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Characterization of Expression of the Gene for Human Pterin Carbinolamine Dehydratase/Dimerization Cofactor of HNF1

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ABSTRACT

Pterin carbinolamine dehydratase/dimerization cofactor of HNF1 (PCD/DCoH) is a dual-function protein. In the cytoplasm it acts as a dehydratase in the regeneration of tetrahydrobiopterin, the cofactor for aromatic amino acid hydroxylases. In the nucleus, it functions as a dimerization cofactor of HNF1 and increases the transcriptional activity of HNF1. To deepen our understanding of this protein, we characterized its expression in human tissues and cells. Human PCD/DCoH was present predominantly in liver and kidney, with significant amounts in testis and ovary, trace amounts in lung, and undetectable levels in whole brain, heart, and spleen. It was expressed in all of the cells that were examined. Importantly, it was also present in the nucleus of HeLa cells, which lack HNF1, and in the cytoplasm of fibroblasts that have little or no tetrahydrobiopterin. The expression of human PCD/DCoH in the liver and nonhepatic cells was compared at both the mRNA and protein levels. Although the mRNA level in liver was only fourfold higher than that in keratinocytes and fibroblasts, the hepatic PCD/DCoH protein level was 20-fold higher than that in normal human epidermal keratinocytes and dermal fibroblasts. Cloning of the 5′ and 3′ untranslated region (UTR) of human keratinocyte PCD/DCoH revealed that it has 53 bp more of GC-rich 5′ untranslated sequence than the published liver PCD/DCoH. *In vitro* transcription and translation analysis showed that the longer 5′ UTR resulted in about a 35% decrease in translation efficiency. These data show that human PCD/DCoH is not only present in cells where tetrahydrobiopterin is synthesized or HNF1 is present but is a widely distributed protein. Its differential expression in different tissues and cells is regulated not only at the transcriptional level but also at the translational level.

INTRODUCTION

As its name implies, pterin carbinolamine dehydratase/dimerization cofactor of HNF1 (PCD/DCoH) has at least two functions. First, it is a pterin 4α-carbinolamine dehydratase that is involved in the regeneration of tetrahydrobiopterin (Huang and Kaufman, 1973; Lazarus *et al.*, 1983), the cofactor for the aromatic amino acid hydroxylases. During the hydroxylation of the aromatic amino acids catalyzed by these hydroxylases, tetrahydrobiopterin is initially converted to pterin 4α-carbinolamine. This pterin product then undergoes PCD-catalyzed dehydration to produce quinonoid dihydrobiopterin, which is eventually reduced back to tetrahydrobiopterin by the NADH-dependent dihydropteridine reductase. Although the dehydration of pterin 4α-carbinolamine also occurs nonenzymatically at physiologic pH *in vitro*, patients with a deficiency of this enzyme suffer from a newly recognized form of hyperphenylalaninemia characterized by the urinary excretion of relatively large amounts of a chemically rearranged form of biopterin, 7-biopterin (Adler *et al.*, 1992; Citron *et al.*, 1993), which is a dead-end product, uncoupled cofactor (Davis and Kaufman, 1991), and a potent inhibitor of phenylalanine hydroxylase (Adler *et al.*, 1992; Davis *et al.*, 1992b), indicating an important physiological role of PCD in the regeneration of tetrahydrobiopterin and metabolism of phenylalanine. This function is further supported by the *in vitro* studies showing

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that the nonenzymatic rearrangement of 6-biopterin to 7-biopterin occurs in the absence of PCD and can be suppressed in the presence of PCD (Curtius *et al.*, 1990; Davis *et al.*, 1991).

The second identified function of PCD/DCoH is that of a dimerization cofactor of HNF1. It was originally copurified with HNF1 from rat liver nuclear extracts (Mendel *et al.*, 1991). Recently, it was shown that cotransfection of DCoH with an HNF1-expression plasmid into a human cell line modifies the intranuclear localization of DCoH (Sourdive *et al.*, 1997), suggesting that interaction of DCoH and HNF1 occurs in their natural cellular environment. *In vitro* studies showed that DCoH stabilizes HNF1 dimers by forming DCoH-HNF1 heterotetramers (Mendel *et al.*, 1991). It was also observed that DCoH increases the transcriptional activity of HNF1 by cotransfection assays (Mendel *et al.*, 1991; Pogge von Strandmann and Ryffel, 1995). Understanding of the effects of DCoH on the expression of HNF1-regulated genes under physiologic conditions awaits DCoH knockout mice analysis.

Recent crystal structural studies have led to speculation about other potential functions of PCD/DCoH. The crystal structure of PCD/DCoH reveals that this protein is a tetramer containing two saddles that have the potential to bind macromolecules such as proteins and nucleic acids (Endrizzi *et al.*, 1995; Ficner *et al.*, 1995). The overall pattern of PCD/DCoH folding is similar to that seen in the TATA-binding protein, a general transcription factor. These findings have raised the possibility that PCD/DCoH might be a general transcription factor or have partners other than HNF1. Additional information about the expression of PCD/DCoH would appear to be crucial for the investigation of the possible additional functions of this protein. In this study, we have characterized the expression pattern of PCD/DCoH with both human tissue and cell cultures and compared its hepatic and nonhepatic expression.

MATERIALS AND METHODS

Cell culture and tissues

Cultures of normal adult human epidermal keratinocytes (NHEK) and normal human dermal fibroblasts (NHDF) were purchased from Clonetics. Induction of differentiation of NHEK was carried out as previously described (Lei *et al.*, 1997). The NHDF were maintained in Fibroblast Growth Medium 2 (Clonetic) containing recombinant human fibroblast growth factor-basic 1 ng/ml, 2% fetal bovine serum, and gentamicin 50 μg/ml and amphotericin B 50 ng/ml in the basal medium. Passage 3 or 4 of these cells was used. Human white blood cells (HWB) were isolated from the buffy coat fraction of blood prepared by spinning whole blood at 4000 rpm for 6 min, taking out the middle layer of HWB, and mixing them with a small amount of plasma. The remaining red blood cells in the buffy coat fraction were selectively lysed by adding three volumes of Red Blood Cell Lysis Solution (PGC Scientific Corp.). White blood cells were then pelleted by centrifuging at 800 rpm for 5 min with a bench centrifuge (Sorvall RT 6000D; Dupont) in a 50-ml culture tube. Pelleted HWB were washed three times with phosphate-buffered saline (PBS). Human liver was provided by the National Disease Research Interchange (Philadelphia). Electrophoresis-ready Human Tissue Protein Medley was purchased from Clontech. HeLa cell nuclear extracts, isolated according to the protocol of Dignam *et al.* (1983), were purchased from GIBCO/BRL.

Immunoprecipitation

Immunoprecipitation was carried out as previously described (Lei *et al.*, 1997). The amount of cell extract used for immunoprecipitation and the amount of immunoprecipitated protein loaded for SDS-PAGE are indicated in the text.

Western blot analysis

Cell or tissue extracts (amounts indicated in the legends) were loaded for 18% SDS-PAGE. After electrophoretic separation, proteins were electrically transferred to a PROTEIN nitrocellulose membrane (Novex). The membrane was first incubated in TBST buffer (20 mM Tris HCl, pH 7.5; 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. After a 1-h incubation, the rabbit anti-rat dehydratase antibody developed by our laboratory was added in a 10,000-fold dilution, and incubation was continued overnight. After thorough washes with TBST buffer, the secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (Promega), was diluted 5000 fold in TBST buffer containing 5% nonfat dry milk and incubated for 2 to 3 h. The Chemiluminescent detection kit (NEN Life Science Products) was used for signal development.

Preparation of liver nuclear extract

Nuclei were prepared from fresh rat liver by the sucrose gradient centrifugation protocol as described in the *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1987). The process was monitored by phase-contrast microscopic observation. After isolation of nuclei, nuclear proteins were extracted by ammonium sulfate precipitation following the protocol of Wang *et al.* (1994).

Isolation of total RNA

Total RNA was isolated from human liver, NHEK, and NHDK using Trizol reagent (Life Technologies) according to the manufacturer's protocol. Total RNA from HWB was prepared using a Purescript whole-blood RNA isolation kit (PGC Scientifics Corp, Frederick, MD). The RNA concentration was determined by measuring optical absorbance at 260 nm.

Reverse transcriptase-polymerase chain reaction

The first-strand cDNA was synthesized with Superscript II RNase H$^-$ reverse transcriptase (RT) (GIBCO/BRL) from 5 μg of total RNA in a final volume of 20 μl using poly(dT) as a primer. The synthesized cDNA was diluted 10 fold, and 2 μl of the diluted fraction was subjected to PCR amplification of the PCD/DCoH coding region. The PCR products were cloned into pUC18 as before (Lei *et al.*, 1997) for sequencing. A 983-bp fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) coding region was PCR amplified for 25 cycles from 1 μl of the above dilution with 5′ primer (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′) and 3′ primer (5′-CATGTGGGCCATGAGGTCCACCAC-3′) (Clontech). The cycling conditions were as follows: denaturation at 94°C for 45

sec, annealing at 60°C for 45 sec, and extension at 72°C for 2 min.

Cloning of 5′ and 3′ UTR of the human keratinocyte PCD/DCoH by rapid amplification of cDNA ends

The 5′ and 3′ RACE were performed with kits from GIBCO/BRL following the provided protocols with some modification. The first-strand cDNA was synthesized with 5 pmole of GSP1 (5′-AGTTCAGTCACCCTTTCCCC-3′) (Thony *et al.*, 1995) at 50°C for 50 min, rather than at 42°C. After purification of the first-strand cDNA with GlassMax DNA Isolation Spin Cartridges (GIBCO/BRL), terminal deoxynucleotidyl transferase (TdT)-catalyzed dC tailing of the cDNA was carried out in the presence of 10% dimethylsulfoxide. The dC-tailed cDNA was PCR amplified with abridged anchor primer (AAP) (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIG-GGIIG-3′) (GIBCO/BRL) and the hPHS 3′ primer (5′-CGGGATCCTATGTCATGGACACTGCTAC-3′) (Thony *et al.*, 1995) under the same condition as PCR amplification of PCD/DCoH coding sequence. For 3′ RACE of the keratinocyte 3′ UTR, the first-strand cDNA was synthesized using adapter primer (AP) (5′-GGCCACGCGTCGACTAGTACTTTTTTT-TTTTTTTTTT-3′) (GIBCO/BRL). Abridged Universal Amplification Primer (AUAP) (5′-GGCCACGCGTCGACTAGTAC-3′) (GIBCO/BRL) and the hPHS 5′ primer (5′-CGGA-ATTCATATGGCTGGCAAAGCACACAG-3′) (Thony *et al.*, 1995) were used for subsequent PCR amplification. Touchdown PCR with hot start at 80°C was employed to elicit a single PCR band. Cycling conditions were as follows: denaturation at 95°C for 40 sec, annealing at 60°C for 60 sec, extension at 72°C for 75 sec, and the following 4 cycles with 1°C decrease at annealing, followed by 25 cycles with annealing at 55°C. The 5′ RACE products were digested with *Sal*I and *Bam*HI and then ligated with similarly digested pUC18. The 3′ RACE products were digested with *Eco*RI and *Sal*I and ligated with similarly digested pUC18. The published human liver PCD/DCoH sequence showed that there is an *Eco*RI site downstream of the stop codon (Mendel *et al.*, 1991); therefore, only the fragment containing the 3′ UTR could be cloned into pUC18.

Construction of pSI-PCD73 and pSI-PCD20

The plasmids pSI-PCD73 and pSI-PCD20 were constructed to perform *in vitro* transcription and translation, in which the 73- or 20-bp 5′ UTR and the complete coding sequence of the keratinocyte PCD/DCoH were inserted at the *Nhe*I and *Eco*RI sites of the mammalian expression plasmid pSI (Promega). The generation of pSI-PCD73 and pSI-PCD20 is shown in Figure 7. A 5′ RACE clone, pUC-PCD73, containing the complete coding sequence and 73-bp 5′ UTR of human epidermal keratinocyte PCD/DCoH inserted into pUC18 at the *Sal*I and *Bam*HI sites was digested with *Bss*HII/*Eco*RI. The small *Bss*HII-*Eco*RI fragment contained the complete coding sequence and the 53-bp 5′ UTR. To extend the 5′ UTR to 73 bp and convert the *Bss*HII site to an *Nhe*I site, the adapter *Bss*HII → *Nhe*I (the upper strand was 5′ phosphorylated; see Fig. 7B) was designed. The *Bss*HII-*Eco*RI fragment, *Bss*HII → *Nhe*I adapter, and *Eco*RI/*Nhe*I-digested pSI were ligated to generate pSI-PCD73. Similarly, pUC-PCD73 was digested with *Nco*I/*Eco*RI to produce a proximate 340-bp fragment that contains 1 bp of the 5′ UTR and the complete coding sequence. Adapter *Nco*I → *Nhe*I (the upper strand was 5′ phosphorylated; Lofstrand Labs Limited, Fig. 7B) was used to extend the 5′ UTR to 20 bp and to convert the *Nco*I site to *Nhe*I. The *Nco*I-*Eco*RI fragment, *Nco*I → *Nhe*I adapter, and *Eco*RI/*Nhe*I-digested pSI were ligated to generate pSI-PCD20.

In vitro *transcription and translation*

In vitro transcription and translation were carried out with TNT Coupled Wheat Germ Extract Systems (Promega) following the provided protocol. *Eco*RI-digested pSI-PCD73 or pSI-PCD20 (1 μg) was used, and the reaction mixture was incubated at 30°C for 90 min. The PCD/DCoH protein produced was analyzed by Western blotting.

Data processing

Agarose gels for PCR product analysis were captured by digital camera. Western blot images were scanned with Silverscan II controlled by Photoshop software. Image data were analyzed with the software of NIH Image.

RESULTS

Western blot analysis of tissue distribution of human PCD/DCoH

The answer to the question of tissue distribution pattern depends on the sensitivity of the assay used. With the purified polyclonal dehydratase antibody raised against the purified rat dehydratase in rabbit, we were consistently able to detect as little as 0.5 ng of dehydratase with second-signal development using the enhanced chemiluminescent (ECL) system. This sensitivity is much higher than that of our previous dehydratase-dependent phenylalanine hydroxylase stimulation assay. Therefore, Western blot analysis was used to determine the tissue distribution of the human PCD/DCoH and to compare the relative levels of PCD/DCoH in several nonhepatic cells and in liver. As shown in Figure 1, human PCD/DCoH was present predominantly in liver and kidney, which is consistent with the results of the mRNA assays in mouse (Mendel *et al.*, 1991),

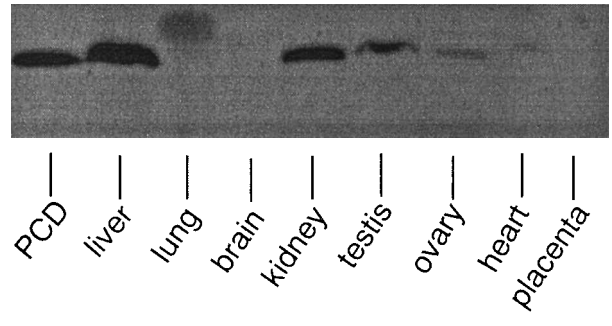


FIG. 1. Western blot analysis of the tissue distribution of PCD/DCoH. Analysis was carried out as described in Materials and Methods. Human tissue extract, 50 μg, was analyzed by 18% SDS-PAGE. A purified rat PCD standard, 2 ng, was included as a positive control.

PCD activity assays (Davis *et al.*, 1992a), and Western blot assays in rat (Strandmann *et al.*, 1998). Surprisingly, there was a striking signal band in testis and a less significant but apparently clear signal band in ovary. Among other human tissues tested, a trace amount of PCD/DCoH was detected in lung; there was no detectable amount in whole brain, heart, or spleen. The observation that significant amounts of PCD/DCoH protein were present both in human testis and ovary is a novel and interesting one. Previously, trace amounts of PCD/DCoH mRNA were detected in mouse ovary (Mendel *et al.*, 1991), but whether the protein is present in rodent testis or ovary has not been examined.

Cellular compartment distribution of PCD/DCoH

It is known that PCD/DCoH is present in both hepatic cytoplasm and nuclei (Huang *et al.*, 1973; Mendel *et al.*, 1991). However, the ratio is not known. In order to estimate this ratio, we prepared nuclear extracts (total volume 1.1 ml) and cytoplasmic extracts (total volume 24 ml) from fresh liver of an adult rat (fresh human liver was not available). With loading of the same volume of cytoplasmic extract (Fig. 2, lane 2) and nuclear extract (lane 3), Western blot analysis showed that the amount of cytoplasmic PCD/DCoH was 2.5-fold higher than that of nuclear PCD/DCoH. Normalized to total volume, the amount of hepatic nuclear PCD/DCoH was about 2% that of cytoplasmic PCD/DCoH. This result is consistent with our observation that most PCD activity was found in the cell extract of Chinese hamster ovary cells transfected with a PCD-expressing plasmid (data not shown). During purification of PCD, it was also found that the pellet fraction of liver homogenate did not contain detectable PHS/PCD activity (Hauer *et al.*, 1993). However, we cannot exclude the possibility that because of its small size, some DCoH might migrate to the cytoplasm during isolation of the nuclei.

Because PCD/DCoH is also present in some other nonhepatic tissues, another interesting question concerning the compartment distribution of PCD/DCoH is whether it is also present in the nuclei of these tissues. We used human cervical carcinoma (HeLa) cells as a representative of nonhepatic cells. This cell line has often been used as a model in studying the transactivation activity of HNF1 by means of transfection assay, because it has no detectable endogenous HNF1 (Nicosia *et al.*, 1992; Yamagata *et al.*, 1998; Powell and Suwanichkul, 1993). Western blot analysis (Fig. 2, lane 4) provided a positive answer that PCD/DCoH protein is present in the HeLa nuclear extracts. Immunoprecipitation further confirmed this result (data not shown). This result complements an early observation that an 11-kD protein in HeLa cells could be coimmunoprecipitated and copurified with exogenously expressed HNF1 in HeLa cells (Nicosia *et al.*, 1992).

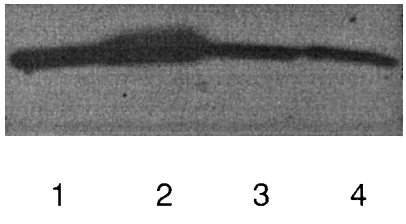


FIG. 2. Detection of nuclear PCD/DCoH by Western blot. Rat liver nuclear extract (10 μl; lane 3) or the supernatant fluid (10 μl; lane 2), HeLa nuclear extract (64 μg; lane 4), and PCD standard (2 ng; lane 1) were loaded for Western blot analysis.

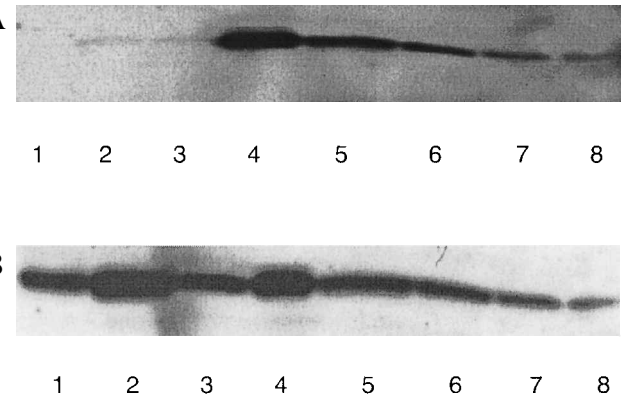


FIG. 3. Detection of PCD/DCoH protein in cells. Human liver extract (50 μg; lane 4 in **A** and **B**) and cell extract (50 μg) of HWB, NHDF, and NHEK (lanes 1–3, respectively) (**A**) or immunoprecipitated protein from 500 μg of cell extract of HWB, NHDF, and NHEK (lanes 1–3, respectively) (**B**) were loaded for Western blot analysis. A PCD standard (4, 2, 1, 0.5 ng; lanes 5–8 in **A** and **B**) was included.

Comparison of PCD/DCoH expression in liver and nonhepatic cells

We have previously detected PCD/DCoH protein and mRNA in NHEK (Lei *et al.*, 1997) and PCD/DCoH mRNA in HWB (Lei and Kaufman, 1998) by using RT-PCR and immunoprecipitation. Because these methods are very sensitive, especially the RT-PCR, by which illegitimate transcription of any gene could be detected in any cell type (Chelly *et al.*, 1989), the importance of this protein in these cells could not be evaluated without quantitative analysis. Therefore, we compared the expression of PCD/DCoH in these nonhepatic cells with that in liver. Another cell line, NHDF, was also included in the analysis. It has been shown that fibroblast cells synthesize very little, if any, tetrahydrobiopterin(BH4) under normal growth conditions, although the synthesis of tetrahydrobiopterin is induced by cytokines (Werner *et al.*, 1990; Bencsics *et al.*, 1996). We were very interested in the question of whether PCD/DCoH is also present in these cells, and if so, how high the expression level might be. Western blot analysis results are shown in Figure 3. The comparison of PCD/DCoH protein levels in the tissue and cells was reliable because the Western blot analysis was quantitative under the conditions used, as demonstrated with the purified rat PCD/DCoH standard (lanes 5–8). The PCD/DCoH protein was detectable and was at the same level in NHEK (lane 3) and NHDF (lane 2) cell extracts. The amount is about one twentieth of that in human liver homogenates (lane 4). The PCD/DCoH protein was not detectable in HWB extracts (lane 1) by the assay. However, immunoprecipitation followed by Western blot analysis (Fig. 3B) demonstrated the presence of PCD/DCoH protein in HWB and confirmed the presence of this protein in NHEK and NHDF.

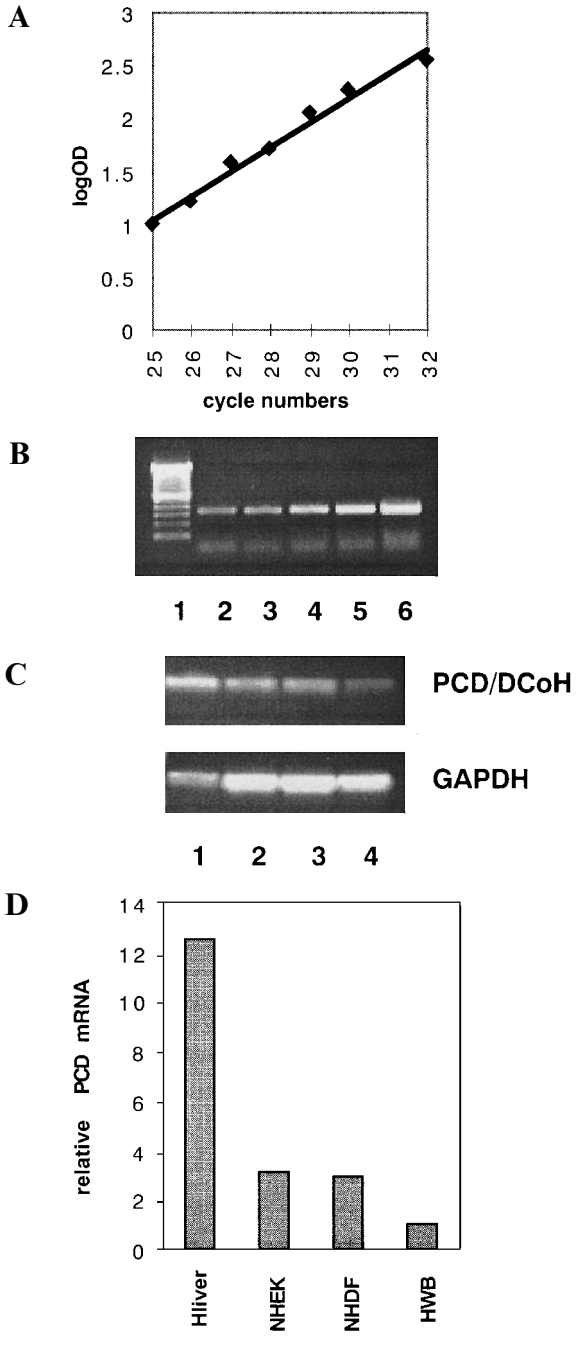


FIG. 4. Comparison of PCD/DCoH mRNA by RT-PCR. **A.** Determination of the linear range of the PCR. Reverse transcription was performed with human liver RNA. The product was diluted 10 fold, and 2 μl of the dilution was subjected to PCR amplification for 25 to 32 cycles. The PCR products were separated on an agarose gel and stained with ethidium bromide. **B.** Testing the PCR linear relation setting cycle number at 30. A series of diluted reverse-transcribed products (2, 4, 8, 16, and 32 μl; lanes 2–6, respectively) was subjected to PCR amplification for 30 cycles. The same volume of each PCR product was loaded for agarose gel analysis. Lane 1 shows the 100-bp DNA ladder. **C.** Total RNA (5 μg) isolated from human liver, NHEK, NHDF, and HWB (lanes 1–4) was reverse transcribed. The products were diluted 10 fold, and 2 μl or 1 μl of each dilution was subjected to PCR amplification of PCD or GAPDH cDNA. The normalized relative PCD/DCoH mRNA level is shown in **D.**

We also compared the PCD/DCoH mRNA levels in human liver and human nonhepatic cells by RT-PCR. To make the RT-PCR assay comparable, we first determined the PCR linear range. A plot of log value of PCR product (Log Product) against PCR cycle number (n) (Fig. 4A) showed that within 32 cycles, the PCR efficiency remained constant. Considering that the PCR linear range also depends on the initial amount of cDNA used, we tested whether the PCR is quantitative with different amounts of cDNA, setting the cycle number at 30. Figure 4B shows that with 30 cycles, the PCR is comparable within a broad range of cDNA used.

After establishing the PCR conditions, we compared the PCD/DCoH mRNA levels in human liver and nonhepatic cells. All cells tested, as well as the liver, showed a clear RT-PCR band with the predicted size (Fig. 4C). Cloning and sequencing of each band confirmed that each one had the coding sequence identical to that of human liver PCD/DCoH. Chromosomal DNA contamination was excluded by RT-minus RT-PCR control and the finding that the PCR products had no intron sequences. Carry-over contamination has been looked for in every assay by including a RT-PCR control without adding RNA or a PCR control without adding cDNA. The normalized relative PCD/DCoH mRNA levels are shown in Fig. 4D. The mRNA amount in NHEK and NHDF is almost equivalent and is higher than that in HWB, consistent with the result of Western blot analysis of the PCD/DCoH protein in these cells. Surprisingly, the mRNA level in human liver is only fourfold higher than that in NEHK and NHDF cells. This finding is in contrast to the Western blot results that showed that the PCD/DCoH protein level in human liver way 20-fold higher than that in NHEK and NHDF cells.

Cloning of the 5′ and 3′ untranslated region of the PCD/DCoH gene of NHEK cells

The nonparallel synthesis of the mRNA and protein of the human *PCD/DCoH* gene in hepatic and nonhepatic cells suggests that post-transcriptional regulation is involved in the tissue- or cell-differential expression of this gene. To investigate whether the post-transcriptional regulation is at the translational level, we cloned the 5′ and 3′ UTR of the *PCD/DCoH* gene of NHEK cells and compared the sequence with that of the published liver counterpart. As shown in Figure 5, agarose gel analysis of the 5′ RACE of PCD/DCoH mRNA from both undifferentiated and differentiated NHEK cells showed a broad band with the same size. A control 5′ RACE without dC tail-

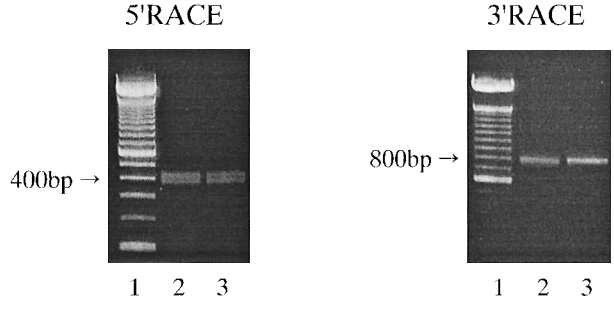
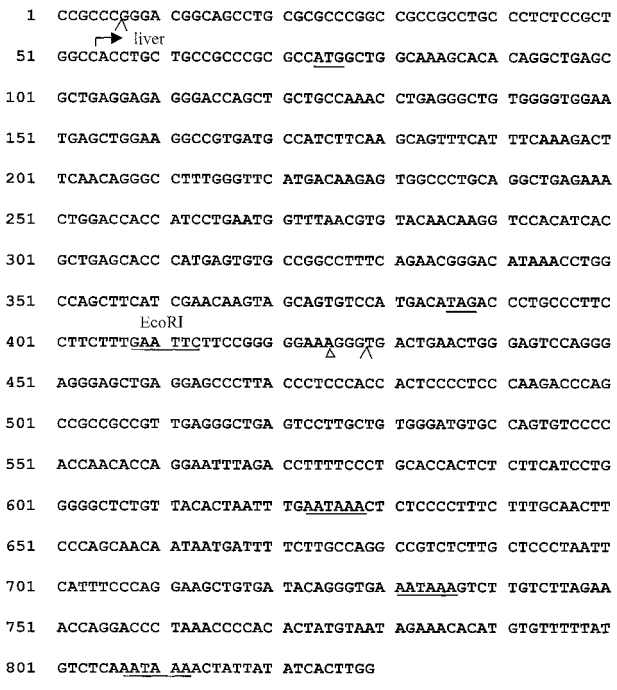


FIG. 5. Agarose gel analysis of 5′ and 3′ RACE products. The RACE products with undifferentiated (lane 2) and differentiated (lane 3) NHEK total RNA were analyzed by agarose gel. Lane 1 shows a 100-bp DNA ladder.

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  1  CCGCCCGGGA CGGCAGCCTG CGCGCCCGGC CGCCGCCTGC CCTCTCCGCT
 51  GGCCACCTGC TGCCGCCCGC GCCATGGCTG GCAAAGCACA CAGGCTGAGC
101  GCTGAGGAGA GGGACCAGCT GCTGCCAAAC CTGAGGGCTG TGGGGTGGAA
151  TGAGCTGGAA GGCCGTGATG CCATCTTCAA GCAGTTTCAT TTCAAAGACT
201  TCAACAGGGC CTTTGGGTTC ATGACAAGAG TGGCCCTGCA GGCTGAGAAA
251  CTGGACCACC ATCCTGAATG GTTTAACGTG TACAACAAGG TCCACATCAC
301  GCTGAGCACC CATGAGTGTG CCGGCCTTTC AGAACGGGAC ATAAACCTGG
351  CCAGCTTCAT CGAACAAGTA GCAGTGTCCA TGACATAGAC CCTGCCCTTC
401  CTTCTTTGAA TTCTTCCGGG GGAAAGGGTG ACTGAACTGG GAGTCCAGGG
451  AGGGAGCTGA GGAGCCCTTA CCCTCCCACC ACTCCCCTCC CAAGACCCAG
501  CCGCCGCCGT TGAGGGCTGA GTCCTTGCTG TGGGATGTGC CAGTGTCCCC
551  ACCAACACCA GGAATTTAGA CCTTTTCCCT GCACCACTCT CTTCATCCTG
601  GGGGCTCTGT TACACTAATT TGAATAAACT CTCCCCTTTC TTTGCAACTT
651  CCCAGCAACA ATAATGATTT TCTTGCCAGG CCGTCTCTTG CTCCCTAATT
701  CATTTCCCAG GAAGCTGTGA TACAGGGTGA AATAAAGTCT TGTCTTAGAA
751  ACCAGGACCC TAAACCCCAC ACTATGTAAT AGAAACACAT GTGTTTTTAT
801  GTCTCAAATA AAACTATTAT ATCACTTGG
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FIG. 6. Complete cDNA sequence of NHEK *PCD/DCoH*. The sequence was obtained by sequencing 5′ and 3′ RACE clones, except for the segment from the stop codon to the *Eco*RI site, which was from the genomic DNA sequence (Thony *et al.*, 1995). Three base differences between cDNA and genomic DNA are indicated (caret, insertion of C or G in the genomic DNA; triangle, deletion of A in the genomic DNA). Three segments identical to the polyadenylation signal consensus sequence, as well as the start and stop codons and an *Eco*RI site, are underlined. The arrow showed the transcription start site of human liver *PCD/DCoH*.

ing of the synthesized first-strand cDNA did not show any product (data not shown), indicating that internal mispriming by the 5′ RACE AAP at the potential C-rich region did not occur. Cloning and sequencing of the 5′ RACE products showed that the coding sequence of the *PCD/DCoH* gene of both undifferentiated and differentiated NHEK is identical to that of the human liver counterpart. It also showed that the 5′ RACE products were heterogeneous. Among 14 analyzed clones, 2 clones had a 73-bp 5′ UTR, 2 had 71 bp, 1 had 66 bp, 1 had 49 bp, 1 had 27 bp, 4 had 26 bp, 1 had 22 bp, 1 had 19 bp, and 1 had 15 bp. The longest one (73 bp) had 53 bp more of 5′ UTR than the human liver counterpart (Thony *et al.*, 1995). We also did 5′ RACE with human liver total RNA purchased from Clontech. The products were also heterogeneous, with a maximum of 20 bp of 5′ UTR among the analyzed clones. To determine the transcription start site accurately, we tried RNA ligation-mediated PCR, which has the potential to locate the exact transcription start site. Probably because of the low abundance of PCD/DCoH mRNA, this method did not work.

In contrast to 5′ RACE, 3′ RACE with total RNA from both undifferentiated and differentiated NHEK cells as well as from human liver produced a sharp product band. The agarose gel analysis showed that 3′ RACE products with NHEK (undifferentiated or differentiated) total RNA were of the same size (Fig. 5). A separate 3′ RACE with human total liver RNA produced a band with the same size as that of NHEK (data not shown). Sequencing eight clones (NHEK) showed that the 3′ RACE products were homogeneous. All of them had a 440-bp 3′ UTR, which is 151 bp longer than the human liver 3′ UTR identified earlier by screening human hepatoma cDNA library (Mendel *et al.*, 1991). The complete cDNA sequence, including the 5′ and 3′ UTRs, is shown in Figure 6.* Although three polyadenylation signal consensus sequences were found in the 3′ UTR, sequencing eight clones showed that the 3′ UTR is homogeneous, suggesting that differential poly (A) choice might not occur.

Effects of 5′ UTR on translation efficiency

The 5′ UTR of the NHEK cell PCD/DCoH, especially the region which has not been found in human liver PCD/DcoH, is a GC-rich sequence. Computer analysis showed that it has potential to form secondary structure. Therefore, we investigated the potential effects of the 5′ UTR on the translation efficiency. For this purpose, we constructed the plasmid pSI-PCD73 or pSI-PCD20 in which the coding sequence along with 73- or 20-bp 5′ UTR was inserted into the mammalian expression plasmid pSI and under the control of the T7 RNA polymerase promoter for *in vitro* transcription. To minimize the addition of multiple cloning site sequences of the plasmid pSI to the 5′ UTR of the transcripts, the 5′ end of the PCD cDNA was adapted to an *Nhe*I site, and the cDNA was inserted at *Nhe*I, where T7 transcription starts (Fig. 7C). The *in vitro* transcription and translation with pSI-PCD73 and pSI-PCD20 had been done three times. Figure 8 shows the result of a typical experiment. The pSI-PCD20 generated 35% more product than the pSI-PCD73.

DISCUSSION

One characteristic of the expression of human PCD/DCoH demonstrated in the present study is that it shows a wider distribution pattern than that of either aromatic amino acid hydroxylases or NHF1. In addition to human liver and kidney, where relatively high levels of phenylalanine hydroxylase and HNF1 have been found, significant amounts of PCD/DCoH were detected in human testis and ovary. Neither aromatic amino acid hydroxylases nor HNF1 has been reported in these tissues. Whether PCD/DCoH plays a role in reproduction is not known. The presence of PCD/DCoH in human epidermal keratinocytes, dermal fibroblasts, and WB is another example of its wide distribution, as those cells do not belong to the tissues known to have HNF1. As far as aromatic amino acid hydroxylases are concerned, except for keratinocytes, in which high levels of tyrosine hydroxylase activity have been detected, the cells do not have significant aromatic amino acid hydroxylase activity. Although mRNA for phenylalanine hydroxylase has

*The sequence has been submitted to GenBank Accession No. AF082858.

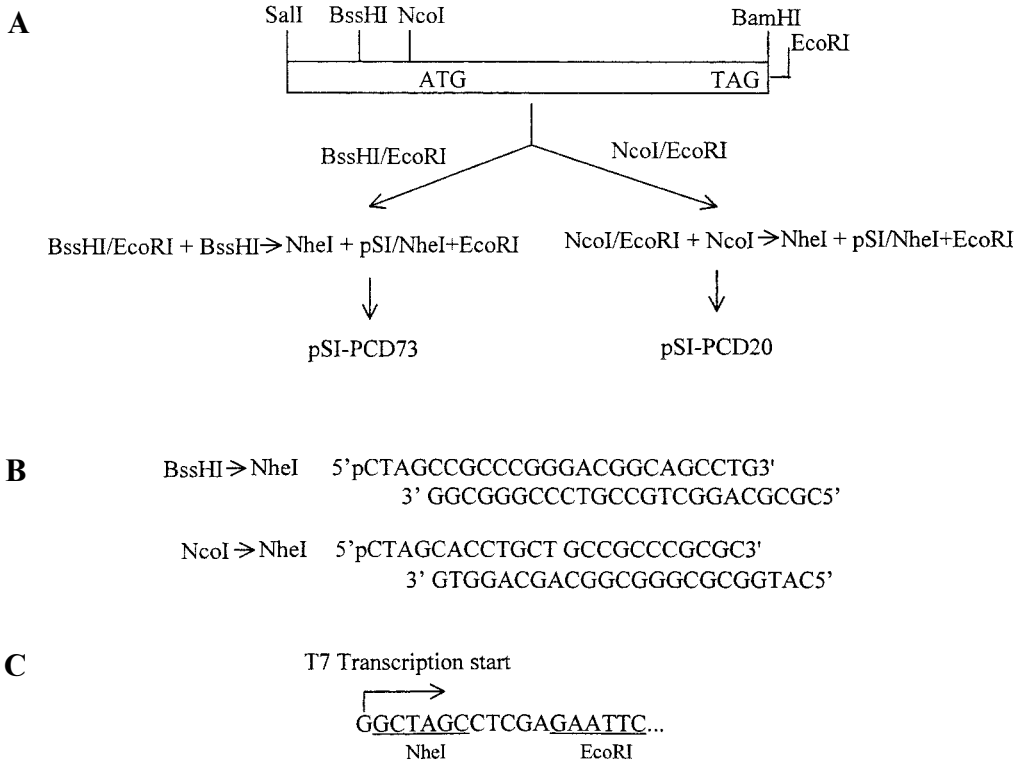

FIG. 7. Construction of pSI-PCD20 and pSI-PCD73. **A.** Insert of PUC-PCD73, which contains the 73-bp 5′ UTR and the coding region. The *Eco*RI site shown is from the vector pUC18. For detailed description, refer to Materials and Methods. Also shown are the sequences of the adaptors (**B**) and the T7 transcription start site in the vector pSI (**C**).

been detected in these cells, this transcription has been determined to be illegitimate, as phenylalanine hydroxylase activity has not been detected. In contrast, PCD/DCoH protein is detectable in extracts of keratinocytes and fibroblasts by Western blot analysis even without specific accumulation by immunoprecipitation; its enzymatic activity is also detectable, although at very low levels (data not shown). More convincingly, comparison of the expression of PCD/DCoH in nonhepatic cells and in liver showed that the mRNA levels of PCD/DCoH in the NHEK and NHDF cells are one fourth of that in the liver, demonstrating that the transcription of the *PCD/DCoH* gene in NHEK and NHDF is not illegitimate. The amount of PCD/DCoH protein in these cells is one twentieth of that in liver, further supporting the conclusion that the detected PCD/DCoH in these cells is unlikely to be secondary to illegitimate transcription of its gene.

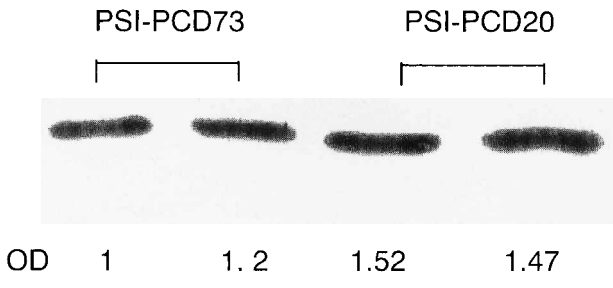

FIG. 8. Western blot analysis of the *in vitro* transcription and translation products. *In vitro* transcription and translation were carried out in duplicate. The product of each reaction (15 μl) was loaded for Western blot analysis. The bands visualized by ECL were scanned, and the relative optical densities are shown below.

As described in the Introduction, the PCD function of PCD/DCoH appears to take place in cytoplasm, whereas its DCoH function occurs in the nucleus. Therefore, the presence of PCD/DCoH in human dermal fibroblasts and in the nuclear extracts of HeLa cells is especially noteworthy, because fibroblasts have little or no tetrahydrobiopterin under normal growth conditions (Werner *et al.*, 1990; Bencsics *et al.*, 1996); and available evidence suggests that HeLa cells do not express HNF1 (Nicosia *et al.*, 1992; Yamagata *et al.*, 1998; Powell and Suwanichkul, 1993). Therefore, it is unlikely that PCD/DCoH functions as PCD in the cytoplasm of fibroblasts or as DCoH in the nuclei of HeLa cells. These findings indicate that PCD/DCoH may have functions other than those attributable to either its PCD or DCoH activities. This speculation is also supported by the finding that PCD/DCoH or its putative homolog is present in a wide array of species, ranging from bacteria to humans. Figure 9 shows the multiple alignment of PCD/DCoH amino acid sequences from different species. Of those listed, PCD/DCoH from *Xenopus* (Pogge von Strandmann and Ryffel, 1995), *Pseudomonas aeruginosa* (Zhao *et al.*, 1994) and the livers of human, rat, mouse (Mendel *et al.*, 1991; Huang *et al.*,

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Human                                          MAGKAHRLS AEERDQLLPN LRAVGWNELE
Rat                                            MAGKAHRLS AEERDQLLPN LRAVGWNELE
Mouse                                          MAGKAHRLS AEERDQLLPN LRAVGWNEVE
Xenopus                                        MAGKVHRLS GEEREQLLPN LRAVGWHELD
Synechocystis SP                                   MATPQ RLTDPEIQTA LGELGGWSLQ
P. aeruginosa                 MTALTQAHCE ACRADAPHVS DEELPVLLRQ IPDWNIEVRD
M. tuberculosis   MTVSTPEQHE QRASHDASEG KHNVCQGRLA ALADAAVSEK LGALPGWQLL
Yeast                            MHNKIVR IASSALTGGK LLEKLKPLTR WEVQWDPNKT

Human             GRDAIFKQFH FKDFNRAFGF MTRVALQAEK LDHHPEWFNV YNK.VHITLS
Rat               GRDAIFKQFH FKDFNRAFGF MTRVALQAEK LDHHPEWFNV YNK.VHITLS
Mouse             GRDAIFKQFH FKDFNRAFGF MTRVALQAEK LDHHPEWFNV YNK.VHITLS
Xenopus           GRDAICKEFH FKDFNRAFGF MTRVALQAEK LDHHPEWFNV YDK.VHITLS
Synechocystis SP  G.NKLHRQFK FANFNQAFGF MTRLALVAET LNHHPEWSNV YNR.VTIDLT
P. aeruginosa     GIMQLEKVYL FKNFKHALAF TNAVGEISEA EGHHPGLLTE WGK.VTVTWW
M. tuberculosis   DM.RLSRAFQ CTNFDQSIDF MNRVASIAND INHHPDIAVL DKRSVRVTAW
Yeast             KCLGITREVT FKDYETTWAF LTRVSMRSHL WGHHPLIHTS YT.WVKLELH

Human             THECAGLSER DINLASFIEQ VAVSMT
Rat               THECAGLSER DINLASFIEQ VAVSMT
Mouse             THECAGLSER DINLASFIEQ VAVSMT
Xenopus           THDCGGLSER DINLASFIEQ IAASLS
Synechocystis SP  THDAGGITEL DVKFATKANS FAD
P. aeruginosa     SHSIKGLHRN DFIMAARTDE VAKTAEGRK
M. tuberculosis   TRKLGYLTDI DFDLAASVEA MYATEFADRP AR
Yeast             THDIDPKDGA HSQLSDIDVR MAKRIDSYID EMTT
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FIG. 9. Alignment of PCD/DCoH amino acid sequences deduced from cDNA or genomic DNA sequences. Sequences were aligned using the Pileup program of the GCG package. The residues critical for enzymatic activity are highlighted.

1973; Hauer *et al.*, 1993) have been demonstrated. The amino acid sequences shown were deduced from the cDNA sequences. Others, such as PCD/DCoH in yeast (Johnston *et al.*, 1994), *Mycobacterium tuberculosis* (Philipp *et al.*, 1996), and *Synechocystis* sp. (Kaneko *et al.*, 1996), are still hypothetical proteins. Their amino acid sequences were deduced from the ORF of the genome. As can be seen in Figure 9, this protein is highly conserved. The residues that have been found to be crucial for the dehydratase activity (highlighted in Fig. 9) are completely conserved.

Another characteristic of the expression of human PCD/DCoH that has been demonstrated in this work is that the synthesis of its mRNA and its protein do not appear to proceed in a parallel fashion. The amount of PCD/DCoH mRNA in human liver is only four times that in human epidermal keratinocytes and dermal fibroblasts. However, the protein level in the former is 20-fold higher than in the latter two cell types, suggesting that post-transcriptional regulation might be involved. In higher organisms, one of the strategies employed to achieve tissue-specific or developmentally specific expression of a gene is the use of alternative promoters. For this reason, we cloned the 5′ UTR of human epidermal keratinocyte PCD/DCoH. It was found to be 53 bp longer than that of the published liver counterpart. *In vitro* transcription and translation analysis with the wheat-germ extract system showed that the longer 5′ UTR resulted in only a 35% decrease in translation efficiency. Because we have not set up an *in vitro* translation system with keratinocyte extracts, the exact effects of the 5′ UTR in the translation efficiency in keratinocytes is still not known. We also realize that we cannot completely exclude the possibility that human liver and keratinocytes have the same length of 5′ UTR and that the reason we cloned a longer 5′ UTR might be that we were using fresh cells to isolate total RNA, whereas it is difficult to get fresh human liver for this purpose. However, because we also detected no more than 20 bp of 5′ UTR with human liver RNA, this possibility seems rather remote. Nevertheless, further studies are needed to clarify whether the extra 53 bp found in the 5′ UTR of the cDNA of keratinocytes in the present study is involved in the tissue-differential expression of PCD/DCoH in keratinocytes.

In conclusion, the present study demonstrated that human PCD/DCoH is not only present in cells where tetrahydrobiopterin is synthesized or HNF1 is present but is a widely distributed protein. Its differential expression in different tissues and cells is probably regulated, not only at the transcriptional level, but also at the translational level.

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