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The Promoter of the Human 25-Hydroxyvitamin D_3 1α-Hydroxylase Gene Confers Positive and Negative Responsiveness to PTH, Calcitonin, and 1α,25$(OH)_2D_3$

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25-Hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase) catalyzes hydroxylation, mainly in the kidney, of 25-hydroxyvitamin D_3 [25(OH)D_3] into 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], a hormonal form of vitamin D, acting as a key enzyme of vitamin D biosynthesis. Reflecting its biological significance, this enzymatic activity is differentially regulated by several factors involving calcium homeostasis, though the molecular mechanism is poorly understood. In our recent study (K. Takeyama, *et al.*, 1997), we cloned the cDNA of mouse 1α-hydroxylase, and this led us to investigate the regulation of gene expression and the function of the promoter of this gene. Here we report the isolation of the 5′ flanking region of the human 1α-hydroxylase gene and the identification of the human 1α-hydroxylase promoter by a primer extension assay. We found that in the identified promoter, a positively regulatory region to parathyroid hormone (PTH) and calcitonin and a negatively regulatory region to 1α,25$(OH)_2D_3$ are located around -4 and -0.5 kb, respectively. Thus, we provide direct evidence that the positive and negative regulation of 1α-hydroxylase gene expression by hormones takes place at transcriptional levels through two distinct promoter regions.

1α-Hydroxylase catalyzes hydroxylation of 25(OH)D_3 into 1α,25$(OH)_2D_3$, a hormonal form of vitamin D, acting as a final and critical key enzyme in the vitamin D biosynthesis pathway (1, 2). The predicted amino acid sequences from the cloned cDNAs of 1α-hydroxylase showed that this enzyme is one of the P450 enzymes (3-8) and is thought to localize in the inner membrane of mitochondria (9-12). The marked expression of this gene is seen only in the kidney (3-6, 8), though there are several reports describing its enzymatic activity in extrarenal tissues (13-16). As 1α,25$(OH)_2D_3$ plays a prime role in calcium homeostasis, the activity of this enzyme is differentially regulated by several hormones involving calcium homeostasis (17-20). Several lines of evidence show that calciotropic hormones such as PTH and calcitonin induce enzymatic activity, and in sharp contrast, 1α,25$(OH)_2D_3$ reduces such activity. Through such complex regulation in 1α-hydroxylase activity, serum levels of 1α,25$(OH)_2D_3$ are strictly regulated responding to altered levels of serum calcium (1, 2, 21).

More recently, we found that the regulations of 1α-hydroxylase by PTH, calcitonin and 1α,25$(OH)_2D_3$ occur at transcriptional levels in intact animals as well as in cell line derived from proximal tubule cells of the mouse kidney using cloned cDNAs (Murayama, A. *et al.* submitted) in agreement with previous reports describing enzymatic activities.

These findings, taken together, suggest that there are positive and negative regulatory elements in the 1α-hydroxylase promoter. We have previously determined the exon-intron organization of the human 1α-hydroxylase gene (8). To search for putative regulatory elements, we cloned the promoter to approximately 12 kb upstream, and analyzed the function of this promoter with the segmented regions. Consequently, we found that the region around -4 kb of the human 1α-hydroxylase gene confers positive responsiveness to PTH, calcitonin as well as forskolin, and the proximal region (up to about -0.5 kb) confers negative responsiveness to 1α,25$(OH)_2D_3$.

Abbreviations used: 25-hydroxyvitamin D_3 1α-hydroxylase, 1α-hydroxylase; 1α,25-dihydroxyvitamin D_3, 1α,25$(OH)_2$ D_3; parathyroid hormone, PTH; VDR, vitamin D receptor; nVDRE, negative vitamin D response element; PKA, protein kinase A.

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MATERIALS AND METHODS

Isolation and sequence analysis of the 5′ flanking region of the human 1α-hydroxylase gene. A λ DASH genomic library from normal human leukocyte was purchased from Clontech. A total of 1×10^6 plaques were screened, hybridizing them with a human 1α-hydroxylase N-terminal genomic fragment (1.7 kb) (8). Two positive plaques were subcloned into pBluescript SK(-) and sequenced automatically in a Prism 377 DNA sequencer (Applied Biosystems), with AmpliTaq DNA polymerase FS (Perkin-Elmer) and dye terminator.

Primer extension analysis. Total RNA was extracted from the normal human kidney by using the acid guanidinium thiocyanate/phenol/chloroform (AGPC) method (22). Polyadenylated RNA [poly(A)$^+$-RNA] was further purified by oligo(dT) affinity chromatography (22).

One μg of poly(A)$^+$-RNA or 20 μg of total RNA was then mixed with 2 pmol (about 1×10^4 cpm) of 5′ ^{32}P-labeled primer (5′-GGATGTCTGCCAAGCTCCGG-3′: corresponding to the nucleotide position 101-120 from the translational start site), denatured by heating at 60°C for 40 min, then annealed at room temperature for 60 min (23). Primer extension proceeded at 37°C for 60 min with 10 U/μl of SuperScript II reverse transcriptase (GIBCO BRL) in a buffer [50 mM Tris-HCl (pH-8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.4 mM dNTPs]. After ethanol precipitation, the products were analyzed on 8 % acrylamide-7 M urea sequence gel. The subcloned 5′ flanking region was sequenced using the same primer by the Sequencing PRO DNA Sequencing Kit (TOYOBO) and electrophoresed on the same gel.

RT-PCR. One μg of total RNA was reverse transcribed using the Superscript preamplification system (GIBCO BRL) according to the instruction manual (24). PCR was performed in 20 μl reaction medium with 1 μl of 1/100 RT mix solution, 0.5 μM forward and reverse primer, 0.2 mM deoxynucleotide triphosphates, and 0.2 U AmpliTaq polymerase (Perkin-Elmer). After 1 min of preincubation at 95°C, amplification was performed for 35 cycles consisting of 40 sec of denaturing at 95°C, 40 sec of annealing at 58°C, and 40 sec extension at 72°C. The list of primers is as follows: mouse PTH receptor, 5′-GCAATGTGACAAGCTGCTCAAGG-3′ and 5′-GGCCAAGGTTGCTC TGACTCCG-3′ ; mouse calcitonin receptor, 5′-GAAGTGATAAGTTGCAAGG-3′ and 5′-CAATTCATTAGAGCACTTTGG-3′; mouse vitamin D receptor, 5′-TTCCCA GGAGAGCACCCTTGG-3′ and 5′-GCGTAGAGCTGGTGGCTGCCCGG-3′.

Northern blot analysis. Northern blot analysis was performed, as previously described (3, 22). Mouse 1α-hydroxylase cDNA N-terminal fragment (700 bp: 1-700 of the mouse cDNA) (3) was used as probe.

Construction of reporter plasmids. The transient expression vector pGL thymidine kinase (tk)-chloramphenicol acetyltransferase (CAT) containing the tk promoter was used to test for human 1α-hydroxylase gene promoter activity. A PCR-amplified fragment, corresponding to nucleotides -889 to -30 relative to the determined transcriptional start site of the gene, was subcloned into the HindIII and BamHI site of a pGL tk-CAT vector (pGL-tk1α/ 0.5 K). A fragment corresponding to nucleotides about -4400 to -3200 with HindIII site at both ends was subcloned into the HindIII site of the vector (pGL-tk1α/4 K).

Transfection and CAT assays. MCT cells, a SV40-transformed mouse proximal tubule cell line (25) were transfected with 1 μg of pGL-1α promoter or empty pGL tk-CAT vector as a negative control, together with or without 0.5 μg of VDR expression vector (26), as indicated, using Lipofect AMINE plus (GIBCO BRL). The DNA-Lipofect AMINE mixture was removed after 3 h, and cells were fed with DMEM containing 5% FCS and treated with rat PTH (Sigma), salmon calcitonin (Sigma), forskolin (Sigma), 1α,25$(OH)_2D_3$ (provided by Chugai Pharmaceutical Co., LTD) or vehicle as indicated for an additional 24 h. Normalization of CAT enzyme activity for transfection efficiency was determined by co-transfection with 1 μg of pCH 110 (Pharmacia), a β-galactosidase expression vector. Cell extracts were prepared by freezing and thawing and assayed for CAT after normalization for β-galactosidase activity as described elsewhere (3).

RESULTS

Cloning of the human 1α-hydroxylase gene and its 5′ flanking region. In a previous study, we cloned the cDNA encoding mouse 1α-hydroxylase from vitamin D receptor knock-out mice (VDR KO mice) (3), and subsequently isolated the human 1α-hydroxylase cDNA (8). Furthermore the exon-intron organization of the human 1α-hydroxylase gene was determined to find genetic mutations in patients with vitamin D-dependent rickets type I (8). Using the cloned genomic fragment of the human 1α-hydroxylase gene, we first cloned the 5′ flanking region up to about 12 kb (Fig. 1A).

The transcriptional start site localizes 40 bp upstream of the translational start site. To determine the transcriptional start site of this gene, a primer extension analysis was performed with purified RNA from normal human kidney. As shown in Fig. 2, a major transcriptional start site was identified 40 bp upstream of the translational start site though this result is different from another finding (27). Quite reasonably, a TATA box is present 26 bp upstream of the identified transcriptional start site, and in the further upstream regions, there are several binding sites for known transcriptional factors such as AP-1 and NF-κB (Fig. 1B). We then analyzed the function of the 5′ flanking region of the TATA box as the promoter.

The mouse 1α-hydroxylase gene is expressed in the MCT cell line. For functional analysis of the 1α-hydroxylase gene promoter, we searched the kidney cell line expressing the 1α-hydroxylase gene, since the 1α-hydroxylase gene is expressed significantly only in the kidney. We found that the 1α-hydroxylase mRNA is present in the MCT cell line derived from the proximal tubules of mouse kidney (Murayama, A. *et al.* submitted). This cell line was confirmed to possess endogenous receptors for 1α,25$(OH)_2D_3$, PTH and calcitonin (Fig. 3B).

As expected from the animal experiments (Murayama, A., *et al.*, submitted), 1α,25$(OH)_2D_3$ decreased the mRNA levels of 1α-hydroxylase in MCT cells, whereas forskolin induced 1α-hydroxylase gene expression (Fig. 3A). Treatment with PTH, which, like forskolin, induces accumulation of cAMP, up-regulated the 1α-hydroxylase mRNA levels of this gene (data not shown).

The human 1α-hydroxylase gene promoter confers positive and negative responsiveness to PTH, calcitonin and 1α,25$(OH)_2D_3$. As the MCT cell line expresses the endogenous 1α-hydroxylase gene and the receptor genes for PTH, calcitonin and 1α,25$(OH)_2D_3$, we analyzed the human 1α-hydroxylase gene promoter (up to about -4 kb) in this cell line by a transient expression

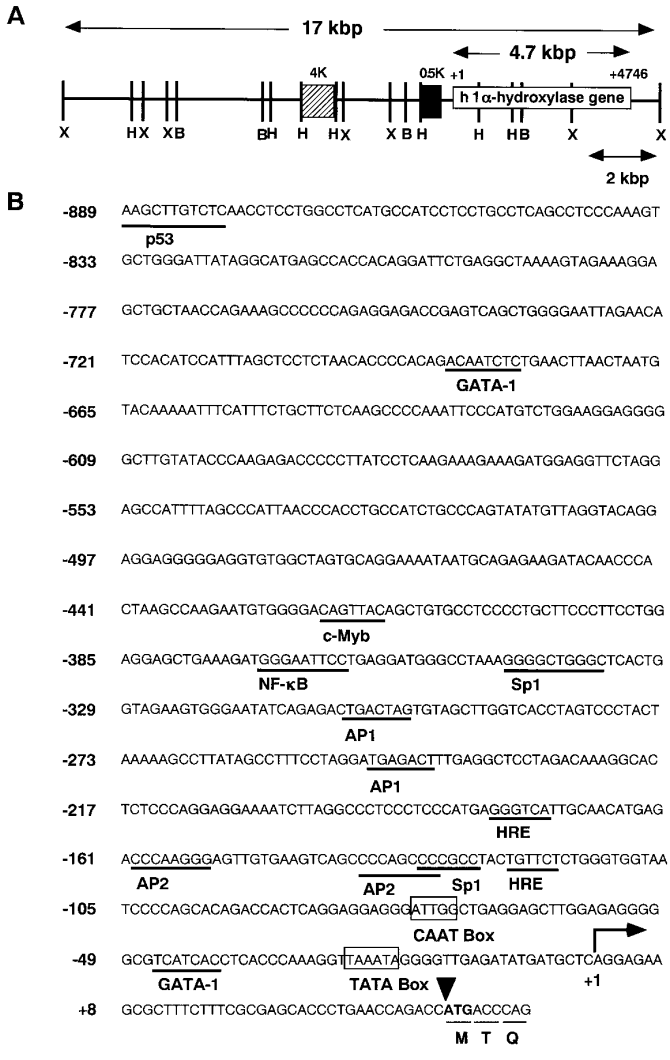

FIG. 1. Genomic organization of the human 1α-hydroxylase gene and its 5′ flanking region. (A) Restriction enzyme map of the human 1α-hydroxylase gene and its 5′ flanking region. X, XbaI; B, BamHI; H, HindIII. (B) Nucleotide sequence of the 5′ flanking region (~-889) of the human 1α-hydroxylase gene. The region upstream of -889 are under sequencing. The transcriptional start site is designated as +1. Putative cis regulatory elements are underlined. The translational start codon is indicated by closed triangle (+ 41). HRE; hormone response element.

assay. A basal activity of the human 1α-hydroxylase gene proximal promoter was observed in the MCT cell line even in the absence of hormones (data not shown). With the transfected cells treated with PTH, calcitonin and forskolin, we further tried to delineate the positive region with the segmented promoters by replacing a weak basal human 1α-hydroxylase promoter into the tk basal promoter (26), and consequently the region around -4 kb was turned out to confer the positive responses to these agents (see Figs. 4B and 4C, right panel), explaining the findings of positive regulations by these agents in cultured cells and in intact animals (Murayama, A. *et al.* submitted). Most notably, we found that a proximal promoter around -0.5 kb negatively responds to 1α,25$(OH)_2D_3$ (see Figs. 4B and 4C, left panel).

DISCUSSION

In previous studies, we cloned the cDNAs encoding the mouse and human 1α-hydroxylase (3, 8), and deter-

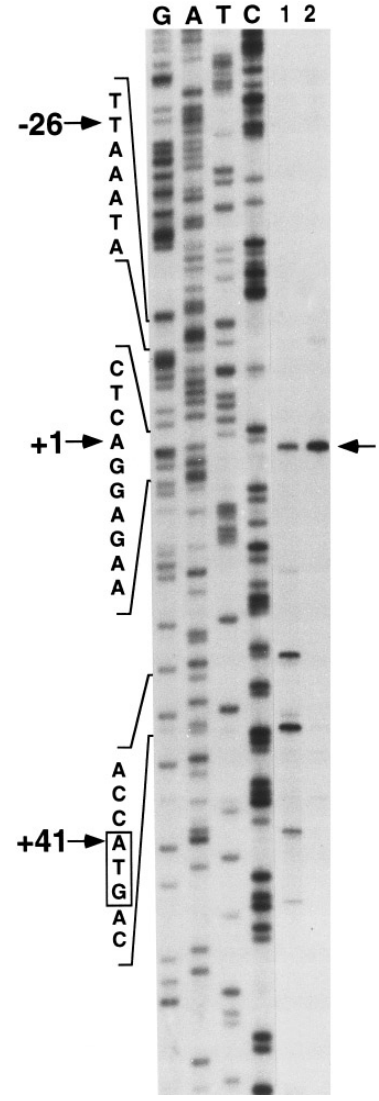

FIG. 2. Identification of the transcription start site of the human 1α-hydroxylase gene. A primer extension analysis of human kidney 1α-hydroxylase mRNA population was performed with 20 μg of total RNA (lane 1) or 1 μg of poly$(A)^+$-RNA (lane 2) from human kidney using a labeled primer. The primer-extended products obtained using a labeled primer were co-electrophoresed with sequencing reaction products using the same primer to determine the precise start site. The major transcriptional start site (+1) is indicated by an arrow in the right. The position of TATA box (-26) and the translational start site (+41) are indicated in the left.

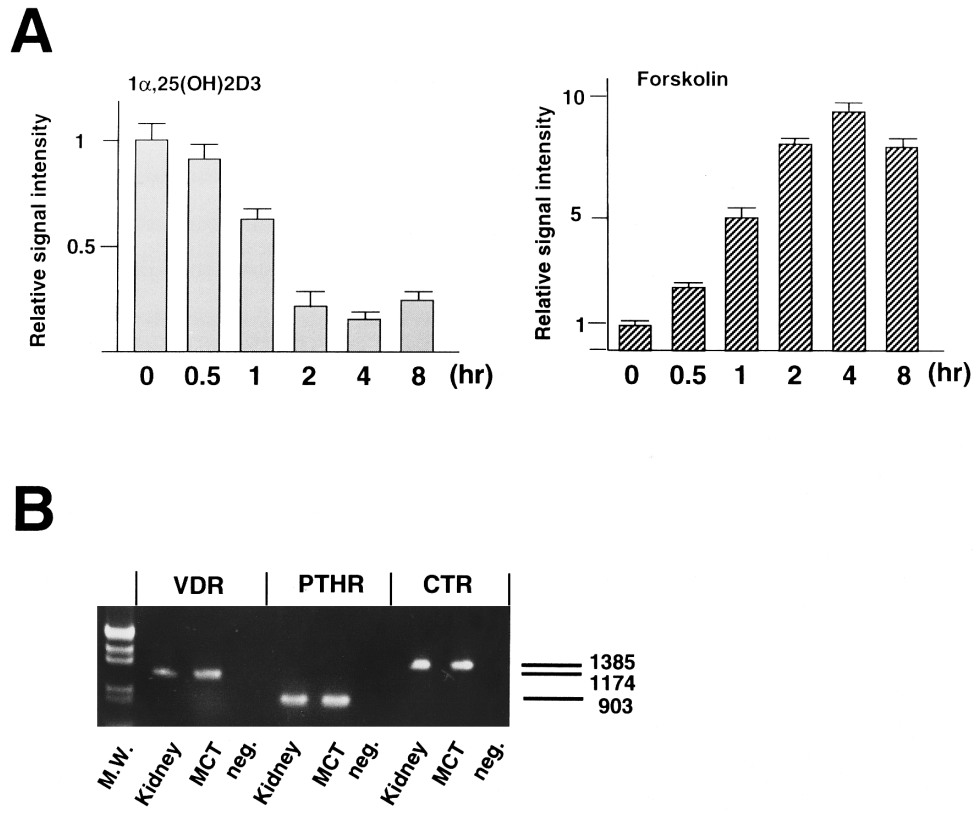

FIG. 3. Regulations of 1α-hydroxylase gene expression by forskolin and 1α,25$(OH)_2D_3$ in MCT cells. (A) The expression of 1α-hydroxylase gene was analyzed using Northern blotting from the time course of 10^{-8} M 1α,25$(OH)_2D_3$ and 10^{-7} M forskolin-treated cells. We calculated the relative abundance of the 1α-hydroxylase gene transcripts normalized with the β-actin transcript from more than three for one group. Five μg each of poly $(A)^+$-RNA from MCT cell lines was electrophoresed and the 700 bp cDNA fragment corresponding to the N-terminal region of mouse 1α-hydroxylase was used as a probe. (B) Expression of PTH receptor (PTHR), calcitonin receptor (CTR) and VDR genes in MCT cells and in mouse kidney by RT-PCR. Agarose gel (1%) electrophoresis revealed 903 bp, 1385 bp and 1174 bp DNA fragments, respectively. The marker (M.W.; molecular weight) is λ DNA digested with HindIII-EcoRI. Negative control (neg.) are RT-PCR products performed without any templates.

mined the organization of the human gene (8). More recently, we showed that the differential regulation of 1α-hydroxylase activity by PTH, calcitonin and 1α,25$(OH)_2D_3$ occur at transcriptional levels in intact animals as well as in MCT cell line (Murayama, A., *et al.*, submitted). To better understand such complex regulation of hormones, we investigated the function of the human 1α-hydroxylase gene promoter by cloning and identifying the 5′ flanking region of this gene. We found that the proximal promoter (-0.5 kb) of the human 1α-hydroxylase gene retains a basal activity in the MCT cell line, where the endogenous 1α-hydroxylase gene is expressed. We further tried to delineate the regulatory region in this promoter, replacing the natural promoter into the tk basal promoter, since the basal promoter of the human 1α-hydroxylase gene seems not to be strong enough to further identify upstream regulatory regions. Treatment with PTH, calcitonin and forskolin to the transfected cells led us to find out the positively regulatory region around -4 kb. In contrast, the negative regulatory region to 1α,25$(OH)_2D_3$ was mapped to the proximal region around - 0.5 kb. From these findings, it is most likely that the transcriptional regulation of the 1α-hydroxylase gene by PTH, calcitonin and 1α,25$(OH)_2D_3$ are mediated through these regulatory regions of the promoter. However, when both regions were fused together, the positive and negative responsiveness were abrogated to some extents (data not shown), raising a possibility that additional counteracted elements and silencer elements are present in these regions. Thus, it is valuable to identify each of the regulatory elements in order to understand the molecular mechanism underlying these regulations.

PTH is well known to induce accumulation of cAMP by activating PKA (19, 28). As a PKA activator, forskolin, induced the 1α-hydroxylase gene expression and activated the promoter, it is most likely that the action of PTH on the 1α-hydroxylase gene expression is exerted through this PKA signaling pathway. The

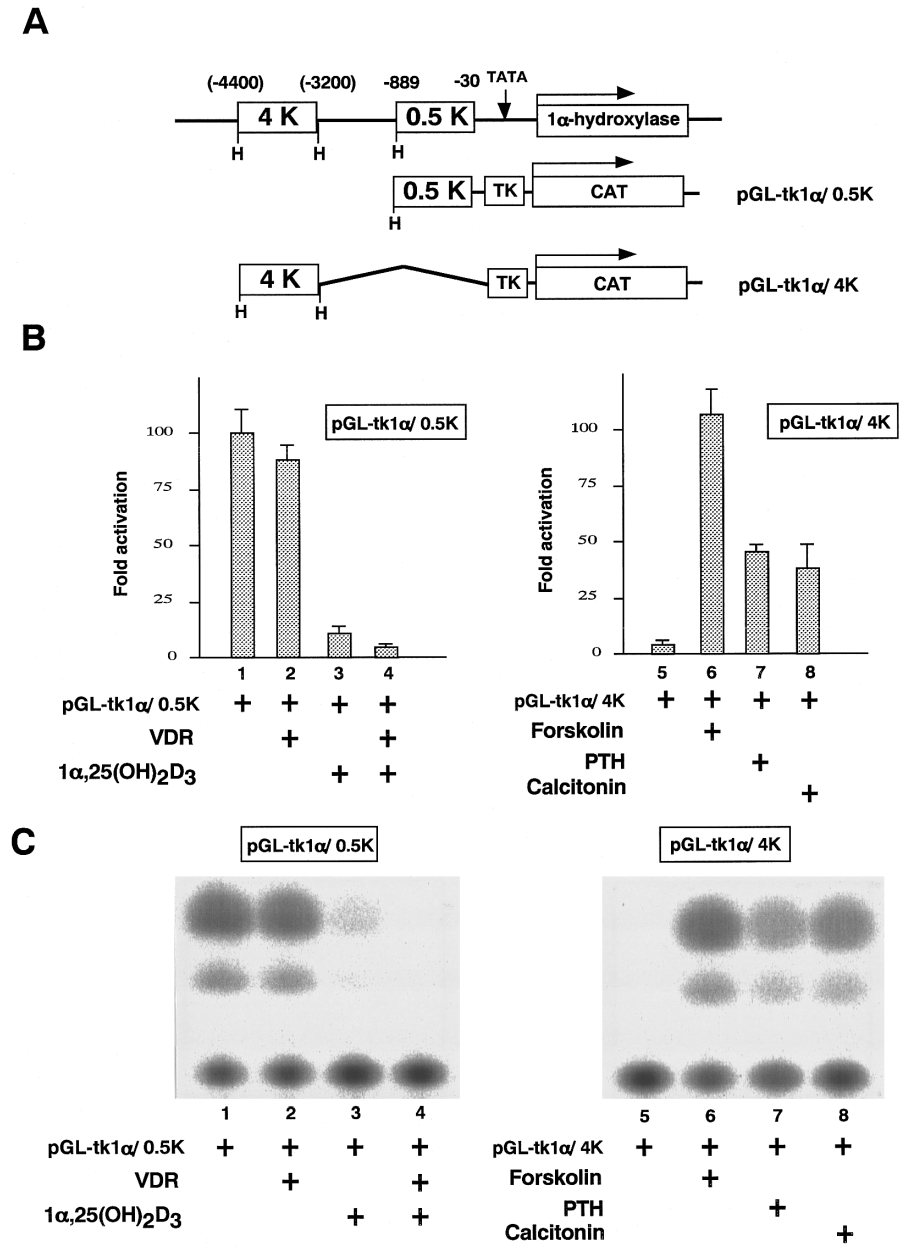

FIG. 4. Identification of a positive regulatory region to PTH and calcitonin, and a negative regulatory region to $1\alpha,25(OH)_2D_3$ in the human 1α-hydroxylase gene promoter. MCT cells were transfected with segmented promoters in the pGL tk-CAT vector as illustrated (A). Transfected cells were treated with 10^{-8} M $1\alpha,25(OH)_2D_3$, 10^{-7} M forskolin, 10^{-7} M PTH, 10^{-7} M calcitonin or vehicle for 24h. Experiments were performed at least three times. Values are mean ± SD (B). The represented results of CAT assay are shown in (C).

exact positive response element for cAMP will be identified by a further study. In this respect, it is necessary to clarify whether the positive element for cAMP responds to calcitonin, since the signaling pathway downstream of this hormone receptor remains to be elucidated (29).

Several negative vitamin D response element (nVDRE) have been identified (30-32), however, a consensus nVDRE has not yet been reached in spite of much effort. In the human 1α-hydroxylase gene promoter, we found no element related to the reported nVDREs in the proximal promoter region (0∼-889), though the inhibitory effect of $1\alpha,25(OH)_2D_3$ was marked in this proximal promoter, even when compared to $1\alpha,25(OH)_2D_3$ action in the mouse 1α-hydroxylase promoter (33). The identification of nVDRE in this promoter is thus very valuable for understanding the molecular mechanism of transcriptional repression by

$1\alpha,25(OH)_2D_3$ through VDR. By the same line of reasoning, a tissue-specific regulatory element in this promoter would be of great interest, since this gene is mainly expressed in a limited site of the kidney, the proximal tubule cells (34).

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