

Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D₃-1 α -hydroxylase gene

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Communicated by Vernon R. Young, Massachusetts Institute of Technology, Cambridge, MA, April 6, 1999 (received for review December 23, 1998)

ABSTRACT The human 25-hydroxyvitamin D₃-1 α -hydroxylase (1 α -OHase) gene has been cloned. It contained nine exons and eight introns spanning \approx 6.5 kb and a 1.4-kb 5'-flanking region. The 5'-flanking region contains consensus or highly conserved sequences for TATA, Pu, and CCAAT boxes, four cAMP response elements, two activator protein-1 (AP-1) response elements, two AP-2 response elements, three specific protein-1 (Sp1) response elements, and four NF- κ B binding sites, but no vitamin D response element. By using luciferase reporter gene constructs of truncated forms of the 1 α -OHase promoter transfected into a modified pig kidney cell line, AOK-B50, we identified regulatory regions of the 1.4-kb 1 α -OHase promoter for parathyroid hormone 1–34 [PTH(1–34)], forskolin, and 1,25-hydroxyvitamin D₃ [1,25(OH)₂D₃]. The 1.4-kb 1 α -OHase promoter (AN1) modestly (1.7-fold) induced luciferase activity, whereas 1,100- (AN2), 827- (AN3), 672- (AN4), 463- (AN5), and 363-bp (AN6)-truncated promoters greatly stimulated luciferase activity by 494-fold, 18.4-fold, 55.3-fold, 643-fold, and 56.4-fold, respectively. PTH(1–34) and forskolin stimulated the activity of all constructs to varying degrees with significantly greater responsiveness for both compounds on AN2 and AN5. 1,25(OH)₂D₃ suppressed PTH(1–34)-induced activity on AN2 and AN5 constructs by 58% and 52%, respectively, but had no effect on the other constructs. These studies characterize the regulatory regions of the human 1 α -OHase gene and provide insight into the physiologic basis for regulation of the expression of this gene by PTH and 1,25(OH)₂D₃.

Vitamin D is metabolized by sequential hydroxylations in the liver to 25-hydroxyvitamin D₃ [25(OH)D₃] and in the kidney to form 1,25-hydroxyvitamin D₃ [1, 25(OH)₂D₃] (1). 1,25(OH)₂D₃ is the biologically active form and plays an essential role in the maintenance of calcium homeostasis (1–4). 1,25(OH)₂D₃ is synthesized in the proximal renal tubule by the 25-hydroxyvitamin D-1 α -hydroxylase (1 α -OHase) (5). This enzyme has also been found in nonrenal tissues, such as macrophages and keratinocytes (6–8). The renal synthesis of 1,25(OH)₂D₃ is tightly regulated by circulating parathyroid hormone (PTH), insulin-like growth factor I, calcium, phosphorus, and 1,25(OH)₂D₃ (1, 2, 9–13). The nonrenal synthesis of 1,25(OH)₂D₃ is not tightly regulated (6, 8). The expression of the renal 1 α -OHase has been shown to be inhibited by its end product, 1,25(OH)₂D₃ (1, 2). Mice lacking the 1,25-hydroxyvitamin D₃ receptor (VDR) developed an abnormally high serum concentration of 1,25(OH)₂D₃ (14), suggesting that the expression of 1 α -OHase was regulated by the 1,25(OH)₂D₃ through liganded VDR. The cDNA encoding the mouse 1 α -OHase was cloned by Takeyama *et al.* (14) in 1997, followed by the cloning of rat and human cDNAs (15–18). The human renal and nonrenal 1 α -OHase cDNA sequences have been

cloned and are 100% identical (18). The complete mouse gene and a portion of the 5'-flanking region of the human 1 α -OHase gene have been cloned (19–22).

PTH appears to be a critical up-regulator of 1 α -OHase activity *in vivo*. Serum calcium levels are sensed in the parathyroid glands through the calcium receptor (23, 24). When calcium levels drop, PTH is secreted, which targets the PTH/PTH-related peptide (PTHrP) receptor in bone to stimulate resorption and in the proximal tubule to stimulate 1 α -OHase activity (19, 25, 26). PTH/PTHrP receptor agonists stimulate intracellular cAMP levels activating the cAMP-dependent protein kinase A, which phosphorylates cAMP-responsive element (CRE)-binding protein that subsequently modulates transcription through CRE in the promoters of target genes (19, 27, 28). Additional pathways of cAMP-dependent modulation of transcription may include activator protein (AP)-1, AP-2, and specific protein-1 (Sp1) sites in target genes (29). The capacity of a PTH/PTHrP receptor agonist to induce the expression of a reporter construct driven by a portion of the mouse 1 α -hydroxylase promoter has been demonstrated in the modified porcine kidney cell line, AOK-B50 (19).

To better understand the role of PTH and other factors in the regulation of the metabolism of 25(OH)D₃ to 1,25(OH)₂D₃, we cloned the human 1 α -OHase gene, including the promoter region. A series of truncated luciferase reporter gene constructs was transfected into AOK-B50 cells and evaluated for their transcription-promoting activity in the presence of PTH and other factors known to modulate serum 1,25(OH)₂D₃ levels *in vivo*.

MATERIALS AND METHODS

Genomic Library Screening and Sequence Analysis. A human genomic DNA library constructed by introduction of *Sau*3AI partially digested genomic DNA from normal placenta lymphocytes into the site of the phage vector EMBL3 was purchased from CLONTECH. The library was screened by plaque hybridization (1 \times 10⁶ plaques) using the 5'-most fragment (700 bp) of the human 1 α -OHase cDNA as a probe, which was labeled with [α -³²P]dCTP (NEN) by random priming (Prime-A-Gene, Promega). Nylon membrane replicas of \approx 1 \times 10⁶ plaques were prehybridized at 42°C for 1 hr with 50% formamide/5 \times Denhardt's solution/0.75 M NaCl/0.05 M NaH₂PO₄, pH 7.4/3 mM EDTA/0.1% SDS/0.1 mg/ml salmon sperm. Hybridization was carried out at 42°C overnight under the same conditions. Membranes were washed for 30 min with 1 \times SSC/0.1% SDS at 42°C for 30 min at room temperature

Abbreviations: 1 α -OHase, 25-hydroxyvitamin D₃-1 α -hydroxylase; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; PTH, parathyroid hormone; CRE, cAMP response element; AOK-B50, modified pig kidney cell line; AP, activator protein; Sp1, specific protein-1; VDR, vitamin D receptor; PTHrP, PTH-related peptide.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF072470).

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and exposed to x-ray film at -70°C overnight. Positive plaques were isolated and rescreened. Purified phage DNA digested with *SacI* generated four fragments of 5.5 kb, 3.5 kb, 3.0 kb, and 1.0 kb. These four fragments were separately subcloned into the pGEM 7 (Promega) vector to perform the sequence analysis in a Prism 373 DNA sequencer (Applied Biosystems) with AmpliTaq DNA polymerase FS (Perkin-Elmer). Sequence analysis using T7 and SP6 promoter primers mapped the fragment order and showed that the 5' end of the sequence is 1,369 kb from ATG.

Computer-Based Sequence Analysis. The promoter sequence was analyzed by the Transcription Element Search System (TESS of the Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, <http://agave.humgen.upenn.edu/tess/index.html>) by using a 5-bp minimum element size limit, a 10% mismatch allowance, a minimum log-likelihood of homology of 10, and a secondary log-likelihood density threshold of 1.6.

Construction of Reporter Plasmids. PCR was used to generate several truncated forms of the 1α -OHase promoter: the primers used and product sizes are summarized in Table 1. By using genomic clone DNA as a template, PCR was performed at 94°C for 3 min of denaturing, annealing for 1 min at 60°C , and extension for 10 min at 72°C by using *Taq* polymerase (GIBCO/BRL). PCR products of 5' flanking fragments of 1α -OHase gene were inserted into the *KpnI* site of the luciferase basic plasmid vector, pGL2 (Promega). The subcloned PCR products were sequenced by using T7 and SP6 promoter primers to confirm that the products were the authentic promoter fragments.

Cell Culture and Treatment. AOK-B50 cells were maintained in DMEM with 7% FCS. Cells in 35 mm² dishes at 50–60% confluence were transfected with 2 μg of constructed reporter gene plasmid DNA or with pGL2 plasmid DNA as a negative control for 3 hr in serum-free DMEM by using 4 μl of Lipofectamine (Life Technologies). Media was replaced with DMEM with 7% FCS, and cells were grown for 24 hr. Cells were then treated with PTH agonists, forskolin, 1,25(OH)₂D₃, or appropriate vehicle controls as indicated for an additional 24 hr in serum-free DMEM.

Luciferase Assays. After treatment, cells were harvested in 150 μl of lysis buffer (Promega). The protein content of the extracts was determined by the Bradford procedure (Bio-Rad). Extracted samples (50 μg of protein) were subjected to the luciferase assay in duplicate by using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego).

Chemicals. PTH(1–34) and PTHrP(1–34) were from Bachem; forskolin, insulin-like growth factor I, and cell culture reagents were from Sigma, restriction enzymes were from Life Technologies, and 1,25(OH)₂D₃ was a kind gift from Milan Uskokovic (Hoffman-LaRoche).

Statistical Analyses. Significant differences were identified by using Student's *t* test.

RESULTS

Sequence Analysis of Human 1α -OHase 5'-Flanking Sequence. A human genomic library was screened with the most 5' fragment (700 bp) of the 1α -OHase cDNA as a probe. A positive genomic clone of 13 kb was digested with *SacI*, which generated 3.5-kb, 3.0-kb, 1.0-kb, and 5.5-kb fragments. These fragments were subcloned into pGEM 7 vector (Promega). Sequence analysis confirmed that the 5.5-kb fragment encoded intron 8 to exon 9, the 1.0-kb fragment encoded exon 6 to intron 8, the 3.5-kb fragment encoded the 5'-flanking region to exon 6, and the 3.0-kb fragment encoded a portion of the 5'-flanking region. The complete sequence analysis showed that there are 1,369 bp of 5'-flanking region upstream from ATG. The TATA box was located 64 bp upstream from ATG, and a CCAAT box was identified 116 bp upstream from ATG. Additional putative regulatory elements including CRE-like sequences, consensus or highly conserved AP-1, AP-2, Sp1, and NF- κ B sites were identified by computer search (30). The complete sequence and a map of the landmarks of the human 1α -OHase promoter are shown in Fig. 1.

Promoter Activity of Human 1α -Hydroxylase Gene. The transcriptional activity profile of 1α -OHase 5'-flanking sequences was studied. A series of truncated portions of the promoter were created extending up to 1.4 kb from the transcription start site of the human 1α -OHase gene. These constructs were inserted upstream of a luciferase reporter gene in the pGL2 basic vector and designated AN1, AN2, AN3, AN4, AN5, and AN6, the lengths of which are enumerated in Table 1 and illustrated by alternating outlined blocks in Fig. 1. Each construct was transiently transfected into AOK-B50 cells (a pig kidney cell line) and subsequently subjected to a luciferase activity assay. The basal luciferase activity of cells transfected with the 1.4-kb construct (AN1) was 1.7-fold greater than the promoter-free construct. The cells transfected with the shorter (AN2–6) constructs exhibited markedly increased promoter activity with the AN2 (490-fold) and AN5 (650-fold) constructs relative to cells transfected with the promoterless vector (Table 2, Fig. 2A).

Regulation of the Promoter Activity of the Human 1α -Hydroxylase Gene. The effects of several factors suspected to be involved in the regulation of the expression of the 1α -OHase gene were examined. The luciferase reporter gene activity was determined in cells transfected with each construct and treated with 10 μM forskolin, a stimulator of cAMP, or vehicle (sterile water). All six promoter constructs responded to forskolin, with AN2 and AN5 being the most responsive (Table 2). Transfected AOK-B50 kidney cells also were tested for PTH responsiveness by treating with PTH(1–34) or vehicle (sterile water) for 24 hr. Induced luciferase reporter gene activity was observed for each of the constructs tested with 100 nM PTH(1–34), with significantly greater induction noted in the AN2 (\approx 2,300-fold) and AN5 (\approx 1,500-fold) constructs

Table 1. Primers used for human 1α -OHase

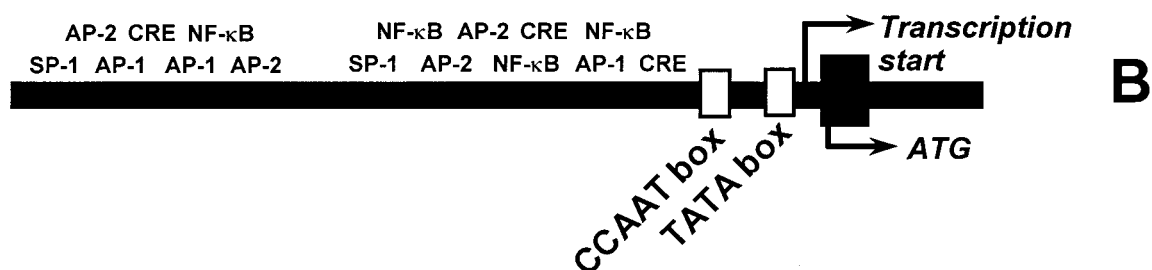
Human 1α -OHase promoter form	Primers (5' → 3')	Direction	Fragment size, bp
AN1	CACGGTACCTTGGGAAGGTGAGGGGGT	F	1,469
	CACGGTACCTCGGGCGCCAGCGGACG	R	
AN2	AN1 cut with <i>KpnI</i> and <i>SacI</i>		1,100
AN3	CACGGTACCAATTAGAACATCCACATC	F	827
	CACGCTAGCTCGGGCGCCAGCGGACG	R	
AN4	CACGGTACCAAGAAAGAAAGATGGAGG	F	672
	CACGCTAGCTCGGGCGCCAGCGGACG	R	
AN5	CACGGTACCTGAAAAGATGATGGGGGAATTCC	F	463
	CACGCTAGCTCGGGCGCCAGCGGACG	R	
AN6	CACGGTACCGCCTTATAGCCTTTCCTA	F	363
	CACGCTAGCTCGGGCGCCAGCGGACG	R	

F, forward; R, reverse.

A

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-1369 CTTGGGAA GGTGAGGGGG TGGAGACCT CTCCTTTTTC ACATTGACCT TCAATTCCAG AACTTCAGAG
          Sp1
-1301 CTAGCTGACT GGCACAGAGC CTGATAGAGT TGCAGTGGGG AATGAGGGAG TAAGGAGCAG AGAGGTAAAC
          AP-2
-1231 TGTGGGAGAT TATTTTATTT TAATTTTTTTT TAGAGACAGG CCCTCTCTCT GTTGCCTAGG CTGGAGTGCA
-1161 GTGGTACAAT CATAACTCAC TGCAGCCTTG ACCTCCCGGG CTCATGTAAT CCACCTCAGC CTCTCAAGGC
          AP-1
-1091 ACTACAGGCA TACGCCACCA TGCCTGGCTA ATTTTTGTAT TTTTTGTAGA GACAGTTCTGA CTATGTTGCC
          CRE
-1021 CAGGCTGGTA CTCAGACTCC TGAGCTCAAG CAGTCTGCC ACCTTAGCCT CCCAAAGTGT TGGGATTACA
-951 GTCATGAGCT GCCGTGCCCA GCCTAATTTT TAAACTTCTT CTGTAGAGAC AGTCTTGCTA TGTTGCTCAA
-881 GCTTGTCTCA ACCTCCTGGC CTCATGCCAT CCTCCTGCTT CAGCCTCCCA AAGTGCTGGG ATTATAGGCA
          CRE
-811 TGAGCCACCA CAGGATTCTG AGGCTAAAAG TAGAAAGGAG CTGCTAACCA GAAAGCCCC CAGAGGAGAC
          NF-κB
-741 CGAGTCAGCT GGGGATTAG AACATCCACA TCCATTTAGC TCCTCTAACA CCCACAGAC AATCTCTGAA
          AP-2
-671 CTTAACTAAT GTACAAAAAG TTTCAATTTCT GCTTCTCAAG CCCCAAATTC CCATGTCTGG AAGGAGGGGG
-601 CTTGTATACC CAAGAGACCC CCTTATCCTC AAGAAAGAAA GATGGAGGTT CTAGGAGCCA TTTTAGCCCA
-531 TTAACCCACC TGCCATCTGC CCAGTATATG TTAGGTACAG GAGGAGGGGG AGGTGTGGCT AGTGCAGGAA
          Sp1 NF-κB
-461 AATAATGCAG AGAAGATACA ACCCACTAAG CCAAGAATGT GGGGACAGTT ACAGCTGTGC CTCCCCTGCT
-391 TCCCTTCTCTG GAGGAGCTGA AAGATGGGGA ATTCCTGAGG ATGGGCCTAA AGGGGCTGGG CTCACTGGTA
-321 GAAGTGGGAA TATCAGAGAC TGACTAGTGT AGCTTGGTCA CCTAGTCCCT ACTAAAAAGC CTTATAGCCT
          NF-κB CRE
-251 TTCCTAGGAT GAGACTTTGA GGCTCCTAGA CAAAGGCACT CTCCCAGGAG GAAAATCTTA GGCCCTCCCT
          AP-1 NF-κB Pu box
-181 CCCATGAGGG TCATTGCAAC ATGAGACCCA AGGGAGTTGT GAAGTACGCC CCAGCCCCGC CTACTGTTCC
          CRE Sp1
-111 TGGGTGCTAA TCCCAGCAC AGACCACTCA GGAGGAGGGA TTGGCTGAGG AGCTTGGAGA GGGGGCGTCA
          CCAAT box Sp1
-41 TCACCTCACC CAAAGGTTAA ATAGGGGTTG AGATATGATG CTCAGGAGAA GCGCTTTCTT TCGCGAGCAC
          TATA box
27 CCTGAACCAG ACCATGACCC AGACCCTCAA GTACGCCTCC AGAGTGTTC ATCGCGTCCG CTGGGCGCCC
97 GAGTTGGGCG CCTCCCTAGG CTACCGAGAG TACCACTCAG CACGCCGGAG CTTGGCAGAC ATCCCAGGCC
167 CCTCTACGCC CAGCTTTCTG GCCGAACTTT TCTGCAAGGG GGGGCTGTCG AGGCTACACG AGCTGCAG
    
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B

FIG. 1. Structure of the human 1 α -OHase promoter. A 13-kb genomic clone of the human 1 α -OHase gene was sequenced, mapped, and analyzed for consensus sequences to known mammalian transcription factors. (A) The nucleotide sequence for the promoter region is given. Underlined sequences correspond to conservative homologous matches to the noted transcription factor or promoter element. The alternating blocked and unblocked sections indicate the truncated promoters AN1–6. (B) A map of the 1 α -OHase promoter showing the relative locations of the putative cAMP-inducible transcription factor response elements.

Table 2. Luciferase activity in AOK-B50 cells

Human 1α -OHase promoter form	Size, bp	Basal promoter activity	Forskolin-induced promoter activity
AN1	1,469	1.7 \pm 0.6	3.5 \pm 1.8
AN2	1,100	494.0 \pm 211.6	1,414 \pm 369
AN3	827	18.4 \pm 10.4	41 \pm 20
AN4	672	55.3 \pm 36.1	57 \pm 26
AN5	463	643.4 \pm 118.8	806 \pm 252
AN6	363	56.4 \pm 23.1	129 \pm 40

Activity data are given as mean \pm SEM of fold induction vs. promoterless (basal) or untreated (forskolin-induced) vector.

(Fig. 2B). The PTH/PTHrP receptor agonist PTHrP(1–34) also induced comparable luciferase activity with each of the constructs (data not shown). The enhancement of luciferase reporter gene activity in the AN2 and AN5 constructs by PTH(1–34) was concentration-dependent (Fig. 3). Neither $1,25(\text{OH})_2\text{D}_3$ (Fig. 2C) nor insulin-like growth factor I alone (data not shown) had significant stimulatory or suppressive effects with any of the constructs. However, $1,25(\text{OH})_2\text{D}_3$ suppressed PTH-stimulated luciferase reporter gene activity by 58% in the AN2 construct and 52% with the AN5 construct. No significant effect was observed on any of the other promoter constructs (Fig. 2D).

DISCUSSION

The results of structure–function and regulatory analyses of the human 1α -OHase gene promoter are reported here. We have mapped the 1α -OHase gene from -3 kb $5'$ to $+5$ kb $3'$, including 5 kb of coding sequence. A 1.4-kb segment of the promoter was sequenced, and several putative cAMP-responsive regulatory elements were found. Functional analyses identified distinct regions of the promoter that regulate expression of this gene. Our studies document strong induction of the expression of the 1α -OHase gene by PTH/PTHrP agonists and show that $1,25(\text{OH})_2\text{D}_3$ is antagonistic to PTH-induced transcription. The presence of putative cAMP-responsive regulatory elements and PTH-inducible and $1,25(\text{OH})_2\text{D}_3$ -suppressible regions suggests a complex regulatory pathway. Because the regulation of 1α -OHase activity in renal and nonrenal tissues appears to be different, this complex promoter structure may also be related to the tissue-specific expression of this gene. A role for additional transcription factors or a secondary pathway of action in the regulation of the 1α -OHase promoter activity is indicated by the observation that $1,25(\text{OH})_2\text{D}_3$ suppresses PTH-stimulated induction despite the lack of a classical vitamin D response element in the promoter for this gene.

Sequence analysis revealed that the 1α -OHase promoter contains putative CRE as well as AP-1, AP-2, Sp1, and NF- κ B response elements. Each of these transcription factors has been independently demonstrated to be induced by cAMP (33–34). The presence of conserved cis elements for these factors suggests additional pathways of cAMP regulation of 1α -OHase expression through AP-1, AP-2, Sp1, and NF- κ B sites in the 1α -OHase promoter (33–35). It is significant to note that our computer-based sequence analysis of the promoter revealed several sequences with high homology to cAMP-responsive transcription factors. In contrast, cloning and analysis of the 1α -OHase promoter by Murayama *et al.* (22) showed fewer of these response elements, only two of which correspond to ones we identified.

The 1.4-kb 1α -OHase promoter (AN1) had modest endogenous transcriptional activity. However, because a $5'$ portion of the promoter was truncated, activity was dramatically increased. Truncation constructs induced significant transcrip-

tional activity, with AN2 and AN5 inducing the highest activity. AN1 induced modest transcriptional activity over baseline, AN2 and AN5 showed dramatic basal activity, and AN3, AN4, and AN6 exhibited more basal activity compared with AN1 but significantly less basal activity compared with AN2 and AN5. Therefore, we conclude that AN1 and AN4 possess suppressive cis regulatory elements and that AN6 contains few positive regulatory elements.

The low basal activity of AN1 sharply contrasts the relatively high endogenous activity of the mouse 1α -OHase promoter in the same system (19). Only $\approx 44\%$ sequence identity exists between the human and mouse promoters, which is probably the basis for these observations. It is possible, therefore, that the mouse promoter clone does not include a suppressor element(s) homologous to those in the $5'$ region of AN1.

PTH is one of two major regulators of 1α -OHase activity. In our system, PTH/PTHrP receptor agonists and forskolin modulated 1α -OHase promoter activity. These results, in combination with the identification of four putative CRE and several cis elements for cAMP-inducible factors in the promoter, suggest a direct role for PTH in the regulation of 1α -OHase expression. PTH/PTHrP receptor agonists significantly stimulated 1α -OHase promoter activity from basal activity in all truncation constructs; however, constructs AN2 and AN5 were significantly more sensitive than AN1, AN3, AN4, and AN6. CRE and several other putative cAMP-sensitive regulatory elements appear to be present in AN2 and AN5, whereas none were found in AN3 and few are present in AN1, AN4, and AN6. These observations suggest a degree of bifunctional regulation of the 1α -OHase promoter. The comparatively low promoter activity of AN1, which contains the most $5'$ portions of the 1α -OHase promoter, supports our hypothesis that the $5'$ region of AN1 contains suppressor sequences.

The other major regulator of 1α -OHase activity is $1,25(\text{OH})_2\text{D}_3$. Similar to the mouse promoter, $1,25(\text{OH})_2\text{D}_3$ did not suppress basal 1α -OHase promoter activity. However, $1,25(\text{OH})_2\text{D}_3$ did suppress luciferase reporter gene expression stimulated by PTH(1–34), and this suppression was exclusive to the two high-activity regions (AN2 and AN5) of the 1α -OHase promoter. Sequence analysis of the 1α -OHase promoter indicates that there are no canonical vitamin D response elements in either region. Together, these observations suggest that $1,25(\text{OH})_2\text{D}_3$ suppresses 1α -OHase expression by an indirect cascade that interferes with the PTH-signaling pathway. However, it is also possible that an atypical suppressive vitamin D response element(s) is/are located in the 1α -OHase promoter. These results indicate a sophisticated degree of regulation that may not be entirely dependent on PTH or $1,25(\text{OH})_2\text{D}_3$ levels.

Our results differ significantly from those presented for another genomic clone of the human 1α -OHase promoter (22). Those investigators found induction by PTH in a region 4 kb $5'$ to the transcription start site and suppression by $1,25(\text{OH})_2\text{D}_3$ in 0.9 kb of the $3'$ -most region of the promoter. No mention is made as to whether any PTH sensitivity was found in the $3'$ -most region or whether any suppression by $1,25(\text{OH})_2\text{D}_3$ was found in the -4 -kb region. More significantly, suppression by $1,25(\text{OH})_2\text{D}_3$ was shown in the absence of PTH. Major differences between the results may be because of experimental differences. Our constructs contained up to -1.4 kb of the 1α -OHase gene promoter, including the native transcription and translation start sites and the first 19 residues of the 1α -OHase, and our transfections were performed in a porcine kidney cell line. In contrast, Murayama *et al.* (22) substituted the $3'$ -most region of the promoter with the thymidine kinase promoter, presented analyses of fragments -0.9 kb and ≈ 4 kb upstream of the promoter, used a mouse kidney cell line, and cotransfected with VDR. The use of the thymidine kinase promoter could be responsible for the ob-

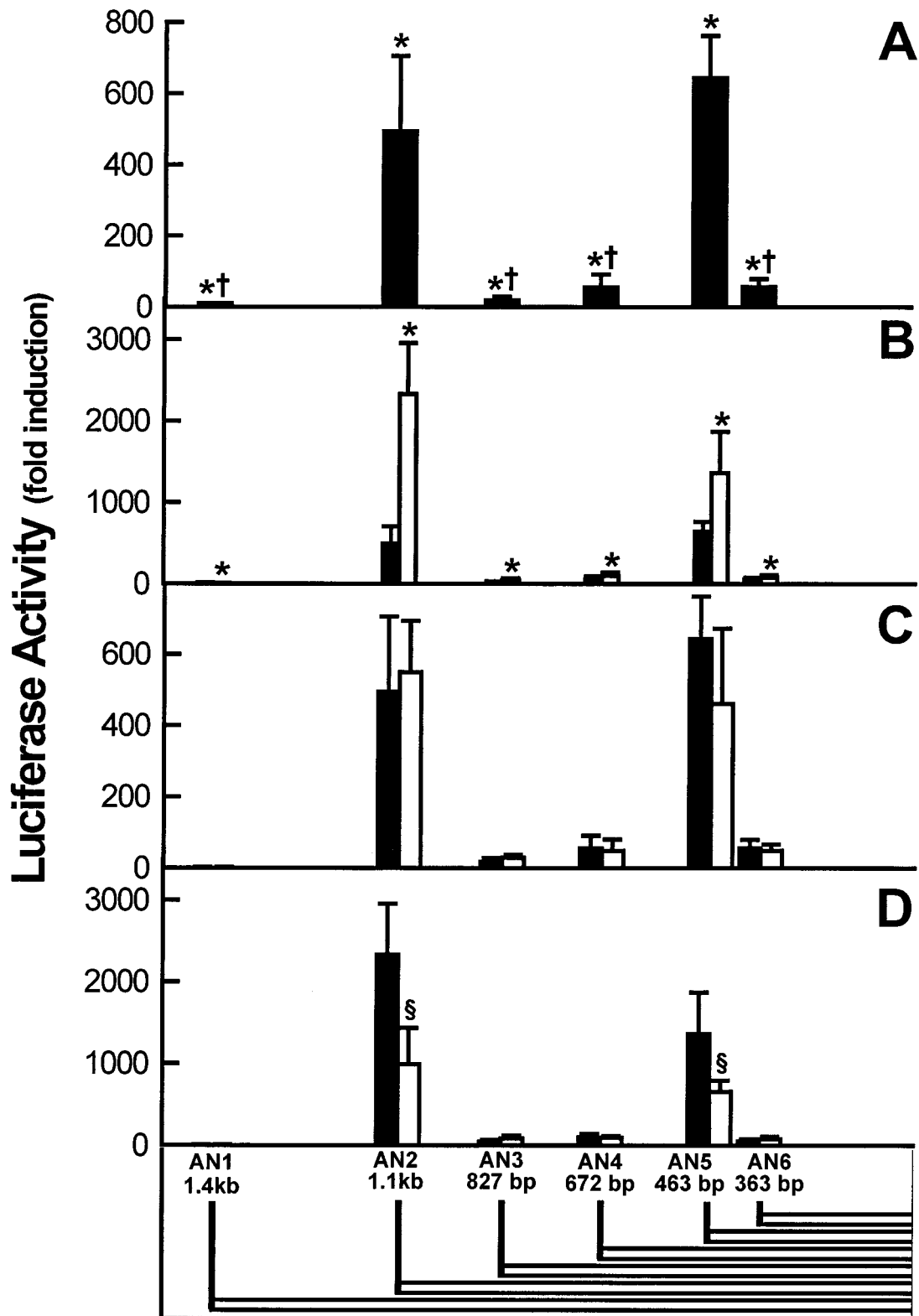


FIG. 2. Activity of the human 1α -OHase promoter. A 1.4-kb clone and several truncated forms of the human 1α -OHase promoter were inserted into a luciferase reporter expression vector, transfected into AOK-B50 kidney cells, grown for 24 hr with and without factors, and then assayed for luciferase activity. (Bottom) The length of the various truncated promoter constructs. The graphs represent the luciferase activity of the constructs relative to that of the promoterless vector. Bars represent the mean \pm SEM of three independent determinations. (A) Basal activity of the human 1α -OHase promoter. Transfected cells were assayed for luciferase activity. *, $P < 0.01$ vs. promoterless vector; †, $P < 0.001$ vs. AN2 and AN5. (B) PTH induction of the activity of the human 1α -OHase promoter. Transfected cells were treated with vehicle alone (filled bar) or 100 nM PTH (1–34) (open bar) for 24 hr and assayed for luciferase activity. *, $P < 0.01$ vs. vehicle alone. (C) Effect of $1,25(\text{OH})_2\text{D}_3$ on the activity of the human 1α -OHase promoter. Transfected cells were treated with vehicle alone (filled bar) or with 10 nM $1,25(\text{OH})_2\text{D}_3$ (open bar). (D) Effect of $1,25(\text{OH})_2\text{D}_3$ on the PTH (1–34)-inducible activity of the human 1α -OHase promoter. Transfected cells were treated with 100 nM PTH (1–34) with vehicle alone (filled bar) or with 10 nM $1,25(\text{OH})_2\text{D}_3$ (open bar). §, $P < 0.01$ vs. the same construct in the absence of $1,25(\text{OH})_2\text{D}_3$.

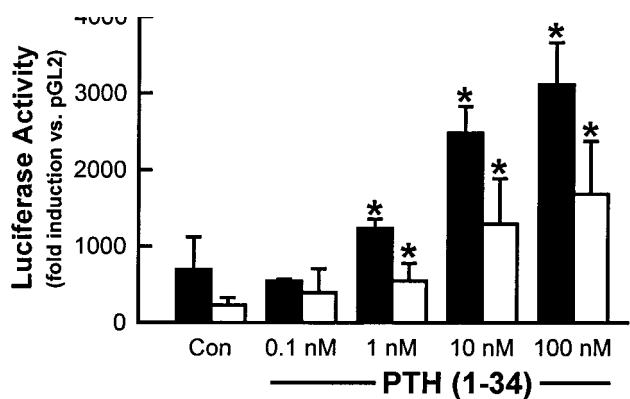


FIG. 3. Dose-dependent induction of the AN2 and AN5 truncated forms of the human 1α -OHase promoter by PTH (1–34). AN2- (1,100 bp, filled bar) and AN5- (463 bp, open bar) truncated forms of the human 1α -OHase promoter were inserted into a luciferase reporter expression vector, transfected into AOK-B50 kidney cells, and treated with increasing concentrations of PTH (1–34) for 24 hr. The AOK-B50 cells were then assayed for luciferase activity. Each bar represents the mean fold induction in luciferase activity relative to that of the promoterless construct in the absence of PTH \pm SEM of three independent determinations. *, $P < 0.01$ vs. the same construct in the absence of PTH.

servation that $1,25(\text{OH})_2\text{D}_3$ independently suppressed 1α -OHase promoter activity. Although this is consistent with our results, it has been shown that thymidine kinase promoter-driven expression may give rise to aberrant results (36).

It has been shown that $1,25(\text{OH})_2\text{D}_3$ suppresses PTH/PTHrP receptor number (37). In VDR knockout mice, the 1α -OHase transcript was present at 50 times the levels of wild-type littermates (14). It has also been demonstrated that PTH agonist treatment led a 2- to 3-fold increase in VDR expression in MC3T3-E1 cells (29). Regulation of receptor number influences the biologic activity of that receptor's ligand. In this context, it is plausible that the combinatorial regulation of 1α -OHase and PTH/PTHrP receptor expression exerted by $1,25(\text{OH})_2\text{D}_3$ are the fine controls for PTH responsiveness, which ultimately maintain serum calcium homeostasis. Although the crosstalk between these receptors and their ligands remains unclear, these observations strongly support the hypothesis that PTH and $1,25(\text{OH})_2\text{D}_3$ are critical physiologic regulators of 1α -OHase expression in the kidney. The regulation of 1α -OHase expression in nonrenal tissues may use different regions of the promoter dependent on the expression of receptors for PTH and $1,25(\text{OH})_2\text{D}_3$. This work provides valuable insights into the regulation of the expression in the kidney of the most critical enzyme in the biosynthetic pathway for $1,25(\text{OH})_2\text{D}_3$, which is primarily responsible for systemic calcium homeostasis, and therefore, of great physiologic relevance.

We thank Drs. F. Binghurst, J. Potts, and H. Kronenberg of the Massachusetts General Hospital for the AOK-B50 cells and Kelly Persons for assistance with cell culture. This work was made possible in part by National Institutes of Health Grant RO1 AR36963.

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