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Biochemical and Biophysical Research Communications 358 (2007) 259–265
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Role of distal upstream sequence in vitamin D-induced expression of human *CYP24* gene

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Received 4 April 2007
Available online 24 April 2007

Abstract

The level of *CYP24* mRNA in cultured human fibroblasts increases up to 20,000-fold in response to 1,25-dihydroxyvitamin D_3. Two vitamin D-responsive elements (VDREs) located immediately upstream of the *CYP24* gene are primarily responsible for the induction. We studied roles of other regions in the 5′-flanking sequence of this gene. A series of deletion constructs between nucleotides −1918 and +209 of the gene were examined for their promoter activities employing the luciferase reporter assay. We found that the VDREs were not sufficient to account for the extent of induction. The sequence between nucleotides −548 and −294, which is located immediately upstream of the VDREs and includes three potential Sp1 sites, acted synergistically with the VDREs for the induction. Further upstream sequence and the 5′-untranslated region did not appear to play a major role in the vitamin D response.

Keywords: Vitamin D; CYP24; VDRE; Sp1; Reporter assay

CYP24 (25-hydroxyvitamin D_3 24-hydroxylase) is a mitochondrial P450 enzyme that plays a key role in the metabolism of 1,25-dihydroxyvitamin D (hereinafter referred to as $1,25(OH)_2D_3$). It carries out hydroxylation of $1,25(OH)_2D_3$ at 24-C, leading to a reduction of the $1,25(OH)_2D_3$ level in blood. The gene encoding this enzyme is expressed in response to $1,25(OH)_2D_3$ [1,2]. We previously reported that in human skin-derived fibroblasts, the induction reaches 20,000-fold 12 h after addition of $1,25(OH)_2D_3$ [3]. Studies of the regulatory sequences upstream of rat and human *CYP24* genes indicated that two VDREs (vitamin D-responsive elements) are responsible for the induction [4–7]. It is known that a heterodimer of vitamin D and retinoid X receptors (the former designated hereinafter as VDR) binds to the VDRE upon association of $1,25(OH)_2D_3$ with VDR [8]. This in turn recruits various coactivators, basal transcription factors, and RNA polymerase II to the promoter region of the gene (reviewed in [9]). Simultaneously, the chromatin structure is converted to an active form by chromatin remodeling complexes such as BRG1/hBrm and WINAC [9,10].

Despite the wealth of knowledge accumulated in the past on the transcriptional regulation of this gene, reporter assays examining the promoter activity of the upstream sequence by transfection have not been able to demonstrate more than 50-fold induction [4–7]. In considering the extremely large fold induction observed with endogenous *CYP24* gene [3], it would be reasonable to assume an involvement of additional regulatory mechanisms that were previously overlooked.

Past studies of the *CYP24* gene promoter were focused on the proximal upstream sequence including VDREs. In the case of human *CYP24* gene, two VDREs are located within 293 bp from the transcription start site. Although in some cases, studies were extended to nucleotide −1262, the reporter gene expression was only increased twofold

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doi:10.1016/j.bbrc.2007.04.103

[7]. These results prompted us to ask whether any other element than those in the proximal upstream sequence would be involved in the regulation of the *CYP24* gene. Here we report that, in addition to the two VDREs, further upstream sequence between nucleotides −548 and −294 is synergistically involved in the regulation of human *CYP24* gene transcription.

Materials and methods

Preparation of deletion constructs of the CYP24 gene's upstream sequence. Human genomic DNA was isolated from skin-derived fibroblasts TIG-112 (Health Science Research Resources Bank (HSRRB), Osaka, Japan), using DNeasy tissue kit (Qiagen) according to the manufacturer's instruction. Various regions of the *CYP24* gene's upstream sequence were amplified by polymerase chain reaction (PCR). The reaction was carried out in a 50 μl mixture containing 1 U KOD-Plus DNA polymerase (TOYOBO), 5 μl of (10×) PCR buffer supplied with the KOD-Plus DNA polymerase, 0.2 mM dNTP, 0.8 mM $MgSO_4$, 0.3 μM each primer as indicated, 30 ng of genomic DNA, and 5% dimethyl sulfoxide. Sequences of the primers used for the PCR are listed in Table 1. Sense primers contained a SacI site as a 5′ add-on sequence. Likewise, antisense primers carried a XhoI site. After initial denaturation for 2 min at 94 °C, 35 cycles of the following steps were performed; 94 °C for 15 s, 60 °C for 30 s, 68 °C for 1 min per 1000 bp of the target size. The final extension was carried out at 68 °C for 7 min. PCR products were digested with SacI and XhoI, and inserted between the SacI and XhoI sites of the pGL3-basic vector (Promega).

Constructs −548ΔGC/+209 and −548ΔVDRE/+209 carrying deletion in the middle of the upstream sequence were prepared by ligating independently amplified distal and proximal upstream fragments. For this purpose, the distal upstream fragment was amplified with primer pairs each carrying an add-on SacI (primer −548s) or MboI (primers −382a and −286a) site, whereas primer pairs for the proximal upstream fragment contained an extra sequence for MboI (primers −294s2 and −150s) or XhoI (primer +209a) as shown in Table 1. These two fragments were then ligated together at their MboI sites. Constructs −548/+13 and −1203/+13, which carry deletion in the 5′-untranslated region (5′-UTR), were generated by amplifying the genomic sequence using primer +13a instead of primer +209a (Table 1 and Fig. 3A).

All recombinant plasmids were isolated using QIAprep spin miniprep kit (Qiagen) and subjected to the cycle sequencing reaction using BigDye Terminator cycle sequencing-ready reaction kit v1.1 (Applied Biosystems). We determined nucleotide sequences employing ABI Prism 310 Genetic Analyzer (Applied Biosystems). Whole sequences of at least one strand were determined and compared with those obtained from the GenBank Accession No. AL138805.

Preparation of a reporter gene construct carrying 3′-flanking sequence of the CYP24 gene. A 2035 bp genomic region, including 777 bp of the last exon of the *CYP24* gene as well as 1258 bp of its downstream sequence, was amplified with KOD-Plus polymerase. Primers used are designated as 3′ENDs and 3′FLNKa (Table 1). Primer 3′ENDs contained a SpeI site originally present in the genome, whereas primer 3′FLNKa includes an add-on BamH1 site. The amplified DNA fragment was digested with SpeI and BamH1, and inserted between the XbaI and BamH1 sites present in the downstream region of the luciferase expression vector. Note that XbaI generates a cohesive end compatible with the SpeI end. This reaction resulted in replacement of the SV40 enhancer and the polyadenylation site originally present in the luciferase expression vector pGL3-basic with the 3′-flanking sequence of the *CYP24* gene. The sequence of the 2035 bp insert was only confirmed in the 350 bp regions from both ends. The 5′-flanking sequence of this reporter construct is identical to that of the construct −548/+209.

Cell culture, transfection, and reporter gene assay. Human skin-derived fibroblasts TIG-112 and HeLa cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. Human skin-derived melanoma cells G-361, obtained from HSRRB, were grown in McCoy's 5A medium (Sigma–Aldrich) supplemented with 10% Serum Plus (JRH Science). About 5×10^5 cells were inoculated into a 35 mm dish. On the following day, they were transfected with 1 μg of the luciferase expression vector and 3 μg of the β-galactosidase expression vector pSV-β-galactosidase (Promega) as an internal control for assessing the transfection efficiency. Lipofectamine 2000 (Invitrogen) was used to promote transfection according to the manufacturer's protocol. $1,25(OH)_2D_3$ dissolved in dimethyl sulfoxide was added to a concentration of 1 μM 24 h after transfection. Control cultures received same volumes of the vehicle. Cells were grown for additional 12 h and then harvested. They were washed twice with phosphate-buffered saline, and then lysed in 200 μl of the reporter lysis buffer (Promega) by three freeze-and-thaw cycles. Luciferase and β-galactosidase activities in cell lysates were assayed using the luciferase assay reagent (Promega) and the Galacto-Light Plus reporter gene assay kit (Applied Biosystems), respectively, following manufacturers' protocols, and detected by a CCD camera linked to the ARGUS-20 image processor (Hamamatsu Photonics). The luciferase activity was normalized to the β-galactosidase activity of the same lysate.

RNA extraction and RT-PCR to quantify luciferase mRNA. Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. RNA samples were digested with DNase I prior to cDNA synthesis in order to reduce residual genomic and plasmid DNA.

Table 1
Primers used for deletion constructs

Constructs	Sense primers		Antisense primers	
	Names	5′——Sequences——3′	Names	5′——Sequences——3′
−294/+209	−294s1	CATGAGCTCAAGCACACCCGGTGAACTCC	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−382/+209	−382s	TATGAGCTCGGCATCGCGATTGTGCAAGC	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−496/+209	−496s	TATGAGCTCTCACTTCAGTCCAGGCTGGG	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−548/+209	−548s	TTGGAGCTCCGCAGAAAGCCAAACTTCCT	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−906/+209	−906s	AGCGAGCTCAGCTCTGTGTGCCCCTCCTT	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−1203/+209	−1203s	CATGAGCTCGGATTTGCCAACACTTGGGG	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−1918/+209	−1918s	GATGAGCTCTCACACTGCCCCACACATAC	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−548ΔGC/+209	−548s	TTGGAGCTCCGCAGAAAGCCAAACTTCCT	−382a	GGCGATCGCTTGCACAATCGCGATGCC
	−294s2	CATGATCAAGCACACCCGGTGAACTCC	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−548ΔVDRE/+209	−548s	TTGGAGCTCCGCAGAAAGCCAAACTTCCT	−286a	TTAGATCGGTGTGCTTCGAACGCGCCT
	−150s	TGAGATCACTCCATCCTCCTTCCACCC	+209a	GTACTCGAGACAGCCTCAGAGCATTGGTG
−548/+13	−548s	TTGGAGCTCCGCAGAAAGCCAAACTTCCT	+13a	AAGCTCGAGCTGTCCCTCTCCATGTTCCTA
−1203/+13	−1203s	CATGAGCTCGGATTTGCCAACACTTGGGG	+13a	AAGCTCGAGCTGTCCCTCTCCATGTTCCTA
3′END/3′FLNK	3′ENDs	AGACTAGTGATTCACTGGGG	3′FLNKa	GTAGGATCCACCTTCTGGCTACTATAGCC

cDNA was synthesized in a 20 μL reaction mixture containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 2 μg total RNA, 0.25 μM oligo-dT primer, 200 U M-MLV reverse transcriptase (Invitrogen), 20 U RNasin (Promega), and 1 mM dNTP. The reaction was carried out for 1 h at 37 °C. Luciferase cDNA was subsequently amplified by PCR in a 12.5 μL mixture containing an indicated amount of cDNA, 0.2 μM each primer described below, and 6.25 μL HotStarTaq Master Mix (Qiagen). After initial activation of the Taq polymerase at 95 °C for 15 min, 26 cycles of the following reactions were performed; 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min. The product was assessed by 8% polyacrylamide gel electrophoresis followed by ethidium bromide staining. The mRNA of the cotransfected β-galactosidase gene was also quantified in the same fashion, and the data were used to normalize the levels of the luc mRNA. Primers used to amplify luc cDNA were 5′-ATGGAAGAC GCCAAAAACAT-3′ (sense primer) and 5′-CTGTTGAGCAATTCACG TTC-3′ (antisense primer), which generate 350 bp DNA representing the N-terminal region of luciferase (GenBank Accession No. U47295). Primers to amplify β-galactosidase cDNA were 5′-GTCGTTTT ACAACGTCGTGA-3′ (sense primer) and5′-GGAACAAACGGCGGATTGAC-3′ (antisense primer), which produce 320 bp DNA corresponding to the N-terminal region of β-galactosidase (GenBank Accession No. X65335).

Results

Deletion analysis of the upstream sequence of the human CYP24 gene

Various regions of the upstream sequence of the human *CYP24* gene were inserted into the promoter site of the luciferase gene in the expression vector pGL3-basic. These constructs were transfected into TIG-112 cells, and transient expression of the luciferase gene was monitored 12 h after addition of $1,25(OH)_2D_3$ (Fig. 1A). Although the construct −294/+209 includes both of the two VDREs, it was not enough to fully respond to $1,25(OH)_2D_3$. An extension of

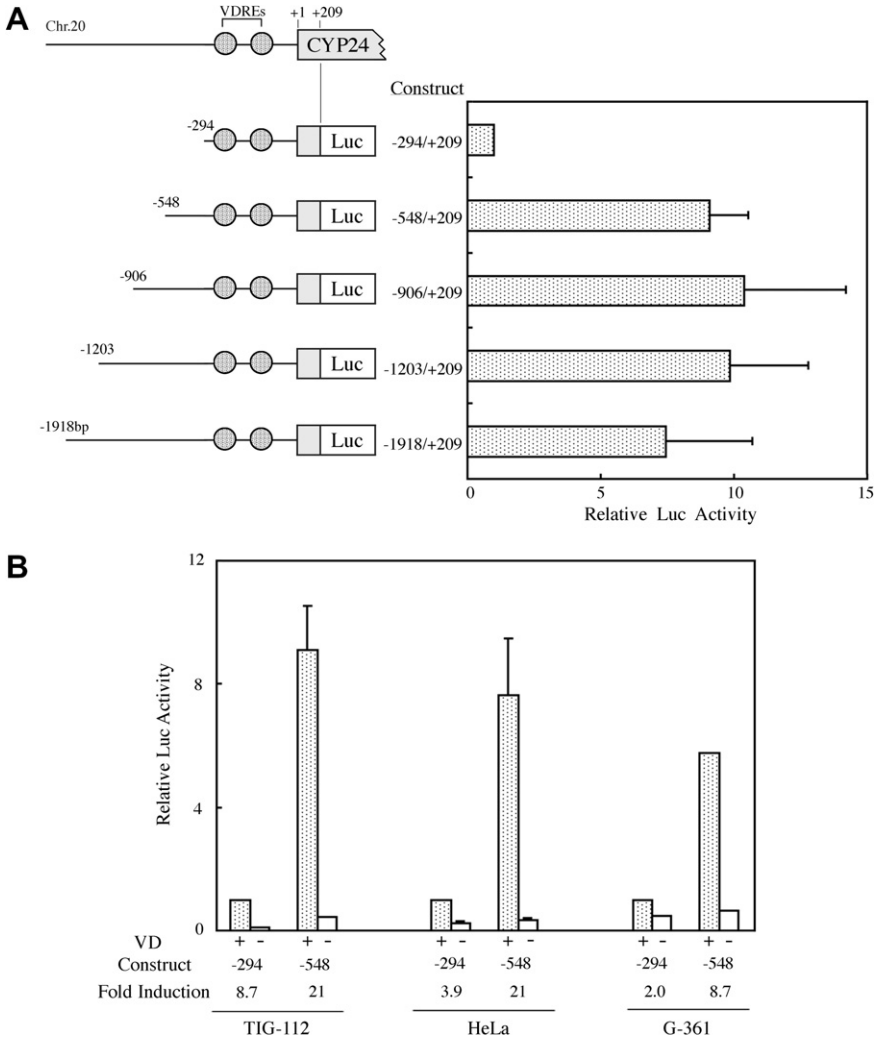

Fig. 1. Sequences immediately upstream of the VDREs are required for activation of the reporter gene expression. (A) Constructs with varying lengths of the distal upstream sequence were ligated to the luciferase gene as schematically depicted on the left, and tested for their promoter activities in transfected TIG-112 cells as described in Materials and methods. Luciferase activities, normalized to those of β-galactosidase from the cotransfected gene, are indicated as ratios to that of construct −294/+209. Bars represent standard deviations (hereinafter SD) of three independent experiments. Luc, luciferase. (B) Two additional human cell lines were examined for the requirement of the distal upstream sequence from nucleotides −548 to −294. The construct, either −548/+209 or −294/+209 as indicated, was transfected into these cells, and expression levels were compared before and after induction with $1,25(OH)_2D_3$. The data were compiled within each cell line as relative activity to that of construct −294/+209 expressed in the presence of $1,25(OH)_2D_3$. Bars, where indicated, represent SD of two independent experiments.

the insert to nucleotide −548 increased the expression level by almost 10-fold (compare constructs −294/+209 and −548/+209), which indicates that the sequence between nucleotides −294 and −548 contains as yet unidentified essential element(s) for the expression. Further upstream sequences did not cause appreciable effects on the expression.

Since roles of the region upstream of VDREs were not described previously, we examined whether the effect described above was due to the particular cell type we used. Two additional human cell lines HeLa and G-361 were tested for the effect of the distal upstream sequence (Fig. 1B). Stimulatory effects of the sequence were observed in both cell lines, supporting the generality of such an effect. Note that the fold induction, i.e., a ratio of expression in the presence and the absence of $1,25(OH)_2D_3$ also increased several fold. Thus the sequence of this region not only promotes transcription but also increases the response to $1,25(OH)_2D_3$.

Deletion analysis of the upstream sequence between nucleotides −548 and −294

Inspection of the distal upstream sequence between nucleotides −548 and −294 revealed the presence of the Sp1-binding core element GGGCGG at three locations as indicated on the top of Fig. 2. In addition, there is a motif similar to the polyadenine element [11] between nucleotides −526 and −497. To examine a role for this region, we made six deletion constructs lacking these elements to varying extents as illustrated in Fig. 2 (left). The luciferase reporter assay indicated that the adenine stretch does not contribute significantly to the *CYP24* gene expression (compare constructs −496/+209 and −548/+209 in Fig. 2). On the other hand, deletion from nucleotides −382 to −294 including two of the potential Sp1 sites, caused an about 70% reduction of expression (compare constructs −548/+209 and −548ΔGC/+209). Likewise, removal of the region containing the most distal potential Sp1 site also led to a significant decrease of expression (compare constructs −382/+209 and −496/+209). Thus the distal upstream sequence between nucleotides −496 to −294 as a whole is involved in the vitamin D-dependent *CYP24* expression. The data shown here further demonstrated that VDREs and the distal upstream sequence are synergistically involved in the activation of *CYP24* expression, because deletion of either one caused a drastic reduction of the reporter gene expression (see constructs −294/+209 and −548ΔVDRE/+209 in Fig. 2).

Deletion analysis of the 5′-UTR

Studies described above was based on the reporter gene constructs whose original 5′-UTR was mostly replaced with the sequence corresponding to nucleotides +1 to +209 of the *CYP24* gene. We examined roles of this region by removing the sequence between nucleotides +13 and +209 in the two constructs −548/+209 and −1203/+209 (Fig. 3A). Regardless of the length of the upstream sequence, the 5′-UTR deletion led to a relatively minor reduction (∼50%) of the luciferase activity.

Since deletion of the 5′-UTR could potentially interfere with the initiation step of translation rather than reduce the mRNA level, we quantified the luc mRNA directly. In this analysis, we needed to use HeLa cells instead of TIG-112, because in the latter cells, expression of the transfected gene was not high enough to generate PCR signals well over the background that was caused by residual transfected DNA. We assessed mRNA levels

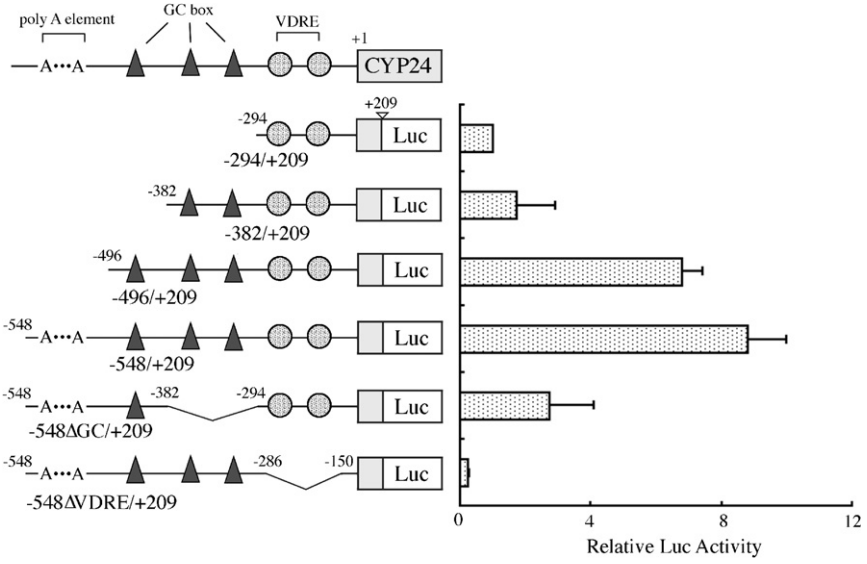

Fig. 2. Synergism of VDREs and the distal upstream sequence. Constructs with deletions of various regions between nucleotides −548 and −150 of the *CYP24* gene were ligated to the luciferase gene, and tested for their promoter activities after transfection into TIG-112 cells. Vitamin D-induced luciferase activities relative to that of construct −294/+209 were compiled as means ± SD ($n = 3$). Note that the middle GC-box was not originally described in Ref. [7] due presumably to a sequencing error or sequence variability in the region of nucleotides −359 to −354.

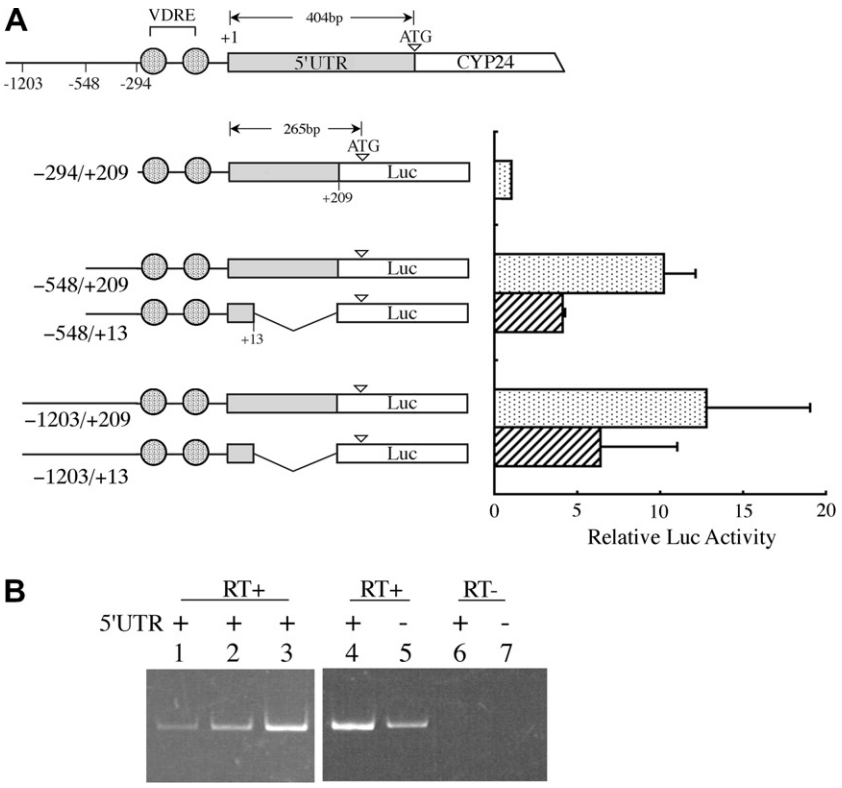

Fig. 3. Effect of the 5′-UTR deletion on the luciferase expression. (A) The 5′-UTR of the human *CYP24* gene consists of 404 bp as depicted on top of the figure. The first 209 bp portion of the 5′-UTR was connected to the last 55 bp portion of the luciferase gene's 5′-UTR to generate the construct −548/+209 as illustrated on the left. On the other hand, construct −548/+13 has only the first 13 bp portion of the CYP24 gene's 5′-UTR. The other construct −1203/+13 was prepared in a similar fashion. These constructs were transfected into TIG-112 cells, and the luciferase activities were assayed 12 h after addition of $1,25(OH)_2D_3$. Fold activities relative to that of construct −294/+209 are shown in the graph. Data shown are means ± SD ($n = 3$). (B) HeLa cells were transfected with construct −548/+209 (lanes under 5′-UTR+) or −548/+13 (lanes under 5′-UTR−), and the levels of luciferase mRNA were assessed by RT-PCR. Lanes 1–3, Luc cDNAs synthesized from 6.25, 12.5, and 25 ng total RNA, respectively, were then amplified by PCR to confirm a roughly linear response of the reaction; lanes 4–7, luc mRNA in 25 ng total RNA was subjected to RT-PCR with (RT+) or without (RT−) reverse transcription as indicated.

under conditions where the PCR signals are roughly proportional to the input of total RNA (Fig. 3B, lanes 1–3), and found that the 5′-UTR deletion resulted in a decrease of the luciferase mRNA level (Fig. 3B, lanes 4 and 5) to about 64% after normalization to the β-galactosidase mRNA level (data not shown). This reduction level more or less paralleled the decrease of the luciferase activity in the same cells (66%). Thus the 5′-UTR of the *CYP24* gene may modulate luc mRNA levels, but does not appear to play a major regulatory role for the *CYP24* transcription.

Role of the 3′-flanking sequence of the CYP24 gene

Although the endogenous *CYP24* gene displays a 20,000-fold increase of expression mediated by $1,25(OH)_2D_3$, the upstream sequence so far examined only accounts for a 20-fold induction at most (Fig. 1B). We searched for additional regulatory sequences in the 3′-flanking sequence of the *CYP24* gene. The SV40-derived poly(A) signal was originally present downstream of the luciferase gene in the pGL3-basic vector. We replaced it with a 2035 bp genomic sequence that origi-

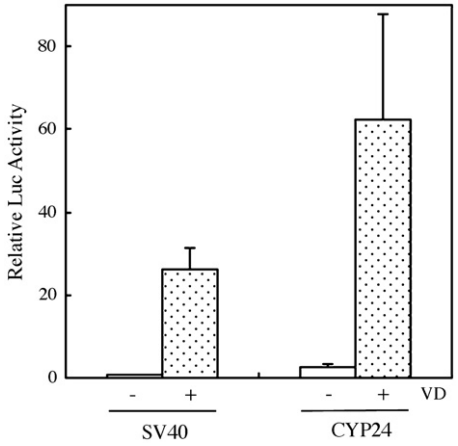

Fig. 4. Effect of the *CYP24* gene-derived 3′-flanking sequence on the luciferase expression. The 3′-flanking sequence of the luciferase gene in the construct −548/+209 contained the SV40-derived polyadenylation signal. This region was replaced with the 3′-flanking sequence of the human *CYP24* gene (construct 3′END/3′FLNK in Table 1). Both of the constructs were transfected into TIG-112 cells, and examined for the luciferase activity expressed in the presence or absence of $1,25(OH)_2D_3$. Data are shown as fold activity relative to that of the construct −548/+209 observed in the presence of $1,25(OH)_2D_3$. Values are means ± SD ($n = 2$). SV40, SV40-derived 3′-flanking sequence; CYP24, *CYP24* gene-derived 3′-flanking sequence.

nates from the 777 bp region of the 3′-UTR along with the adjacent 1258 bp region downstream of the human *CYP24* gene. In addition, a fragment between nucleotides −458 and +209 of the *CYP24* gene was placed upstream of the reporter gene in this construct. Transient transfection assays indicated that the luciferase expression was only increased about twofold by the *CYP24* gene-derived 3′-flanking sequence. In addition, the fold induction did not change due to a simultaneous increase of the basal expression (Fig. 4). Thus we did not find any major regulatory elements in the 3′-flanking region.

Discussion

We have described deletion analyses of the 5′-flanking sequence as well as 5′-UTR of the human *CYP24* gene. Most of our effort was directed to the analyses of the region between nucleotides −548 and −294, which is located immediately upstream of the two VDREs, and was not fully studied previously. We found that this region contains sequence elements that synergistically function with the nearby two VDREs. Neither of the sequences was dispensable for the expression of the *CYP24* gene. The sequence between nucleotides −548 and −294 contains three Sp1 site-like elements and the whole area appears to be required for the expression of the gene. We suggest that these elements might indeed work as Sp1 sites based on the following reasons.

Synergism between a nuclear receptor and Sp1 appears to be common in many genes driven by nuclear receptors. For example, it is known that the estrogen-responsive elements have nearby Sp1 sites to cooperate with estrogen-receptors (ERs) for stimulation of the cognate gene expression (reviewed in Ref. [12]). Furthermore, some Sp1 sites are known to act as an indirect estrogen- or vitamin D-responsive element upon association with the Sp1/ER or Sp1/VDR complex, respectively [12,13].

Although it is not clear why previous work failed to reveal a role of the distal upstream sequence of the *CYP24* gene, we suggest that the heterologous system, where the human *CYP24* regulatory sequence was tested for its function in transfected murine cells, might not have worked properly [7]. It is also possible that the slightly inhibitory effect of the far upstream sequence between nucleotides −1918 and −548 (Fig. 1) masked the role of the sequence between nucleotides −548 and −294.

In sum, our data indicate an important role of the distal upstream sequence in the regulation of the human *CYP24* gene expression. Presence of three Sp1 site-like elements in this region suggests that formation of a complex of Sp1, VDR, and DNA might be involved in the initiation of transcription. Such a complex could also contain the Ets1 factor that is implicated in the activation of vitamin D-responsive genes [14].

Finally we should point out that, while a number of regulatory mechanisms underlying human *CYP24* gene expression have been elucidated [1,2], none of them can fully account for the extremely high induction ratio of this gene. In transient expression systems described to date displayed only several-tens fold induction of the reporter gene. Aberrant binding of transcription factors to the vector sequences might partially contribute to increasing basal transcription and thereby lowering the induction ratio. Nevertheless, the major mechanisms of the remaining 100- to 1000-fold induction is still open to question. It would be of interest to uncover additional regulatory mechanisms apparently operating in this system.

Acknowledgment

This work was supported in part by a grant awarded to M.R. from the Hiroshima Prefectural Government in Japan.

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