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# Isolation and Characterization of the Chicken Vitamin D Receptor Gene and Its Promoter

**Zhongjian Lu, Frederic Jehan, Claudia Zierold, and Hector F. DeLuca***

Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin–Madison, Madison, Wisconsin 53706

**Abstract** The sequences from several independent cDNA clones encoding the chicken vitamin D receptor as well as primer extension assay have clearly delineated the 5′ terminus and the transcriptional start site. Screening a chicken genomic library produced genomic clones containing vitamin D receptor (VDR) gene fragments. Restriction map of clone 8 showed that the 18.6-kb chicken VDR fragment has exons 1 and 2, intron 1, part of intron 2, and 7-kb 5′ flanking region. Exons 1, 2 , and 3 found in the chicken VDR gene shares low homology with its mammalian counterparts (i.e., E1A, E1B, and E1C in human). By contrast, the fourth exon and following exons for the coding region of VDR gene are highly conserved between avian and mammalian species. While the fourth exon bears the ATG sites for translation initiation in mammals, the third exon in birds has two extra ATG sites for leaky translation as determined previously. Thus, the avian VDR has more N-terminal sequence than the mammalian VDR and is found in two distinct forms. The 5′ flanking region from genomic clone 8 shares considerable homology in several regions with the human and mouse VDR promoters. Moreover, the 5′ flanking region of chicken VDR gene possesses promoter activity, as shown by its ability to drive the luciferase reporter gene in cell transfection assays. Like other steroid receptor promoters, the chicken VDR promoter contains no TATA box but possesses several GC boxes or SP1 sites. A series of deletional promoter constructs established that the proximal GC boxes are the major drivers of gene transcription, while the more upstream sequences have repressive elements. J. Cell. Biochem. 77:92–102, 2000. 

**Key words:** VDR; gene transcription; tissue-specific expression

The vitamin D receptor (VDR) is a central component of the vitamin D endocrine system maintaining calcium and phosphorus homeostasis in the body [Darwish and DeLuca, 1996; DeLuca and Zierold, 1998]. As a member of the nuclear hormone receptor superfamily, VDR has a highly conserved DNA binding domain composed of two zinc fingers, a variable hinge region, and a well-conserved ligand binding domain [Christakos et al., 1996; Mangelsdorf et al., 1995; Perlmann and Evans, 1997]. So far VDR has been cloned from three mammalian species, rat [Burmester et al., 1988a,b], human [Baker et al., 1988], and mouse [Kamei et al., 1995]; two avian species, chicken and Japanese quail [Elaroussi et al., 1994; Lu et al., 1997]; and one amphibian species, *Xenopus laevis* [Li et al., 1997a]. The importance of VDR is clearly demonstrated by VDR knock-out experiments [Li et al., 1997b; Yoshizawa et al., 1997] and vitamin D-dependent rickets type II [Hawa et al., 1996; Whitfield et al., 1996; Wiese et al., 1993], To carry out its function, VDR must first bind its ligand and, together with the retinoid × receptor (RXR), bind to the vitamin D response elements (VDREs) in the promoters of the target genes [Lin et al., 1996; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995; Munder et al., 1995; Ross et al., 1992]. VDREs have been discovered in many genes, including (1) 25-OH-$D_3$ 24-hydroxylase [Chen and DeLuca, 1995; Ohyama et al., 1996; Zierold et al., 1994, 1995]; (2) PTH/PTHrP [Demay et al., 1992b; Kremer et al., 1996; Liu et al., 1996]; (3) calcium binding protein 9k or 28k, osteocalcin, osteopontin [Darwish and DeLuca, 1992; De-

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Frederic Jehan is currently at CNRS UPR 1526 Hôpital, Saint Vincent de Paul, 82 Avenue Denfert-Rochereau, F-75014 Paris, France.

*Correspondence to: Hector F. DeLuca, Department of Biochemistry, University of Wisconsin-Madison, Biochemistry Addition, 433 Babcock Drive, Madison, WI 53706-1544. E-mail: deluca@biochem.wisc.edu

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may et al., 1992a; Noda et al., 1990; Takeda et al., 1994]; and (4) interleukin-2 (IL-2), IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [Alroy et al., 1995; Harant et al., 1997; Towers and Freedman, 1998].

Of great interest is the regulation of the vitamin D receptor itself. Many factors including calcium, $1,25(OH)_2D_3$, parathyroid hormone (PTH), growth hormone (GH), glucocorticoid, and estrogen have been reported to alter VDR levels or its mRNA [Brown et al., 1995; Chen et al., 1997; Denda et al., 1996; Ishibe et al., 1995; Lee et al., 1991; Sandgren and DeLuca, 1990; Solvsten et al., 1997; Strom et al., 1989; Uhland-Smith and DeLuca, 1993]. The mechanisms of VDR regulation remain undefined except for VDR protein stabilization by its ligand [Arbour et al., 1993; Santiso-Mere et al., 1993; Wiese et al., 1992]. To understand VDR regulation clearly at the transcriptional level, the VDR gene and its promoter must be cloned. This effort has been largely hampered because of inadequate information on the 5′ end sequence of the VDR cDNA [Hughes et al., 1988]. Previously we reported cloning the full-length chicken VDR cDNA, including more 5′ end sequence [Lu et al., 1997]. While the work on chicken VDR gene and its promoter neared completion, VDR gene promoters for human and mouse have been cloned [Jehan and DeLuca, 1997; Miyamoto et al., 1997]. This article reports the organization of the chicken VDR gene at the 5′ end and its promoter.

## MATERIALS AND METHODS

### Determining the 5′ End Sequence of VDR cDNA

A λ gt11 +D kidney library constructed by both oligo(dT) and random primer methods was extensively screened [Lu et al., 1997]. A 200-base pair (bp) region corresponding to the known 5′ end sequence of cVDR cDNA was amplified by polymerase chain reaction (PCR) with a set of primers covering this region. The 200-bp PCR product was separated and excised from the agarose gel for $^{32}P$ probe preparation by random labeling (Promega, Madison, WI). About 1,000,000 plaques grown on an *Escherichia coli* lawn of Y1090 strain were screened. Pure positive phage clones were further analyzed. The cDNA inserts excised from the phage DNA by *Eco*RI digestion were transferred into Bluescript (BS) plasmid for sequencing.

### Genomic Library Screening

A chicken genomic library constructed from chicken liver DNA was purchased from Clontech (Palo Alto, CA). This EMBL3 library with an actual titer of $1.5 \times 10^{10}$ pfu/ml was probed with a 500-bp fragment corresponding to the 5′ end sequence of the cDNA. Specifically, phage plaques were grown on 150-mm plates of *E. coli* k802 strain. Plaques were then transferred onto Hybond-N nylon membranes (Amersham, Arlington Heights, IL) for DNA denaturation, and ultraviolet (UV) cross-linking. After overnight prehybridization at 42°C in the buffer containing 50% formamide, 6× SSPE, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml salmon sperm DNA, the lifted membranes were hybridized with the VDR probe labeled with $^{32}P$ by random primer method (Prime-a-Gene kit, Promega). After overnight hybridization, membranes were washed in successively stringent solutions: 2× SSC, 0.05% SDS, 25°C, 15 min; 1× SSC, 0.1% SDS, 60°C, 1 h, twice; and finally 0.2× SSC, 0.1% SDS, 60°C, 15 min. The membranes with desirable backgrounds were exposed to Kodak X-OMAT AR film at −70°C with an intensifying screen. Positive phage plaques were picked up by Pasteur's pipettes and released into SM phage buffer. Subsequent two or three more rounds of plating and screening produced pure genomic clones for further analysis.

### Subcloning, Restriction Mapping, and Sequencing

The positive genomic clones were used to prepare λ phage DNA by Wizard® lambda preps kit (Promega). *Xho*I digestion of the phage DNA gave rise to genomic fragments subsequently subcloned into pBluescript $SK^+$ plasmid. The BS plasmid with an 18.6-kb VDR genomic insert was analyzed by extensive restriction digestions with many enzymes, including *Bam*HI, *Sac*I, *Sma*I, *Pst*I, and *Xho*I. To locate the exon positions within the genomic fragments, Southern blots were also performed with several oligonucleotide probes corresponding to the VDR cDNA sequences in the 5′ to 3′ direction. The exon-intron borders were further determined by direct sequencing. The 5′ flanking region of exon 1, which represents the putative promoter region of chicken VDR gene, was revealed by an ALF™ DNA sequencer. All sequence data processing and analysis were done on the GCG

computing platform supported by the Genetics Computer Group (Madison, WI).

### Primer Extension

Antisense primers corresponding to bases +80 to +46 (Luc 67), +115 to +81 (CCV 1), and +95 to +61 (CCV 2) of the chicken VDR cDNA sequence were synthesized by IDT (Coralville, IA) as follows:

1. Luc 67: 5′ ACT GTT GGA GGA CCG CAG ACA GGT CGG GTC GGC AG 3′
2. CCV1: 5′ TGG AGA TGC CAG ACT GGA GGC ACT CCC AGC GTC CG 3′
3. CCV2: 5′ CAC TCC CAG CGT CCG ACT GTT GGA GGA CCG CAG AC 3′

All extension primers were end-labeled with T4 polynucleotide kinase by $\gamma^{32}$P-ATP. 5 µg of poly(A)$^+$ RNA from chicken intestine were mixed with 100 fmol of $^{32}$P-labeled primers in 1× primer extension buffer provided by the Primer Extension System-AMV Reverse Transcriptase kit (Promega). The primer-RNA mixtures were heated to 65°C for 20 min and gradually cooled to room temperature for specific hybridization. The annealed primers were then extended by AMV reverse transcriptase at 42°C for 1 h. After completion of the reaction, samples were mixed with an equal volume of loading dye and analyzed on a denaturing 8 M urea, 8% polyacrylamide sequencing gel. Also included were the ϕX174 *Hin*fI DNA markers and a control reaction supplied by the Promega kit.

### Reporter Constructs

The 5′ end flanking region of exon 1 was transferred into the pGL2-basic reporter plasmid (Promega) containing the firefly luciferase gene. The 6.5-kb construct defined by two convenient *Bam*HI sites includes 149 bp of intron 1, the entire exon 1 (27 bp), and the rest of 5′ end flanking region. In addition, the 6.5-kb genomic fragment was inserted into pBluescript for manipulations later on. *Sma*I digestion of the 6.5-kb construct and subsequent insertion of the *Sma*I fragment (3.7 kb) into the pGL2-basic plasmid in right direction generated the 4.2-kb construct. *Pst*I digestion of the 6.5-kb construct gave rise to the 1-kb construct. A second series of reporter constructs were made by use of the *Hae*II site inside exon 1. The previous 1-kb construct deleted the intron 1 sequence and part of the exon 1, produced the new 800-bp construct. *Sac*I, *Sma*I, and *Ban*I digestions shortened the construct to 590, 270, and 134 bp, respectively. To extend the construct, the immediate upstream *Sac*I fragment of 1,710 bp was added back to the 590-bp construct in right direction so as to create the 2.3-kb construct. Finally, elimination of a 1.2-kb fragment from the 2.3-kb construct resulted in the 1.1-kb construct. As a result, two series of deletional constructs were made. The first series of constructs with some intron 1 sequence are 6.5, 4.2, and 1 kb. The second series of constructs with only 16 bp exon 1 sequence are 2.3 kb, 1.1 kb, 800 bp, 590 bp, 270 bp, and 134 bp.

### Cell Preparation and Culture

COS-1 cells (green monkey kidney cell line) were maintained at 37°C, 6% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Typically the six-well plates seeded with cells from the master plate took 16–18 h to reach 70–80% confluency.

### Transient Transfection and Luciferase Assay

Cells grown in six-well plates to a subconfluent state were transfected with plasmid DNA by the lipofectin reagent (1 mg/ml, Gibco-BRL). For cells in one well, 3 µl of lipofectin resuspended in 100 µl medium for 30 min was mixed with 1 µg reporter plasmid DNA in 100 µl medium. After 10-min incubation, the DNA-lipofectin mixture was brought up to 1 ml and delivered to the cells in one well. Cells transfected for 14–16 h were supplied with fresh medium containing 10% FCS and incubated for additional 48 h before harvesting. The harvested cells were washed in phosphate-buffered saline (PBS), pH 7.4, and lysed in 100 µl lysis buffer (1% Triton X-100, 25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM CDTA, and 10% glycerol). For determination of luciferase activity, 14 µl of lysate was mixed with 100 µl of luciferase assay reagent (20 mM Tricine, 1.00 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.70 mM $MgSO_4$, 0.1 mM EDTA, 1 mM dithiothreitol, 270 µM acetyl CoA, 470 µM D-luciferin, and 526 µM ATP). Luminescence was measured for 10 s in the luminometer Monolight® 2010 (Analytical Luminescence Laboratory, San Diego, CA).

## RESULTS

### Determination of the 5′ End Sequence of the Chicken VDR Transcript

The +D chicken kidney cDNA library was screened to produce many independent clones of VDR sequences (Fig. 1). While the origin of the extra variable 5′ ends remains uncertain, the consensus sequence clearly delineates the 5′ terminus of the cVDR cDNA. Moreover, primer extension assay was carried out to determine the +1 site for VDR gene transcription (Fig. 2). Three 35mer primers were used in the reverse transcription with 5 μg poly(A)$^+$ RNA from the chicken intestine. With all three primers, extension products were made in doublets with three nucleotides (nt) apart. The lengths of the primer extension products are 78 nt and 81 nt for Luc 67; 114 nt and 117 nt for CCV 1; and 94 nt and 97 nt for CCV 2. Since the distances between the downstream primers and the first base of the consensus sequence are 80, 115, and 95 bp, respectively, it is obvious that transcription of the chicken VDR gene starts from the very beginning of the consensus sequence (Fig. 1).

### Cloning the Chicken VDR Gene

With the confirmation of the 5′ end sequence of the cDNA, chicken VDR genomic clones were isolated. While clones 6 and 19 have downstream exons and introns, clone 8 of a 18.6-kb genomic fragment has two new cVDR exons, intron 1, and part of intron 2 (Fig. 3). The restriction map was constructed by extensive restriction digestion coupled with Southern blot analysis with specific oligo probes (Fig. 3A). Partial sequencing of this genomic clone established the exon-intron borders, which agree well with the GT. . .AG rule of the 5′ and 3′ splice sites. In the human VDR gene recently reported by Miyamoto et al. [1997], two additional 5′ exons were also found and named 1A and 1B, with 1C for the original exon 1, in line with the previous nomenclature of the human VDR gene [Hughes et al., 1988]. Most significantly, exons 1, 2, and 3 of the chicken VDR

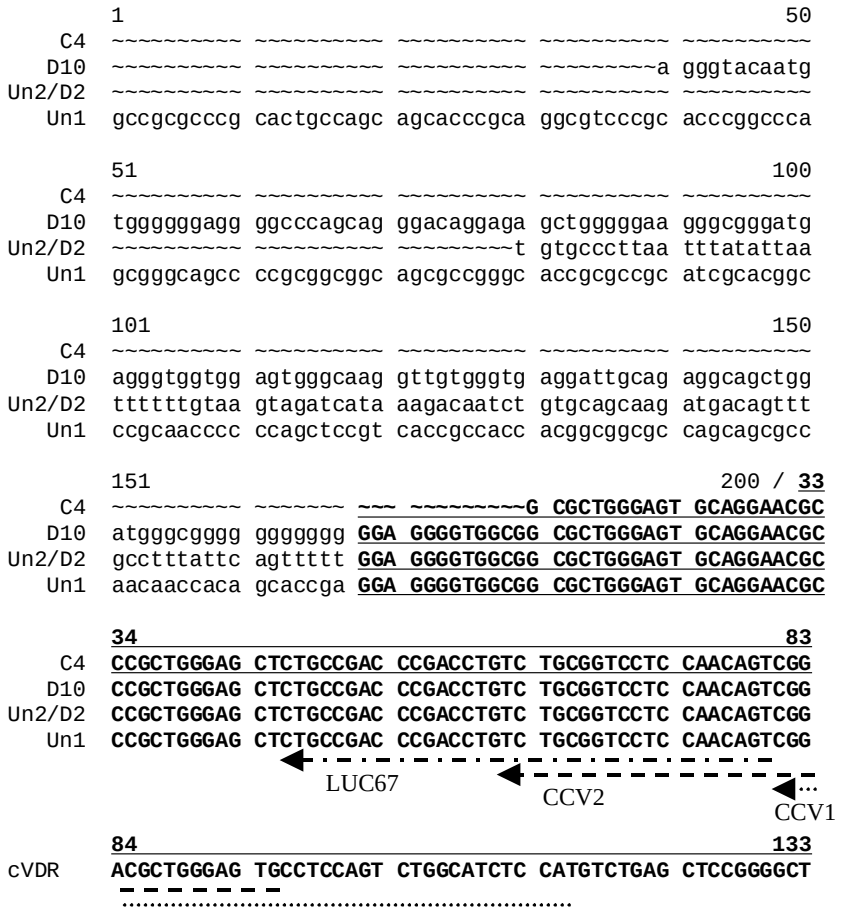


**Fig. 1.** 5′ end consensus sequence for the chicken VDR cDNA. Uppercase and underlined sequence is obtained by lining up four independent cDNA clones. While the consensus sequence represents the true cVDR sequence, the origin of variable 5′ ends remains uncertain. Arrows represent primers used in primer extension assays as described under Materials and Methods.

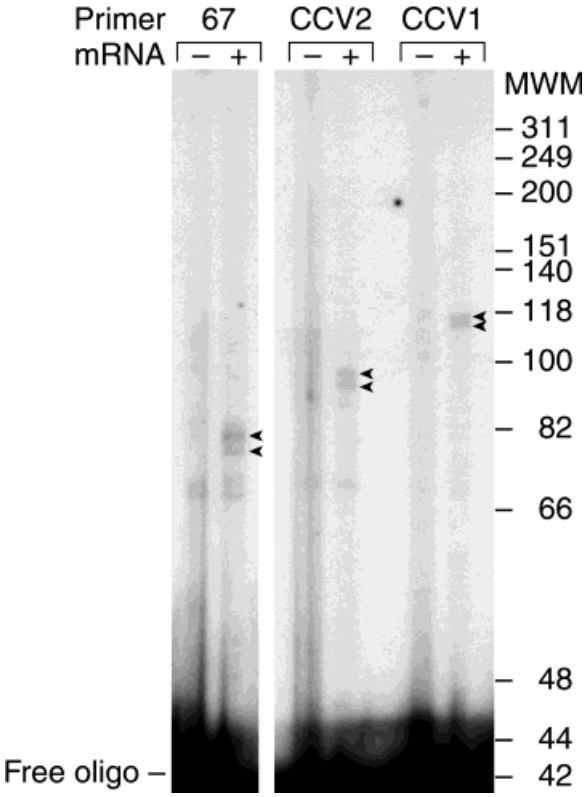


**Fig. 2.** Primer extension analysis of the chicken VDR transcripts. Poly(A)$^+$ RNA prepared from the chicken intestine was used in the extension experiments. Primer Luc 67, CCV 1, and CCV 2 were designed from the beginning of the consensus sequences by 80, 115, and 95 bp, respectively. Molecular-weight markers (MWM) indicate the sizes of the extension products.

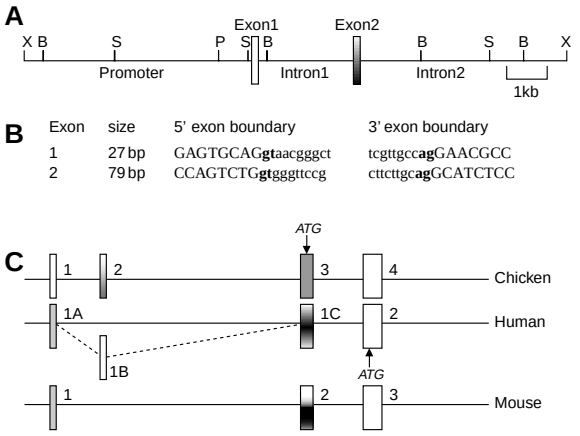


**Fig. 3.** Genomic organization of the chicken VDR gene. **A:** Restriction map of the 18.6-kb genomic fragment from Clone 8. The promoter region, exon 1, intron 1, exon 2, and part of intron 2 are illustrated. B, *Bam*HI; P, *Pst*I; S, *Sma*I; X, *Xho*I. **B:** The 5′ exon and intron borders are in agreement with the gt. . .ag general rule for the exon-intron boundary. **C:** Comparison of the 5′ exon organization of the VDR gene from chicken, human, and mouse. The first three exons are poorly conserved between species. Two extra in frame ATG sites are gained in exon 3 of the chicken VDR gene. The fourth exon with the conventional ATG site is well conserved across the species.

gene share low homology with exons 1A, 1B, 1C of the human counterpart. By contrast, exon 4, and thereafter exons for the coding region, are highly similar to exon 2 and following exons in human. While the translation start site ATG is located in exon 2 in the human VDR gene, this sequence is also conserved in exon 4 in chicken; exon 3 of the chicken VDR gene has two more upstream inframe ATG sites for initiating protein translation (Fig. 3C).

## Characterization of the Chicken VDR Gene Promoter

In front of exon 1 of the chicken VDR gene, the genomic clone 8 has a 7-kb 5′ flanking region, which is evidently the gene promoter, as proved by several observations. First, there are no more upstream exons, since the 5′ end sequence of the cDNA is exhausted. Second, the putative promoter of the chicken VDR gene shares considerable homology in several regions with the VDR promoters found in human and mouse (Fig. 4). Third and also the most critical proof is that the putative promoter is capable of driving reporter gene expression. About 1.2 kb of the chicken VDR promoter region was sequenced (Fig. 4). It shows that this promoter does not have a TATA box, or a CAAT box; instead, it has several GC boxes/SP1 sites. Interestingly, the GC boxes/SP1 sites are conserved in the VDR promoters across species. An AP2 site is also present in the chicken VDR promoter. So is a site for binding to Krox-20, which is a transcriptional factor involved in bone remodeling and brain development [Lemaire et al., 1990].

## Functional Analysis of the Chicken VDR Promoter

Transient transfections of COS-1 cells demonstrated that this 5′ flanking region can actively drive the transcriptional expression of luciferase reporter gene (Fig. 5). When the 6.5 kb inverted in front of the luciferase gene (−6.5 kb), the promoter activity is minimum as shown by the luciferase assay. The +6.5-kb construct with the right direction in relation to the reporter gene elicits more than threefold activity as that of the −6.5-kb construct. Deletion of upstream sequences in the 4.2-kb and 1-kb

**A**

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   1  CGAGGACCTG CAGGGCAGCC ACACCCCACC TGCAGTCCCT GACCCCCAAA CATGTGAGGG CATTGCACGG GCTCAGCTCA CGTGCAGGAT CTGGAGCCCC
 101  AGCCATGATG GGGGTGAGCG GAGAGTCCAA GCAGACTGGA GGCTGCAAAC AGCCCCGACT GCTGGGAGCC CACACTGATC CTTGTGCATG GAGTTGTGCT
 201  TGGGCACTTG CAGATGTTTC TGTCTCTGGT CTGGAGGAAG GGGATGAATC AGCTCTCCTT CTGCCCTTAA TCTGATCTAG GCATCAGAAA AATCCATGAA
 301  CACTCCAAGA GATGCAATCA GGGAGAGGAA CTGCTAGTCT GCAGCGGCAG CTCTTCTGGG TGTAGTCCYA AAAACGGGAG AGGATGGAGA CCAGCTCTGC
 401  TAGATGCTGC AGGCGTATAA ATGAAATGAA TTTCATTTGC CAGGTGATGA ATGCAGCTCT TGCTCCCAGT ACACAGCGGA TTGTTACTTA GAACTCCGGA
 501  AGAAAGCAGC CTCTGAGCCA CTGCCAGCTC TGTAGACAGG GCCATGTGAC ACCTGAGCTT CCAGAAGCCA CCAGAGCAAA GGTGAAACAC TGCGGGGACA
                    SacI
 601  CCTGGATGTC CTCAAGAGCT CTTATAACAG CGAGATGTTT GCCCAGGACT TCTGGGAGGG CACTGTTGTA CTGGTGGAGT TTGCTCAGTC CGGTAAGCAG
 701  CTAGGAGGGA AGGCCAGGAG GAAGCAAAAC TGCATGCCCC ACTGCACTTC CTAGAGTGTC TGTGTCCCCA AAAATGCCAG GCAGCAGCAC CCCAAAAGCT
 801  CAAAGCAGAA GCAGCAGCAG CTGCCCAGAA GCCCCTGGCA GAGCATAGGG CACCCCACAA GGCTCAGGCG GGGAGCAGCA CAGGCAGAAG CTGGGTCGAA
        Krox-20                          AP2 / SmaI                    SP1
 901  AGCGGGGGGG TGCCGCGCTG CCGCCTTCTG GGGTCCCCGG GCCGGCAGCC ACGTGCAATC TCCCGCCAGC CCACACCACG CCCATAGTGG GGGCCTTTAC
                                                                SP1              BanI
1001  TACAATTCTC ACCTTATATG GTAACGGTAG GCGGTGTCCG GGCGGGTTCA CCCCTTCGGG GCGGTGGCCG AGGAGGTGCC GCGTTGTCAT TGGTGGGCCG
                                                 SP1          SP1                GC Box/SP1
1101  GGGAGGGGGC GTGGCGGAGA GGGGGCGTGG CGAGGGAGGG GGCGGGATGC GGAGCGGCGG TTCCGTCAAA AGGCGGCTCC GGGGCGGAGG GGAGGGAGGG
        HaeII                                                                                          Exon 1
1201  GTGGCGGCGC TGGGAGTGCA Ggtaacgggc tgtggggggt ggggagggct ctgggctcag tgtgtggtgg gagacagctt cgtatcaatg tcagattgtg
                           Intron 1                                        BamHI
1301  ggggargagg tcgcagttct ggtggttgcg gggggcttct atggggactc ttcggtgtag aaggggatcc
```

**B**

```
Chicken    1065 TGGCCGAGGAGGTGCCGCGTTGTCATTGGTGGGCcGGGGAGGGGgCGTGG 1114
                |||| |||| |  ||| || | |  |||| |||| | |
Human      1405 TGGCAAGAGAGGACTGGACCTGTGGGCGGGGCGGTGGGGCGGGGCGGGGC 1454
                |||  ||||||||| || |||||||| ||  | ||||| ||||||  |
Mouse      1368 GGGCTTCAGAGGACTGAACTTGTGGGCGTGGTTGAGGGGCAGGGCGGTCC 1418

Chicken    1115 CGGAGAGGGGGCGTGGCGAGGGAGGGGGCGGGATGCGGAGCGGCGGTTCC 1164
                ||| | |||| |   |||| |  |||||  | ||| | || |    | |
Human      1455 CGGGGCGGGGCCTGACCGAGAGGCGGGGCCAGGTGCTGGGCTGTCT CTGC 1504
                |||||||||||  |   | |||||||||||||||||| || ||||||||
Mouse      1419 GGGGGCGGGGCCTTGC..GGGGGCGGGGCCAGGTGCTGAGCAGTCTCTGC 1466
                 Promoter sequence

Chicken    1165 ..GTCAAAAGGCGGCTCCGGGGCGGAGGGGAGGGAGGGGTGGCGGC GCT 1211
                  |||||||||||||  ||| || | | |  |   || | ||   | |||
Human      1505 TTGTCAAAAGGCGGCAGCGGAGCCGTGTGCCGCCGGGAGCGGGAACAGCT 1554
                |||||||||||| || || ||||| |  ||    |       || | |
Mouse      1467 TTGTCAAAAAGCTGCTGCCGAGCCCTAAGCACGGGAAGCGCCGAGCTGTG 1516
                                        Exon 1

Chicken    1212 GGGAGTGCAG..... 1221
                |     | |
Human      1555 TGTCCACCCGCCGGCCGGACCAG 1577
                ||||||||  | | || |    |
Mouse      1517 TGTCCACCGCCAGACCAGGTGCG 1539
```

**Fig. 4.** The promoter for the chicken VDR gene. **A:** Promoter sequences of the chicken VDR gene show several GC boxes/SP1 sites, a Krox-20 site, and an AP-2 site. Restriction sites used for deletional promoter constructs are also indicated. **B:** The chicken, human, and mouse VDR promoter sequences are compared in the proximal region immediately upstream of the first exon. The proximal region and several other upstream regions in the VDR promoters are highly conserved across species.

constructs resulted in even higher activity. To better define the chicken VDR promoter, we made a second series of constructs without any intron 1 sequence (Fig. 5A). Deletion of the intron 1 and part of the exon 1 sequence from the previous 1-kb construct generated the new 800 bp construct with a 3–4-fold increase of the luciferase activity. From the pattern of the luciferase activity elicited by constructs 2.3 kb, 1.1 kb, 800 bp, 590 bp, 270 bp, and 134 bp, it is

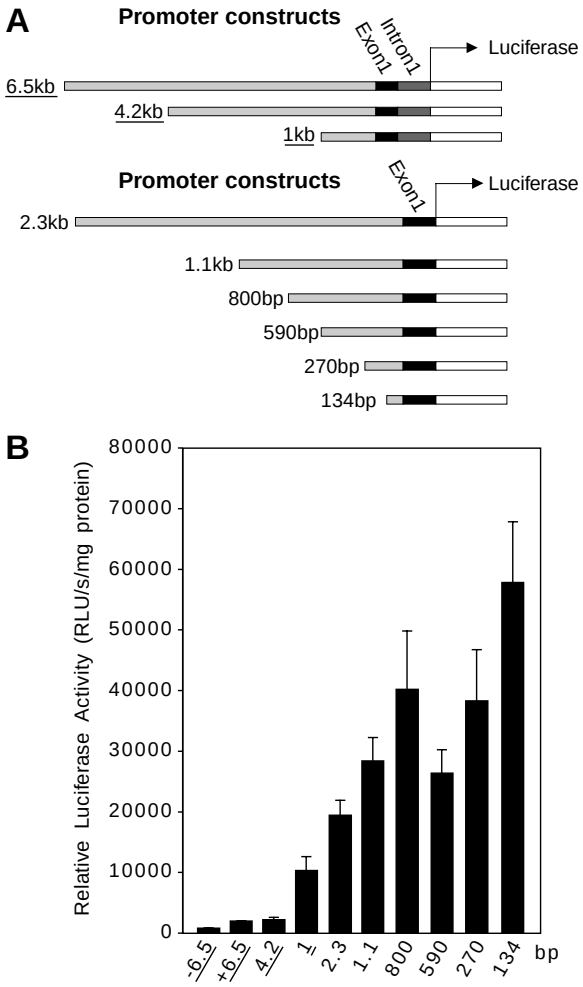


**Fig. 5.** Functional analysis of the chicken VDR promoter. **A:** Underlined promoter constructs (−/+ 6.5 kb, 4.2 kb, and 1 kb) include some intron 1 sequence and the entire exon 1. The second series of promoter constructs are defined by the *Hae*II site inside exon 1 and upstream promoter sequences. **B:** Transfection assays in COS-1 cells. 1 μg construct plasmid DNA was introduced into the cells by lipofectin and luciferase activity was assayed after 48 h post-transfection. All values were calculated from triplicate experimental data.

possible that there exist some suppressive regions from −2.3 kb to −800 bp and from −590 bp to −134 bp (Fig. 5B). Likewise, some activating element may lie between −800 bp to −590 bp. Notably, the 134-bp construct showed the highest activity. Therefore, the proximal GC boxes/SP1 sites are the major elements to drive gene expression in the chicken VDR promoter while the two distal SP1 sites contribute to the promoter activity slightly.

## DISCUSSION

In the current research, the 5′ end cDNA sequence of chicken VDR has been elucidated. Although the unknown sequences at the 5′ end of the cDNA prevented our cloning the VDR promoter, the consensus sequence derived from several independent cDNA clones has enabled us to define the genomic organization at the 5′ end and successfully isolate the promoter for the chicken VDR gene. An extra unknown sequence at the 5′ end of the chicken VDR cDNA was also observed in a clone from a λ ZAP chicken kidney library. In search of mouse VDR isoforms, variable 5′ cDNA ends were reported [Ebihara et al., 1996]. The source of these extra unknown sequences at the 5′ end of a cDNA needs to be addressed. Using the Blast search program for nucleotide sequence, the 128-bp sequence from the cDNA clone 10 exactly matched a part of the cDNA sequence of chicken major histocompatibility complex (MHC) class I B-FIV-B12 α-chain gene from 4018 to 3960 in the antisense direction [Altschul et al., 1997]. Consequently, the VDR cDNA with an unknown 5′ end may be an artifact by combining the VDR cDNA fragment with an unknown cDNA fragment in the sense or antisense direction. The combination process leading to the artifact likely occurred at the step of ligating cDNA fragments with the short adapter sequences during the construction of the cDNA library.

From the chicken VDR gene, we retrieved three small 5′ exons interspersed within exceedingly large introns. While the mammalian ATG site for protein translation is located in the fourth exon (exon 2 in human), the avian species has two more upstream ATG sites in the third exon (exon 3 in chicken versus exon 1C in human). Two forms of chicken VDR protein result from the two ATG sites in exon 3 due to leaky translation (Lu et al., 1997). Although the mammalian VDR has a much longer transcript than that of avian (4.4 kb vs 2.7 kb), the avian VDR has a coding sequence with a long N-terminus [Baker et al., 1988; Burmester et al., 1988b]. Interestingly, the VDR has a very short A/B domain 5′ to the DNA binding domain as compared with A/B domains of 100–300 amino acids in other nuclear hormone receptors such as RAR, TR, and GR [Mangelsdorf et al., 1995]. It is known that A/B domain is responsible for the activation function (AF-1) in the absence of ligand [Lees et al., 1989]. While the mammalian VDR has a short A/B domain of about 20 amino acids, the avian VDR has a larger A/B

domain: 42 amino acids for Form A protein and 28 amino acids for Form B. Recently, human VDR gene polymorphism at the two adjacent consecutive ATG sites has been reported to be related to bone mineral density Arai et al., 1997; Gross et al., 1996]. T to C change in the first ATG site makes the second ATG site located two codons downstream accessible. Individuals using the second ATG site (mm allele) produce a two-amino acid-short VDR protein with a better transactivation function and also have a higher bone mineral density than those using the first ATG site (MM allele).

The physiological features of avian species such as aviation skeleton, feathers, and eggshell may be related to the distinct avian VDR gene. Certainly it is of interest to investigate the functional difference of two forms of chicken VDR. In the evolution of avian and mammalian species adapting to distinct ecological niches, the first three 5′ exons, especially the third exon of the VDR gene, have undergone substantial changes as shown by low homology between species. Nevertheless, the following exons starting from the fourth are highly conserved between these two classes of animals. The highly conserved DNA binding domain and ligand binding domain of avian and mammalian VDRs reflect the constraint and essence of the vitamin D endocrine system [DeLuca, 1988, 1992].

The promoter for the chicken VDR gene does not bear a TATA box but possesses several GC boxes/SP1 sites, which are the typical features of the steroid receptor promoters [Encio and Detera-Wadleigh, 1991; Huckaby et al., 1987]. Two distinct promoters are present in the human progesterone receptor gene to produce two classes of hPR mRNA [Kastner et al., 1990]. Differential splicing of some exon(s) in the single VDR pre-mRNA can certainly lead to multiple forms of mature VDR mRNA. It is interesting to note two VDR transcripts (2.2 kb and 1.8 kb) in the amphibian species, *Xenopus laevis* [Li et al., 1997a]. At the translational level, alternative ATG sites can be used to produce two forms of VDR proteins in the chicken [Lu et al., 1997]. Despite of these minor modifications at the RNA processing and translational levels, distinct major subtypes present in other nuclear hormone receptors, such as RAR α, β, γ, RXR α, β, γ, ER α, β, TR α, and β are not applicable to the VDR gene [Paech et al., 1997; Pemrick et al., 1994; Sjoberg and Vennstrom, 1995].

Transfection assays demonstrated that the promoter for the chicken VDR gene is able to drive expression of the luciferase reporter gene. Further deletional analysis indicated that the proximal GC boxes/SP1 sites in the promoter are the strong elements to drive gene expression, while the more upstream promoter sequence negatively modulates the expression of the gene. Isolation of the VDR promoter from three species will enable direct studies on the transcriptional regulation of the VDR gene. Many hormones, such as glucocorticoid, estrogen, PTH, and GH, and other factors may regulate VDR gene at the transcriptional level [Brown et al., 1995; Chen et al., 1997; Pierce and DeLuca, 1988; Reinhardt and Horst, 1990], yet not a single agent has been found to modulate the gene promoter definitely in an on/off manner. Perhaps the most important regulation of the VDR gene is reflected by its developmental and tissue-specific expression [Halloran and DeLuca, 1981; Lee et al., 1991]. In the intestine of infant rats, VDR can only be detected around weaning [Huang et al., 1989]. It will be of great interest to identify the DNA elements responsible for this temporal and spatial expression in the VDR promoter.

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