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Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 527–532
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Towards a complete picture of splice variants of the gene for 25-hydroxyvitamin $D_3$1α-hydroxylase in brain and skin cancer☆

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Abstract

Recently, we reported amplification of the gene encoding the P450 Cytochrome 25-hydroxyvitamin $D_3$1α-hydroxylase (CYP27B1) in 25% of human malignant glioma. Additionally, we reported the first alternative splice variants of CYP27B1. Here, we developed and employed a highly specific approach that combined nested and touchdown PCR to clone full length CYP27B1. In addition, we identified several new splice variants in human melanoma, glioblastoma multiforme (GBM), cervix carcinoma and kidney cell lines. All of the examined cell lines showed a similar expression pattern of the CYP27B1 variants. The new splice variants that were termed Hyd-V5, -V6, -V7, and -V8 were cloned and sequenced. All but one of the new variants showed an insertion of intron 1 leading to a premature termination signal and to truncated proteins without ferredoxin and haem-binding site of the P450 protein. There was no influence of 1α,25$(OH)_2D_3$ on the expression pattern of the splice variants in melanoma cell line SkMel28.

Keywords: Glioblastoma multiforme; Melanoma; CYP27B1; Extra-renal expression; Alternative splicing; 1α,25$(OH)_2D_3$

1. Introduction

1,25-Dihydroxyvitamin D3 (1α,25$(OH)_2D_3$), the biologically active metabolite of Vitamin D, has been shown to regulate the growth of various cell types, including human melanocytes [1,2] and glioma cell lines [3]. Analysis of numerous normal and cancer cell lines demonstrates that at high concentrations (10^{-9} to 10^{-7} M) 1α,25$(OH)_2D_3$ inhibits the growth of tumor cells in vitro and it has also been shown that 1α,25$(OH)_2D_3$ has beneficial effects in several in vivo models of various types of cancer [4,5].

There are two principal enzymes involved in the formation of circulating 1α,25$(OH)_2D_3$ derived from Vitamin D. The hepatic microsomal or mitochondrial Vitamin D 25-hydroxylase (25-OHase) catalyzes the hydroxylation of Vitamin D and the renal mitochondrial enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase, CYP27B1) catalyzes 25$(OH)D_3$, respectively [6,7]. The 1α-OHase catalyzes the synthesis of the biological active form of Vitamin D, 1,25-dihydroxyvitamin D_3, calcitriol. This secosteroid hormone is best known for its role in calcium– and bone metabolism. In addition, calcitriol provides antiproliferative and differentiating effects through binding to the vitamin D receptor (VDR) belonging to the steroid/thyroid/retinoic acid receptor family which functions as a ligand dependent transcription factor [8].

Here we describe the expression of full length 1α-OHase in human melanoma, GBM, cervix carcinoma, and kidney cells and report several new splice variants in addition to the already in GBM known variants [9].

2. Materials and methods

2.1. Cell lines

Cell line TX3868 was established and cultured as described previously [10]. HEK293 and HeLa cell lines were purchased at ATCC.

2.2. RNA-isolation and plasmid-isolation

RNA and mRNA isolation was carried out according to manufacturer's instructions (RNeasy Mini, Oligotex mRNA,

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

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doi:10.1016/j.jsbmb.2004.03.062

QIAgen, Germany). The RNA was quantified spectrophotometrically, and its integrity was controlled by agarose gel electrophoresis in MOPS buffer. In order to screen *Escherichia coli* transformants, plasmid DNA from bacterial cultures was isolated using alkaline lysis without a further purification step [11]. A column purification was added for plasmids that were sequenced (Plasmid Mini Kit, QIAgen, Germany).

2.3. RT-PCR

Prior to utilization, RNA was DNaseI (Promega) treated. First-strand cDNA was synthesized with Omniscript reverse transcriptase (QIAgen, Germany) using oligo-d$(T)_{15}$ primer. The existence of genomic DNA contaminants was excluded via RT-minus reaction. After the first PCR using primers Sp1aFor (5′-GGA GAA GCG CTT TCT TTC G-3′) and Sp1aRev3 (5′-AAA CCA GGC TAG GGC AGA TT-3′) with 10 cycles (10 s at 94 °C, 20 s at 62 °C, 4 s at 72 °C) the PCR reaction was purified (PCR purification kit; QIAgen, Germany) and 2 μl were used as template for the second PCR using primers Sp1aRev3 (5′-TGG GGC AAA CCC ACT TAA TA-3′) and HE1 (5′-CAG ACC CTC AAG TAC GCC-3′). This PCR consisted of first 12 cycles with a touchdown from 68 to 62 °C (10 s at 94 °C, 20 s at 68 °C with touchdown in 0.5 °C intervals to 62 °C, 4 s at 68 °C) followed by 18 cycles (10 s 94 °C, 20 s at 62 °C, 4 s at 8 °C). Both PCR reactions were performed with RedAccuTaq (Sigma). The PCR products were separated on a 1% agarose gel.

Cloning of PCR products in vector pCR4-TOPO was performed using TOPO TA Cloning Kit for sequencing (Invitrogen) and carried out to manufacturer's instructions.

2.4. Sequence analysis

Sequencing was performed according to the manufacturer's instructions using the Amersham Thermo Sequenase labeled primer cycle sequencing kit with 7-deaza-dGTP. Plasmid inserts were sequenced with an automated sequencer (Licor 4000L, MWG Biotech, Ebersberg, Germany). The obtained sequences were edited using the SEQUENCHER^{TM} 3.0 program (GeneCodes, Michigan, USA). Homology search was done with the BLASTN and BLASTX algorithms [12].

3. Results

3.1. Expression of splice variants in several cell lines from different origin

Previously, our attempts failed to amplify the entire 1α-OHase cDNA. We recently reported partial PCR-amplification of the 1α-OHase cDNA including variants that lack exons 4 and 5 [9]. This approach was, however, restricted to identification of truncated 1α-OHase splice variants. To adequately describe the full length expression of 1α-OHase gene, we now developed a highly specific approach that combined nested and touchdown PCR (Fig. 1). Using this method, we confirmed the expression of the normal enzyme (2.15 kb) in cell lines originating from several tissues including human melanoma, GBM, cervix carcinoma, and kidney (Fig. 1B). In addition, we describe the appearance of splice variants others than the previously reported variants at 1.78 kb (Hyd-V2), 1.97 kb (Hyd-V3), and 2.23 kb (Hyd-V4). All of the examined cell lines appear to have a complex, but similar expression pattern of the

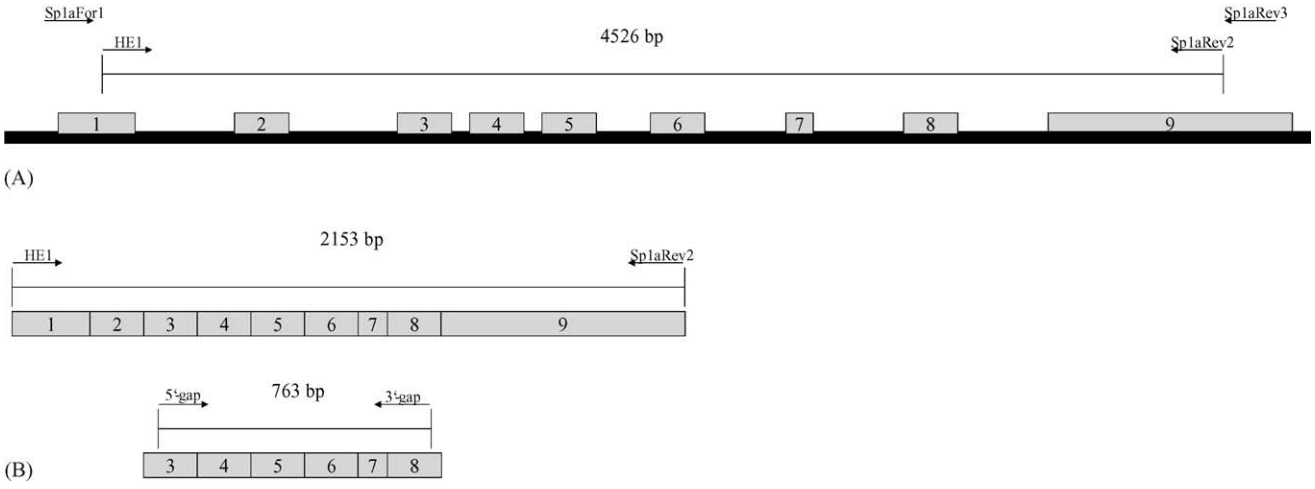

Fig. 1. (A) Primer localization for nested-touchdown PCR in the 1α-OHase gene, showing exon/intron structure (see Section 2). (B) RT-PCR products of the 1α-OHase cDNA generated by nested-touchdown PCR (HE1 and SplaRev2) vs. partial PCR-amplification including variants that lack exons 4 and 5 (5′-gap and 3′-gap).

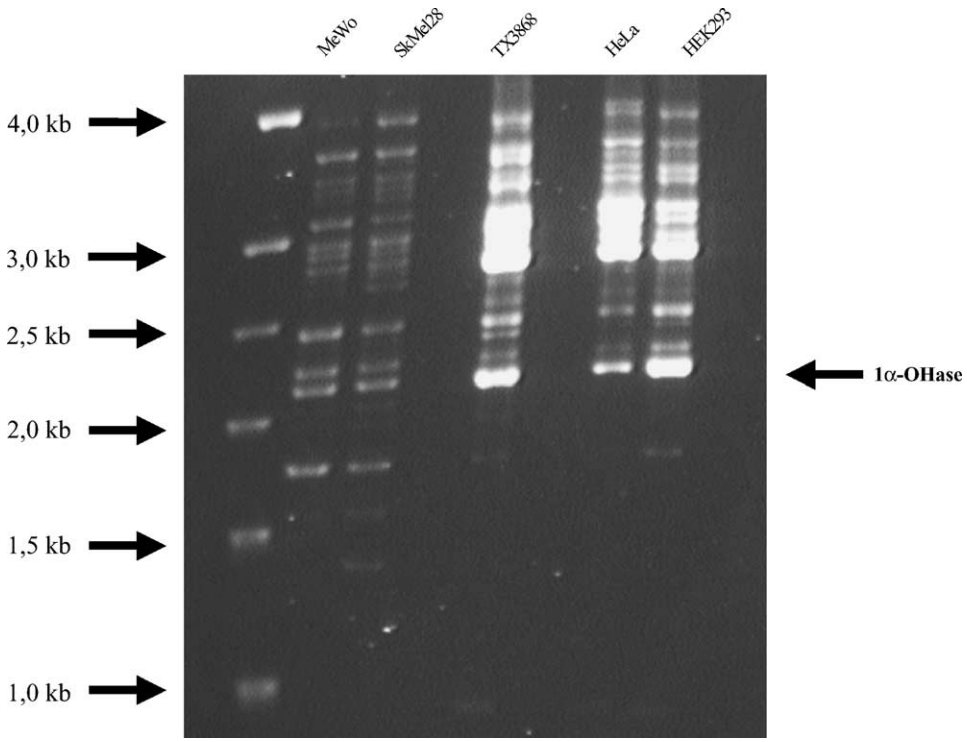

Fig. 2. Two-step RT-PCR (nested-touchdown PCR) with mRNA from melanoma cell lines MeWo, SkMel28, glioblastoma cell line TX3868, from which the first splice variants were described, cervix carcinoma cell line HeLa and embryonal kidney cell line HEK293. The band according to the normal 1α-OHase cDNA sequence is marked.

1α-OHase gene (Fig. 2). Using specific primers from exons 1 and 9, we detected up to 20 PCR products (1–4 kb). This approach did not allow quantitative measurements, due to the high product variety.

3.2. Identification and cloning of new splice variants of the 1α-OHase gene

In order to analyze the newly detected splice variants, we cloned an aliqot of the described nested-touchdown-PCR products in pCR4-TOPO (TOPO TA Cloning Kit for sequencing, Invitrogen). Examining 24 clones, we exclusively detected 1α-OHase specific inserts, underlining the high specificity of this approach.

Restriction analysis of the resulting transformants revealed at least four different inserts. Sequence analysis of the subcloned cDNAs and comparison with the known sequence for 1α-OHase and the already known splice variants indicated that these clones represent further splice variants. They were named hydroxylase-variant 5, 6, 7, and 8 (Hyd-V5, -V6, -V7, and -V8; Fig. 3). A portion of variant Hyd-V5 showing an insertion of intron 5 was already detected with the RT-PCR with primers from exons 3 and 8. This insertion results in a frameshift leading to a termination codon after the first 84 bp of exon 6. In silico translation results in an truncated protein with 418 amino acids. Variant Hyd-V6 shows an insertion of intron 1 resulting in a very short protein of 81 amino acids in contrast to 508 amino acids encoded by the full-length 1α-OHase. This is due to a termination codon that maps after the first 48 bp in intron 1 and is caused by a frameshift. Variant Hyd-V7 has an insertion of intron 1 and lacks exons 4 and 5, resulting in the same termination codon as Hyd-V6. Hyd-V8 contains two insertions of intron 1 as well as intron 5. This variant also contains the described termination codon in exon 1. All of newly detected splice variants result in proteins that neither contain the ferredoxin nor the haem-binding site of the P450 protein, and therefore probably represent inactive variants of the enzyme.

Searching the human EST database, we confirm the variable expression pattern of the 1α-OHase gene. We found three EST's containing intron 1 that is retained in Hyd-V6, -V7, and -V8 (BX111995, 964 bp; AL433907, 917 bp; AL698396, 797 bp). A further variant is indicated by another EST (BG395700) containing intron 3. The variant Hyd-V5 is represented as full length sequence in GenBank entries BC020267 and BC001776.

3.3. Vitamin D treatment of melanoma cell line SkMel28

We treated SkMel28 with 10^{-7} M 1α,25$(OH)_2D_3$ to investigate the possible influence on the auto-regulation of 1α-OHase activity via the product of the alternative splicing in melanoma cell lines. After different times we isolated mRNA and performed the described nested-touchdown-PCR (Fig. 4). SkMel28 shows response to the antiproliferative

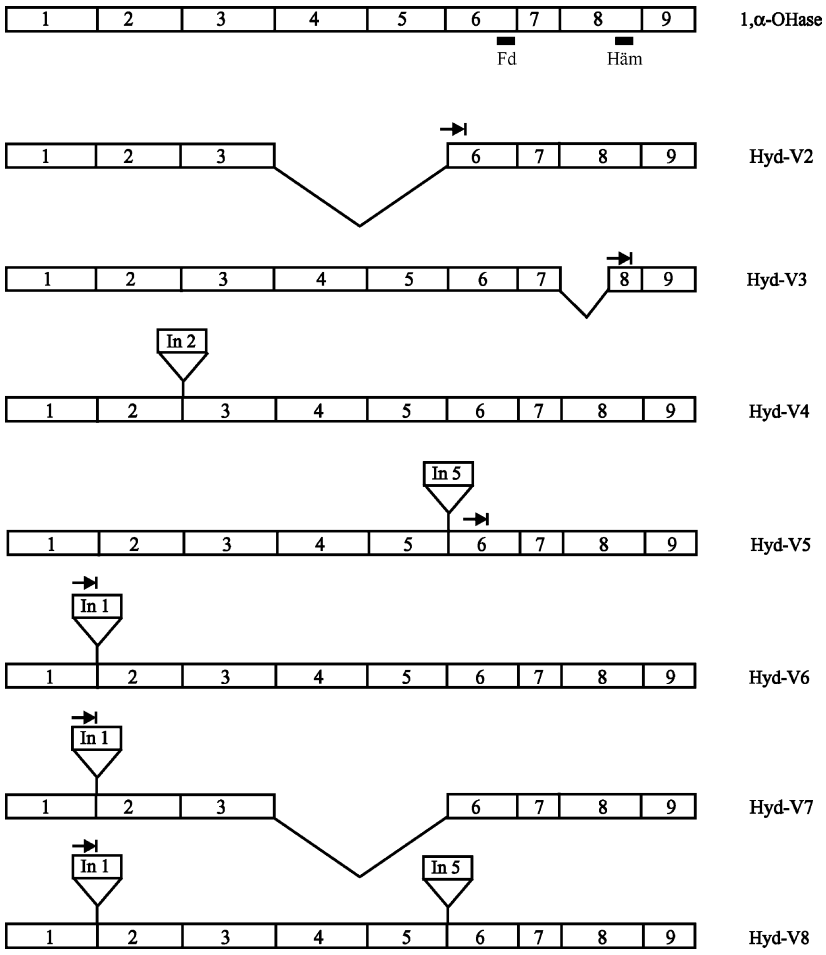

Fig. 3. Structures of the normal and the alternatively spliced 1α-OHase mRNAs. The coding regions for the ferredoxin-binding site in exon 6 and for the haem-binding site in exon 8 are indicated by barrs in the normal 1α-OHase mRNA. Premature stop codons are marked by arrows. Newly detected splicing variants are Hyd-V5, -V6, -V7, and -V8.

effect of 1α,25(OH)$_2$D$_3$, as shown by WST proliferation assay (preliminary data, not shown). Expression analysis of the 25-hydroxyvitamin D$_3$ 24-hydroxylase shows a strong increase of expression after treatment with 1α,25(OH)$_2$D$_3$ (preliminary data, not shown). All various time points showed similar expression pattern of the 1α-OHase gene. As above, this approach does not allow quantitative analysis.

4. Discussion

We show the expression of full length 1α-OHase in human melanoma, GBM, cervix carcinoma, and kidney cells. As of yet, extra-renal expression of 1α-OHase has been reported for a wide variety of tissues [13]. In vitro, many non-renal cells, including bone, prostate [14], kidney [15], keratinocytes [16], macrophages, T-lymphocytes [17] and microglial cells [18] produce 1α,25(OH)$_2$D$_3$ from its precursor. Also, several cancer cells from prostate, nonsmall cell lung carcinoma [19] and skin show 1α-OHase activity.

We report the expression of different 1α-OHase splice variants. All investigated cell lines show a very complex expression pattern of the 1α-OHase gene (Fig. 2). Using an approach to detect full length clones we found up to 20 PCR products (1–4 kb). Cloning of these products showed, in addition to already known splice variants, three variants, all of which containing intron 1. Insertion of intron 1 causes an early stop codon and the resulting mRNA will not be translated in a functional protein. The non sequenced PCR-products most likely represent alternatively spliced variants containing intron 1 combined with different other introns. This finding is supported by three EST GenBank entries containing a part of intron 1. In addition, most of these PCR products are greater than the normal full length transcript and the remaining introns of the 1α-OHase gene are small (Fig. 1A). We furthermore describe a variant that contains intron 5 and that is most likely not enzymatically active. Only one of the described variant, Hyd-V4, has the potential to encode an active enzyme.

It has been reported that the expression and regulation of 1α-OHase in extra-renal tissue is different from that

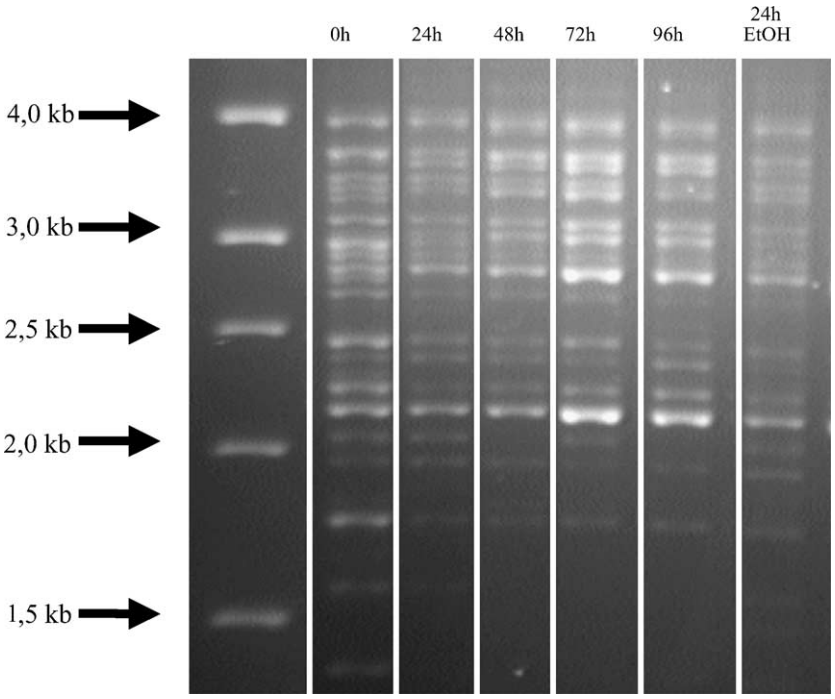

Fig. 4. Treatment of SkMel28 cells with 1α,25(OH)$_2$D$_3$ (10^{-7} M) and vehicle alone (ethanol). Isolation of mRNA after different times of treatment and nested-touchdown PCR. No significant differences can be detected.

observed for the kidney enzyme. Prior to the cloning of the renal 1α-OHase gene, it seemed likely that extra-renal production 1α,25(OH)$_2$D$_3$ was due to a separate enzyme. However, it is now confirmed that extra-renal 1α-OHase activity is due to a single gene product. Classically, the addition of 1α,25(OH)$_2$D$_3$ inhibits the renal 1α-OHase [20]. Addition of exogenous 1α,25(OH)$_2$D$_3$ did not inhibit macrophage 1α-OHase as observed in its renal counterpart. Alternative splicing of the 1a-OHase gene may be an important factor in regulating the enzyme activity and may cause the differences between renal and extra-renal tissue. Decreased 1α,25(OH)$_2$D$_3$ activity, as shown in several tumor cell lines, e.g. CA-derived prostate cells [21] may be caused by alternative splicing. So far, treatment of melanoma cell lines with 1α,25(OH)$_2$D$_3$ (10^{-7} M), showed no differences in the expression pattern indicating that the expression pattern is independent from 1α,25(OH)$_2$D$_3$ (Fig. 4).

It is well documented that alternative splicing plays a biologically important function. Alternative splicing is a frequent feature in the expression of many P450 genes. It is considered as an important factor in regulating the enzyme level and may be the cause for tissue-specific variation [22]. Several studies show that alternative splicing occurs frequently in human cancers cells, e.g. breast and ovarian cancer although the cause of the increase is not known [23,24]. Alternative splicing has been suggested to have the potential as a diagnostic marker for cancer [25].

The effect of the expression of alternative transcripts on the 1α-OHase activity level in cancer and normal tissues has to be investigated in further experiments.

Acknowledgements

We thank Gertrud Schwär for technical assistance. We thank Lise Binderup (LEO Pharmaceuticals, Denmark) for kindly providing 1α,25(OH)$_2$D$_3$.

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