *bold*. Accession numbers for these sequences are AB002157–AB002168.

organization of the three transcripts are illustrated in Fig. 1B relative to the sequence of Baker *et al.* (42). The nature of these transcripts, one of which contains an insertion and the other a deletion, suggests that the 5′-end of the hVDR gene contains four exons, three of which are exclusively noncoding exons, and that at least two of these exons may undergo unique splicing events in the kidney. Based upon these data, a fourth unique transcript containing a deletion of exon 1C is possible.

λ-Phage Genomic Clones

Having characterized the 5′-end of the hVDR mRNA, we used the 5′-RACE-derived DNA fragments to screen a λ-phage genomic library and recovered several positive clones. Three clones were mapped using the restriction enzymes *Bam*HI, *Eco*RI, and *Sac*I, and relevant probe-reactive fragments were sequenced. As observed in Fig. 2, λ-clones 1 and 2 represent overlapping clones that contain exons 1A and 1B, which are in turn separated by an intron of approximately 5 kb. λ-Clone 3 did not overlap λ-clone 2 but contained exon 1C and exon 2. Exon 2 contained the start site of translation beginning 3 bp downstream of the 5′-end of the exon. The intron located between exons 1C and 2 was determined to be approximately 4 kb, but the lack of contiguity between λ-clones 2 and 3 prevented determination of the size of the intron located between 1B and 1C. The presence of these four exons is entirely consistent with the data obtained from analysis of the hVDR mRNA. We designated the first three exons 1A, 1B, and 1C to be consistent with a

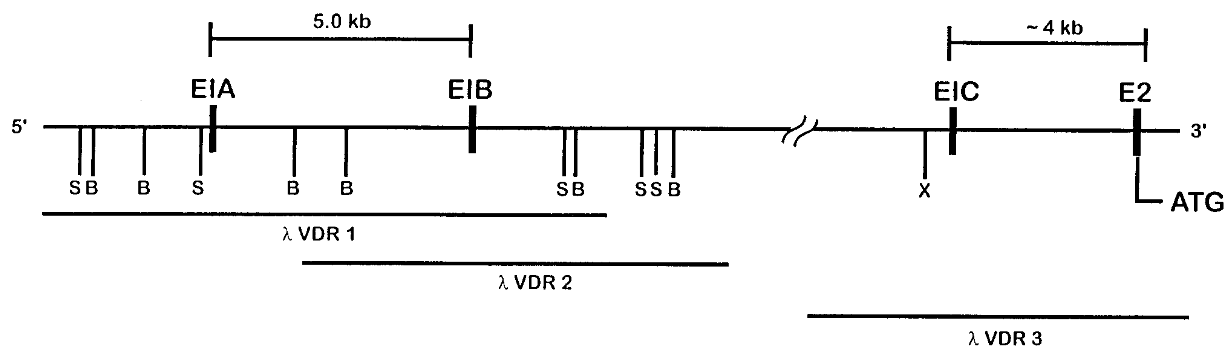

Fig. 2. Organizational Map of Exons 1A, 1B, 1C, and 2 Comprising the 5′- End of the hVDR Chromosomal Gene

A λ-phage genomic library was screened using 5′-RACE products to obtain three genomic fragments containing the hVDR gene. Each clone was restriction mapped and relevant portions sequenced. Restriction mapping and sequence analysis revealed that two overlapping clones (λ-VDR1 and λ-VDR2) contained sequence corresponding to proposed exons 1A and 1B (type II) separated by 5 kb (intron A). A downstream noncontiguous clone (λ-VDR3) contained exons IC and exon 2 (type II) separated by 4 kb (intron B). The distances between exons 1A and 1B and between exons 1C and 2 were determined by restriction mapping followed by Southern blot analysis. S, *Sac*I; X, *Xho*I; B, *Bam*HI. ATG represents the first translation codon.

preliminary description of the organization of this gene (43).

Cosmid Genomic Clones

In view of the lack of contiguity between λ-clones 2 and 3, and to recover the remainder of the hVDR gene, we screened a human liver genomic library prepared in the cosmid vector pCV109 (44) sequentially with several nick-translated hVDR cDNA probes. Screening resulted in the recovery of four unique individual human DNA cosmid clones designated phVDRG1, 3, 4, and 11. As illustrated in Fig. 3A, *Eco*RI, *Kpn*I, *Xho*I, and *Sal*I restriction endonucleases were used initially to map each of the respective clones and revealed that they represent overlapping fragments of 40 to 50 kb which together span approximately 93 kb of contiguous genomic DNA.

Three unique hVDR cDNA probes (see *Materials and Methods*) were used to determine the extreme 5′- and 3′-boundaries of the VDR gene within this locus and to determine the relative orientation of individual DNA restriction fragments located within these four clones with respect to the hVDR mRNA. Southern blot hybridization analysis with these respective VDR cDNA probes revealed that clones G1, G3, and G11 likely contained exons comprising the entire VDR chromosomal gene. Subsequent detailed hybridization analysis of G3 and G11 DNA digested with *Eco*RI, *Kpn*I, or *Eco*RI and *Kpn*I utilizing hVDR cDNA probe 3 (nt $^{+}$10 to $^{+}$2012 which includes the entire normal hVDR open reading frame) revealed the presence of eight hybridizable DNA fragments spanning a total of 55 kb (Fig. 3B, *left*). The locations of these fragments (identified as B4.2, C2.2, D4.4, E8.0, F5.5, G5.6, H2.7, and I7.0) in relationship to the genomic cosmid clones are depicted in Fig. 3A. Importantly, each of these DNA restriction fragments was unequivocally identified in human genomic DNA after high-stringency Southern blot hybridization analysis with a combination of the cDNA probes indicated above. Six of these fragments (C-H) are detected after analysis of *Eco*RI- and *Kpn*I-digested human DNA with probe 3 (Fig. 3B, *right*).

The position of exon 1A of 77 bases in genomic clone phVDRG1 was verified through hybridization screening with an oligonucleotide corresponding to a portion of exon 1A. DNA fragment A was sequenced to identify the first exon located as illustrated in Fig. 3A. Exons 1B (121 bases), 1C (81 bases), and 2 (148 bases) located in this clone were mapped relative to exon 1A, further defining the approximately 20 kb size of the intron located between exons 1B and 1C (see figure). DNA fragments B and C (see Fig. 3A) were cloned and sequenced to identify the intron/exon boundaries of exons 1C and 2. Exon 1B was positioned relative to exon 1A in λ-clone 1.

Additional DNA fragments identified through hybridization analysis were isolated, subcloned, and either partially or completely sequenced to determine the precise location of downstream VDR exons. As observed in Fig. 3C, the gene is split into seven additional exons (exons 3 through 9) of 131, 185, 121, 172, 152, 117, and 3466 bp, which, together with the upstream exons, form the authentic full-length hVDR gene. The gene itself, however, spans some 75 kb of DNA, the majority consisting of introns whose sizes, boundaries, and locations within the mature hVDR mRNA are documented in Table 2. A comparison of the sequences of the intron/exon boundaries indicates that each conforms to a canonical splice consensus sequence typical of most eukaryotic genes.

The relative organization of the exons and their locations with respect to domains located in the hVDR protein (45) is depicted in Fig. 3C. Exon 2 contains 2 bp of noncoding sequence, the translation initiation codon, and the N terminal of the two dissimilar DNA-binding Zn^{++} finger modules. Exon 3 lies downstream of a 15-kb intron and encodes the second DNA-binding zinc module. Exons 4–6 are clustered together; exons 4 and 5 encode a region that serves a hypothetical hinge function between DNA- and steroid-binding portions of the receptor protein, while exon 6 encodes the remainder of the hinge and the first portion of the steroid-binding domain. Finally, clustered exons 7–9 encode the C-terminal half of the VDR, with exon 9 containing the final 85 amino acids as well as the remaining approximately 3200 bp that constitute the large 3′-noncoding sequence.

An Exon 2 Translation Site Polymorphism

Exon sequences within the VDR gene derived here were compared with those of the human VDR cDNA recovered from the T47D breast cancer cell line and reported by Baker *et al.* (42). Exon sequences corresponded to those found in the hVDR cDNA with one exception, a T to C transition that eliminated the most 5′-ATG codon within the T47D VDR mRNA (see Table 2). The likely result of this mutation, which creates a potential polymorphic *Fok*I site within exon 2, is the utilization of a second in-frame translation codon beginning 10 nucleotides downstream that ultimately encodes a potentially foreshortened receptor protein of 424 amino acids. Evaluation of additional human DNA samples via DNA amplification techniques revealed that this feature of the cloned gene was not unique to the genomic library from which the hVDR gene was recovered (data not shown). More importantly, perhaps, the existence of an hVDR mRNA of the latter type has been demonstrated recently by Saijo *et al.* (46). Despite these observations, however, it will be necessary to confirm that two proteins that differ in molecular mass by only three amino acids are in fact translated in human tissues or cells.

The hVDR Promoter

We sequenced a DNA fragment extending 5′ of the start site of transcription containing the putative promoter for hVDR (Fig. 4). While this promoter lacks consensus TATA or CAAT boxes, the region is GC rich with five binding motifs for the transcription factor SP1 lying between

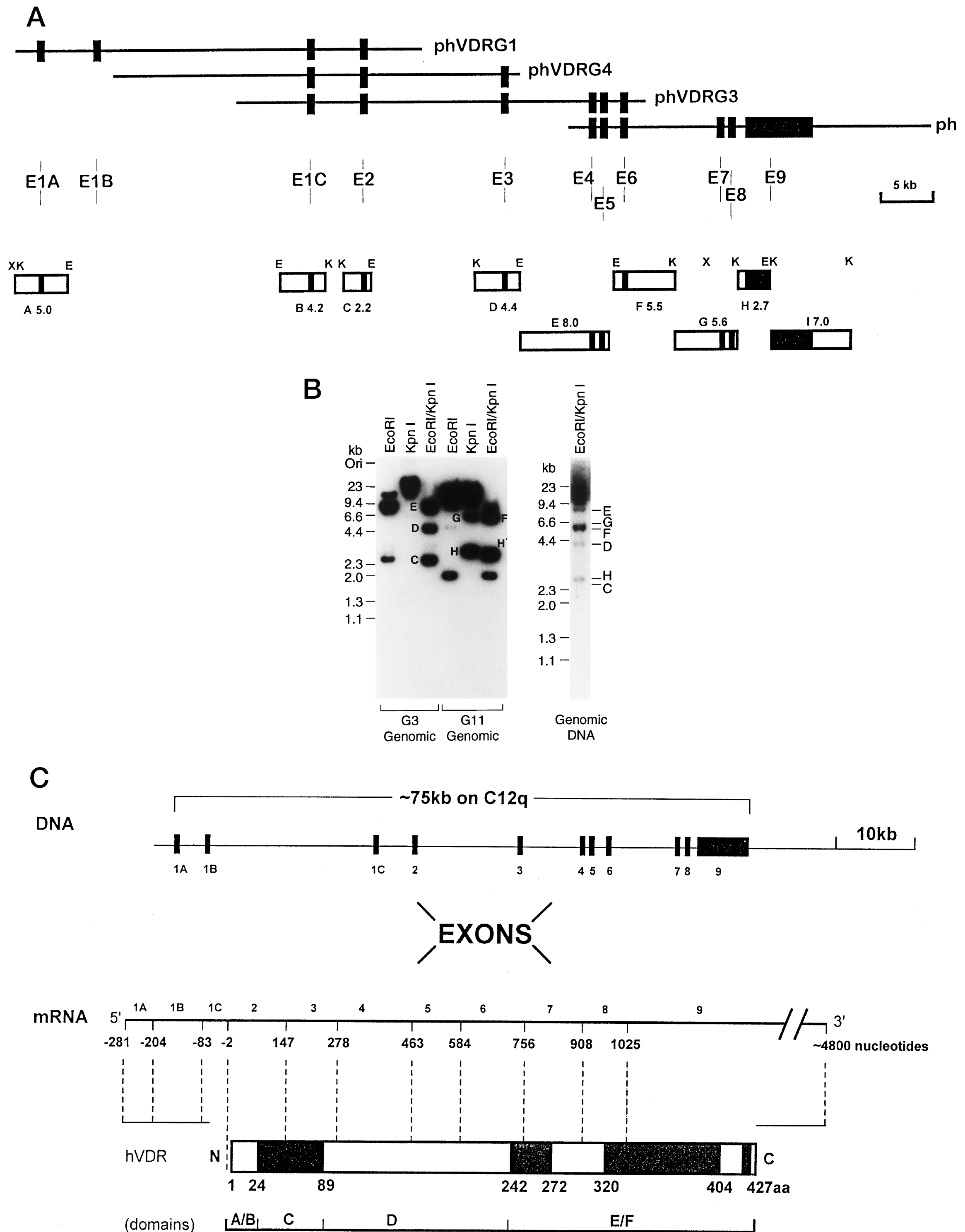

Fig. 3. Organizational Map of the Complete hVDR Chromosomal Gene

A, Organizational map of the complete hVDR chromosomal gene. A human liver cosmid library was screened with hVDR cDNA probes to obtain four clones (phVDRG1, phVDRG4, phVDRG3, and phVDRG11) that spanned the entire hVDR gene. Restriction mapping and Southern blot analysis revealed the overlapping nature of the clones as well as the relative position of each exon within the clone as indicated. Relevant restriction fragments (A5.0, B4.2, C2.2, D4.4, E8.0, F5.5, G5.6, H2.7, and I7.0) indicated as *rectangles* were subcloned and subjected to high resolution restriction mapping and sequencing. X, *Xho*I; K, *Kpn*I; E, *Eco*RI. The positions of the hVDR exons, which are numbered E1A, E1B, E1C, E2-E9, are indicated by the *black bars*. B, Southern blot analysis of phVDRG3 and phVDRG11 DNA and human genomic DNA. Overlapping phVDRG clones 3 and 11 were subjected to restriction digestion with *Eco*RI, *Kpn*I, or both enzymes as indicated (*left*). Human genomic DNA was subjected to simultaneous

Table 2. Features of the hVDR Gene

Exons			Exon/Intron Sequence		
No.	Size (bp)	Position	5′-Exon Boundary	Intron (Size, kb)	3′-Exon Boundary
1A	77	−281/−205	CGGACCAG**gt**gcgaaccc	· · · A (4.95) · · ·	tatcctta**ag**GCTCCTGAACCTAGC
1B	121	−204/−84	TCTTTGGG**gt**aagtacag	· · · B (∼20) · · ·	ttcatttc**ag**AAGCCTTTGGGTCTG
1C	81	−83/−3	TCCTTCAG**gt**aggtgttt	· · · C (∼4) · · ·	gttcttac**ag**GG **ATG** GAG GCA **ATG** Met Glu Ala Met 1
2	148	−2/146	TTC AG**gt**gagccctc Phe Ar 49	· · · D (13.2) · · ·	ctctcggc**ag**G CGA AGC ATG g Arg Ser Met 49
3	131	147/277	GAG T**gt**gagtgtcc Glu P 93	· · · E (8.4) · · ·	caccccac**ag**TC ATT CTG ACA he Ile Leu Thr 93
4	185	278/462	TTC CGG**gt**atgtctgc Phe Arg 154	· · · F (0.252) · · ·	tcccttcc**ag**CCT CCA GTT CGT Pro Pro Val Arg 155
5	121	463/583	TCA G**gt**aagcagga Ser A 195	· · · G (1.32) · · ·	tctctccc**ag**AC ATG ATG GAC sp Met Met Asp 195
6	172	584/755	TTC AG**gt**aagaaacc Phe Ar 252	· · · H (9.2) · · ·	tcccctgc**ag**A GAC CTC ACC g Asp Leu Thr 252
7	152	756/907	AAA G**gt**atgcctag Lys A 303	· · · I (0.205) · · ·	tctctcac**ag**CC GGA CAC AGC la Gly His Ser 303
8	117	908/1024	CCA G**gt**atggggcc Pro A 342	· · · J (∼1.4) · · ·	gtgcccac**ag**AT CGT CCT GGG sp Arg Pro Gly 342
9	3466	1025/3′ END			

The size and position of each of the 11 exons relative to the hVDR mRNA type II transcript are documented. The intron/exon boundaries as well as the approximate size of the introns are indicated. The canonical splice site consensus sequences are indicated in *bold*. The polymorphism associated with the two start sites of translation are *underlined* and shown in *bold.*

nucleotide (nt) −72 and −34 relative to the transcription start site (47). Potential binding sites for other transcription factors are also evident (48). In addition, five AGGTCA-like sequences, which represent potential nuclear receptor-binding element half-sites (49), are located between nt −1394 and −949. The functional relevance of these, as well as of additional interesting sequences, will need to be determined.

Activity of the 5′-Flanking Region of the hVDR Promoter

To evaluate the transcriptional capacity of the hVDR promoter, we cloned a series of 5′-deletion fragments of the gene into a luciferase reporter gene and transfected them individually into two mammalian cell lines. We tested constructs beginning at −1.935, −1.479, −1.221, −0.586, −0.464, −0.103, −0.034 kbp relative to the start site of transcription. The downstream boundary of each construct was located at the 3′-boundary of exon 1A at +71 relative to the start site of transcription [−89 relative to the start site of translation reported by Baker *et al.* (42)]. As observed in Fig. 5A, pVDE1–1.93 was capable of directing significant luciferase expression when introduced by transient transfection into HeLa cells. Each deletion construct likewise exhibited

digestion with *Kpn*I and *Eco*RI (*right*). Southern blot analysis was carried out as described in *Materials and Methods* and hybridized to the hVDR3 probe. Bands D-H identified both in genomic DNA and within the clones represent the DNA fragments schematically illustrated in A. Band C was weakly evident on the autoradiogram but not reproducible in the figure. C, Structural organization of the human chromosomal vitamin D receptor gene. The structural organization of the human VDR gene locus (DNA) comprising 11 exons (1A, 1B, 1C, 2 through 9) spanning approximately 75 kb of DNA is depicted. A 10 kb scale is indicated to the *right*. The location of exons relative to the mRNA transcript of ∼4800 nucleotides (mRNA) and the encoded VDR protein of 427 amino acids (hVDR) is illustrated. With regard to the hVDR mRNA, negative numbers indicate 5′-noncoding nucleotides, and positive numbers indicate protein encoding nucleotides beginning with +1 indicated by Baker *et al.* (42) as well as 3′-untranslated sequences. Numbers *below* the hVDR protein indicate the amino acid residue boundaries of *shaded* homology domains. Regions of functionality are designated A/B, C, D, and E/F as described in Ref. 76.

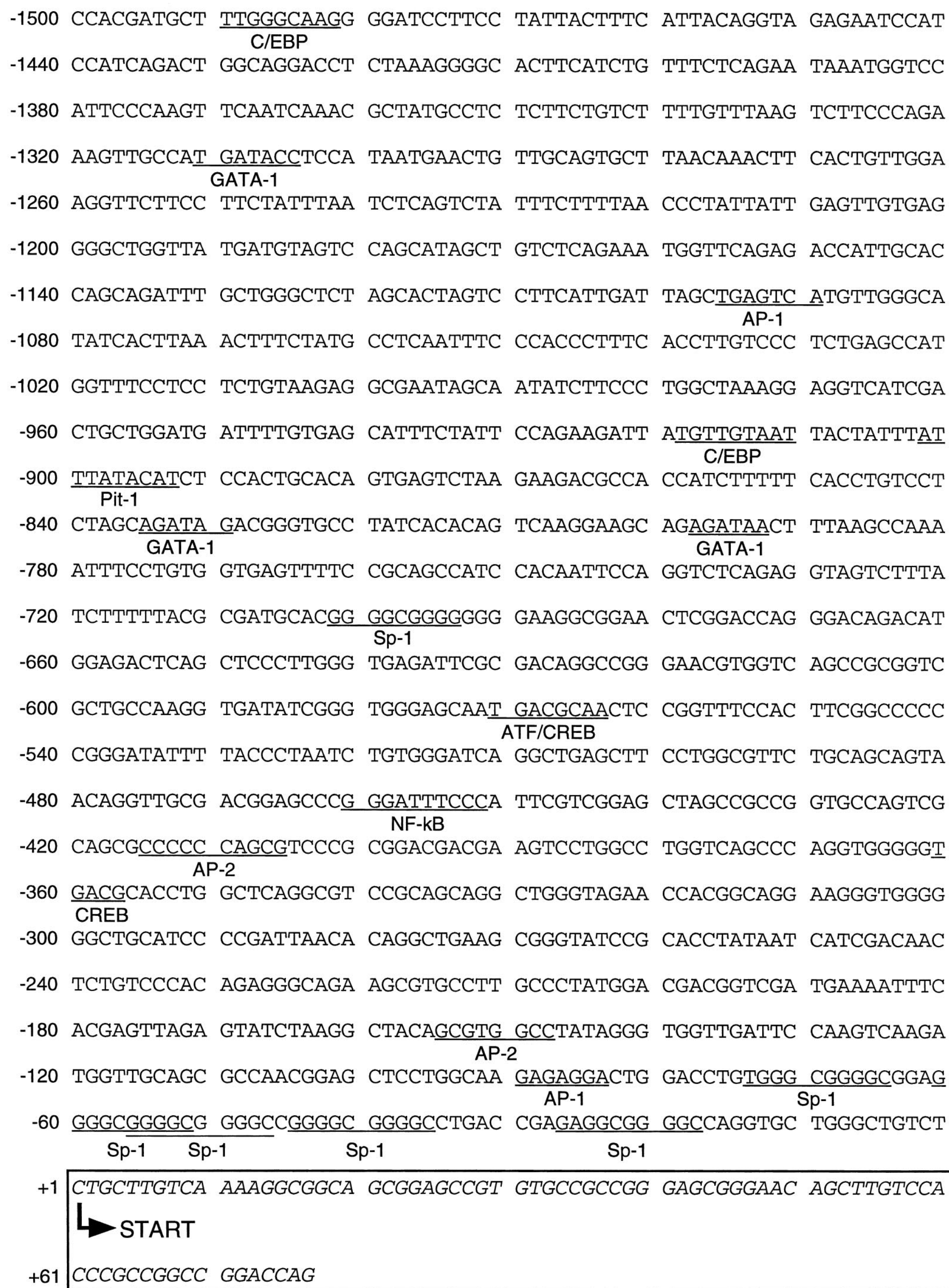

Fig. 4. Sequence Determination of the hVDR Gene Promoter

The nucleotide sequence of the hVDR gene promoter is documented. The transcription start site is indicated as +1 beginning within the *boxed sequence*. The locations of potential binding sites for specific transcriptional regulators are *underlined* and indicated. Transcription factors indicated include SP-1, AP-1, AP-2, NFkB, GATA-1, Pit-1, and C/EBP. Potential binding sites for nuclear receptors are also present.

substantial transcriptional activity, although this activity varied and did not exhibit an obvious pattern. Constructs containing proximal elements of the promoter (−464 and −103 relative to the start site) displayed the most activity, suggesting the possibility that elements upstream of −0.464 kb may confer a negative regulatory function. 5′-Deletion of the hVDR promoter from −103 bp to −34 bp that removed four of the five GC boxes near the initiator resulted in a 10-fold drop in activity. The latter result substantiates the hypothesis that these elements play an important role in the activity of the hVDR promoter and provide the basis for future studies. Preliminary studies in Hela cells suggest that the 5′-flanking region of the gene is not responsive to 1,25-$(OH)_2D_3$ (data not shown).

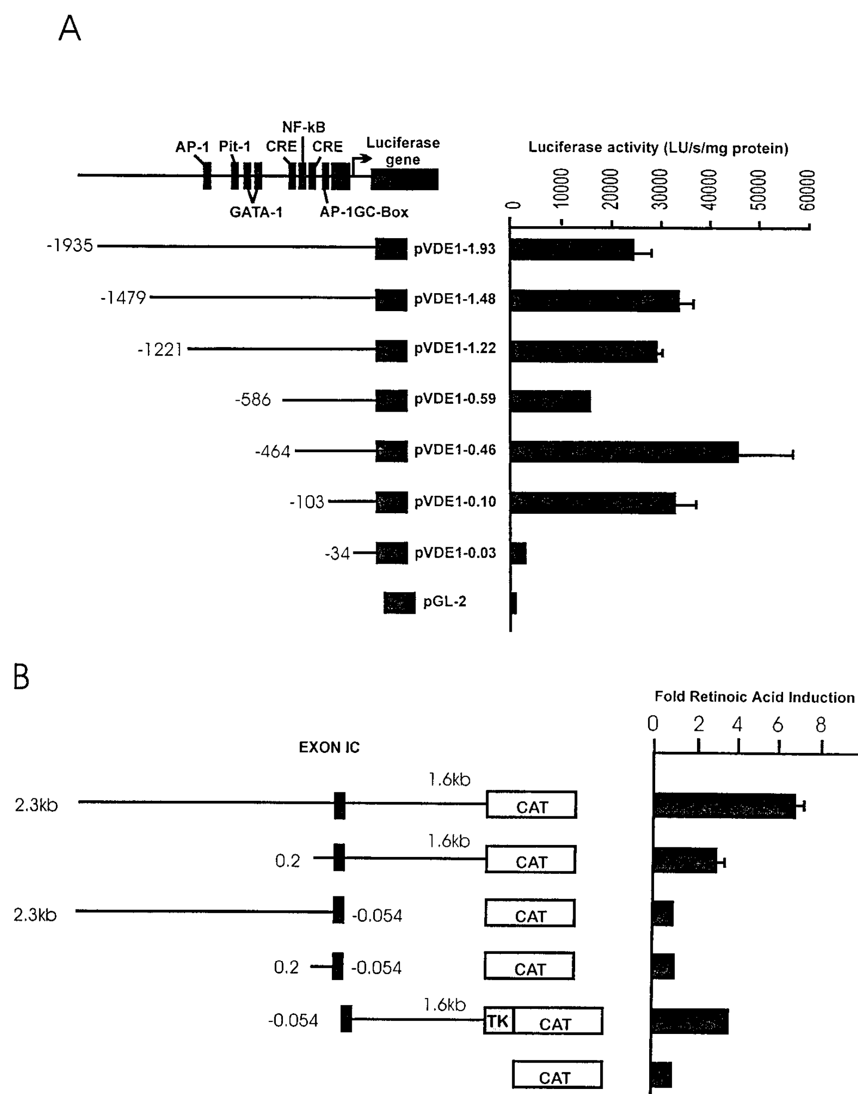

Fig. 5. Functional Analysis of the Human VDR Gene Promoter

A, hVDR promoter constructs pVDE1–1.93, pVDE1–1.48, pVDE1–1.22, pVDE1–0.589, pVDE1–0.46, pVDE1–0.10, pVDE1–0.03, and the promoterless control plasmid (pGL-2) were individually cotransfected together with the normalization vector pCMV-β-galactosidase as indicated in *Materials and Methods*. The activities of these constructs in Hela cells were determined after 64 h and normalized to the activity of β-galactosidase. Luciferase activity is expressed as light units per second per mg of protein. Data are means $\pm$ SEM of four independent experiments. B, DNA fragments surrounding exon 2 as indicated in the figure were fused to the pBL CAT2 or pBLCAT3 chloramphenicol acetyltransferase expression vectors and transfected into rat ROS 17/2.8 cells as described in *Materials and Methods*. Transfected cells were treated with either vehicle or retinoic acid (10^{-6} M) for 48 h after which the cells were harvested and extracts (100 μg protein) assayed for CAT activity. Each assessment represents the mean $\pm$ SEM of a triplicate determination. The data are representative of three separate experiments and are expressed as the activity of the construct in the presence of retinoic acid compared with the activity of the construct in the ligand's absence (fold induction).

A Retinoid-Responsive Region Lies Downstream of Exon 2

In view of the complexity of the hVDR gene, we examined several DNA fragments surrounding exon 1C for their capacity to direct transcriptional activity or to be regulated by hormones such as 1,25-$(OH)_2D_3$ or retinoic acid. All clones examined exhibited transcriptional activity when cloned into the promoterless chloramphenicol acetyltransferase (CAT) vector pBLCAT3 (data not shown). As observed in Fig. 5B, however, several constructs, each containing intron sequence downstream of exon 2, were responsive to treatment with retinoic acid (10^{-6} M). The constructs were uniformly unresponsive to 1,25-$(OH)_2D_3$ in this cell line (data not shown). The ability of this fragment of DNA to transfer retinoic acid response to the viral thymidine kinase promoter provides further support for the possibility that the previously identified regulation of VDR expression by retinoic acid (33, 34) is direct and mediated via a *cis*-element located a sig-

nificant distance downstream of the authentic promoter. Further work will be required to define the exact location of this *cis*-element.

DISCUSSION

We report here the structural organization of the human VDR gene derived from several λ-clones and four overlapping cosmid clones that span the entire gene locus. The human gene comprises 11 exons that together with the associated introns cover approximately 75 kb of DNA. Three exons (exons 1A, 1B, and 1C) make up the 5′-noncoding leader sequence of the largest of the hVDR mRNA species (type 2). An additional eight exons (exons 2–9) encode the structural portion of the gene product. This numbering system allows retention of the numbering system assigned in an earlier preliminary analysis of the gene (43) as well as that found in a body of literature related to the existence of hVDR gene polymorphisms that has evolved recently (see below). The multiple exonic structure and general organization of the hVDR gene are comparable to that of many of the other steroid receptor genes that have been characterized including the thyroid receptor (18), progesterone receptor (11), estrogen receptor (12), androgen receptor (14), and glucocorticoid receptor (13, 17). The promoter for this gene is TATA-less and GC rich.

The organization of the hVDR gene indicates that separate exons encode each of the zinc finger modules and that the 3′-boundaries of each of these exons appear to be generally conserved within the nuclear receptor family of genes. One exception may be chicken ovalbumin upstream promoter-transcription factor α and β, for which both finger modules appear to be encoded by a single exon (16). Although the two zinc modules within these proteins appear highly related structurally, they are not equivalent topologically (50). Furthermore, the function of each module is substantially different; the first zinc module determines the specificity of DNA binding whereas the second is more intimately involved in the protein-protein contacts that stabilize the association through dimerization (51). Although it is possible that the two exons encoding these modules evolved from a common ancestral gene through duplication and subsequently diverged under a different set of selective pressures, it is also possible that they evolved independently. Three exons encode the hVDR hinge, whereas only two encode this flexible region within the sex steroid receptor genes. It is noteworthy that the VDR appears to contain an extended hinge region relative to many of the other members of this gene family (4). The function, if any, of these additional hVDR residues that are encoded by insertion of exon 5 is unknown, although this region appears to be the least conserved among VDRs from different species. Finally, exon 2 of the hVDR is restricted to 21 amino acids upstream of the first zinc module, placing the hVDR DNA-binding domain near the amino-terminal end of the protein. This is unlike several of the other family members where one or more relatively large exons lie upstream, relegating the DNA-binding domain to a more central location within the protein (4). The absence of this extended region within the hVDR, whose presence in the larger receptors is associated with an important activation domain(s) (52–54), implies a more complete reliance of the hVDR on the carboxy-terminal activation function (AF2) or on an, as yet, undescribed activation region.

Amplification of the 5′-end of the hVDR mRNA revealed the existence of three separate transcripts in human kidney RNA. Because each contains the identical start site and first exons, the transcripts are likely derived from alternative splicing of two of the 5′-noncoding exons, 1B and 1C, one of which eliminates exon 1B and the other which eliminates both exons 1B and 1C. It is hypothetically possible that a fourth transcript that does not contain exon 1C also exists, perhaps in tissues other than kidney. Alternative splicing of the RAR, retinoid X receptor, thyroid hormone receptor, and other receptors is a common feature of this gene family of proteins. Unlike that which was observed here, some splicing events lead to the productions of different proteins with unique functions (19, 20). The relative abundance of each of the three hVDR transcripts within the kidney is unknown; indeed, the similarity in their size prevented their earlier detection by Northern blot analysis. The existence and relative abundance of these hVDR transcripts in other tissues as well as a determination of their possible individual functions remain for future studies.

The promoter region of the hVDR lies in a GC-rich island and does not contain a TATA box. In that respect, the hVDR gene is like certain other steroid receptor gene promoters. Our analysis of this promoter indicates a substantial capacity to direct transcription of a chimeric reporter function. The most proximal region (-103 to -34) imparts more than 80% of the activity of the promoter in HeLa cells and appears to contain strong positive elements. A possible negative contribution is evident between -586 and -464. The hVDR promoter contains an array of putative binding sites for transcription factors that mediate the activities of multiple pathways that serve to transduce a variety of extracellular signals. For example, it is known that the VDR is regulated by both PKA and PKC pathways (27–29, 55, 56) that are in turn known to converge on several specific transcription factors. These and other studies support the possibility that binding sites upstream of the hVDR promoter may play modulatory roles in its regulation, although future functional studies will be necessary to confirm this hypothesis. At the very least, the presence of five GC boxes immediately upstream of the start site of transcription suggests a fundamental role for the transcription factor SP1 in the activation of this gene (47). SP1 is known to interact both with a number of cellular and viral promoters as well as with other transcription

factors such as NFkB (57, 58). Interestingly, the hVDR gene is induced with retinoic acid at a site that lies downstream of exon 1C. While a potential binding site for one or more of the retinoid receptors has not been definitively localized, it is likely that this site mediates the recognized ability of RA to induce the transcription of VDR (33, 34). The existence and, more importantly, the location of this site, together with the size of the introns lying between exons 1A, 1B, and 1C, suggest that it may prove difficult technically to identify additional regulatory sequences. The inability to detect vitamin D inducibility of the VDR gene at the promoter level currently suggests that the autoregulatory actions of vitamin D are indirect. For example, a recent study suggests that 1,25-$(OH)_2D_3$ can stimulate the expression of c-*fos* in osteoblastic cells that might in turn stimulate hVDR gene expression (59). Based upon the noted complexity at the 5′-end of the VDR gene, however, it is possible that 1,25-$(OH)_2D_3$-responsive portions of the gene have yet to be defined.

During the analysis of the hVDR gene, we identified the existence of a polymorphism associated with exon 2. This polymorphism leads to synthesis of two different hVDR mRNAs which we detected utilizing single-strand conformational analysis (46). One corresponds to the mRNA sequence of Baker *et al.* (42) wherein the start site of translation is situated at codon 1 and the second is associated with a T to C conversion in this codon that results in potential translation beginning at an in-frame ATG located downstream at codon 4. Translation of such an mRNA would result in the production of an abbreviated protein of only 424 amino acids. Two studies have recently reported the distribution of these alleles in the human population (60, 61); the ATG to ACG allele, which can be detected by the presence of a *Fok*I site (60), represents the more common form. Interestingly, the frequency of this allele correlates with an increase in bone density in two different human female populations (60, 61). This observation suggests that the VDR products of these two alleles may exhibit unique activities. Validation of this hypothesis, however, will require additional population studies as well as the demonstration that the two VDR alleles are indeed expressed in human tissues and that they exhibit quantitative and/or qualitative differences in their activity on bone or other tissues. As a first step, Arai *et al.* (61) have shown that both proteins can be produced through recombinant means in transfected COS cells. More importantly, the capacity of these two gene products to direct transcription of a vitamin D-sensitive chimeric gene in cotransfected cells is quantitatively different. The smaller protein, whose corresponding allele appears to correlate with increased bone density, exhibited greater transcriptional activity. Additional studies will be necessary to confirm these functional studies as well as the correlation between bone mineral density and VDR alleles. Interestingly, some plasticity is also apparent in these two codons in other species; while the mouse VDR mRNA contains both ATGs (62), the rat VDR mRNA contains only the most 5′-ATG (63). Perhaps unique activities are also associated with these mRNA products.

Additional polymorphisms have been identified within the hVDR gene in the intron between exons 7 and 8 (*Bsm*I and *Apa*I) and within the 3′-noncoding sequences lying in exon 9 (*Taq*I) (64) (see Table 3). The presence of these restriction fragment length polymorphisms has been reported to be associated with population bone mineral density (65) and more recently with prostate cancer (66). In the former case, this finding has not been widely reproduced, suggestive of relatively weak linkage, and remains highly controversial (67). In contrast to the *Fok*I polymorphism located in exon 2, it is unclear how the *Bsm*I and/or *Taq*I polymorphisms located in noncoding portions of the hVDR gene might influence hVDR function. It is possible that they are linked in some way either to the potentially functional exon 2 start site polymorphism, to as yet unidentified allelic differences located within the hVDR gene promoter, or to unrelated genes that impact bone mineralization directly. Both the validity of the proposed associations between hVDR polymorphisms and disease and, if proven true, the mechanism(s) by which they impart disease remain to be established.

In conclusion, we report on the structural organization of the hVDR chromosomal gene. The availability of this gene locus as well as identification and cloning of its promoter will enable future studies aimed at identifying molecular determinants of the VDR's expression. A wealth of studies that describe the regulation of expression of the VDR provide the backdrop and rationale for these impending studies.

MATERIALS AND METHODS

Messenger RNA Analysis

The sequence of the 5′-end of the human VDR mRNA was determined utilizing the 5′-RACE system obtained from Life Technologies, Inc, (Gaithersburg, MD) (68). Total RNA was isolated from human kidney (69, 70) and used to synthesize first-strand cDNA utilizing random hexamers and oligo d(T) primers and Superscript RT (Life Technologies, Inc). Two sequential PCR amplification reactions were employed to identify the 5′-ends of the hVDR mRNAs and to generate DNA fragments useful for initial sequencing. As illustrated in Fig. 1A, the first was performed using common anchored primer A (5′-CTGGTTCGGCCCACCTCTGAAGGTTCCA-GAATCGATAG-3′) and primer B2 (5′-CTTCCGCTTCAGGAT-CATCTCCCGC-3′), which corresponds to nt +309 to +333 relative to the translation start site of the human VDR cDNA of Baker *et al.* (42). The second was performed with a portion of the reaction products from the first amplification and used primer A and primer B1 (5′-GCAGGGGGCAGGTAAGTGG-3′), which corresponds to nt −29 to −11 relative to the start site of translation reported by Baker *et al.* (42). Sequence determination of the PCR products of these sequential reactions established the 5′-end of the hVDR mRNA transcript, enabling a subsequent amplification of kidney RNA using primer A1, which corresponds to the first 22 bases of the

authentic 5′-end of the transcript and primer B2 (see Fig. 1A). All amplified DNA products were subcloned into pBluescript II SK (Strategene, San Diego, CA) and sequenced using the SequiTherm Long-Read Cycle Sequencing Kit-LC (EpiCentre Technologies, Madison, WI). All DNA sequencing was performed using the fluorescence-based LI-COR model 4000L sequencer.

Recovery and Analysis of hVDR Genomic Clones

A human EMBL 3 genomic library was screened with ^{32}P-labeled PCR probes obtained after 5′-RACE of human kidney mRNA using high-stringency hybridization conditions. Nylon membrane replicas of approximately 1.5×10^6 plaques were incubated for 2 to 3 h in 50% formamide, 5× Denhardt's solution, 0.75 M NaCl, 0.05 M NaH_2PO_4, pH 7.4, 3 mM EDTA, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Hybridization was performed overnight at 42 C with 5×10^5 cpm/ml labeled probe. Membranes were washed for 1 h at 30 C in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0, and 0.05% SDS and then twice for 1 h at 68 C in 0.15 M sodium citrate, pH 7.0, and 0.1% SDS. After exposure of the membranes to Kodak X-Omat film, positive plaques were isolated, rescreened twice, and purified, and then the inserts were isolated and subcloned into pBlueScript II SK. DNA inserts were verified by Southern blot analysis, restriction enzyme-mapped by routine methods, and portions were then sequenced using the strategy outlined below.

A human liver genomic library prepared in the cosmid vector pCV109 (44) was screened via colony hybridization techniques (71) using nick-translated hVDR cDNA probes. HVDR probe 1 extended from −115 to +145 nt relative to the sequence of Baker *et al.* (42); hVDR probe 2 contained nucleotides +10 through +576 relative to the sequence of Baker *et al.* (42); and hVDR probe 3 contained nucleotides +10 through +2012 nt relative to the sequence of Baker *et al.* (42). The latter probe represents the entire open reading frame of the normal hVDR cDNA together with 731 nucleotides of noncoding 3′-sequence. Nylon filter-immobilized DNA was prehybridized at 68 C overnight in 6× NaCl-sodium citrate (SSC) (1× = 0.15 M $NaCl_2$, 15 mM sodium citrate, pH 7), 2 mM EDTA/0.5% nonfat dry milk (wt/vol), and then hybridized with ^{32}P-labeled probes (initially probes 2 and 3) under identical conditions for 16 h. Filters were washed for 2 h with several changes of 0.15 M $NaCl_2$, 15 mM sodium citrate, pH 7, 0.5% SDS at 68 C and then autoradiographed overnight. Positive phVDRG cosmid clones were isolated through three to four rounds of additional screening.

Human genomic DNA was prepared as described (71). The latter as well as cosmid phVDRG DNA were transferred to nylon membranes, prehybridized for 6 h at 68 C in 6× SSC, and then hybridized with the appropriate ^{32}P-labeled hVDR cDNA probes overnight. Filters were washed for 2 h as above, and autoradiographed for 4 h (cloned DNA) or 48 h (human genomic DNA). phVDRG DNA was isolated via alkaline lysis and polyethylene glycol precipitation techniques as previously described (71). The isolated DNA was mapped with restriction endonucleases by routine techniques. The relative orientation of specific DNA fragments of related size within the clones was determined by sequential probing with hVDR cDNA probes 1, 2, and 3. DNA fragments that hybridized with the hVDR cDNA probes were isolated, subcloned into the pGEM 3 cloning vector (Promega Biotech, Madison, WI), and then subjected to standard sequencing methods using Sequenase (US Biochemical Corp., Cleveland, OH). The sequencing strategy we employed involved determining the ends of each DNA fragment utilizing the T7 and SP6 sequencing primers followed by extension of this sequence with synthetic oligonuclotides complementary to the newly identified sequence. As the relative position of hVDR gene exons emerged within the DNA clones, synthetic oligonucleotides corresponding to hVDR cDNA sequence either 5′ or 3′ to the identified exon were used as sequencing primers. Intron/exon boundaries were identified using pairs of primers that generated sequence from both strands. Orientation of the exon(s) within the DNA fragment was achieved by identifying sequence overlaps within the clone or by mapping a unique restriction site identified within the newly obtained sequence.

hVDR Gene Promoter Analysis

hVDR promoter constructs were prepared beginning with an approximately 3.2 kb *Sac*I-*Hin*dIII fragment of the hVDR gene extending from exon 1 upstream approximately −3200 bp. This fragment (pVDE1–3.2) as well as additional fragments were cloned into the *Hin*dIII site of the luciferase expression vector pGL-2 basic (Promega). Plasmids pVDE1–2.6, pVDE1–1.93, pVDE1–1.48, pVDE1–1.22, pVDE1–0.59, pVDE1–0.46, pVDE1–0.10, and pVDE1–0.03 were similarly constructed from promoter fragments that contained common 3′-ends but extended 2.6, 1.93, 1.22, 0.586, 0.462, 0.10, or 0.03 kb upstream, respectively. DNA restriction fragments were also isolated from hVDR gene DNA surrounding exon 1C. These fragments include exon 1C and the length of intron sequence lying both upstream and/or downstream of exon 1C as indicated in the figure. The activity of these fragments of DNA was assessed in promoterless pBL-CAT3 and thymidine kinase promoter-containing pBL-CAT2 chloramphenicol acetyltransferase expression vectors (72). The orientation and cloning boundaries of all constructs were verified through DNA sequence analysis.

Transient Transfection Analysis of hVDR Promoter Sequences

Hela cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and nonessential amino acids. Rat osteosarcoma ROS 17/2.8 cells were grown in Ham's F12 medium supplemented with 10% FCS containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), and nonessential amino acids as previously described. Cells were transfected with DNA 24 h after passage using either polybrene (73) (Sigma Chemical Co., St. Louis, MO) (ROS 17/2.8) or lipofectAMINE (Life Technologies Inc.) (HeLa cells). hVDR gene DNA reporter plasmids (5 or 10 μg) were cotransfected together with 1 μg β-galactosidase normalization vector (1 μg), and the activities of the enzymes CAT, luciferase, and/or β-galactosidase were evaluated in cellular extracts prepared 64 h following transfection. Luciferase or CAT activities were determined as previously described (65, 74) and normalized to the activity of β-galactosidase (75). All plasmids used for cellular transfections were purified on Qiagen ion exchange columns.

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