Cloning and tissue expression of two cDNAs encoding the peroxisomal 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase in the guinea pig liver

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Abstract The 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) is the second enzyme of the peroxisomal β-oxidation pathway. In human and rat, only one HD mRNA has been so far detected in the liver. This paper reports for the first time in a mammal species, the guinea pig, the cloning and sequencing of two cDNAs encoding an HD. The 3,274 nucleotide-cDNA is a strictly identical but longer copy of the 2,494 nucleotide-form. A 2,178 bp-open reading frame encodes a protein of 726 amino acids (M_r 79.3 kDa) with the peroxisomal-targeting signal (tripeptide SKL) at the carboxyterminus. Northern blot analysis of HD mRNA identified three mRNAs of respective sizes 3.5, 2.6 and 1.6 kb in the guinea pig liver and kidneys.

Key words: Bifunctional enzyme; cDNA; 2-Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; Peroxisome; Guinea pig

1. Introduction

In mammal liver, the peroxisomal β-oxidation system is composed of three enzymes: the acyl-CoA oxidase, the 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (HD) that catalyses the second and third steps, and the 3-ketoacyl-CoA thiolase (for review see ref. [1]). The administration of compounds known to be peroxisome proliferators causes a marked induction of these three enzymes in rats [2,3], but not in guinea pigs. On the contrary to the extensive studies done on the regulation of the rat peroxisomal β-oxidation enzymes expression, and particularly the HD [4,5] nothing has been reported on the guinea pig counterpart yet. Nevertheless, we think that the guinea pig, which is like human, a non-responsive species to peroxisome proliferators could be a better model than the rat to understand the regulation of the peroxisomal β-oxidation by these compounds in non-responsive species. We initiated a study on the guinea pig model and discovered not only one like in rat or human, but two highly homologous HD cDNAs. Furthermore, not only two but three transcripts are coexpressed in the guinea pig liver and kidneys. This typical pattern of HD mRNA expression in the guinea pig confirms the interest to further investigate the regulation of the β-oxidation in this non-responsive species.

2. Materials and methods

2.1. cDNA library screening and rapid amplification of cDNA ends

Poly(A)$^+$ RNAs were isolated from a frozen guinea pig liver using the Fast Track mRNA Isolation kit (Invitrogen, San Diego, CA). A 200 nucleotide (nt)-cDNA probe was obtained by RT-PCR using these poly(A)$^+$ RNAs and primers deduced from the human HD cDNA sequence [6]. The two human oligonucleotides were centered at positions 741 and 941, respectively, and the cDNA was amplified by PCR (35 cycles: 1 min at 94°C, 1 min at 51°C, 2 min at 72°C and a final elongation (15 min at 72°C) in a PCR buffer containing 2 mM of $MgCl_2$ and 1 μM of each primer). This 200nt cDNA probe was then used to screen a guinea pig adult liver cDNA λgt 10 library (Clontech Lab., Palo Alto, CA) according to standard procedures [7]. To obtain cDNA from 3′- and 5′-ends, the RACE extension method was applied using 3′-Amplifinder Race kit and 5′-Amplifinder Race kit protocols (Clontech Lab., Palo Alto, CA) and four specific primers from guinea pig HD sequence: P15 and P25 (5′-TGCTGCCTCCGCATCTTCTCGACGGCTTCC-3′ and 5′-GCCGTCCTGAGGTTATTAAGTCAAGTGCAG-3′, respectively centered at positions 656 and 455) for the 5′-end determination; P13 and P23 (5′-GCACAAGGGCGGGCCCATGTTCTATGCTGC-3′ and 5′-CCTCAGTTGGGTTGCCCACAGTTCT-3′, respectively centered at positions 1,988 and 2,015) for the 3′-end determination. The 5′-end amplification was performed according the manufacturer's recommendations: with 10 μM of P15 to synthesize the first cDNA strand, and in the following PCR conditions: 35 cycles (45 s at 94°C, 45 s at 60°C, 2 min at 72°C) and a final elongation (7 min at 72°C) with 1 μM of primer P25. The 3′-end amplification was performed according the manufacturer's recommendations and in the following PCR conditions: 1st PCR: 28 cycles (45 s at 94°C, 45 s at 63°C, 2 min at 72°C) and a final elongation (7 min at 72°C) with 0.2 μM of primer P13. 2nd PCR: 27 cycles (45 s at 94°C, 45 s at 65°C, 2 min at 72°C) and a final elongation (7 min at 72°C) with 0.2 μM of primer P13 and cDNA from the first PCR (0.5 μl).

2.2. DNA sequencing

The cDNA inserts of positive phages and the PCR-amplified 5′- and 3′-ends cloned into pCRII vector using the TA cloning kit (Invitrogen, San Diego, CA) were sequenced several times on both strands using T7 and SP6 fluorescent primers and an Auto Read Sequencing kit (Pharmacia Biotech, USB Corp, Cleveland, OH) according to the manufacturer's recommendations, and analyzed on an A.L.F. DNA Sequencer (Pharmacia LKB, Uppsala, Sweden).

2.3. RNA blotting

Total RNAs (20 μg) obtained from various freshly excised tissues of a male guinea pig (IFFA Credo, L'Arbresle, France) were electrophoresed and Northern blot analysis performed according to Cherkaoui-Malki et al. [8]. The HD cDNA clone ranging from nt 263 to 1,260 was used as a probe. The Northern blot was normalized with the 18S and 28S ribosomal RNAs.

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Abbreviations: bp, base pair(s); HD, hydratase/dehydrogenase; kDa, kilodalton; nt, nucleotide; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

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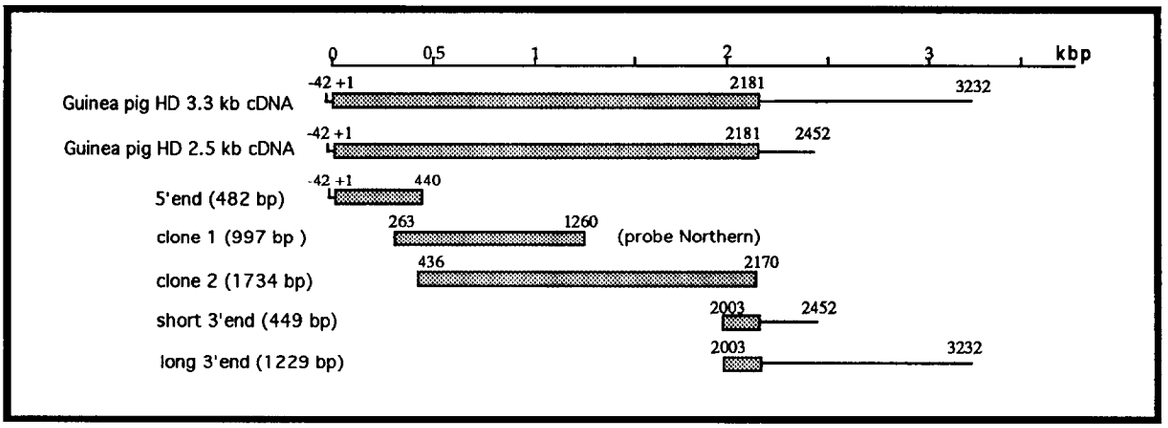

Fig. 1. Schematic representation of the two HD cDNAs isolated from a guinea pig liver cDNA library. The two overlapping cDNA HD clones 1 and 2 (clone 1 being used as a probe in Northern blot analysis) as well as the 3′ and 5′ PCR-obtained ends are represented.

3. Results and discussion

3.1. Isolation of two highly homologous cDNAs encoding a guinea pig peroxisomal hydratase/dehydrogenase

By screening 5×10^5 clones of an adult guinea pig liver cDNA λgt 10 library we have isolated two overlapping clones (Fig. 1). These two clones contain cDNA inserts ranging from nucleotides 263 to 1,260 (clone 1) and from 436 to 2,170 (clone 2), respectively, as deduced by comparison with human HD cDNA sequence. The nucleotide sequences of these two clones are strictly identical in the common part ranging from 436 to 1,260. By application of the RACE protocol, both the 3′- and 5′-ends

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-42                                aggcagagtttggcagtggccccctcggcagcaacagacagag
  1 ATGGCGGAGTACCTGAGGCTGCCCCACTCCCTGGCGCTGATCCGCCTCCGAAACCCGCCT
  1  M  A  E  Y  L  R  L  P  H  S  L  A  L  I  R  L  R  N  P  P
 61 GTCAACGCCATCAGTCCGGCTGTAATCCATGGAATAAAGGAAGGACTACAGAAGGCTATG
 21  V  N  A  I  S  P  A  V  I  H  G  I  K  E  G  L  Q  K  A  M
121 TCAGACTACACAATAAAAGGAATTGTGATTTCTGGAGCAAATAATATCTTTGTGCAGGT
 41  S  D  Y  T  I  K  G  I  V  I  S  G  A  N  N  I  F  C  A  G
181 GCAGATATCCATGGCTTCAGTGCACCTCTATCCTTTGGCACTGGCAGTGGATTGGGACCC
 61  A  D  I  H  G  F  S  A  P  L  S  F  G  T  G  S  G  L  G  P
241 ATAGTAGATGAAATGCAGAGATATGAGAAGCCAGTGGTGGCTGCTATCCAAGGCATGGCT
 81  I  V  D  E  M  Q  R  Y  E  K  P  V  V  A  A  I  Q  G  M  A
301 CTTGGAGGGGGACTGGAGCTGTCCCTGGGCTGTCACTACAGGATTGCGCATGCAGAGGCT
101  L  G  G  G  L  E  L  S  L  G  C  H  Y  R  I  A  H  A  E  A
361 CGAATTGGTTTCCCAGAAGTCACGCTAGGGATTCTTCCTGGTGCAAGAGGAACCCAGCTT
121  R  I  G  F  P  E  V  T  L  G  I  L  P  G  A  R  G  T  Q  L
421 CTCCCCAGACTCATTGGAGTTCCTGCTGCACTTGACTTAATAACCTCAGGACGGCATATT
141  L  P  R  L  I  G  V  P  A  A  L  D  L  I  T  S  G  R  H  I
481 ACAGCAGGTGAAGCACTCAAGCTGGGTATTCTGGATAAAGTCGTTAATTCAGCCCCAGTT
161  T  A  G  E  A  L  K  L  G  I  L  D  K  V  V  N  S  A  P  V
541 GAAGAAGCAATCAAATTCGCTCAGAAAATTTTAAATCAACCCCTAGAACCTCGCAGAATC
181  E  E  A  I  K  F  A  Q  K  I  L  N  Q  P  L  E  P  R  R  I
601 CTCAACAGGCCAGTCTCCAGCTTGCCCAACATGGACGCCATTTTTGGGGAAGCCGTCGAG
201  L  N  R  P  V  S  S  L  P  N  M  D  A  I  F  G  E  A  V  E
661 AAGATGCGGAGGCAGCACCCAGGGCAGCTTGCTCCGGAGACCTGTGTCCGTTCAGTCCAG
221  K  M  R  R  Q  H  P  G  Q  L  A  P  E  T  C  V  R  S  V  Q
721 GCCTCTGTGCAGTACCCCTATGAAGGGGGCATCATGAAGGAGAGAGAACTGTTTCTGAAC
241  A  S  V  Q  Y  P  Y  E  G  G  I  M  K  E  R  E  L  F  L  N
781 CTTCAGCACTCCGGGCAGGCCAAAGCCCTGCAGTATGCCTTCTTCGCTGAGAGAAGTGCA
261  L  Q  H  S  G  Q  A  K  A  L  Q  Y  A  F  F  A  E  R  S  A
841 CCCAAGTGGTCAACCCCATCTGGAGCCTCCTGGAAAACAGCTGCTGCGCGCCCAGTCTCC
281  P  K  W  S  T  P  S  G  A  S  W  K  T  A  A  A  R  P  V  S
901 TCCGTTGGTGTTCTTGGCTTGGGAACAATGGGCCGAGGCATCGCCATTTCTTTGCAAGG
301  S  V  G  V  L  G  L  G  T  M  G  R  G  I  A  I  S  F  A  R
961 GTCGGGATCCCTGTGATTGCTGTGGAATCAGACCCAAAGCAGCTAGAAACTGCACAGAAG
321  V  G  I  P  V  I  A  V  E  S  D  P  K  Q  L  E  T  A  Q  K
1021 TTAATAACTTCAATCTTGGAGAAAGAAGCATCCAAAAGCAGGCAACAGTGTGGCCAGCAA
341  L  I  T  S  I  L  E  K  E  A  S  K  S  R  Q  Q  C  G  Q  Q
1081 AGGTCAGGACCAAAACCTAGGTTCAGCTCATCTATGAAGGACCTAGCCAGTGTCGATCTA
361  R  S  G  P  K  P  R  F  S  S  S  M  K  D  L  A  S  V  D  L
1141 GTCGTCGAAGCAGTATTTGAGGACATGAACCTGAAGAAGCGGGTCTTTGCTGAACTGTCA
381  V  V  E  A  V  F  E  D  M  N  L  K  K  R  V  F  A  E  L  S
1201 GCTGTGTGCAAGCCAGAAGCTTTTCTGTGCACCAATACTTCAGCCCTGGATGTGGATGAA
401  A  V  C  K  P  E  A  F  L  C  T  N  T  S  A  L  D  V  D  E
1261 ATTGCTACTTCTACCAATCGCCCTCAGCAGGTCATCGGCACTCACTTCTTCTCCCCAGCT
421  I  A  T  S  T  N  R  P  Q  Q  V  I  G  T  H  F  F  S  P  A
1321 CATGTTATGAAGTTATTAGAGGTTATTCCCAGTCGACACTCATCCCCCACCACCATTGCC
441  H  V  M  K  L  L  E  V  I  P  S  R  H  S  S  P  T  T  I  A
1381 ACTGTTATGGACCTAGCAAAGAAAATTAAAAAGGTCGCAGTTGTTGTAGGCAACTGTTAC
461  T  V  M  D  L  A  K  K  I  K  K  V  A  V  V  V  G  N  C  Y
1441 GGTTTTGTTGGGAATCGCATGTTGAGATCTTATTATGAACAGACTAATTTCTTATTGGAA
481  G  F  V  G  N  R  M  L  R  S  Y  Y  E  Q  T  N  F  L  L  E
1501 GATGGCAGTAAGCCAGAGGACATAGATCAGGCACTGGAAGAGTTTGGTTTTAGAATGGGA
501  D  G  S  K  P  E  D  I  D  Q  A  L  E  E  F  G  F  R  M  G
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1561 CCCTTCAGAGTGTCAGACCTCGCAGGGCTAGATGTGGGTTGGAAAATTCGCAAGGGGCAA
521  P  F  R  V  S  D  L  A  G  L  D  V  G  W  K  I  R  K  G  Q
1621 GGCCTTACTGGACCATCATTGCAAGGTACAGCCCCTGCCCGAAAGCGAGGCAATGCCAGG
541  G  L  T  G  P  S  L  Q  G  T  A  P  A  R  K  R  G  N  A  R
1681 TACAGCCCCATTGCTGATATGCTGTGTGAATTGGGAAGATTTGGCCAGAAGACTGGTCAG
561  Y  S  P  I  A  D  M  L  C  E  L  G  R  F  G  Q  K  T  G  Q
1741 GGTTGGTATAAATATGACAAGCCATTGGGTAGGATTCACAAACCTGACCCCTGGCTTTCC
581  G  W  Y  K  Y  D  K  P  L  G  R  I  H  K  P  D  P  W  L  S
1801 AAATTCCTGTCAGAATACAGAGAGACCCATCACATCAAACCGCGTGTCATTGGGCGGGAT
601  K  F  L  S  E  Y  R  E  T  H  H  I  K  P  R  V  I  G  R  D
1861 GAGATCCTGGAGCGCTGCTTGTATGCACTCATCAATGAAGCCTTCCGCATCTTGGGGGAA
621  E  I  L  E  R  C  L  Y  A  L  I  N  E  A  F  R  I  L  G  E
1921 GGGATAGCTGCTAGTCCAGAGCACATTGATGTGATCTACTTGCATGGGTATGGCTGGCCG
641  G  I  A  A  S  P  E  H  I  D  V  I  Y  L  H  G  Y  G  W  P
1981 CGGCACAAAGGTGGGCCCATGTTCTATGCTGCCTCAGTTGGGTTGCCCACAGTTCTAGAG
661  R  H  K  G  G  P  M  F  Y  A  A  S  V  G  L  P  T  V  L  E
2041 AAGTTGCAGAAGTATTACCAGCAGAATCCTGATATTCCCCACCTGGAGCCCTGCAACTAT
681  K  L  Q  K  Y  Y  Q  Q  N  P  D  I  P  H  L  E  P  C  N  Y
2101 CTAAAAAAGCTGGCTTCCCAGGGAAACCCTCCTCTGAAAGAATGGCAGAGCCTGGCAGGG
701  L  K  K  L  A  S  Q  G  N  P  P  L  K  E  W  Q  S  L  A  G
2161 CTACCCAGCAGTAAATTGTGAttcagccttctcagttgggctcacatactggagtcagaa
721  L  P  S  S  K  L
2221 aaaaaaaagaaaacaaaaaccggaaattcaacctaaattgcaatctttagaatgtgcttc
2281 ctatacttctgagtctgtccctattattaaatttcagtgaatgaacttgtgttacacaatg
2341 ccatgctatctgacgaatatgttaaggttcccaagtgctttgatcgttggagaaatatgt
2401 catcaatttataaatgacatgcatctgttaaatatatttaagtgataaactgattttgtc
2461 aattaagtcataaactcatttttggcccttagcgtcccatacgcagtatgagtcacacac
2521 attcactgagtctctcctgaacatctctgaccatcagagagcacctagcagacctacagt
2581 ccagtggggcattaaaccaacatgggaacctgcattggattctcgtggagaaggttggca
2641 gggcagagtcggcagtggcaacctctgtgtgtaggtagcaaagctgcctacagacaggta
2701 gaggtagtaacttgacagaggcaagacacatgcaggaattgccaacagagtcaagaatgt
2761 ctggccagaatgggaactggagagcacctgggtatgtcatgctcagcctgtatctatgca
2821 aggtccaatctcatgtaagtagtcatttcttgatgcttagagagggcttagtgatttttga
2881 taatttccgtaagacagacagacagacaaccccctgccacccagtctttacttttctattc
2941 aaagtgggacagagacagtaagttgttgacccagtgtaagaagggattatagggaaggca
3001 caatcttcagagattccttgattgtgaatgtgtgatgacgccatttttcactggcaggata
3061 agccttcctgatattgtctgtggaaacagttaaatcttgaaacatctaaaatttattccaa
3121 aacaaaatttttatacttttcattgtaactataagaattcttctgtttgccaaggatttagc
3181 tcagtggcataagcatatgcctggcaagcacaaggtcatgagttgatcccag
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Fig. 2. Nucleotide sequence of two cDNAs and deduced amino acid sequence of the guinea pig peroxisomal hydratase/dehydrogenase bifunctional enzyme (EMBL Accession nos. X85112 and X92742). By analogy with the human HD cDNA sequence, the deduced coding sequence is written in capital letters, and the nucleotides are numbered beginning with the first encountered ATG codon encoding the initiator methionine. This ATG translation initiation codon, the TGA stop codon and three putative polyadenylation signals (two in the short cDNA and one in the long one) are underlined. The 5′ and 3′ non-coding regions are written in lower case. In the 3′-untranslated region, nucleotides specific of the long cDNA are indicated in bold type, while the nucleotides shared by the two cDNAs are in normal type. The NADH-binding site of the dehydrogenase, identical to the rat one, is indicated in bold type.

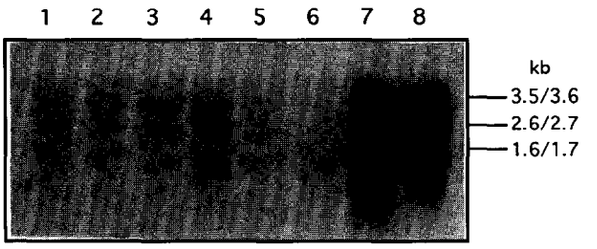

Fig. 3. Size and tissue distribution of the HD mRNA: total RNAs (20 μg) from a male adult guinea pig spleen (lane 1), lung (lane 2), muscle (lane 3), intestine (lane 4), brain (lane 5), heart (lane 6), kidney (lane 7), liver (lane 8) were electrophoresed, transferred onto nylon filter and hydridized with a cDNA derived probe ranging from nt 263 to nt 1,260. The size of the three detected transcripts (kb) are indicated on the right. The membrane was exposed 48 h to XAR films (Kodak) at −70°C. For details, see section 2.

have been obtained. The PCR-amplified 5′-end is a fragment ranging from nt −42 to 440 (position of primer P25). But surprisingly, two 3′-ends have been detected by application of the RACE protocol. Indeed, two fragments of different length have been PCR-obtained with both P23 and anchor primers. These two 3′-ends share a common, strictly identical part: from nucleotide 2,003 (position of primer P23) to nucleotide 2,452, which is the end of the shortest fragment. The longest fragment ranges from 2,003 (position of P23) to 3,232, which is the end of this second 3′-end.

The two cDNA sequences are shown in Fig. 2. The short HD cDNA is composed of 2,494 nucleotides and the long one of 3,274 nucleotides. Beginning with the first encountered ATG codon encoding the initiator methionine, the cDNA sequence exhibits an open reading frame of 2178 nucleotides encoding an enzyme of 726 amino acids (M_r 79.3 kDa) showing 79.4% of homology with human HD, and containing the peroxisomal import signal tripeptide SKL at the carboxyterminus. In that reading frame, the cDNA sequence includes 42 bp at the 5′-noncoding region and in the first case 274 bp or in the second case 1,054 bp at the 3′-noncoding region, excluding the poly(A) tails. Sequence comparison of HD coding region between human and guinea pig shows an 82% homology at the nucleotide level. Both the short and the long cDNAs are shorter than the human HD cDNA (3,779 nt). This difference is mostly due to smaller 3′ untranslated regions which exhibit little overall homology with the human one (1,607 nt). No perfect putative polyadenylation signals (AATAAA) like those found in human and rat sequence were found, but two highly homologous signals are located after nucleotide 2,451 (TATAAA) and 2,486 (CATAAA) in the short cDNA and one after nucleotide 3,149 (ATAA) in the long one.

3.2. *Three mRNAs are detected in various tissues of guinea pig*

Northern blot analysis of HD mRNA, using the cDNA clone 1 as a probe, identified two mRNAs of respective approximate sizes 2.6/2.7 and 3.5/3.6 kb in the liver and kidneys (Fig. 3). The length of these transcripts is in accordance with the two cDNA sequences described in this paper, when taking poly(A) tails into account. But surprisingly, another transcript of 1.6/1.7 kb is also detected in the same tissues. These findings highly differ from those obtained in human [6] and rat [9] where only one mRNA has been so far detected in the liver. These three mRNAs are mainly expressed in the liver and kidneys, but are also present in lower amounts in the intestine and muscles and faintly detectable in the spleen. No mRNA is detected, at least in our experimental conditions, in the brain and heart.

3.3. *The two cDNAs probably encode the same peroxisomal protein*

Some genes can produce more than one mRNA by the use of alternative transcription initiation sites (alternative promoters), splicing, cleavage, polyadenylation and nucleotide editing, and it is also well-known that frequently, the production of multiple mRNAs has no functional consequence [10]. In our case, the two transcripts are strictly identical in the common part (i.e. the first 2,494 nucleotides), suggesting that obviously they are encoded by the same gene whose transcription leads to two transcripts: a long one and a short one. In that case, a variable pre-mRNA processing (splicing and cleavage polyadenylation) probably gives rise to these two transcripts. Furthermore, their common 5′-end suggests that the transcription starts at a single transcription initiation site.

According to Kozak [11], there is a limited possibility that these two mRNAs encode two proteins of different length. Indeed, even if there are a number of well-characterized mRNAs in which translation is not limited to the AUG codon nearest the 5′-end, these 'exceptional' mRNAs must adhere to specific rules. Initiation at downstream AUG codon occurs, not haphazardly, but under three specific conditions [11] which are not combined in our case. Thus, the two cDNAs most probably encode the same protein and in that case, their untranslated 3′-ends of different length might contribute to a different stability of the messenger. Further investigations are required to elucidate that last point. The protein encoded by these two messengers possesses a C-terminal tripeptide SKL, known to be a peroxisome targeting signal and a NADH-binding site of the dehydrogenase identical to the rat one (Fig. 2).

Thus, the present work suggests that, in addition to a genuine peroxisomal HD of 726 amino acids encoded by the 2.5 and the 3.3 kb cDNAs described in this paper, protein which is highly homologous to the rat and human counterparts, another 3-hydroxyacyl-CoA dehydrogenase or 2-enoyl-CoA hydratase might exist in the guinea pig liver, as shown by northern blot analysis.

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