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Identification of a vitamin D-response element in the rat calcidiol (25-hydroxyvitamin D_3) 24-hydroxylase gene

(calcium/bone/hydroxylase/steroid receptor)

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ABSTRACT **The calcidiol (25-hydroxyvitamin D_3) 24-hydroxylase is one of the key enzymes in the metabolism of vitamin D. This enzyme acts on both calcidiol and calcitriol (1,25-dihydroxyvitamin D_3) to initiate degradation of these potent vitamin D metabolites and is tightly regulated. Calcitriol itself induces this enzyme and acts at the transcriptional level. Transcriptional regulation of genes by calcitriol has been shown to occur via the vitamin D-receptor binding to a vitamin D-response element located upstream of the transcription start site. We now report a vitamin D-response element located between nt −262 and nt −238 of the rat calcidiol 24-hydroxylase gene. This sequence binds the calcitriol receptor and confers vitamin D-dependent transactivation of transcription to its own, as well as heterologous, promoter.**

Vitamin D_3 (calciol or cholecalciferol) is important for the maintenance of calcium homeostasis in higher animals. It is not active in its native form and must be hydroxylated first on C25 and subsequently on C1 in the liver and kidney, respectively, to form the hormone calcitriol, also known as 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (1). Hydroxylation on C24 occurs in the kidney to form two much less active metabolites [calcitriol or 24,25-$(OH)_2D_3$ and calcitetrol or 1,24,25-$(OH)_3D_3$]. This 24-hydroxylation is thought to be the first step in the catabolic pathway (2, 3). *In vivo* studies have shown that the 24-hydroxylase is induced by 1,25-$(OH)_2D_3$ (4), essentially inducing its own inactivation. An understanding of this regulation at the molecular level is crucial to providing insight into the regulation of vitamin D metabolism. The cDNA and gene for this enzyme were reported for the rat (5, 6) and the human (7).

1,25-$(OH)_2D_3$ regulates transcription of target genes by means of the vitamin D-receptor (VDR) binding to specific vitamin D-response elements (DREs). DREs for the human osteocalcin (8), rat osteocalcin (9), mouse osteopontin (10), rat calcium-binding protein (11), human parathyroid hormone (12), and mouse calbindin-D_{28k} (13) genes have previously been identified and characterized. In this paper we report the cloning of the promoter and the identification of a DRE in the rat 24-hydroxylase gene.†

EXPERIMENTAL PROCEDURES

Cloning and DNA Sequencing. A 9.5-kbp *Eco*RI fragment containing the 24-hydroxylase 5′-upstream region was obtained by screening a rat λ Charon 4A genomic library (Clontech) and a synthetic oligonucleotide coinciding with the first 150 bp of the reported 24-hydroxylase cDNA (14). This fragment was then subcloned into pUC18 by using the *Eco*RI sites. Sequencing analysis was done with sequential primers starting from the first exon going into the promoter region by the dideoxynucleotide chain-termination method (15). A 1.5-kbp *Stu* I fragment excised from +76 to −1399 was cloned into the *Bam*HI site (after filling in with Klenow fragment) of the promoterless pBLCAT3 (16). Furthermore, a double-stranded synthetic oligonucleotide (25-mer) corresponding to nt −238 to −262 made with *Xba* I ends was subcloned into the thymidine kinase promoter containing pBLCAT2 vector (16). All plasmids were purified by using a CsCl gradient and standard protocols (17).

Gel-Retardation Assays. All promoter DNA fragments used as probes (see *Results*) in the binding assay with the VDR were labeled by filling in recessed ends using [^{32}P]dCTP or [^{32}P]dTTP (DuPont/NEN) and the Klenow fragment of DNA polymerase (Promega). Probe purification and the binding assays were run as explained (11). A VDR-specific monoclonal antibody, XVIE10 (18), was used in the mixture for the supershift test.

Cell Transfection and Chloramphenicol Acetyltransferase (CAT) Assay. NRK rat kidney cells (American Type Culture Collection) were cultured to ≈50% confluency 24 hr before transfection. The cells were transfected with 2–10 μg of test plasmid by using the standard lipofectin method (GIBCO/BRL). The DNA/lipofectin solution was left on the cells for 24 hr after which the medium was changed to DMEM (GIBCO/BRL)/10% fetal calf serum (HyClone), and the cells were dosed with 40 nM of 1,25-$(OH)_2D_3$ or vehicle and incubated for an additional 24–36 hr at 37°C. The cells were harvested by scraping them into 0.25 M Tris·HCl, pH 8, and lysed by three cycles of freezing and thawing; the CAT enzyme was assayed as described (19) by using 100 mg of lysate protein, as measured by the Bradford protein assay (20). The acetylated chloramphenicol was quantitated by using the Betascope model 603 blot analyzer (Betagen, Waltham, MA).

RESULTS

Fig. 1 shows the sequence of the 5′ upstream region of the 24-hydroxylase gene from −1749 to +76. Bases +76 to −516 coincide with the sequence published by Ohyama *et al.* (6). The position of the identified DRE is marked by asterisks and lies between nt−262 and nt−238 from the transcription start site, and sequences of possible cAMP-response elements lie between nt−368 and nt−126. The position of the DRE is closer to the transcription start site than previously identified positive DREs (8–11, 13).

Because all known DREs lie within the first 500 bases upstream of the transcription start site, the search for the DRE in the rat 24-hydroxylase gene was performed by a

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Abbreviations: 1,25-$(OH)_2D_3$, calcitriol (1α,25-dihydroxyvitamin D_3); VDR, vitamin D receptor; DRE, vitamin D response element; CAT, chloramphenicol acetyltransferase.

*No reprints will be available from the authors.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U03112).

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( -1749) GGGGTCTTTCACATGTTTTTCACGTTGAGCTGAGGTGAAATTTGCCCCCCCCCAACCCCACGCA
( -1685) TATACAACAGAGAGCTGCTTTGTCTGGCTTCAGTGGGAGAGGATGTGCCTGATCCTACAGAGAC
( -1621) TTAATACACCAGGGTGAGGGGATATGGCGGGGGGGGGGGGCACCCTCTCAGAAGGGAAGGGGAA
( -1557) ATGGTAGGAGGAACCTGTGAGGGCACACGGGGAGGCGGCAGCACCGGGATGTAAACACTAAAAT
( -1493) AAATTATTAATAAATAAGAAGCATTGGCGTGAAATTCCTGGTTTATCACAGGCTCTCAGAAGCA
( -1429) TAGCATGTGGTACCCTCATGCAGTTATAAggccTACACTGTATTACATAGAGAATAAAATGTAT
                                        StuI
( -1365) GCTCCACAGACACACAAAAGACTGTGTAAGCTTACAGTGTGTGTGCCGTACACActgcagTGCA
( -1301) TATTGTAAAAGATCTGATGGATTTCAGCTAAATATTATTGCTTATGTGCAGCTCTTGTATTTTT
( -1237) TGTTTTTTGTTTTTTTTTAATTAGCGCCGACCTCTTAGGGGATATTTGAAGTGACAGCGTTGGAG
( -1173) CACAATCACAAATCGGATTGCAGAAGTTCTCCAGAAGCTGACAGATGTTTACGGAAGACTTTGC
( -1109) TATTGCTTGGGTCACGGTAACCTCAGGTAAGGTGACTGGAAAGCAGGGGAGGAAGACTCTGCCT
( -1045) CCTGCCAGGGTGCTGGTGGCTTTGCATTGGAAAAGCGGGAAACAATATGAATCACTTTGAGTCA
( -0981) GCCGAGACCAATTTCCTTTCCAGAATTGGAAAGCGAGCTCTGTTCTATCCGGCCGCAAAAGCAG
( -0917) GAGTGCGACTGTGATAACCCTGCCTGCTTTAGGTGGGCTTTAGGGAGAAAGTGGGGCTCTTGGG
( -0853) AACCTGTGGGTGCCTCTGCTCTGTGCAGGGTGGCGGTGCGCAAGAAGGAAAGGCCCGAGGGACC
( -0789) TGTGGGAAACAGTCTAAAGGAAACTGAGCTAGTCCCTGTAGGCATTGCACAGTCTCCGGAAGAA
( -0725) CTAAGGCCACTAGTATCCTTTATTGAGGACACACACCTGTGTAGACCTTACATGCGTTCATTTA
( -0661) TTCGATTCCCTAACAAGTCAACCCGAAGCATCGAGGAATCTGGTAAGGAAATTCTGCAAACGCA
( -0597) TTGGGCTTTCTTGCCTAATTTAAGACCCAAGGTAAGAAGATTATTTCCTCTCCCCCTCCCCTCT
( -0533) TTTTGGTATAGCCTAGTAAGTTTCAAGTCCTCTCTTCCTTCAGAAGCTCAAAAAGGCAACTTTC
( -0469) ATCCAAGGGAAGTCTGGCTCAGGCTGCGAAAACCTGTCTCCCAGGATATTGGAAGGCGGACACT
( -0405) CTCCACTTGCTTGGCAAGCGCGGGCGGCCTTCCGCGCtgtcctcaGGGACCTTGCCCGCCCTGC
( -0341) ATGGCGATTGTGCAAGCGCACGTTTGGGCTCCTGCAAGGCCAGCTGCAGCCTGCAGGAGGAGAG
( -0277) CGAGGAGGCGTGCTCGAGCGCACCCGCTGAACCCTGGGCTCGACCCGCCTTTCTCAGGTTATCT
                  ******  ******
( -0213) CCGGGGTGGAGTCCACCGGTGCGTCTGCCGGGCCAGCAGCGTGTCGGTCACCGAGGCCCCGGCG
( -0149) CCCTCACTCACCTCGCtgactccaTCCTCTTCCCACACCCGCCCCCCGCGTCCCTCCCAGCCGG
( -0085) TCCCCCTCCGCCCTGCTCAGCGTGCTCattggCCACTCCAGCATGCCCTGCTTCCataaataCG
( -0021) AGGTCCCTATGCCTGGAATCT>TGGGGAGGTACAACGAGGTACATCAAACCTGGCGGCATCCCT
( +0043) TCGACCCTCCTTGATTCCCCCGTGGCTTTAggcc
                                    StuI
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FIG. 1. Nucleotide sequence of the rat 24-hydroxylase promoter and 5′-flanking region. Transcription start site is indicated by an arrowhead, and TATA and CCAAT boxes are underlined and in lowercase letters; G+C-rich regions are underlined. The DRE is marked by asterisks and is located on the antiparallel strand; putative cAMP response elements are in lowercase letters and italicized. *Stu* I sites used for subcloning into pBLCAT3 are indicated as well.

series of gel-retardation assays on successively smaller pieces of DNA in that region. First, a fragment amplified by PCR extending from −590 to +60 was cut into three smaller pieces by digestion with *Dde* I and then tested for binding to the VDR. As shown in Fig. 2*A*, a fragment extending from −363 to −225 formed a complex in the presence of porcine nuclear extract and a larger complex with an antibody specific to VDR. Next, a double-stranded synthetic oligonucleotide was made from bases −308 to −243 because closer examination of this region indicated two putative DREs, the first between nt−284 and nt−270 (AGGAGA GCG AGGAGG), which resembles the sequence of the mouse calbindin-D_{28k} (13), and the second between nt−245 and nt−259 (GGTTCA GCG GGTGCG), which resides on the antiparallel strand and is similar to that in the mouse osteopontin gene (10). This 66-mer (−308 to −243) contained the sequence binding to VDR because it shifted with receptor and supershifted with antibody (data not shown). To identify the exact location and sequence of the DRE in this region, gel-retardation assays were done separately on the two above-mentioned sequences. Fig. 2*B* shows that the sequence between −262 to −238 is the one that specifically binds VDR, whereas the fragment between −308 to −263 did not bind the VDR. The observed complexes with the latter DNA fragment probably represent other proteins in porcine nuclear extract that bind to this region of the promoter. Fig. 3 shows that the receptor–DNA complexes could be inhibited by the specific unlabeled but not by nonspecific DNA of the same size. As a final test of specificity, porcine nuclear extract stripped of VDR failed to generate the observed complex, except for faint bands that are due to trace amounts of VDR in the stripped preparation.

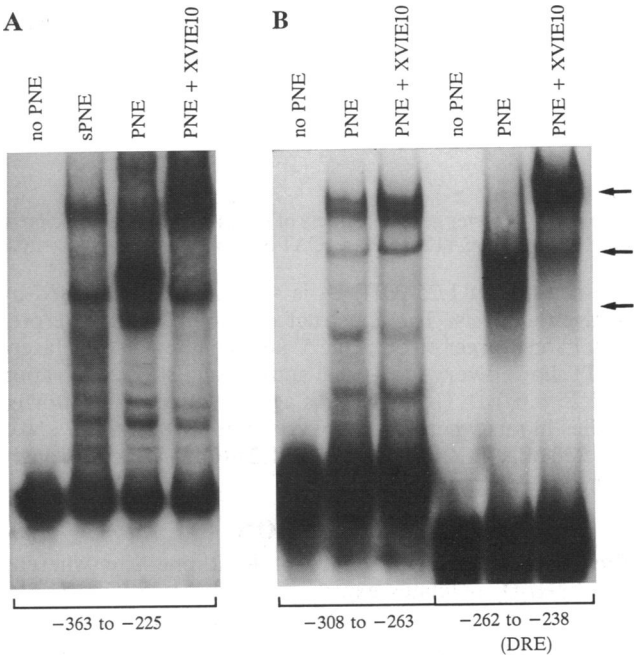

FIG. 2. Gel-retardation assays on fragments of the 5′-upstream region of the 24-hydroxylase gene. (*A*) Binding of a 139-bp fragment extending from −363 to −225 containing a DRE with VDR. (*B*) Binding of two subfragments of the same region, one containing a DRE (−262 to −238) and one that does not (−308 to −263). The arrows indicate VDR-DNA and antibody–VDR-DNA complexes. PNE, porcine nuclear extract (source of VDR); sPNE, PNE stripped of receptor; XVIE10, monoclonal antibody to receptor.

It is interesting to note two complexes are formed with receptor and the putative response-element DNA. Both are diminished by specific unlabeled DNA. Similarly, both complexes are supershifted with specific antireceptor antibody (Fig. 2). These results indicate that two distinct complexes are formed with the same receptor and DNA. The reason for the two complexes is at present not known.

To study the potential transcriptional activity of this sequence in response to 1,25-$(OH)_2D_3$, reporter gene constructs were made as shown in Fig. 4. Insertion of the identified sequence into the pBLCAT2 reporter vector conferred 1,25-$(OH)_2D_3$ responsiveness, as shown by the construct pOHase-CAT when transfected into NRK cells and treated with 1,25-$(OH)_2D_3$ or vehicle (Fig. 5). The increase in transacety-

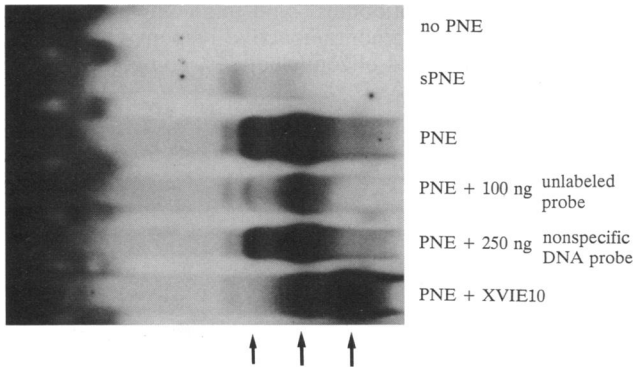

FIG. 3. Competition gel-retardation assay with the DRE-containing sequence (−262 to −238). A 50-fold excess of unlabeled probe or >100-fold excess of nonspecific DNA of the same size were added as indicated. The effects of PNE stripped of VDR are also shown. The arrows indicate VDR-DNA and antibody–VDR-DNA complexes. PNE, porcine nuclear extract; sPNE, PNE stripped of receptor; XVIE10, monoclonal antibody to the porcine receptor.

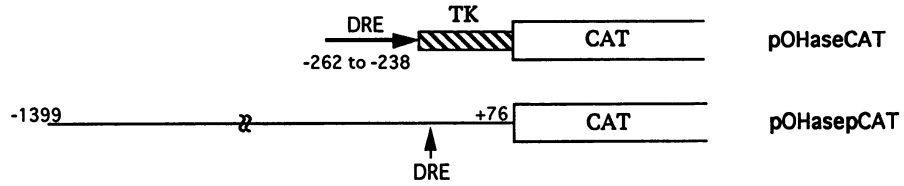

FIG. 4. Reporter gene constructs of the 24-hydroxylase gene promoter. pOHaseCAT contains the DRE sequence with *Xba* I ends cloned at the *Xba* I site of pBLCAT2. pOHasepCAT is a construct with the 24-hydroxylase promoter and 5′-flanking region up to nt-1399. TK, thymidine kinase.

lase activity by 1,25-$(OH)_2D_3$ is ≈4-fold, as determined by Betagen analysis. The construct containing the native promoter and a larger piece of the 5′ upstream region (pOHasepCAT) showed very little basal activity and strong activation by 1,25-$(OH)_2D_3$ (10-fold). This agrees with previous *in vivo* studies in rats where 24-hydroxylase mRNA in kidneys was undetectable in the absence of 1,25-$(OH)_2D$ and strongly induced in its presence (4, 21).

DISCUSSION

The present findings identify at least one site whereby 1,25-$(OH)_2D_3$ regulates expression of the 1,25-$(OH)_2D_3$ 24-hydroxylase gene. This gene expresses an enzyme that begins the degradation of the potent calcemic hormone, 1,25-$(OH)_2D_3$, removing it and its precursor 25-hydroxyvitamin D_3 from any further activity. This is an important physiologic event that allows meaningful regulation of circulating 1,25-$(OH)_2D_3$. Thus, 1,25-$(OH)_2D_3$ programs its own destruction by inducing the 24-hydroxylase.

The important achievement of cloning the rat renal 25-hydroxyvitamin D_3 24-hydroxylase by Ohyama and Okuda (5) has made possible the cloning of the human 24-hydroxylase cDNA (7) and the isolation of the 24-hydroxylase gene including the promoter (ref. 6 and the present report). It is clear that 1,25-$(OH)_2D_3$ activates genes through its receptor interacting with the DREs located in the promoter, causing transcription of the subsequent gene (8–13, 22). This report shows that the rat 24-hydroxylase gene joins this group and provides a specific responsive element at −245 to −259 bases on the antiparallel strand. This response element is completely silent in its natural promoter in the absence of 1,25-$(OH)_2D_3$ and is strongly activated by added 1,25-$(OH)_2D_3$.

The idea that this is a DRE is strongly supported by the fact that it confers strong 1,25-$(OH)_2D_3$ responsiveness to a heterologous promoter in transfection assays using a CAT reporter gene system. The specific binding of this responsive element to the VDR provides additional support. Of great importance is the fact that specific antireceptor antibodies (and not irrelevant antibodies) bind this complex, giving a "supershift." Further, nonradioactive responsive element, but not other unrelated oligonucleotides, compete with the radiolabeled responsive element for the complex. The possible presence of DREs at other sites in the 24-hydroxylase promoter was not supported by this study, although these cannot be completely excluded as yet.

It is known that parathyroid hormone cAMP can downregulate the 24-hydroxylase gene, as revealed by both hydroxylase and mRNA analysis (1, 4). Analysis of the promoter region has revealed two putative cAMP-responsive sites (Fig. 1). Whether they represent true response elements or not remains to be determined.

The isolation of the promoter region of the rat, human, and the chicken 24-hydroxylase genes, a determination of their respective vitamin D- and cAMP-responsive elements should make possible a thorough examination of how the degradation of 1,25-$(OH)_2D_3$ is regulated at the molecular level.

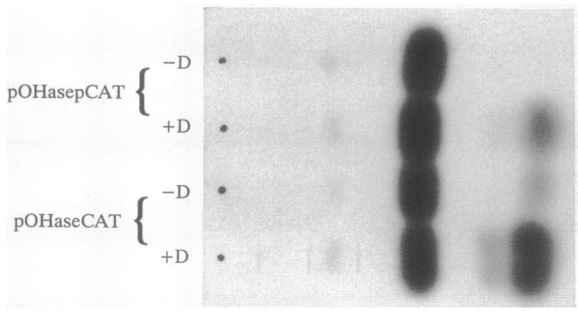

FIG. 5. CAT assay on lysates obtained from NRK cells transfected with the respective constructs. Transfected cells were dosed with either 40 nM of 1,25-$(OH)_2D_3$ (+D) or with vehicle only (ethanol) (−D).

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