Molecular cloning and gene organization of the mouse mitochondrial 3,2-*trans*-enoyl-CoA isomerase

Wilhelm Stoffel*, Maria Düker, Kay Hofmann

Institut für Biochemie, Medizinische Fakultät der Universität zu Köln, Joseph-Stelzmann-Str. 52, D-50931 Köln, Germany

3,2-*trans*-enoyl-CoA isomerase (mECI, E.C. 5.3.3.8) is the key enzyme of mitochondrial β-oxidation of unsaturated fatty acids. A mouse cDNA clone spanning the entire coding region of mECI was isolated and sequenced. Subsequently, two overlapping genomic clones containing the complete mECI gene were isolated and characterized. The mouse mECI cDNA comprises an open reading frame of 867 bp, encoding a protein of 32 kDa. The mECI gene, spanning about 15 kb, consists of seven exons. Multiple transcription starts were determined by primer extension experiments. Knowledge of the gene organization and availability of genomic clones for mouse mECI will facilitate the study of unsaturated fatty acid metabolism in normal and pathological states.

1. INTRODUCTION

Mitochondrial β-oxidation of unsaturated fatty acids leads to intermediates that are not recognized as substrates by the enzymes involved in the β-oxidation of saturated fatty acids [1]. 3-*cis*-Enoyl-CoA esters arise in the oxidative degradation of fatty acids unsaturated at odd-numbered positions while 3-*trans*-enoyl-CoA esters are the product of 2,4-dienoyl-CoA reductase (EC 1.3.1.34), which has been proposed to reduce the 2,4-dienoyl-CoA esters arising in the β-oxidation of fatty acids unsaturated at even-numbered positions [2]. By converting both 3-*cis*-enoyl-CoA and 3-*trans*-enoyl-CoA esters into their 2-*trans*-enoyl-CoA isomers, which are substrates of the mitochondrial 2-*trans*-enoyl-CoA hydratase (mECH, EC 4.2.1.17), the 3,2-*trans*-enoyl-CoA isomerase (mECI, EC 5.3.3.8) is a prerequisite for the β-oxidation of both types of unsaturated fatty acids [3].

A second set of β-oxidation enzymes is located in the peroxisomes. Peroxisomal β-oxidation is of elevated importance in the degradation of very long chain fatty acids and under abnormal conditions such as prolonged fasting [4] and the influence of hypolipidemic drugs [5]. In the mitochondrial β-oxidation, the mECI and mECH activities are located on different proteins. Both of them have been cloned in the rat and shown to be related poly-peptides with molecular weights of about 30 kDa in the mature form [6–8]. In the peroxisomes, however, both activities are located together with a 3-hydroxy-acyl-CoA dehydrogenase on a single protein chain of 78 kDa, the peroxisomal trifunctional enzyme (pTFE) [9]. The mECI and mECH activities are supposed to share the N-terminal half of the pTFE chain, a region that shows sequence homology to both of the mitochondrial enzymes. The same arrangement of β-oxidation acitivities as in the peroxisomes is found in *E. coli* [10] where the N-terminal half of the fadB encoded protein harbours both ECI and ECH activities. Sequence analysis suggests that mECI, mECH and the N-terminal halves of pTFE and fadB have arisen from a common ancestor.

Inborn errors of mitochondrial fatty acid oxidation have only recently been described as a cause of metabolic diseases. Besides 12 already characterized inherited human disorders of mitochondrial β-oxidation [11], there are numerous additional clinical observations referring to an altered fatty acid oxidation. Thus, it is likely that various genetic disorders of this pathway are still unidentified. Studies of the role of unsaturated fatty acids and their mitochondrial degradation will be facilitated by knowledge of the organization of the mouse mECI gene and by the availability of corresponding genomic clones.

2. MATERIALS AND METHODS

2.1. *Isolation of mECI cDNA and genomic clones*

An oligo-dT primed mouse liver cDNA library in λgt11(Clontech) was screened with rat mECI cDNA described previously [6]. Plaques producing positive signals were isolated and the phage DNA prepared by established procedures [12]. The cDNA inserts were obtained by *Eco*RI digestion and characterized by Southern blot analysis and DNA sequencing.

A mouse (Balb/c) leukocyte genomic library in EMBL3 SP6/T7 (Clontech) was screened with the complete mouse mECI cDNA as a probe. The corresponding phage clones were characterized by restriction mapping using single and double digestions with the enzymes SacI, XbaI, BamHI and EcoRI and by Southern blot hybridization.

2.2. Subcloning and sequencing

The full-length cDNA clone and the exon-containing portions of the two genomic mECI clones were subcloned into the pBlueskript SK(+) vector (Stratagene). DNA sequencing of the plasmid subclones was carried out by the didesoxy chain termination method [13] using the T7-sequencing kit (Pharmacia).

2.3. Primer extension analysis

The transcription start site was determined by primer extension using the synthetic antisense oligonucleotide oISO-PE (5'-CAGCA-GAAGGCGACGCGCAGCAGCCAGCGCCAT-3') and poly(A)$^+$ RNA prepared from mouse liver RNA [14]. The 5'-labeled oligonucleotide was hybridized to 2 and 4 μg RNA, respectively. The primer extension reaction was performed following the standard procedure [15] with Moloney murine leukemia virus RNaseH$^-$ reverse transcriptase (Gibco-BRL). Extension products were analyzed on a 7 M urea 5% polyacrylamide gel adjacent to a sequence ladder generated with the same 33nt oligonucleotide primer.

3. RESULTS AND DISCUSSION

3.1. Mouse liver mitochondrial 3,2-trans-enoyl-CoA isomerase cDNA

A 900 bp EcoRI fragment of the previously cloned rat liver mECI [6] was used for screening a mouse liver cDNA library in λgt11. A cDNA clone with a 1 kb insert spanning the whole coding region of mECI were isolated and the sequence determined. The full length mouse liver mECI cDNA has an open reading frame of 867 bp, coding for the uncleaved pre-mECI of 289 amino acids. Comparison of the peptide sequence with the rat liver mECI and the recently cloned human liver mECI (Janßen, Stoffel, submitted) yielded sequence identities of 85% and 74%, respectively.

3.2. Mouse 3,2-trans-enoyl-CoA isomerase gene

The above described cDNA clone was used for screening a mouse leukocyte genomic library in λ-EMBL3 SP6/T7. Two genomic clones, λ-ISO9 and λ-ISO8, with insert sizes of 17.1 and 14.6 kb were isolated and characterized by restriction mapping and partial sequencing. The resulting gene organization is shown in Fig. 1. The mouse mECI gene is organized in 7 exons, 40 to 117 bp long, separated by 6 introns with sizes between 90 bp and 7.7 kb. All coding segments of the gene together with a part of the flanking introns have been sequenced, the result is shown in Fig. 2. The exon/intron boundaries are in agreement with their respective consensus sequences. The mouse mECI gene contains several copies of the dispersed B1 repeat, one copy located in the promoter region, another one immediately downstream of the stop-codon.

3.3. The transcription initiation site

Primer extension experiments with mouse liver mRNA were used for the determination of the transcription start site of the mECI gene. The untranslated region was extended from the 5'-end labeled antisense oligonucleotide oISO-PE, derived from 11 N-terminal amino acid residues of the signal sequence. The extension products were analyzed on a sequencing gel by alignment to a sequence ladder generated by the didesoxy chain termination reaction of a genomic subclone, also using oISO-PE as a primer. As shown in Fig. 3, three clusters of signals were detected. Major bands correspond to the nucleotides C at −72, G at −87 and T at −99 upstream of the ATG (Fig. 3).

Sequence analysis of the 5'-flanking region of the mouse mECI gene showed no typical TATA or CAAT box in proper spacing to the putative transcription start. The immediate upstream region is relatively GC rich
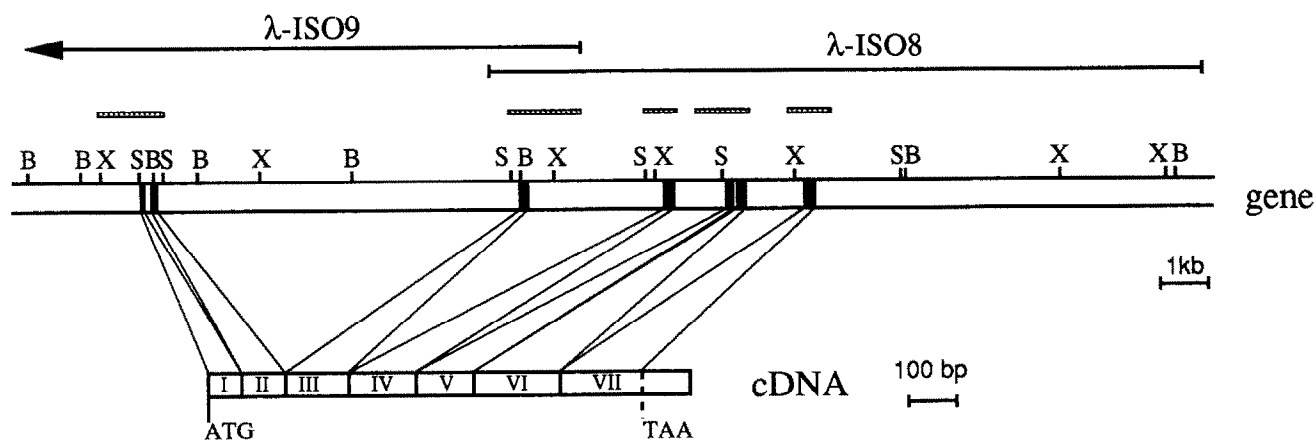

Fig. 1. Gene structure of the murine mECI. In the gene structure, exons are indicated as black boxes and recognition sites of the restriction enzymes BamHI (B), SacI (S) and XbaI (X) are shown. The two genomic clones λ-ISO9 and λ-ISO8 are represented at the top of the figure, regions studied by DNA sequencing are indicated by shaded boxes above the gene.

ggcagatttctgagttcaaggtcagcctggtctataaagtgagttccaggacagccaggactacacagaaaccctgtctcaaaaaaccaaaaaaaaaag

aataaaaggaagacttcttgtgtttgtgccccttgagtattgagtcaaataaaaatgcactatttggggcaaatagcctaggtaaagcctcccagtcacc

cctgtgctggcaaggccgtctgttcttcactacggtgccagccttgatttatggtcctaatcccagctctgcaagtgcctctgagctctggcagctcgcc

ccagcaaaatgaaagcactcttggacacacaccctgccctaactggcacaggggaaacccagtcctctggacgctgcggactggctaaccttggccttgt

ggggcggggccggccggagaggaggagtctgcaaggccgggctggaagcgaacgcgctggctctgtagtcccactgatatccaagATGGCGCTGGCTGCT

                                                                                          MetAlaLeuAlaAla   I

GCGCGTCGCCTTCTGCTGCACGCGGtgcgtggcccgggcgagagcgctggtgcagggcctcacagggctgggtgcgcgggcggcggtggcgagagtctg

AlaArgArgLeuLeuLeuHisAlaG

ggcgtgcttgctaacgtgctccgggctgtctgcagGATCCCGCCTCGGCGCAGGGAAGCGGTGCACGGCGCGCGGCCGTTCGCTAACAAGCGGGTGCTG

                                     lySerArgLeuGlyArgArgGluAlaValAspGlyAlaArgProPheAlaAsnLysArgValLeu   II

GTGGAGACGGAGGGCCCGGCAGgtgagagggagggagggccacatctcgttcatctcctcgggctccgagcccacacggtctgtgaggtcgggctgacga

ValGluThrGluGlyProAlaG

tgagctc
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gagctctacctgtctctgcctcctgaatcctgtgattaaaggcctgtgccaccacttggcctgcctataaacctctaaattctgcagttactgtagccag

actaggaaccaggggtgggcaggtatggcctgggctaagccttgagaggatcccctcttttctaaactagGGGTTGCAGTGATGAAGTTAAGGAACCCTC

                                                                       lyValAlaValMetLysLeuArgAsnProP   III

CCGTGAATTCCCTCAGCTTGGAGTGTCTCACAGAGTTCACCATCAGCCTGGAGAAGCTGGAAAATGACAAGAGCATCCGAGGTGTCATCCTCACGTCGgt

roValAsnSerLeuSerLeuGluCysLeuThrGluPheThrIleSerLeuGluLysLeuGluAsnAspLysSerIleArgGlyValIleLeuThrSer

acgtgcctgttcaagccacactccagcctgactggggaagagcaggaacctagaagatctggagccctctatcatgcaggqctgagtcaccaggcacccc

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agtcagtccaggctgtgtccttgacccctcagttctggactccatgggaagcaccctcttaccagccaaacacggcccaccctgccttctccccgcagGA

                                                                                                    G1

ATGCCCGGGTATCTTCTCGGCTGGGCTGGACTTGCTGGAGATGTATGGCCGGAACCCAGCCCACTATGCTGAGTACTGGAAGAACGTGCAGGAGCTGTGG

uCysProGlyIlePheSerAlaGlyLeuAspLeuLeuGluMetTyrGlyArgAsnProAlaHisTyrAlaGluTyrTrpLysAsnValGlnGluLeuTrp   IV

CTGAGACTCTACACGTCCAACATGATCTTAGTGTCTGCCATCAATgtgagtgccc

LeuArgLeuTyrThrSerAsnMetIleLeuValSerAlaIleAsn
                                |
                             0.4 kb
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tacactgagaacacagtcttgaaacagtgcccaaataaaaatagggtgaaaataacaaccatcaagaaaagaagagggctggtgagatggctcagtgggt

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cagtgtgagcgccttctgccctgcctaaggaatattactggaacacctcctgaccgattctttctctcttcagGGAGCCTCTCCAGCTGGAGGGTGCCT

                                                                                     GlyAlaSerProAlaGlyGlyCysLe   V

CTTGGCTCTCTGCTGTGACTACAGGGTTATGGCTGACAACCCCAAATATACTATAGGATTGAATGAGAGCCTGCTGGGCATTGTTGCCCCCTTCTGgtaa

uLeuAlaLeuCysCysAspTyrArgValMetAlaAspAsnProLysTyrThrIleGlyLeuAsnGluSerLeuLeuGlyIleValAlaProPheTr

tacaaaggactgcacccatactttatatttgcattctggtggaacatcccctcatggctcagaggtaaacgatgtcttcattgcagGTTTAAAGACATGT

                                                                                    pPheLysAspMetT   VI

ATGTGAACACCATCGGGCACCGGGAGGCGGACGGTGCCCTTCAGCTGGGGACCCTTTTCTCACCAGCAGAGGCCCTCAAGGTGGGAGTGGTGGATGAGGT

yrValAsnThrIleGlyHisArgGluAlaAspGlyAlaLeuGlnLeuGlyThrLeuPheSerProAlaGluAlaLeuLysValGlyValValAspGluVa

GGTACCTGAGGATCAGGTACACAGCAAGGCTCGCTCAGTGATGACCAAGTGGTTAGCTATCCCAGgtaaggagcaagcatgctagtccctccatgcagct

lValProGluAspGlnValHisSerLysAlaArgSerValMetThrLysTrpLeuAlaIlePro

ttggagtggctggttaagggaagccttctcattctggttgaccacctggtgaaaccaaa
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tctagatgggatattgctatgtatttcaggctgaccctggactccttagcccaccttagctgccctaatggtttctctgattttctcaactctaggagag

tttatatttcagtacttgaggactcaagtgtagaggagccgggaacgGcccccccacactgcacaggtgagtttcataagtaaccctgttttcttcagACC

                                                                                                     spH

ATTCTCGGCAGCTGACAAAGAACATGATGCGAAAGGCCACCGCAGACAACCTGATCAAGCAGCGAGAGGCTGACATCCAGAATTTCACCAGTTTCATCTC

isSerArgGlnLeuThrLysAsnMetMetArgLysAlaThrAlaAspAsnLeuIleLysGlnArgGluAlaAspIleGlnAsnPheThrSerPheIleSe   VII

CAAAGACTCCATCCAGAAGTCCCTACACATGTACTTGGAAAAGCTCAAGCAAAAGAAGGGCTAAcctaggtggatttctgagttcgaggccagcctggtc

rLysAspSerIleGlnLysSerLeuHisMetTyrLeuGluLysLeuLysGlnLysLysGly *

tacagagtgagttccaggacagccaggggtatacagagaaaccctgttttgaaaaaaaaaataaaaataagggctaacctacggctgccacatatgtgcc

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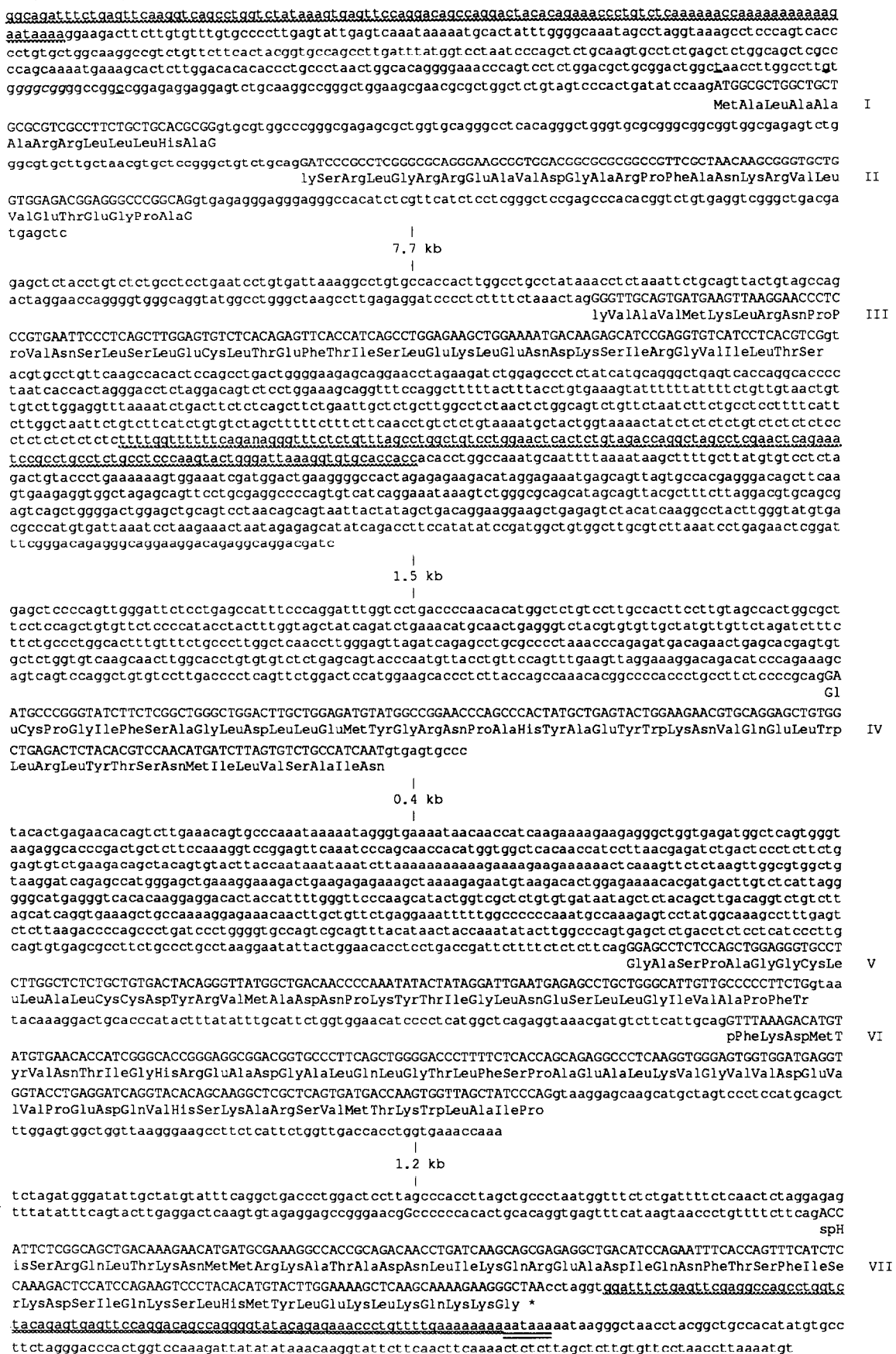
Fig. 2. Partial sequence of the murine mECI gene. Nucleotide sequences of the seven exons (I–VII) and the corresponding amino acid sequences are shown. Distances between the exons were calculated from the restriction map. The three dominant transcription start sites are underlined. The consensus Sp1 recognition sequence GGGCGG is shown in italics. A putative polyadenylation signal is double underlined. Three copies of the dispersed B1-repeat are denoted by dotted lines.
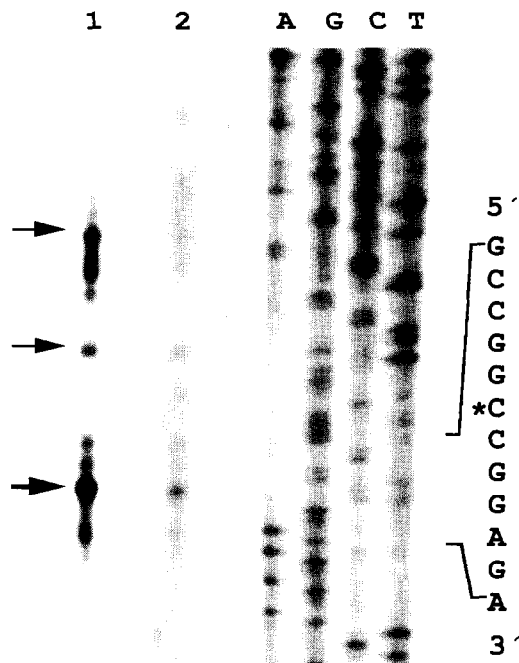
Fig. 3. Primer extension mapping of the transcription start site. The transcription start site was analyzed by primer extension using the oligonucleotide oISO-PE and 4 μg (1) or 2 μg (2) mouse liver poly(A)$^+$ RNA. The exact positions were determined by using the corresponding sequence ladder as a size marker.

and contains one consensus site for Sp1 binding (GC box, GGGCGG) between position −78 and −84 upstream of the start codon. The relative lack of well characterized cis-elements is not uncommon for constitutively expressed proteins like mECI and is in good agreement with the situation in the human mECI gene.

3.4. The intron insertion sites

Consideration of the intron insertion positions of the coding region of mECI is interesting in two different aspects. A comparison of intron insertion sites of mECI and the N-terminal half of pTFE (Fig. 4) shows that these positions are completely independent. It can be concluded that the divergence of the mitochondrial and peroxisomal β-oxidation pathway has taken place earlier than the processes of intron-gain or intron-loss, which are responsible for the actual positions of the intron insertion sites. In the light of the 'exon shuffling' hypothesis [16], the intron insertion points of mECI together with those of the pTFE are suitable to define module boundaries of the supposedly conserved tertiary structure of the known β-oxidation enzymes possessing ECI and/or ECH activity.
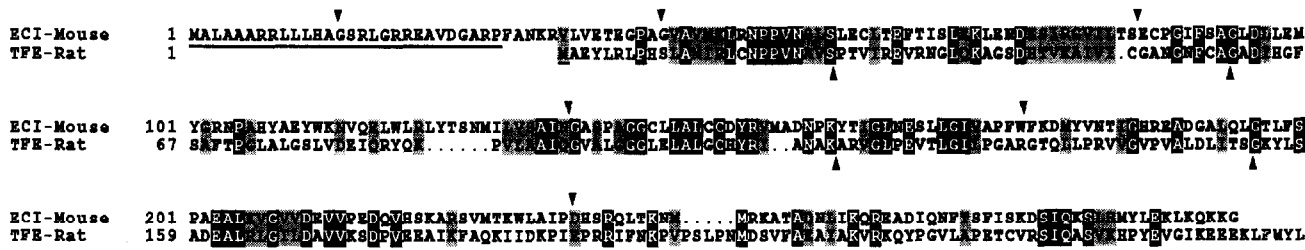

Fig. 4. Comparison of the intron insertion points of mECI and pTFE. The two protein sequences are shown in one-letter representation, sequences not present in the mature polypeptide are underlined. Identical residues are printed in inverse colors, similar amino acids (I,L,V,M), (A,G,S.T), (F,Y), (H,K,R), (D,E), (D,N), (E,Q) are printed on shaded background. Positions of the intron insertions are indicated by arrowheads.

REFERENCES

[1] Stoffel, W. (1966) Naturwiss. 53, 621–630.

[2] Kunau, W.H. and Dommes, P. (1978) Eur. J. Biochem. 91, 533–544.

[3] Stoffel, W., Ditzer, R. and Caesar, H. (1964) Hoppe-Seyler's Zeitschr. Physiol. Chemie 339, 167–181.

[4] Krahling, J.B., Gee, R., Murphy, P.A., Kirk, J.R. and Tolbert, N.E. (1978) Biochem. Biophys. Res. Commun. 82, 136–141.

[5] Lazarow, P.B. and de Duve, C. (1976) Proc. Natl. Acad. Sci. USA 1976, 2043–2046.

[6] Müller-Newen, G. and Stoffel, W. (1991) Biol. Chem. Hoppe-Seyler 372, 613–624.

[7] Palosaari, P.M., Vihinen, M., Mäntsälä, P.I., Alexson, S.E.H., Pihlajaniemi, T. and Hiltunen, J.K. (1991) J. Biol. Chem. 266, 10750–10753.

[8] Minami-Ishii, N., Taketani, S., Osumi, T. and Hashimoto, T. (1989) Eur. J. Biochem. 185, 73–78.

[9] Palosaari, M.P. and Hiltunen, J.K. (1990) J. Biol. Chem. 265, 2446–2449.

[10] DiRusso, C.C. (1990) J. Bacteriol. 172, 6459–6468.

[11] Coates, P.M. and Tanaka, K. (1992) J. Lipid Res. 33, 1099–1110.

[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, New York.

[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.

[14] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.

[15] McKnight, S.L. and Kingsbury, R. (1982) Science 217, 316–324.

[16] Gilbert, W., Marchionni, M. and McKnight, G. (1986) Cell 46, 151–154.