Organization and sequence of the gene for the human mitochondrial dicarboxylate carrier: evolution of the carrier family

Giuseppe FIERMONTE*, Vincenza DOLCE*†, Roberto ARRIGONI*, Michael J. RUNSWICK‡, John E. WALKER‡[1] and Ferdinando PALMIERI*

*Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari, Via E. Orabona 4, 70125 Bari, Italy, †Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Calabria, Arcavacata di Rende, Cosenza, Italy, and ‡The MRC Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2DH, U.K.

The dicarboxylate carrier (DIC) is a nuclear-encoded protein located in the mitochondrial inner membrane. It catalyses the transport of dicarboxylates such as malate and succinate across the mitochondrial membrane in exchange for phosphate, sulphate and thiosulphate. We have determined the sequences of the human cDNA and gene for the DIC. The gene sequence was established from overlapping genomic clones generated by PCRs by use of primers and probes based upon the human cDNA sequence. It is spread over 8.6 kb of human DNA and is divided into 11 exons. Five short interspersed repetitive Alu sequences are found in intron I. The protein encoded by the gene is 287 amino acids long. In common with the rat protein, it does not have a processed presequence to help to target it into mitochondria. It has been demonstrated by Northern- and Western-blot analyses that the DIC is present in high amounts in liver and kidney, and at lower levels in all the other tissues analysed. The positions of introns contribute towards an understanding of the processes involved in the evolution of human genes for carrier proteins.

Key words: evolution, human gene, mitochondria.

INTRODUCTION

The transport of important metabolites across the inner membranes of mitochondria is mediated by specific carrier proteins that span the lipid bilayer [1–4]. One of these transporters, the dicarboxylate carrier (DIC) protein, has been isolated from rat liver [5,6] and yeast [7], and has an apparent molecular mass of 28 kDa. Since 1967, when the existence of the DIC was proposed [8], the properties of this carrier have been investigated extensively. First, it was described as catalysing an electroneutral exchange of dicarboxylates such as malate for P_i [8–11]. Later, certain sulphur-containing compounds, particularly sulphate and thiosulphate, were also found to be transported via this carrier by an antiport mechanism [12,13]. DIC is inhibited by some impermeable dicarboxylate analogues, including butylmalonate [14], and also by bathophenanthroline and thiol reagents [15–17]. The activity of the DIC is high in liver and low in heart [18]. In liver it plays an important role in gluconeogenesis, in urea synthesis and in sulphur metabolism [1]. Similar to many other mitochondrial carriers [3], DIC has a simultaneous (sequential) antiport reaction mechanism [19]. Recently, the cDNA for DIC was cloned from rat [20], and the human gene was mapped to chromosome 17q25.3 [21]. In the present paper we describe the cloning and the nucleotide sequences of the cDNA and gene for human DIC. The gene is spread over 8.6 kb of DNA. It has 11 exons separated by 10 introns, more than described to date for any other gene for a mitochondrial carrier. Their positions help to understand how human genes for mitochondrial carriers have evolved.

EXPERIMENTAL

DNA sequence analysis

An automatic DNA sequencer (Applied Biosystems, Prism 377) was employed with Big Dye Terminator Kits (Perkin–Elmer). Sequence errors from PCRs were avoided by sequencing at least three independent clones in both directions from each reaction. Ambiguities were resolved by sequencing more clones. Databases were compiled and analysed with the program Autoassembler (Perkin–Elmer).

Amplification of human cDNA sequence

Touchdown PCRs [22] were performed using an adaptor-ligated double-stranded human liver cDNA (1 ng, Clontech) as described previously [20]. The full-length cDNA sequence was obtained in two PCR reactions of 5′ and 3′ ends extension using the adaptor primers AP1 and AP2 (Clontech) and a primer set (Figure 1) designed on the basis of the nucleotide sequence of rat cDNA [20] and on the emergent human cDNA sequence. The PCR products were identified and recovered from agarose gels [23], cloned into the pCR2.1 Topo-vector (Invitrogen) and sequenced.

Amplification of human genomic DNA sequence

Overlapping segments of human genomic DNA (Boehringer Mannheim Biochemicals) were amplified in three PCRs, using synthetic oligonucleotide primers with appropriate linkers based on the human DIC cDNA and on the emergent sequences. The

Abbreviations used: HUGO, Human Genome Organisation (http://www.gene.ucl.ac.uk/nomenclature/); GDB, Genome Database (http://gdbwww.gdb.org/); DIC, (the) dicarboxylate carrier (the abbreviation DIC is approved by the HUGO/GDB Nomenclature Committee).

[1] Present address and address for correspondence: Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, U.K. (e-mail walker@mrc-dunn.cam.ac.uk).

The sequence data reported in this paper have been deposited in the EMBL/GenBank® Nucleotide Sequence Databases under the accession numbers AJ131613 (DIC cDNA) and AJ131612 (DIC gene).

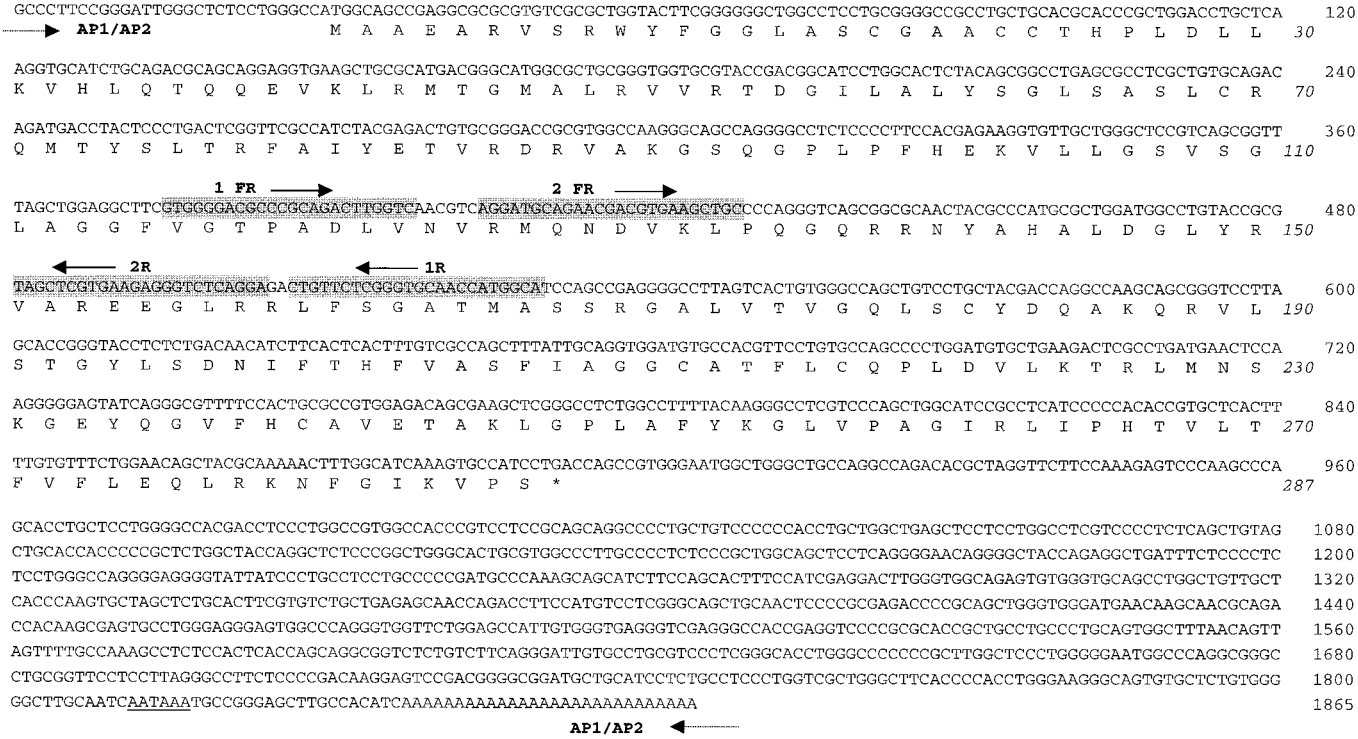

Figure 1 Sequence of the human liver cDNA and the encoded protein sequence of the DIC

The amino acids are numbered from 1–287. An asterisk denotes the stop codon. Primer sequences are shaded. Horizontal arrows pointing to the left or right indicate that the primers were synthesized as either the sequence shown or its complement respectively. The underlined sequence is a potential polyadenylation signal.

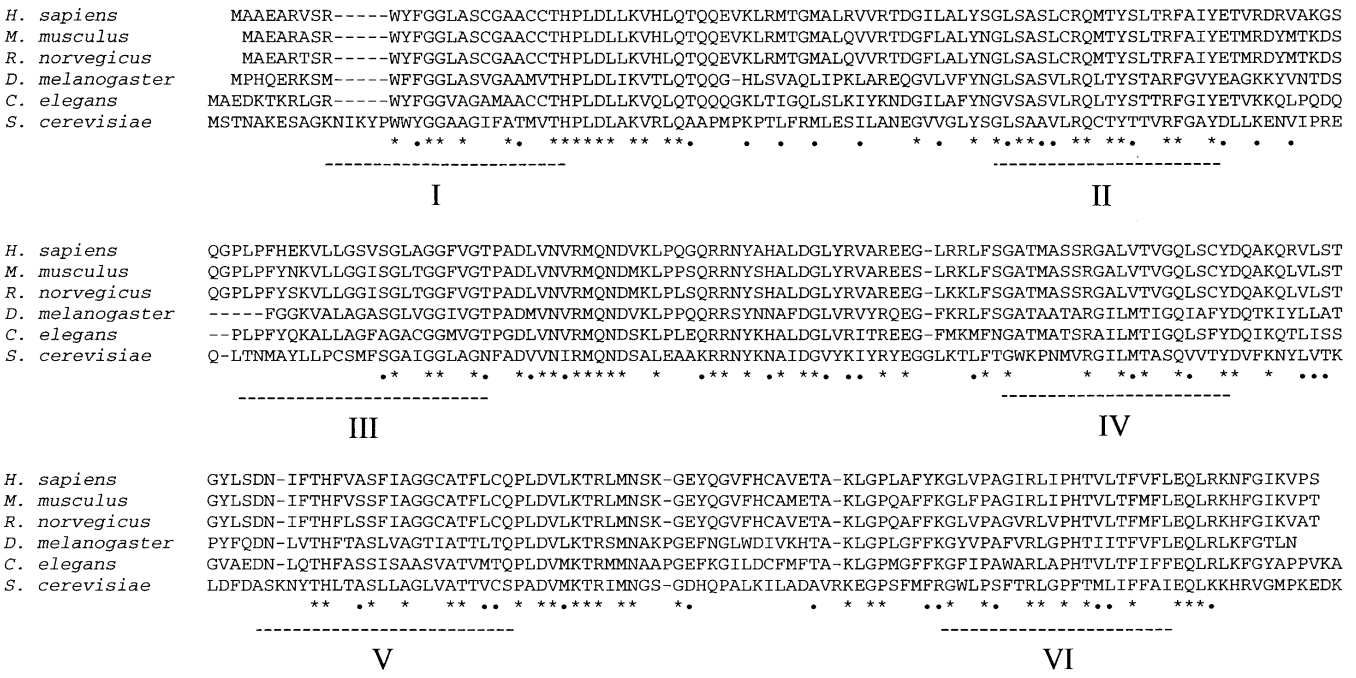

Figure 2 Alignment of the protein sequences of the DIC from human (*Homo sapiens*), rat (*Rattus norvegicus*), mouse *(Mus musculus), D. melanogaster, C. elegans* and *S. cerevisiae*

Asterisks (*) and bold dots (●) indicate identity and conservation respectively of residues in all six sequences. Segments I–VI are regions that are proposed to be folded into transmembrane α-helices.

forward and reverse primers are shown in Figure 6 (below). The reactions were carried out as described previously [23] for 30 cycles and, after addition of fresh portions of enzyme and primers, for an additional 30 cycles. The reaction products were analysed on 0.8 % high-melting-temperature-agarose gels. The DNA fragments were transferred to Hybond-N membranes (Amersham), cross-linked to the membranes by irradiation with UV light, and hybridized with radioactively labelled synthetic oligonucleotides at 5 °C below the minimum dissociation temperature. Fragments that hybridized with the probes (see Figure 6 below) were recovered from the gel, cloned into the pCR2.1 Topo-vector (Invitrogen) and sequenced.

Northern-blot analysis

A human multiple-tissue Northern blot was purchased from Clontech. The hybridization experiments were performed as described previously [24], except that the probe was nts 695–1837 of the human cDNA DIC sequence (see Figure 1). Samples were autoradiographed at −70 °C for 15 days with an intensifying screen. Hybridization signals were normalized with an 850 bp probe encoding part of rat glyceraldehyde-3-phosphate dehydrogenase [25] employed under the same conditions as those described above.

Western-blot analysis

The anti-DIC antiserum was raised against the entire rat DIC obtained by overexpression in *Escherichia coli* [20]. Samples of rat mitochondria were solubilized in SDS sample buffer, boiled for 5 min, analysed by SDS/PAGE [26] and the proteins then transferred to nitrocellulose membranes by electrophoresis. The membranes were exposed for 2 h to anti-DIC antiserum [diluted 1:3000 in PBS-TM, which is PBS containing 0.5 % (w/v) non-fat dried milk and 0.05 % (v/v) Tween-20], washed for 10 min three times in PBS-TM, and then exposed for 2 h to the secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (Pierce) diluted 1:2000 in PBS-TM. Immunodecoration was revealed by 3,3′-diaminobenzidine as substrate for the peroxidase reaction. The immunodecoration was normalized using an antiserum raised against the subunit IV of bovine cytochrome *c* oxidase (kindly given by Dr. B. Kadenbach, Biochemie im Fachbereich Chemie der Philipps-Universität, Marburg, Germany) under the same conditions as those described above.

Database searches

The sequence encoding the *Drosophila melanogaster* DIC was identified with the TBLASTN program by screening the *Drosophila* (Berkeley Fly Database U.S.A) and the National Center for Biotechnology Information ('NCBI') databases with the human DIC protein sequence. The *D. melanogaster* DIC protein sequence was compiled from information in a genomic clone (accession number AC003923) and in two expressed-sequence-tag ('EST') clones (accession numbers AI238956 and AI401861).

RESULTS AND DISCUSSION

Cloning and sequencing of the cDNA for the human DIC

Two forward-nested oligonucleotides 1FR and 2FR (see Figure 1) based on the rat DIC cDNA nucleotide sequence [20] and the AP1 and AP2 adaptor primers were employed to amplify the 3′ extremity of the human DIC cDNA (see the Experimental section). The product gave a single band on a gel of about 1450 bp, which was cloned and sequenced. Two nested reverse primers (see Figure 1) were chosen from this emergent human sequence in order to amplify the 5′ end of the human DIC cDNA. The product of the 5′ end extension reaction gave several bands on a gel. The longest fragment, of about 500 bp, hybridized with probe (2FR) and was sequenced. It overlapped the previous

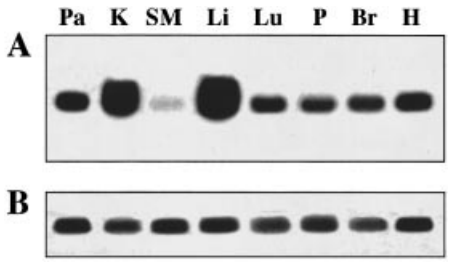

Figure 3 Expression of the DIC mRNA in human tissues

Northern blots were hybridized with the following probes: (**A**) a 1.15 kb probe from the human DIC cDNA; (**B**) a 0.85 kb probe from a rat cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. Each lane contained about 2 μg of polyadenylated RNA. The RNA samples were from pancreas (Pa), kidney (K), skeletal muscle (SM), liver (Li), lung (Lu), placenta (P), brain (Br) and heart (H).

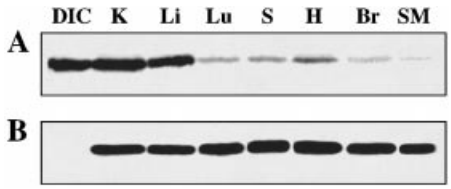

Figure 4 Expression of DIC protein in rat tissues explored by Western-blot analysis

Mitochondria (75 μg of protein) isolated from various rat tissues, and recombinant DIC (0.1 μg), were analysed by SDS/PAGE. The separated proteins were transferred from the gel to nitrocellulose sheets and were treated with the anti-DIC antiserum (**A**) or with anti-(subunit IV of the cytochrome *c* oxidase) antiserum (**B**). DIC denotes recombinant DIC. K, Li, Lu, S, H, Br and SM are samples from kidney, liver, lung, spleen, heart, brain and skeletal muscle respectively.

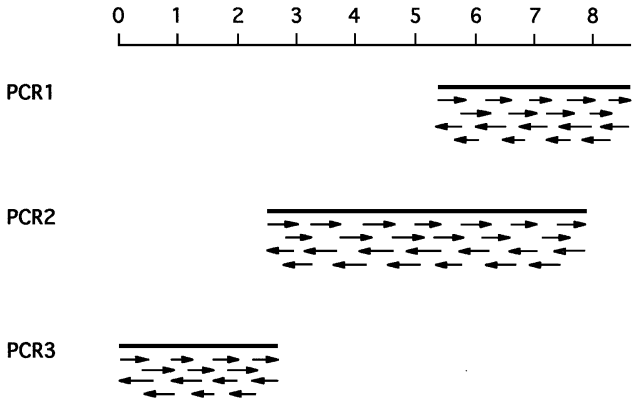

Figure 5 Generation by PCRs and sequence analysis of genomic clones of the human DIC gene

PCR1–PCR3 refer to DNA segments of human DNA amplified by PCRs. The heavy horizontal lines are proportional to the lengths of these DNA segments, and the arrows represent the directions and the extents of the determined DNA sequences. The scale is in kb.

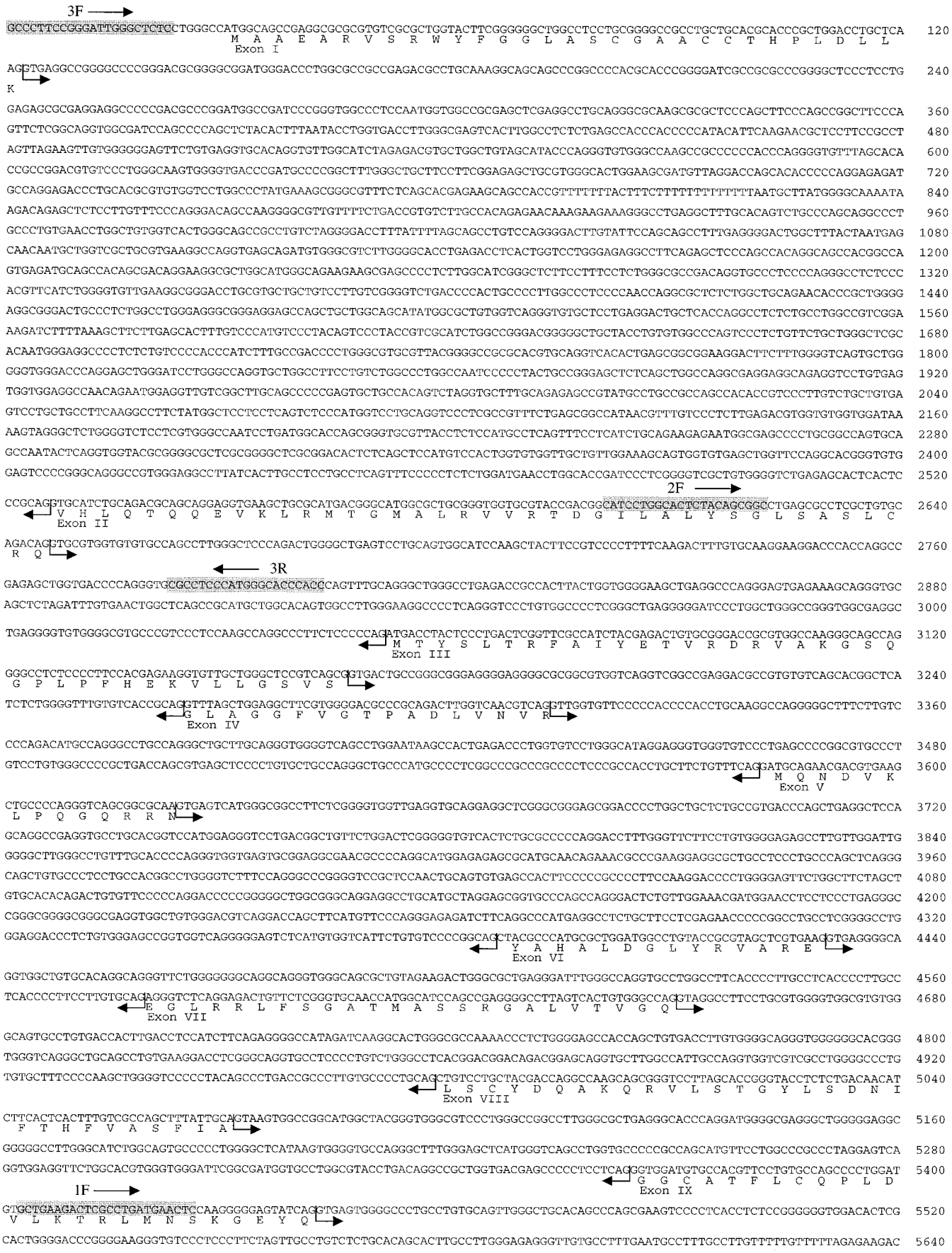

Figure 6 For legend see opposite page

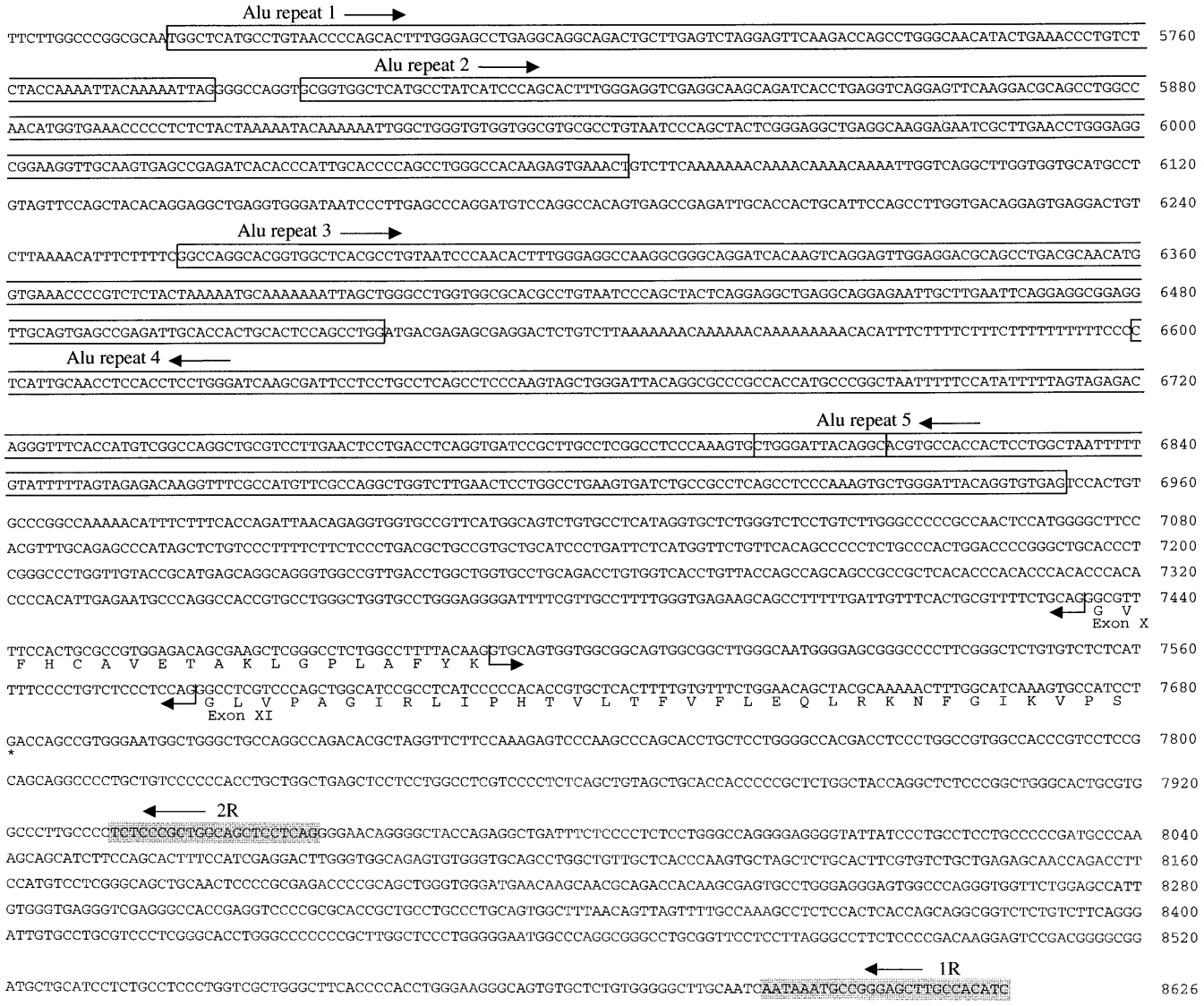

Figure 6 DNA sequence of the human DIC gene

The nucleotide sequence is numbered, and the locations of exons I–XI and the protein sequences they encode are shown. Exon/intron boundaries are denoted by small arrows. The stop codon is denoted by an asterisk (*). Primer sequences used in PCR and hybridization experiments are shaded. Horizontal arrows pointing to the left or right indicate that the primers were synthesized as either the sequence shown or its complement respectively. Large boxes contain Alu sequences.

fragment and contained the putative translational initiator methionine codon. The final human cDNA sequence of 1865 nucleotides (see Figure 1) was determined completely in both directions. The assignment of the proposed initiation codon is supported by the closely related murine sequence, where the corresponding codon is preceded by an in-frame stop codon. The 5′ and 3′ untranslated regions are 29 and 972 bp in length respectively. The 3′ end of the sequence is terminated by a run of A residues separated by a 20 nucleotide sequence from the preceding AATAAA sequence, a typical signal for polyadenylation of mRNA [27].

The cDNA encodes a protein of 287 amino acids, one amino acid longer than the rat and mouse homologues, with a molecular mass of 31 329 Da. The protein sequence of human DIC is 88, 89, 57, 57 and 36% identical to the rat, mouse, *D. melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* homologues respectively (see Figure 2).

Expression of the DIC in various tissues

We studied the distribution of the transcript of the DIC gene by Northern blots performed on mRNA derived from various human tissues. Among the tissues examined, DIC was expressed most strongly in kidney and liver. Lower levels were detected in pancreas, lung, placenta, brain and heart, and even less was found in skeletal muscle (see Figure 3). The DIC transcript, estimated to be about 2.3 kb long, is larger than the cDNA sequence that has been determined, indicating that the transcript may have an extensive 5′ non-coding region. The tissue distribution at the protein level was also investigated by Western-blot experiments performed on rat mitochondrial lysates, using an antiserum raised against the rat DIC protein expressed in *E. coli*. Abundant levels of protein were detected in kidney and liver, whereas lower levels were found in lung, spleen, heart and brain (Figure 4). The lowest level of DIC was detected in skeletal

Table 1 Exon–intron organization of the DIC gene

Exon sequences are shown in UPPER CASE, intron sequences are shown in lower case, and splice sequences ag/gt are in **bold face**. The interrupted amino acids are shown in parentheses with three-letter abbreviations; the location of the slash (/) indicates the location within the codon where the splice junction occurs; and dashes (–) indicate uninterrupted codons.

Exon	Size (bp)	3′ Splice site (acceptor)	5′ Splice site (donor)	Intron	Size (bp)	Interrupted amino acid
1	122		TGCTCAAG**gt**gaggc	A	2404	–
2	120	actcccgc**ag**GTGCA	GCAGACAG**gt**gcgtg	B	405	–
3	115	tctcccccc**ag**ATGAC	CGTCAGCG**gt**gactg	C	98	G/GT (Gly)
4	49	gtcaccgc**ag**GTTTA	AACGTCAG**gt**tggtg	D	268	AG/G (Arg)
5	42	tctgtttc**ag**GATGC	CGGCGCAA**gt**gagtc	E	763	AA/C (Asn)
6	44	tccccggc**ag**CTACG	TCGTGAAG**gt**gaggg	F	149	G/AG (Glu)
7	71	ccttgtgc**ag**AGGGT	TGGGCCAG**gt**aggcc	G	328	–
8	93	gcccctgc**ag**CTGTC	TTATTGCA**gt**aagtg	H	293	–
9	78	ccctcctc**ag**GGTGG	AGTATCAG**gt**gagtg	I	1992	–
10	57	ttttctgc**ag**GGCGT	TTTACAAG**gt**gcagt	L	89	–
11	1046	ctccctcc**ag**GGCCT				

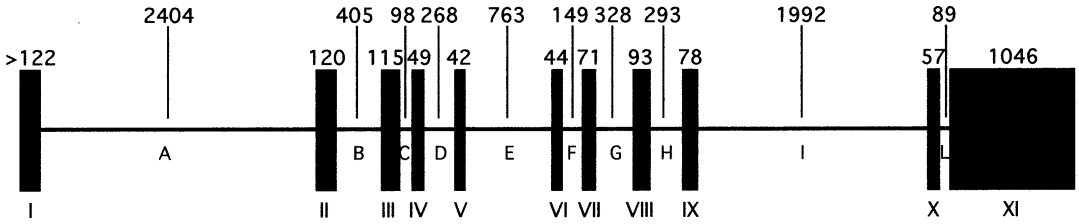

Figure 7 Structure of the human gene encoding the mitochondrial DIC

The exons and introns are shown as filled boxes and continuous lines respectively, and their sizes are given in bp.

muscle, in agreement with the results obtained by Northern blotting. At present, there is no explanation for the presence of DIC in heart and other tissues where gluconeogenesis and ureogenesis do not operate. Thus the DIC may have other functions in these tissues, possibly exclusively catalysing supply of substrates to the tricarboxylic acid cycle.

Cloning and sequencing the human gene for the DIC

The human genomic sequence of the DIC gene was derived from three overlapping clones generated by PCR1, PCR2 and PCR3 (Figure 5) using genomic DNA as template. The primers employed in PCR1, namely 1F and 1R (see Figure 6), corresponded to nucleotides 695–718 and 1812–1837 respectively of the human DIC cDNA sequence. PCR1 gave a single band of about 3200 bp, which corresponded to the 3′ extremity of the DIC gene and contained only the intron L (Figure 6). Using primers 2F and 2R (see Figure 6), corresponding to nucleotides 197–218 and 1143–1164 respectively of the human DIC cDNA sequence, a genomic fragment of about 5300 bp was obtained in PCR2. This fragment, which overlapped the one obtained in PCR1 for 2551 bp (see Figure 5), was much bigger than the corresponding segment of the human cDNA, indicating the presence of introns. In fact, nine introns (B–L) were present in this region (Figure 6). The 5′ extremity of the human DIC gene was completed with the approx. 2800 bp fragment obtained in PCR3 (Figure 5). The primers employed in this third PCR corresponded to nucleotides 1–22 (3F) of the human DIC cDNA and 2783–2803 (3R) of the human DIC gene (Figure 6). The product of PCR3, which overlapped the fragment obtained in PCR2 for 203 bp (Figure 5), was about 2400 bp larger than the corresponding cDNA sequence because it contained the first intron (A) of the gene (Figure 6).

The genomic region cloned by PCR in the present study corresponds to the same gene which has been previously mapped to chromosome 17 [21], since in the latter studies the fluorescence-*in-situ*-hybridization ('FISH') experiments were performed using the same genomic fragment obtained in PCR2 (see Figures 5 and 6).

Gene structure

The exons of the human DIC gene were identified by comparison of the genomic sequence with the human cDNA sequence and by comparison of the deduced amino acid sequence with that of the carrier. Consensus rules for splice sites, which predict conservation of the dinucleotides GT and AG next to the 5′ and 3′ intron boundaries respectively, were also taken into consideration [28]. All the introns in the human DIC gene have the canonical dinucleotides GT and AG at their 5′ and 3′ sites respectively (see Table 1 for a summary of the properties of the introns). In this way it was found that the human DIC gene has 11 exons separated by ten introns. Its structural organization is indicated in Figure 7. The DNA sequence of the eleven exons, ranging in size from 42 to 1046 bp, corresponded to the human cDNA sequence (Figure 1). The junctions of introns A, B, G, H, I, and L are type 0 (splicing between codons), those of introns C and F are type I (splicing after the first base of the codon) and those of introns D and E are type II (splicing after the second base of the codon) (see Table 1). The transcriptional start site of the human

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5658-5782          -------------TGGCTCATGCCTGTAACCCCAGCACTTTGGGAGCCTGAGGCAGGCAG-ACTGCTTGAGTCTAGGAGTTCAAG-AC
5792-6066          ---------GCGGTGGCTCATGCCTATCATCCCAGCACTTTGGGAGGTCGAGGCAAGCAG-ATCACCTGAGGTCAGGAGTTCAAGGAC
6259-6520          -GGCCAGGCACGGTGGCTCACGCCTGTAATCCCAACACTTTGGGAGGCCAAGGCGGGCAGGATCAC---AAGTCAGGAGTTGGAGGAC
6813-6600 (-)      ----------------------GCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCAAGCGG-ATCACCTGAGGTCAGGAGTTCAAGGAC
6952-6799 (-)      ----------------CTCACACCTGTAATCCCAGCACTTTGGGAGGCTGAGGCG-GCAG-ATCACTTCAGGCCAGGAGTTCAAG-AC
Alu consensus      --GCTGGGCGTGGTGGCTCACACCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGGTGG-ATCACCTGAGGTCAGGAGTTCAAG-AC
                     **  ***  *********  *** * * **** ***********    ***   *  * *   *   *    *******  ** **

5658-5782          -CAGCCTGGG-CAACATACTG--AAACCCTGTCTCTACCAAAATTACA--AAAATTAG-----------------------------
5792-6066          GCAGCCTGGC-CAACATGGTG--AAACCCCCTCTCTACTAAAAATACA-AAAAATTGGCTGGGTGTGGTGGCGTGCGCCTGTAATCCC
6259-6520          GCAGCCTGACGCAACATGGTG--AAACCCCGTCTCTACTAAAAATGCAAAAAAATTAGCTGGGCCTGGTGGCGCACGCCTGTAATCCC
6813-6600 (-)      GCAGCCTGGC-CGACATGGTG--AAACCCTGTCTCTACTAAAAATATGGAAAAATTAGCCGGGCATGGTGGCGGGCGCCTGTAATCCC
6952-6799 (-)      -CAGCCTGGC-GAACATGGCG--AAACCTTGTCTCTACTAAAAATACA--AAAATTAGCCAGGAGTGGTGGCACGTGCCTGTAATCCC
Alu consensus      -CAGCCTGGC-CAACATGGTGTGAAACCCCGTCTCTACTAAAAATACA--AAAATTAGCCGGGCGTGGTGGCGCGCGCCTGTAATCCC
                    *******     ****   *  *****   ******* **** *     ****** **  **  *******    ************

5658-5782          ------------------------------------------------------------------------------------------
5792-6066          AGCTACTCGGGAGGCTGAGGCAAGGAGAATCGCTTGAACCTGGGAGGCGGAAGGTTGCAAGTGAGCCGAGATCACACCCATTGCACCC
6259-6520          AGCTACTCAGGAGGCTGAGGCA-GGAGAATTGCTTGAATTCAGGAGGCGGA-GGTTGCAG-TGAGCCGAGATTGCACC-ACTGCACTC
6813-6600 (-)      AGCTACTTGGGAGGCTGAGGCAGGAGGAATCGCTTGATCCCAGGAGGTGGA-GGTTGCAA-TGAG----------------------
6952-6799 (-)      AG--------------------------------------------------------------------------------------
Alu consensus      AGCTACTCGGGAGGCTGAGGCA-GGAGAATCGCTTGAACCCAGGAGGTGGA-GGTTGCAG-TGAGCCGAGATCGCGCC-ACTGCACTC
                   *** ***  ************* *  **** ******     ***** *** *******  ***********  * ** * ***** *

5658-5782          ------------------------------------------------------------------------------
5792-6066          CAGCCTGGCCGAGATCACACCCATTGCACCCCAGCCTGGGCCACAAGAGTGAAACT----------------------
6259-6520          CAGCCTGGCCGAGATTGCACC-ACTGCACTCCAGCCTGG------------------------------------
6813-6600 (-)      ------------------------------------------------------------------------------
6952-6799 (-)      ------------------------------------------------------------------------------
Alu consensus      CAGCCTGGCCGAGATCGCGCC-ACTGCACTCCAGCCTGGGCAACA-GAGCGAGACTCCATCTCAAAAAAAAAAAAAAAAA
                   ***************  * **   ***** *************** *** ** ***
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Figure 8 Summary of human Alu sequences in the DIC gene

The sequences have been aligned with the consensus human Alu sequence by the ClustalW 1.7 program. Insertions, denoted by dashes (–), have been introduced to improve the alignment. Completely conserved residues are indicated with asterisks (*).

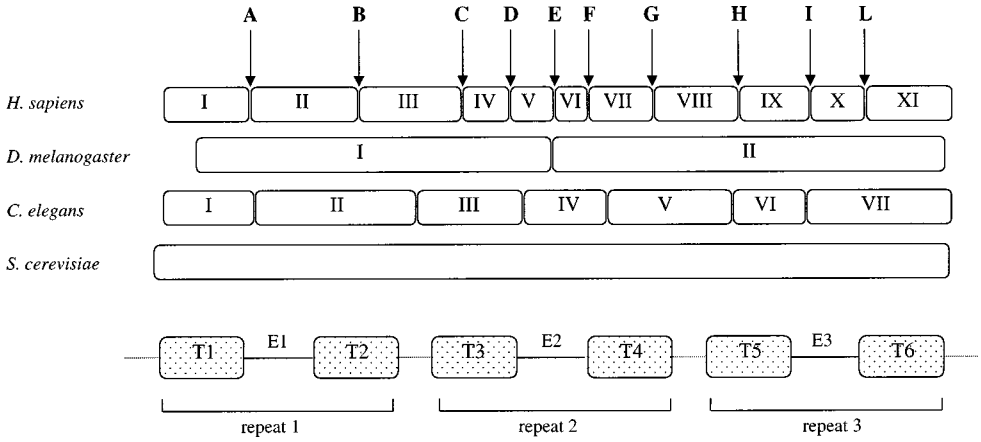

Figure 9 Alignment of exons of DIC genes for various species with a secondary-structural model of carrier proteins

The exons are represented by white boxes containing Roman numerals. The gaps between boxes indicate the positions of introns in the corresponding genes. The capital letters above the arrows correspond to the introns of the human DIC gene. The stippled boxes T1–T6 represent hydrophobic segments in the carrier proteins that are folded into transmembrane α-helices. The consecutive pairs of helices are joined by three hydrophilic sequences, E1, E2 and E3, which are proposed to lie outside the lipid bilayer. Shorter hydrophilic regions are denoted by dotted lines (.....). Each of the three sequence repeats 1, 2, and 3 in the carrier proteins consists of two proposed α-helical regions joined by segments E1, E2 or E3.

gene for DIC has not been mapped. Therefore, the 5′ extremity of exon I is not known, and it is unlikely that the rather short 5′-non-coding sequence present in the human cDNA represents the full extent of this exon.

Repetitive DNA sequence

Human DNA contains two types of middle repetitive DNA sequence: the long and the short interspersed sequences ('LINES' and 'SINES'; [29–31]). No examples of the former have been

detected in the DIC gene sequence described in the present paper, but an example of SINES was detected in intron I. It contains five repeats about 230 nucleotides long that are highly conserved and identical with the Alu consensus [32] (see Figure 8). Similar repeats have been detected previously in other mitochondrial carrier genes, namely in the T2 gene for human ADP/ATP translocase and in the gene for the carnitine carrier [33,34].

Evolution of human genes for mitochondrial carriers

Like other members of the mitochondrial carrier family, the sequence of DIC consists of three homologous tandem repeats of about 100 amino acids that are distantly related to repeats in other mitochondrial carrier proteins. Each repetitive element is made of two anti-parallel transmembrane hydrophobic α-helices linked by an extensive polar region. The repeats are linked by short extramembranous polar sequences, or loops [1–4,35,36], and they are characterized by the conserved sequence motif:

P-h-D/E-X-h-K/R-X-K/R-(20–30 amino acids)-D/E-G-(4 amino acids)-a-K/R-G

where h and a are hydrophobic and aromatic amino acids, respectively. Therefore the evolution of carrier proteins includes two tandem gene duplications of a primordial gene encoding the 100 amino acid repeat. The mammalian genes for the phosphate, adenine nucleotide, carnitine, oxoglutarate and citrate carriers [23,33,34,37,38] and for the uncoupling protein from mitochondria from brown adipose tissue [39] show that there is a tendency for introns to interrupt the coding sequence in or near to the sequences encoding the extramembrane loops, and their presence in these positions suggests that the 100-amino-acid repeat itself has evolved by an earlier linking of DNA sequences encoding single transmembrane segments. Indeed, all mammalian carrier genes sequenced so far have two conserved intron positions, one in the sequence encoding the first hydrophilic loop (intron A of the DIC gene; Figure 9) and the second immediately before the sequence encoding the sixth transmembrane segment (intron L of the DIC gene; Figure 9). Intron A is also found at the same position in *C. elegans* (but not in *D. melanogaster* or *S. cerevisiae*; see Figure 9), and introns H and I are also at common positions in man and *C. elegans*. Therefore these three latter introns were in the ancestral gene before the separation of Pseudocoelomata and Coelomata. The position of intron E, which is conserved in man, *C. elegans* and *D. melanogaster* (where it is the only intron in the gene), may be a relic of the linking of two superdomains representing the N- and C-terminal halves of a primitive DIC. Two other introns in the human DIC gene (introns D and F) and one in *C. elegans* interrupt the two superdomains, lending further support to this suggestion.

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REFERENCES

1 Kramer, R. and Palmieri, F. (1992) in Molecular Mechanisms in Bioenergetics (Ernster, L., ed.), pp. 359–384, Elsevier Science Publishers, Amsterdam
2 Walker, J. E. (1992) Curr. Opin. Struct. Biol. **2**, 519–526
3 Palmieri, F. (1994) FEBS Lett. **346**, 48–54
4 Palmieri, F. and van Ommen, B. (1999) in Frontiers of Cellular Bioenergetics (Papa, S., Guerrieri, F. and Tager, J., eds.), pp. 489–519, Kluwer Academic/Plenum Publishers, New York
5 Bisaccia, F., Indiveri, C. and Palmieri, F. (1988) Biochim. Biophys. Acta **933**, 229–240
6 Lancar-Benba, J., Foucher, B. and Saint-Macary, M. (1994) Biochim. Biophys. Acta **1190**, 213–216
7 Lancar-Benba, J., Foucher, B. and Saint-Macary, M. (1996) Biochemie **78**, 195–200
8 Chappell, J. B. and Haarhoff, K. N. (1967) in Biochemistry of Mitochondria (Kaniuga, Z., Slater, E. C. and Wojtczak, L., eds.), p. 75, Academic Press, London
9 Palmieri, F., Prezioso, G., Quagliariello, E. and Klingenberg, M. (1971) Eur. J. Biochem. **22**, 66–74
10 Palmieri, F., Quagliariello, E. and Klingenberg, M. (1970) Eur. J. Biochem. **17**, 230–238
11 McGivan, J. D. and Klingenberg, M. (1971) Eur. J. Biochem. **20**, 392–399
12 Crompton, M., Palmieri, F., Capano, M. and Quagliariello, E. (1974) Biochem. J. **142**, 127–137
13 Crompton, M., Palmieri, F., Capano, M. and Quagliariello, E. (1974) FEBS Lett. **46**, 247–250
14 Chappell, J.B (1968) Br. Med. Bull. **24**, 150–157
15 Meijer, A. J., Groot, G. S. P. and Tager, J. M. (1970) FEBS Lett. **8**, 41–44
16 Passarella, S., Palmieri, F. and Quagliariello, E. (1973) FEBS Lett. **38**, 91–95
17 Palmieri, F., Passarella, S., Stipani, I. and Quagliariello, E. (1974) Biochim. Biophys. Acta **333**, 195–208
18 Sluse, F. E., Meijer, A. J. and Tager, J. M. (1971) FEBS Lett. **18**, 149–151
19 Indiveri, C., Prezioso, G., Dierks, T., Krämer, R. and Palmieri, F. (1993) Biochim. Biophys. Acta **1143**, 310–318
20 Fiermonte, G., Palmieri, L., Dolce, V., Lasorsa, F. M., Palmieri, F., Runswick, M. J. and Walker, J. E. (1998) J. Biol. Chem. **273**, 24754–24759
21 Pannone, E., Fiermonte, G., Dolce, V., Rocchi, M. and Palmieri, F. (1998) Cytogenet. Cell. Genet. **83**, 238–239
22 Don, R .H., Cox, P. T., Wainwright, B. J., Baker, K. and Mattick, J. S. (1991) Nucleic Acids Res. **19**, 4008
23 Dolce, V., Iacobazzi, V., Palmieri, F. and Walker, J. E. (1994) J. Biol. Chem. **269**, 10451–10460
24 Dolce, V., Fiermonte, G. and Palmieri, F. (1996) FEBS Lett. **399**, 95–98
25 Ness, G. C. and Pendleton, L. C. (1991) FEBS Lett. **288**, 21–22
26 Capobianco, L., Bisaccia, F., Mazzeo, M. and Palmieri, F. (1996) Biochemistry **35**, 8974–8980
27 Proudfoot, N. J. and Brownlee, G. G. (1976) Nature (London) **263**, 211–214
28 Breathnach, R. and Chambon, P. (1981) Annu. Rev. Biochem. **50**, 349–383
29 Kao, F. T. (1985) Int. Rev. Cytol. **96**, 51–88
30 Roger, J. H. (1985) Int. Rev. Cytol. **93**, 187–279
31 Weiner, A. M., Deininger, P. L. and Efstratiadis, A. (1986) Annu. Rev. Biochem. **55**, 631–661
32 Deininger, P. L., Jolly, D. J., Rubin, C. M., Freidmann, T. and Schmid, C. W. (1981) J. Mol. Biol. **151**, 17–33
33 Cozens, A. L., Runswick, M. J. and Walker, J. E. (1989) J. Mol. Biol. **206**, 261–280
34 Iacobazzi, V., Naglieri, M. A., Stanley, C. A., Wanders, R. J. A. and Palmieri, F. (1998) Biochem. Biophys. Res. Commun. **252**, 770–774
35 Saraste, M. and Walker, J. E. (1982) FEBS Lett. **144**, 250–254
36 Kuan, J. and Saier, M. H. (1993) Crit. Rev. Biochem. Mol. Biol. **28**, 209–233
37 Iacobazzi, V., Palmieri, F., Runswick, M. J. and Walker, J. E. (1992) DNA Sequence **3**, 79–88
38 Iacobazzi, V., Lauria, G. and Palmieri, F. (1992) DNA Sequence **7**, 127–139
39 Kozak, L. P., Britton, J. H., Kozak, U. C. and Wells, I. M. (1988) J. Biol. Chem. **263**, 12274–12277