Molecular Genetics and Metabolism **71,** 139–153 (2000)
doi:10.1006/mgme.2000.3055, available online at http://www.idealibrary.com on IDEAL®

MINIREVIEW

Genomics of the Human Carnitine Acyltransferase Genes

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Received July 31, 2000

Five genes in the human genome are known to encode different active forms of related carnitine acyltransferases: *CPT1A* for liver-type carnitine palmitoyltransferase I, *CPT1B* for muscle-type carnitine palmitoyltransferase I, *CPT2* for carnitine palmitoyltransferase II, *CROT* for carnitine octanoyltransferase, and *CRAT* for carnitine acetyltransferase. Only from two of these genes (*CPT1B* and *CPT2*) have full genomic structures been described. Data from the human genome sequencing efforts now reveal drafts of the genomic structure of *CPT1A* and *CRAT,* the latter not being known from any other mammal. Furthermore, cDNA sequences of human *CROT* were obtained recently, and database analysis revealed a completed bacterial artificial chromosome sequence that contains the entire *CROT* gene and several exons of the flanking genes *P53TG* and *PGY3.* The genomic location of *CROT* is at chromosome 7q21.1. There is a putative *CPT1-like* pseudogene in the carnitine/choline acyltransferase family at chromosome 19. Here we give a brief overview of the functional relations between the different carnitine acyltransferases and some of the common features of their genes. We will highlight the phylogenetics of the human carnitine acyltransferase genes in relation to the fungal genes *YAT1* and *CAT2,* which encode cytosolic and mitochondrial/peroxisomal carnitine acetyltransferases, respectively. © 2000 Academic Press

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Key Words: **acetylcarnitine; acetyl-CoA; acyl-CoA; fatty acid; beta-oxidation; peroxisome proliferator; PPRE; mitochondrion; exon boundary; gene phylogeny.**

Carnitine acyltransferases are important enzymes for energy homeostasis and fat metabolism through their modulation of the pools of acetyl-CoA and long-chain acyl-CoA in distinct cell compartments of animals and fungi. These enzymes belong to the family of carnitine/choline acyltransferases and have different properties with respect to intracellular location, substrate specificity, kinetics, and physiological function (1–4). We will give a brief introduction about these enzymes and their role in energy metabolism, and will discuss some basic aspects of the carnitine palmitoyltransferase system, deficiencies of which are among the most common causes of fatty acid oxidation disorders (5). The genomics and genetics of the carnitine acyltransferase genes will be treated in detail, with the emphasis on new data revealed from the Human Genome Project. A comparative gene structure analysis will be concluded with the first results we obtained from a series of phylogenetic analyses. Hence, we will focus on related mitochondrial and peroxisomal carnitine acyltransferases. However, it should be understood that carnitine transferases are also present in the endoplasmic reticulum, the nuclear envelope, and the plasma membrane. Most of those enzyme activities are not genetically characterized, and

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some may be encoded by unrelated genes (reviewed in 2).

Carnitine Acyltransferases

The carnitine acyltransferases yet genetically and unambiguously identified consist of mitochondrial carnitine palmitoyltransferases (CPT), peroxisomal carnitine octanoyltransferase (COT), and mitochondrial/peroxisomal carnitine acetyltransferase (CrAT). CPT and COT transesterify medium- and long-chain fatty acyl chains, whereas CrAT transesterifies short-chain acyl chains. There are three genes in the human genome known to be responsible for mitochondrial CPT activity: *CPT1A* encodes liver-type (L-)CPT I, *CPT1B* encodes muscle-type (M-)CPT I, and *CPT2* (formerly annotated as *CPT1* since it was the first known gene for a CPT enzyme) encodes CPT II. The CPT I and CPT II couple mediate the transport of long-chain fatty acids (6) by transesterification of long-chain acyl-CoA into long-chain acylcarnitine in the cytosol and vice versa in the mitochondrial matrix (Fig. 1A). The acylcarnitine shuttle composed by these enzymes is possible through the transport over the mitochondrial inner membrane via the carnitine/acylcarnitine translocator CACT (7–9). Overall, the net mitochondrial uptake of activated long-chain fatty acids, i.e., long-chain acyl-CoA, is facilitated through the temporary replacement of the CoA group by carnitine. The CPT I and CPT II enzymes therefore have a clearcut function that is essential to allow mitochondrial β-oxidation of long-chain fatty acids. The product of this process, acetyl-CoA, is the central substrate for many processes, most importantly the Krebs cycle and, in the liver, ketogenesis.

Apart from mitochondrial β-oxidation, mitochondrial acetyl-CoA can also come from carbohydrate oxidation (Fig. 1B). The acetyl-CoA/CoA ratio is known to be one of the many determinants of the activity of the pyruvate dehydrogenase (PDH) complex through the action of acetyl-CoA on the PDH-inactivating kinase PDK4 (reviewed in 10). Therefore, CrAT is a potentially important mitochondrial enzyme since it determines the equilibrium between acetyl-CoA (plus carnitine) and acetylcarnitine (plus free CoA) (Fig. 1B), and thereby modulates the acetyl-CoA/CoA ratio. Thus, a buffer is formed when acetyl-CoA production from fatty acids or pyruvate is high, and circulatory acetylcarnitine could possibly facilitate replenishment of acetyl-CoA elsewhere. This buffer may explain many of the beneficial effects of carnitine and propionylcarnitine for cardiac performance in models of ischemia and hypertrophy (11), and of acetylcarnitine for the aging heart (12). CrAT is also active in the peroxisome, where it acts on acetyl-CoA and propionyl-CoA which result from oxidation (chain shortening) of branched-chain and very long-chain fatty acids. Since mammalian peroxisomes do not fully β-oxidize medium-chain fatty acid intermediates, the medium- and long-chain acyl-CoA molecules resulting from chain shortening are further metabolized elsewhere, i.e., in the mitochondria. The first enzyme needed to allow transport of medium- and long-chain acyl-CoA out of the peroxisome is COT (Fig. 1C). Although medium-chain fatty acids can traverse membrane systems without being esterified to carnitine, peroxisomal transesterification is beneficial since the activated state of medium-chain fatty acids as acyl-CoA is energetically preserved as acylcarnitine. Therefore, medium-chain acylcarnitines and also long-chain acylcarnitines are formed from their corresponding CoA esters to be transported to the cytosol and mitochondria. This transport is most probably mediated by the same carrier as the mitochondrial CACT, as has been shown by immunochemical methods (13), but direct genetic evidence of this finding is lacking. Similarly, there is evidence that mitochondrial CPT I is also a peroxisomal and microsomal enzyme (14). However, when human M-CPT I is fused to green fluorescent protein and expressed in human cells, the fusion protein localizes exclusively to mitochondria (15). This exclusive localization of recombinant M-CPT I may be natural, but could also be caused by the fusion as such or may be specific for the muscle-type enzyme only, and additional experiments are now carried out to address the subcellular distribution of CPT I.

There is striking similarity between the intracellular location of human CrAT and its fungal equivalent Cat2p (16–18) in the fact that both enzymes are translated into a mitochondrial form, whereas translation from a different start codon results in the shorter peroxisomal form. Thus, both in yeast and in humans the peroxisomal and mitochondrial carnitine acetyltransferases originate from single genes (17,19). However, in yeast there exists a cytosolic carnitine acetyltransferase Yat1p (20), the function of which is similar to that of mammalian CPT I in the sense that activated acetate (acetyl-CoA) is energetically preserved for transport into the mitochondria. In yeast and other fungi, long-chain fatty acids are β-oxidized in peroxisomes exclusively, and the action of Cat2p in the peroxisome

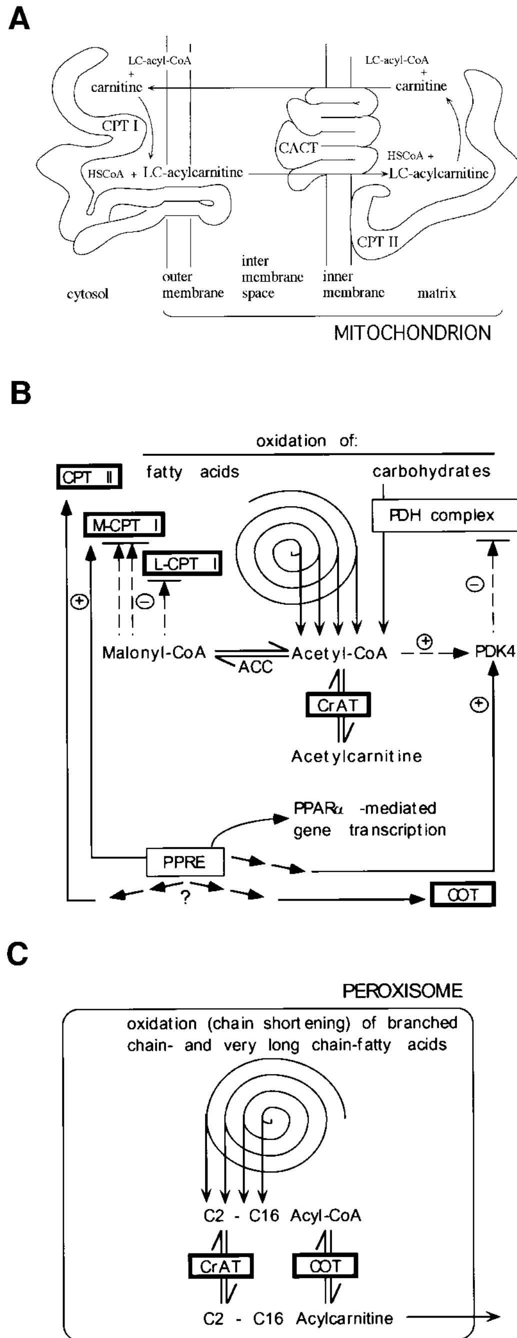

FIG. 1. Distinct roles of mitochondrial and peroxisomal carnitine acyltransferases. (A) The carnitine palmitoyltransferase system consists of CPT I in the outer membrane and CPT II in the matrix of the mitochondrion, which, together with the translocator CACT, form the carnitine shuttle for mitochondrial import of long-chain (LC) acyl-CoA. CPT I is anchored by two transmembrane domains in the N-terminal part of the protein, whereas CPT II is a soluble membrane-attached enzyme. CACT contains six transmembrane helices. (B) M-CPT I and L-CPT I are sensitive for inhibition by malonyl-CoA, the product of the reaction carried out by acetyl-CoA carboxylase (ACC). Acetyl-CoA indirectly affects the pyruvate dehydrogenase (PDH) complex via activation of the kinase PDK4. Carnitine acetyltransferase (CrAT) determines the equilibrium between acetyl-CoA and acetylcarnitine. M-CPT I expression is mediated by peroxisome proliferator-activated receptor alpha (PPARα), and a peroxisome proliferator response element (PPRE) has been identified in its promoter. Expression of CPT II, COT (carnitine octanoyltransferase), and PDK4 may be indirectly PPAR-dependent, as indicated by multiple arrows and a question mark. Metabolic interactions are indicated by dashed arrows, genetic interactions by solid arrows. β-oxidation is shown as a spiral. (C) A simplified scheme of peroxisomal functions of CrAT and COT. Peroxisomal CrAT transesterifies short-chain acyl-CoA into short-chain acylcarnitines for further transport to the mitochondria. COT is required to exert the same transesterification on medium-chain acyl groups, but its substrate specificity includes long-chain (C16) acyl-CoA molecules as well. The oxidation (chain shortening) of branched-chain and very-long-chain fatty acids is carried out by different enzymes than those of the mitochondrial fatty acid oxidation and gains less energy; hence the spiral is represented as a mirror image of the mitochondrial β-oxidation spiral.

and mitochondrion allows further metabolism of acetyl-CoA in the Krebs cycle in the mitochondrion. In this case the transmembrane transport of acetylcarnitine (and carnitine) is facilitated by a homologue of human CACT: Crc1p (18,21,22). Crc1p is a mitochondrial inner membrane carrier with a structure very similar to that of CACT; however, a peroxisomal equivalent of these carriers in yeast has not been identified, but is predicted if the physiological function of peroxisomal Cat2p is similar to that of peroxisomal CrAT (Fig. 3).

The CPT System

Both CPT I isoforms are located in the mitochondrial outer membrane and are sensitive for inhibition by malonyl-CoA, which makes these enzymes important sites for metabolic regulation (23). Malonyl-CoA is the product of a reaction catalyzed by acetyl-CoA carboxylases. This reaction is the first step in fatty acid biogenesis from acetyl-CoA and is preserved in nonlipogenic tissues such as skeletal and heart muscle (24,25). The formation of malonyl-CoA may therefore serve as a main control of fatty acid catabolism through the inhibition of CPT I. The tissue distribution of the two CPT I isoforms and their level of sensitivity for malonyl-CoA inhibition is markedly different, the muscle isoform being more sensitive than the liver isoform (reviewed in 1), thus providing the body the possibility to modulate the degree of control over fatty acid β-oxidation in organs that express both CPT I isoforms, for instance, the heart (26). CPT II is also expressed in the

heart, as it is in many (if not all) tissues. Most molecular data on *CPT2* thus far point to one isoform, encoded for by a single, ubiquitously expressed gene. Recent progress in the structural knowledge of CPT I has pointed out that two transmembrane domains anchor the enzyme and that the active site in the C-terminal domain is at the cytosolic side of the mitochondrial outer membrane (15,27). The fact that this configuration is supported by analyses of the primary protein structures of all known mammalian CPT I proteins, and that this has been experimentally shown for both the rat liver CPT I (27) and a human muscle CPT I fusion protein (15), illustrates that the two isoforms are similar with respect to these main-frame characteristics (Fig. 1A). The identification of the main determinants of the kinetic and inhibitory characteristics of L-CPT I and M-CPT I is a rapidly progressing field of research (28–32). From those studies, the interactions of the C-terminal domain with the highly conserved N-terminus, which also is at the cytosolic side (Fig. 1A), have become evident.

As mentioned already, the action of the CPT proteins would be senseless (as far as acyl-CoA transport is concerned) if there were not a mitochondrial inner membrane translocator. The structural data on this protein point to a triple repeat structure that contains six transmembrane helices (33–35). This structure is predicted on the basis of many related mitochondrial transporters. The question how long-chain acylcarnitines actually enter the intermembrane space is yet unresolved. This may be explained by the fact that before and after 1987, when it was established that CPT I is an outer membrane protein (36), it was thought that the catalytic site of CPT I is in the intermembrane space. However, it is now generally accepted that the CPT I reaction is carried out at the cytosolic face of the mitochondrion (15,27,28). For uptake of acylcarnitine into the intermembrane space, Zammit proposed a diffusion process through porin (2), and Kerner and Hoppel (37) point at the involvement of porin as well. Still, it must be more than a coincidence that the *cytosolic* formation of acylcarnitine resides at the surface of the *mitochondria.* The enzymes needed for fatty acid activation (long-chain acyl-CoA synthetase) and transesterification (CPT I), as well as the "regulator" of CPT I (acetyl-CoA carboxylase II (38)), all reside in the mitochondrial outer membrane, and complementary data suggest that CPT I and CPT II (39,40), and to a lesser extent CACT (13), are in close proximity in contact sites of the mitochondrial membranes. Therefore, it should be considered that CPT I *in situ* acts on acyl-CoA molecules that are supplied by the synthetase through the outer membrane, and that the carnitine esters leave the membrane at the inner side, i.e., in the intermembrane space (Fig. 1A). It would be interesting if the hypothesis could be tested that the transesterification of long-chain acyl moieties, which perhaps only temporarily are removed from the membrane by CPT I, is followed by flipping of acylcarnitine and channeling toward CPT II by CACT. We will not treat the many pros and cons for such a view in this minireview, but emphasize that the differences between the CPT I isoforms and the other transferases could go beyond kinetics and inhibition by malonyl-CoA, and may include a basic difference in enzyme action.

GENOMICS

The five human carnitine acyltransferase genes have all been cloned as cDNA (41–46), and (partial) genomic sequences of these genes are available in the databases (see Table 1). The determination of the genomic sequences should reach completion soon or may have reached completion by the time this article is printed, given the speed of progress of the combined efforts of the Human Genome Project participants and industries. As of July 2000, the sequence information from industrial efforts is not freely accessible and we will limit our overview to the publicly available data (see Acknowledgments).

Chromosomal Localization of Human Carnitine Acyltransferase Sequences

Four of the five genes, i.e., *CPT1A, CPT1B, CPT2,* and *CRAT,* have been localized to their chromosomal positions by different methods. *CPT1A* was localized by hybrid panel mapping (43) and later was fine-mapped by fluorescent *in situ* hybridization (FISH) (46). *CPT1B* was localized by FISH with a cDNA as probe (46) and with a genomic clone as probe (45). The position of *CPT1B* was also readily identified by database analysis (45) since this gene is located in a telomeric region of chromosome 22, the first human chromosome that was entirely sequenced (47). The bacterial artificial chromosome (BAC) clone that contains *CPT1B* was originally oriented in the opposite direction than is now believed to be correct, and one more gene is thought to be positioned between this clone and the telomeric repeats (47). Therefore, there may be four genes

TABLE 1
Genomic Localization of the Human Carnitine Acyltransferase Genes

Gene	Enzyme	Chromosome	Reference	Genbank Accession No.		
				Genomic	cDNA	Protein
CROT	COT	7q21.1	This update, (53)	AC005045	AF168793	AAF03234
					AF073770	AAD41654
CPT1A	L-CPT I	11q13.1-2	(43,46)	AC019166	NM_001876	NP_001867
					L39211	AAC41748
CPT1B	M-CPT I	22q13.3-ter	(45,46)	U62317	NM_004377[1]	NP_004368
				AB003286	Y08682[2]	BAA21492
CRAT	CrAT	9q34.1	(42)	AL158151	NM_004003[3]	NP_003994[3]
				AC007913	NM_000755[4]	NP_000746[4]
CPT2	CPT II	1p32	(48)	AL355483	NM_000098	NP_000089
				AC022728	M58581	P23786
CPT1-like pseudogene		19	This update	AC011495		

Note. The NM_ and NP_ numbers refer to refseq sequences; [1] type II cDNA, [2] type I cDNA, [3] peroxisomal variant, [4] mitochondrial variant.

between the telomere and *CPT1B. CPT1B* genomic information has also been described by Yamazaki *et al.* (44). *CPT2* (48) and *CRAT* (42) were localized by FISH. The localization of *CPT1A* and *CPT2* and possibly also *CRAT* is of direct clinical relevance since metabolic diseases are known to be caused by deficiencies in L-CPT I (5,49) and CPT II (50) and possibly CrAT (51,52), although about CrAT deficiencies no molecular data are known. CPT II deficiency is the most common carnitine transferase disorder (50), various presentations of which are known along with the disease-causing alleles (5).

Only the human gene for COT, for which the symbol is now *CROT* (HUGO/GDB nomenclature committee), was lacking in direct evidence for its chromosomal location. However, Torigoe *et al.* (53) mapped yeast artificial chromosome (YAC) contigs to chromosome 7q21.1, one of which contained an exon with similarity to the rat gene for COT. We recognized that this exon indeed was identical to the human COT cDNA, which was published later (54). However, searches in the high throughput genomic sequences (HTGS) database, which contains much information of the sequencing of genomic clones that is in progress, did not reveal more information. Much to our surprise, the nonredundant (NR) database—which contains finished sequences only—had contained a fully sequenced BAC clone (AC005045) for 2 years. This sequence reveals the full genomic structure of *CROT* and several exons of the flanking genes *P53TG* and *PGY3* (Genome Sequencing Center, personal communication). *PGY3* encodes a multidrug resistance *P*-glycoprotein of the ABC transporter family and was localized by hybrid panel mapping at 7q21.1 (55). Therefore, the localization of *CROT* is now fully established.

Apart from the carnitine acyltransferase genes known to be active and to encode active gene products, there is a putative *CPT1-like* pseudogene at chromosome 19. This gene is transcribed in many tissues, since more than 20 expressed sequence tags (ESTs) of it are known, but it is not likely to encode an active carnitine acyltransferase. This is a putative pseudogene for two reasons. The first and most important reason is that in several of the ESTs as well as the corresponding exons in the genomic sequence, all reading frames, including the reading frame that shows identity with carnitine acyltransferases (highest with *CPT1A*), are interrupted by stop codons, and these cannot be explained by sequencing errors. The second reason is that several of the ESTs are sequenced in the reverse direction than would be expected, and in multiple cases are spliced to exons of an elongation factor gamma-like gene, which probably also resides at chromosome 19. Although post-transcriptional RNA editing cannot be excluded as a possible explanation for the existence of several premature stop codons in the exons, the fact that these nonsense codons remained in the transcripts that served as templates for EST sequencing makes such an explanation unlikely. A more detailed analysis along with that of its mouse orthologue will be presented elsewhere (van der Leij *et al.,* in preparation).

There has been one temporal and preliminary misassignment of a *CPT2* sequence in the databases, which is resolved now (Whitehead Institute, personal communication), but may require some time to be corrected in the database entry AC022728.

STRUCTURAL FEATURES

Promoter and exon–intron structures can reveal clues for various purposes. The knowledge of genomic sequences is of clinical relevance since it allows the design of primers for PCR analysis on genomic DNA, which is often easier to obtain than mRNA from the appropriate human tissue.

Promoter studies often rely on initial computer analyses before experimental designs are possible. Alternatively, or additionally, binding site conservation in orthologous promoters may point at their significance, as we know from the analysis of four mammalian *CPT1B* promoters (56; van der Leij *et al.,* in preparation). Exon–intron structures of related genes are also important since these may reveal some of the evolutionary relationships between these genes. The evolutionary connections in turn can be used to explain structure–function relations of the genes and the proteins they encode. We recently obtained detailed information of some of the mouse genes of this family, since these structures needed to be revealed for gene targeting studies (57). We also obtained relevant information from sheep, since the chronically instrumented lamb is the model of choice for *in vivo* perinatal cardiological studies (58,59). Full reports of these sequence analyses are in preparation and fall beyond the scope of this minireview. However, where appropriate we will relate some aspects of the human genes to those of other mammals.

Promoter Sequences

The present genomic knowledge outlined in this minireview implicates that in principle the promoters of human *CROT, CPT1B,* mitochondrial *CRAT,* and *CPT2* are known, and that those of *CPT1A* and peroxisomal *CRAT* are to be expected. The human *CROT* and *CRAT* promoters are new data. We scanned these sequences for potentially important transcription factor binding sites using TRANSFAC-based matrix algorithms (http://transfac.gbf.de/TRANSFAC/ and http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). We will not list the many candidate motifs that result from these analyses, since functional studies will be needed to show their relevance. However, a few interesting aspects should be discussed.

We particularly searched for putative peroxisome proliferator response elements (PPREs), since peroxisome proliferator-activated receptors (PPARs), especially PPARα (60–65), are important for the constitutive and regulated expression of many genes of fat metabolism, and provide a molecular link between the major pathways of energy metabolism (10,66–68) (see Fig. 1B for a few examples). However, only in the promoter of *CPT1B* do the TRANSFAC-based matrix algorithms readily identify a PPRE, which is known as fat-activated response element (FARE). This element was identified by Mascaro *et al.* (63) and by Kelly and co-workers (62,67), and is a prominent *cis* element in both the "ubiquitous" (upon fasting) and the "muscle-specific" human *CPT1B* promoters (69). No firm indications for putative PPREs were found in the promoters of human *CROT,* mitochondrial *CRAT,* and *CPT2,* and this is also the case for the rat orthologue of *CPT1A* (70) and the mouse orthologue of *CPT2* (71). For *CRAT* and *CPT1A* this may not be surprising, since there is little information (42,72) about *CRAT* transcription, and *CPT1A* transcription is largely PPARα-independent, at least in the mouse liver (67,68). However, from studies in rat for COT (73,74), and in mouse for CPT II (61,65) and also *PDK4* (10) (Fig. 1B), we inferred that *CROT, CPT2,* and *PDK4* are strong candidates for PPARα-dependent transcriptional control. Since no obvious FARE is present in these promoters, a fairly different PPRE must be the target site, or indirect signaling by intermediate gene(s) between PPARα and these promoters may be the case (Fig. 1B). For mouse CPT II the PPARα-mediated regulation of expression may even be only at the post-transcriptional level, since only protein data are known from livers (61) and hearts (65) of PPARα null mice.

The promoter of the rat gene for COT shows a possible PPRE half-site as part of an imperfect inverted repeat (a 53-bp partial palindrome) (74). Comparison of the rat promoter to the human *CROT* promoter reveals that only one degenerate half of this repeat is present in the human sequence. The human *CROT* promoter is different in another aspect as well: the rat promoter sequence of 1140 bp does not contain part of a homologue of the P53 target gene *P53TG.* In the human genome sequence, the first nucleotide of the *P53TG* exon 1, which is

transcribed away from *CROT,* aligns only 229 bp upstream of the first nucleotide of *CROT* exon 1. Although the assignment of the starts of these exons is not based on transcription start site determinations of *P53TG* (75) and *CROT,* the actual transcription start sites would even be closer to each other if the currently known cDNAs are not of full length. Therefore, the promoter region of human *CROT,* and also *P53TG,* is extremely short if it is assumed that no promoter functions overlap with the transcribed parts of these genes. This feature of human *CROT* is similar to the upstream region of human (45,76) (Fig. 2A), rat (70), and mouse (56,77) *CPT1B,* where a choline/ethanolamine kinase gene (*CHKL*) ends less than 0.5 kb from the transcriptional start of the first exons in these species. In conclusion, the *CROT* promoter is far less conserved than the *CPT1B* promoter, but they are similar with respect to genomic limitations caused by adjacent genes. The difference between humans and rats in their liver response to peroxisome proliferators may in part be ascribed to differences in promoter structures between these species, and *CROT* might be one example. Nevertheless there is a conserved stretch of DNA in the *CROT* promoter that is noteworthy: pos. 1022–1084 of the 1140-bp rat promoter shows 84% nucleotide similarity to pos. 76–138 of the 229-bp human promoter. At the start and near end of this stretch the human sequence contains perfect SP1 binding sites, and the rat contains one almost perfect (one mismatch) and one perfect SP1 binding site. Together with the notion that between these conserved motifs there is a candidate NF-Y binding site (pos. 97–110 in the human 229-bp sequence, pos. 1044–1055 in the rat promoter), the mechanisms of basal expression of *CROT* may be highly similar to those of *CPT1A,* which for the rat were indeed shown to involve binding of SP1 and NF-Y (78). The *CPT1* promoters and many of the factors that influence their activity have recently been reviewed (79).

Mitochondrial CrAT is expressed in human liver (80), and high mRNA expression was found in skeletal muscle (42). The pigeon muscle and liver form are likely to be the same (81); however, the mouse shows much lower mRNA levels in the liver and brain compared to heart, kidney, and testis (72). The promoter for mitochondrial *CRAT* messengers shows several potential MyoD binding sites as well as potential binding sites for members of the hepatic nuclear factor (HNF) family. There is also an enrichment of potential SP1 binding sites near the expected start of transcription. However, there are no hints from conserved regions since genomic sequences are only known from the human *CRAT* gene, and genomic information from other species is limited to mapping data of the mouse (82).

The *CPT2* promoter has been shown to be able to drive transcription of a reporter gene, and was suggested to contain an insulin response element (83). Its counterpart from mouse has been called a "cardiac" promoter (71), but it is well established that *CPT2* is active in almost every tissue (1). We have discussed PPARα-related matters of *CPT2* expression above (refer to 1 for further review).

Exon–Intron Structures

The first cDNA sequence of *CROT* was published recently (54) and an unpublished database submission by Kim *et al.* (database Accession No. AF073770, see Table 1) contains a full-length cDNA with minor variations in the coding sequence. The genomic organization of the *CROT* gene points to two untranslated leader exons that are 568 bp apart. The start codon is in the third exon (Fig. 2), and the total number of exons is 18. This is one more exon than for the rat gene for COT (74), which is explained by the fact that in rat only one 5′ untranslated exon is present. All exon–intron boundaries of *CROT* comply with the GT-AG rule, as do all boundaries of the other genes as well, with one functional exception (*CRAT,* see below). Except one splice junction (exon 12–13 in rat and 13–14 in human *CROT*), which shuffled four amino acid residues in the protein alignment, all splice junctions within the coding region are conserved between humans and rats. In the rat it has been shown that this gene is sometimes spliced in a peculiar way (84), such that repeats of the rat exon 2 or exon 2 and 3 occur. The mechanism that best explains these findings is called *trans* splicing since apparently two primary transcripts of the same gene result in a single mature mRNA. Whether this phenomenon also occurs in human *CROT* transcripts is not known. The exons and introns of *CROT* span a region of 54 kb, which makes the size of this gene best comparable to *CPT1A,* which is at least 60 kb (Fig. 2).

The exact size of *CPT1A* is not yet known. There are six gaps in the draft genomic sequence; the first exon has not been identified, and since the cDNA data published thus far do not cover the majority of the 3′ untranslated region (43), the genomic location of the 3′ part of *CPT1A* cannot be fully assigned.

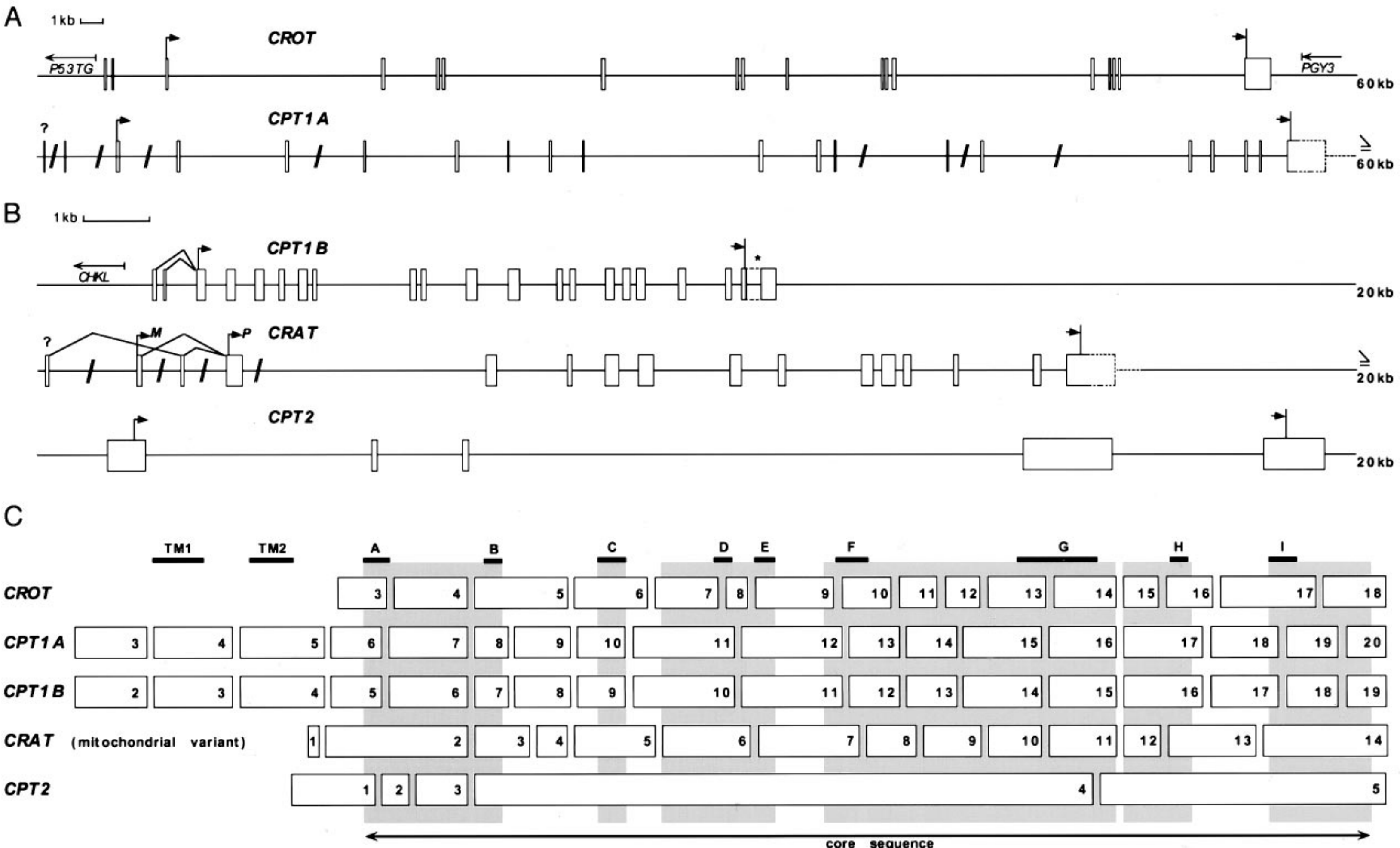

FIG. 2. Genomic organization of the human carnitine acyltransferase genes. Exons are indicated by boxes, start and stop codons by short arrows with solid arrowheads, splice variations of 5′ exons by additional lines, adjacent genes by long arrows, gaps of unknown length by slashes, and exon locations in unsequenced regions by question marks. Unsequenced parts of 3′ mRNA that may be part of sequenced genomic DNA are indicated by dashed lines. The scale of 1 kb is indicated for (A) and (B), minimum sequence lengths are indicated by the ≥ symbol. (A) The genes for carnitine octanoyltransferase (CROT) and liver-type carnitine palmitoyltransferase (CPT1A) cover 54 kb and at least 60 kb, respectively. Immediately upstream of CROT is the p53 target gene P53TG, and immediately downstream, the P-glycoprotein gene PGY3. (B) The genes for muscle-type carnitine palmitoyltransferase I (CPT1B), carnitine acetyltransferase (CRAT), and carnitine palmitoyltransferase II (CPT2) are shown on a 20-kb scale. Alternative polyadenylation, which prevents splicing of CPT1B intron 19, is depicted (*). The first "peroxisomal" CRAT exon has not yet been located, and the gaps in the CRAT sequence may make the total length of CRAT more than 20 kb. The two translational starts of CRAT are indicated by M and P for the mitochondrial and peroxisomal isoform, respectively. (C) Alignment of the exons according to protein similarity. Only the coding exons are shown, without the untranslated regions. Exon numbering of CPT1A is preliminary. The CPT I transmembrane domains TM1 and TM2 are indicated along with nine blocks (A–I) that are conserved throughout all transferases. Regions that align without large gaps are shaded. The core sequence region used for phylogenetic analyses is depicted below.

However, *CPT1A* probably represents the largest gene of the five, which is remarkable since its closest relevant *CPT1B* is the shortest of the five (Fig. 2). The second-last and part of the last *CPT1A* exon with coding sequence were cloned by Britton *et al.* (43) after the first *CPT1A* cDNA had been obtained from rat (85). Three other splice junctions of coding exons were also reported by Britton *et al.* (43), and these were shown to be positionally conserved in *CPT1B* by Yamazaki *et al.* (76). The exon junctions of the coding region are all known now, and alignment of the *CPT1A* and *CPT1B* coding regions, which only differ 6 bp in size, shows that all intron positions are fully conserved (Fig. 2B). This is even true at the nucleotide level (not shown). Obviously, the difference in genomic size between *CPT1A* and *CPT1B* is a consequence of intron size differences. The region containing the first 10 exons of the rat gene has been analyzed in detail (86) and this revealed exon skipping of the second untranslated exon. Whether this mechanism also applies for human *CPT1A* is not known and since the genomic sequence draft does not include the first exon (or exons), a question mark is shown in Fig. 2A, and exon numbering (Fig. 2C) is premature. As mentioned, the 3′ end of the cDNA is not fully known yet, and there may be additional introns in this part of the gene as well. EST analyses point to differential splicing of the 3′ untranslated sequences (F. R. van der Leij, unpublished), which might include features similar to those found for *CPT1B* (45) (see below).

The *CPT1B* gene spans about 11 kb. The first cDNA for *CPT1B* was cloned from rat (87) and was indeed shown to encode muscle-type CPT I (88). Human cDNA sequences were reported later by several groups (44–46,89,90). *CPT1B* contains two alternatively transcribed first exons (45,76,90), i.e., either exon U (90), also called 1A (76), or exon M, also called 1B. Thus far, only a single first exon has been found in other mammalian *CPT1B* genes, i.e., that of the rat (70). There are several examples of alternative splicing of coding exons of *CPT1B* (45,90), but whether these result in active gene products is not known. The alternative splicing in the 3′ untranslated end of the *CPT1B* mRNA is not only at the level of splice site choice (immediately behind the stop codon), but also involves another mechanism. Intron 19 contains a poly(A) signal near the splice acceptor site, and polyadenylation according to this signal prevents splicing of intron 19. Since intron 19 is larger than exon 20, the polyadenylation at this site results in a slightly larger mRNA ("type II") than the majority ("type I"), as judged on EST analyses (45). The functional significance of these variants is not known, and nonselective competition between splicing and polyadenylation may be the fact.

Two genomic drafts of human *CRAT* are near completion (see Table 1), but, similar to knowledge of *CPT1A,* the untranslated parts of *CRAT* are not fully established. Corti *et al.* (19,42) reported the majority of the cDNA sequence (2.3 kb of an estimated mRNA of 2.9 kb) (42), and showed that splice variation at the 5′ end is a likely explanation for differential expression of the peroxisomal and mitochondrial proteins (19). The "mitochondrial" first exon is covered by genomic data; however, there are probably two "peroxisomal" exons, the second of which is present downstream of the "mitochondrial" exon, but the first is not in a genomic draft of chromosome 9. There are transcribed sequences that are almost identical (139 of 142 bp) to the first "peroxisomal" exon though, but these originate from another locus (*BAZ1B* mRNA, AB032253; chromosome 7q11.23 sequence AC005074). Therefore, a cloning artifact as part of the peroxisomal *CRAT* cDNA should not be ruled out; however, the fact that the second "peroxisomal" exon is preceded by a potential 3′ intron sequence (data not shown) suggests that this is not necessarily the case. The presence of GC instead of GT at the 5′ donor site of the peroxisomal (2nd) intron is confirmed, and has been proposed to be part of the underlying mechanism of differential splicing (19). Similar to the status of *CPT1A,* the mRNA 3′ end is extending the length of the currently known cDNA sequences and there may be introns downstream of the stop codon. The coding exons show several conserved exon–intron boundary positions when compared to the other transferases (Fig. 2B).

The *CPT2* genomic structure has been well described from both man (48) and mouse (71), and although full sequences of the introns are not completed, the sizes are well defined and very similar in the two species. *CPT2* has only 5 exons and its gene product differs from those of the other members of the family at the protein level by local stretches that have derived from insertions, deletions, inversions, or intron slipping during evolution. Still, one splice junction, i.e., between exon 3 and 4 (Fig. 2C), is almost preserved and we know from *CPT2* genes from other animals that some splice junctions of these *CPT2* genes are at conserved positions when aligned to the other transferase genes. Together with the preservation of at least

nine "blocks" of protein sequence throughout the carnitine/choline acyltransferase family (http://blocks.fhcrc.org/blocks-bin/), this points to the evolution of all these genes from a single ancestral gene. However, convergent evolution may have occurred from DNA regions that did not take part in duplication events. In addition, duplication events of only a core sequence should be considered to explain the divergence of the protein targeting signals responsible for the different subcellular locations.

PHYLOGENETICS

The subcellular assignments and phylogenetic relation of the different proteins encoded by the human and yeast genes are summarized in Fig. 3. The exon–intron boundaries as outlined in Fig. 2C are part of the information we are currently implementing in alignment studies of various genes of this family, from various organisms. These alignments are made from DNA and protein sequences of the coding sequences or parts thereof. Using different gap penalties (91) and taking the exon junctions into account, we finally aligned the genes by hand and produced unrooted trees from these data by maximum-likelihood- and maximum-parsimony algorithms (92,93) of the PAUP* program (Version 4.0b4a). Although different trees are sometimes obtained by these methods, which depends on the number of genes that are included, and particularly the way these are aligned before the trees are built, the general picture that arises is the one represented in Fig. 3B. The direction of the branches of such a tree is not informative; however, the branch distances are informative as well as the branch point positions. The picture shown in Fig. 3B is from a parsimony analysis of the "core sequences" (Fig. 2C) from 46 genes from various species. From such a tree it can be inferred that of the genes shown, the *CPT1* genes duplicated and diverged most recently, probably when or even before the chordates and vertebrates evolved. Therefore, early in vertebrate evolution the duplication of a *CPT1* ancestral gene has led to a gene that nowadays encodes the liver-type CPT I, and one that encodes muscle-type CPT I. The *CPT1* genes are the closest relatives, which is also apparent from the exon junction conservation.

The *CROT, CRAT,* and *CPT2* genes are more closely related to each other than to the *CPT1* genes, and from many analyses it became apparent that *CPT1* and *CPT2* are in fact the most distant members of the human carnitine acyltransferase genes. At first sight this may be surprising, since CPT I and II in principle catalyze the same equilibrium reaction in opposite directions. However, the mechanism by which this is done may be very different. It is known that CPT II and COT also share similar substrate length specificities, but that the mechanism by which the reaction is carried out is markedly different: an ordered mechanism by CPT II versus a rapid equilibrium random one by COT (94,95). The final alignment used for Fig. 3B did not include the divergent ends that encode the mitochondrial targeting signal of CPT II and the N-terminal anchor domain of CPT I, but started at the first conserved block and ended almost at the C-termini of the proteins (Fig. 2C). Therefore, the evolutionary distance between CPT I and CPT II is not simply explained by the evolution of different targeting signals or the weight of the unique N-terminal part of the CPT I proteins in the alignment (Fig. 2C).

The relation of the human genes to the yeast genes *CAT2* and *YAT1* is also interesting. Cat2p shows a compartmental distribution pattern similar to that of mammalian CrAT, but the underlying mechanisms of alternative sorting are different: the human gene is translated from different start codons as a consequence of alternative splicing (19), whereas in fungi, i.e., *Candida tropicalis,* a differential preference for the two translation start sites is the mechanism as such (96). The tree points at separate branches for CrAT and Cat2p. However, these branches are at central positions and the distance between CrAT and Cat2p is less than the intraspecies distances between Cat2p and Yat1p or between CPT I and CPT II. Therefore, although the root of the tree is not unambiguously defined, a common CrAT-like or Cat2p-like ancestor, shared by all genes of the carnitine/choline acyltransferase family, is a likely candidate for such a root. This would place the root probably somewhere between the COT and Cat2p branches (Fig. 3B). However, many assumptions have to be made to calculate the most probable root (it has to be assumed, for instance, that the speed of the molecular clocks of yeast and human is comparable). If the intuitive rooting between the COT and Cat2p branches is right, it would mean that *YAT1* shares an ancestral gene with *CPT2* that is not shared by the other genes. This would point at possible conservations in protein functions that are not directly apparent because of the differences in subcellular localization and substrate specificity of these proteins. However,

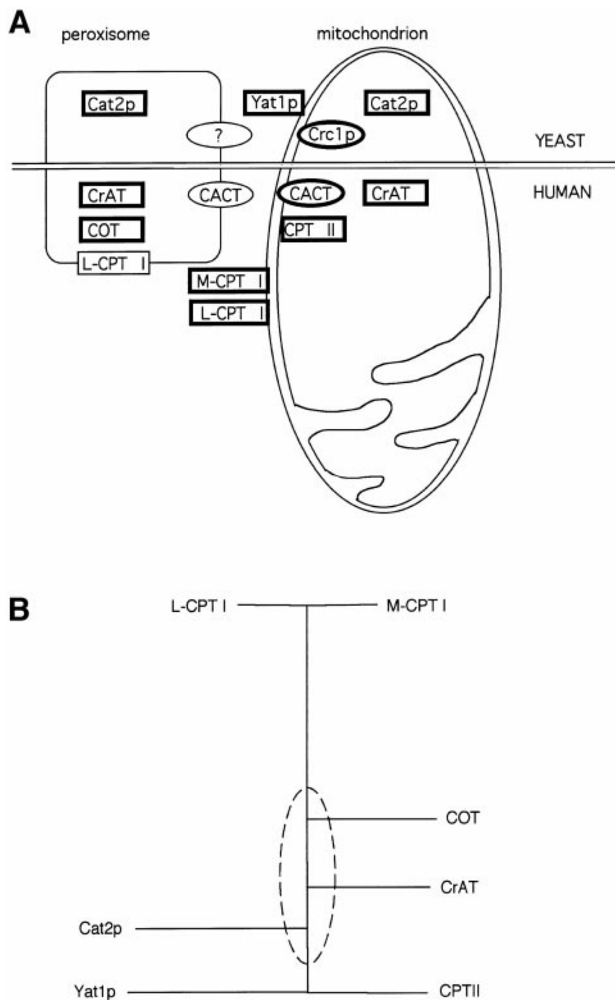

FIG. 3. Subcellular location and phylogenetics of yeast and human carnitine acyltransferases and carnitine/acylcarnitine translocators. Yat1p, yeast carnitine acetyltransferase 1; Cat2p, yeast carnitine acetyltransferase 2 (peroxisomal and mitochondrial isoform); Crc1p, yeast carnitine/acylcarnitine carrier; CrAT, human carnitine acetyltransferase (peroxisomal and mitochondrial isoform); COT, carnitine octanoyltransferase; L-CPT I, liver-type carnitine palmitoyltransferase I; M-CPT I, muscle-type carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; CACT, carnitine/acylcarnitine translocator. (A) A peroxisome and mitochondrion are shown to point out the subcellular location of the yeast (top) and human (bottom) proteins. The genetically and functionally identified transferases are shown in thick-lined boxes, the translocators in thick-lined ovals. Immunologically identified proteins are in thin-lined boxes and ovals, and a hypothetical peroxisomal translocator is represented by a question mark. (B) Phylogenetic relation between the human and the yeast proteins, according to parsimony analysis of core sequence alignments. The dashed oval in gray indicates an area of uncertainty with respect to branch order. The relation between Crc1p and CACT is shown to indicate their homology; the Crc1p-CACT distance is not strictly drawn to scale.

little information on Yat1p is known compared to CPT II.

FUTURE PROSPECTS

The rapid progress in the sequencing of the human genome will soon reveal the complete structures of the genes we have discussed. The academic knowledge of the genomics and structure–function relations of carnitine acyltransferases may have clinical impact for major genetically (co-) determined disorders of energy metabolism, like diabetes and inherited cardiomyopathies, as well as metabolic adaptations during and after ischemia. It will also be interesting to see how choline acetyltransferase relates to the carnitine transferases, and how we can learn from comparative studies within as well as beyond the mammal. Only of *CPT1A* and *CPT2* are the disease-causing mutations known at the molecular level. Mutations in the other genes may be present in the human population; however, malfunction of one of these genes may be detrimental, may be compensated for by other genes, or may have remained undiagnosed (57). One important application of knowledge of some of the human carnitine acyltransferase genes may therefore be to extrapolate their structural features for the design of studies on the orthologous genes from other mammals. For the human genes, promoter studies of *CROT* and mitochondrial *CRAT* are now amenable for research, and should reveal accurate information on the elements involved in their function. Therefore, future studies may shed more light on the versatile aspects of the carnitine acyltransferase genes on human energy metabolism and its regulation.

ACKNOWLEDGMENTS

The authors thank the Genome Sequencing Center, Washington University, St. Louis, for communication of the genomic *CROT* and *CPT1A* DNA sequence data prior to publication. The genomic *CRAT* sequence data were produced by the Human Chromosome 9 Sequencing Group at the Sanger Center. New *CPT2* data were produced by the Human Chromosome 1 Sequencing Group at the Sanger Center (AL355483) and by the Whitehead Institute, MIT (AC022728). The *CPT1-like* genomic sequence data were produced by the DOE Joint Genome Institute. We thank Prof. J. J. Beintema and Dr. W. T. Stam for helpful discussions on the phylogenetic analyses, and The Netherlands Heart Foundation for funding (NHS Grants 94.194 and 97.093).

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