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The Mitochondrial Carrier Family of Transport Proteins: Structural, Functional, and Evolutionary Relationships

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ABSTRACT: Energy transduction in mitochondria requires the transport of many specific metabolites across the inner membrane of this eukaryotic organelle. We have screened the protein sequence database for proteins homologous to the mitochondrial ATP/ADP exchange carrier, and the homologous proteins found were similarly screened to ensure that all currently sequenced members of the mitochondrial carrier family (MCF) had been identified. Thirty-seven proteins were identified, 28 of which were less than 90% identical to any other sequenced member of the MCF, and the latter proteins fell into 10 clusters or subfamilies as follows: (1) ATP/ADP exchangers of mammals, plants, algae, yeast, and fungi (11 members); (2) a bovine oxoglutarate/malate exchanger (one member); (3) mammalian uncoupling carriers (five members); (4) yeast and mammalian phosphate carriers (three members); (5) MRS proteins that suppress mitochondrial splicing defects in *Saccharomyces cerevisiae* (two members); (6) a putative peroxysomal carrier of *Candida boidinii;* (7) a putative solute carrier from the protozoan, *Oxytricha fallax;* (8) a putative solute carrier from *S. cerevisiae;* (9) a putative solute carrier from *Zea mays,* and (10) two putative solute carriers from the mammalian thyroid gland. The specificities of proteins in clusters 5 to 10 are not known. A multiple alignment and an evolutionary tree of the 28 selected members of the MCF were constructed, thus defining the conserved residues and the phylogenetic relationships of the proteins. Hydropathy plots of the homologous regions were determined and averaged, and the average hydropathy plots were evaluated for sequence similarity. These analyses revealed that the six transmembrane spanners exhibited varying degrees of sequence conservation and hydrophilicity. These spanners, and immediately adjacent hydrophilic loop regions, were more highly conserved than other regions of these proteins.

All members of the MCF appear to consist of a tripartite structure with each of the three repeated segments being about 100 residues in length. Each repeat contains two transmembrane spanners, the first being more hydrophobic with conserved glycyl and prolyl residues, the second, preceded by a highly conserved glycyl residue, being more hydrophilic with largely conserved hydrophilic residues in certain positions. Five of the six spanners are followed by the largely conserved sequence (D/E) – Hy (K/R)[– = any residue; Hy = a hydrophobic residue]. Based on both intracluster and intercluster statistical comparisons, repeats 1, 2, and 3 are homologous, but repeats 1 are more similar to each other than they are to repeats 2 or 3 or repeats 2 or 3 are to each other. Taken together the results suggest specific functional roles for the individual spanners and further suggest that segments 1, 2, and 3 exhibit decreasing degrees of structural and functional significance in that order.

KEY WORDS: mitochondrial carriers, exchange transport, antiport, sequence comparisons, evolutionary relationships, phylogenetic tree, intragenic triplication

I. INTRODUCTION

All higher eukaryotes possess mitochondria that function primarily in the conversion of respiratory energy into chemiosmotic and chemical energy (Mitchell, 1979). In order for mitochondria to perform their compartmentalized energy interconversion functions, the continual traffic of metabolites through the two membranes of the mitochondrion is required. Transport across the outer membrane is mediated by regulated channel-forming proteins, the voltage-dependent anion carriers (VDAC), resembling the porin proteins of bacteria (Colombini, 1987; Mannella, 1992; Nikaido, 1992; Cowan et al., 1992; Troll et al., 1992). By contrast, metabolite transport

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across an inner membrane is mediated by a series of carrier-type proteins, the best characterized of which catalyze exchange transport. Among these transporters are ATP/ADP exchangers, phosphate carriers, oxoglutarate:malate exchange carriers, and uncoupling proteins of brown adipose tissue, the sequences of representative members of which have been reported (Table 1). These proteins prob-

TABLE 1
Homologous Protein Members of the MCF.[a]

Cluster	Transport protein	Organism	#Residues	Abbrev.	Accession number
1	ATP/ADP Carrier	*Homo sapiens*	300	AACHsa1	ATHAT_1
	ATP/ADP Carrier	*H. sapiens*	298	AACHsa2	HUMANT1_1
	ATP/ADP Carrier	*H. sapiens*	298	AACHsa3	HUMANT2X_1
	ATP/ADP Carrier	*H. sapiens*	298	AACHsa4	HUMATPC_1
	ATP/ADP Carrier	*H. sapiens*	225	AACHsa5	HUMTLC_1
	ATP/ADP Carrier	*H. sapiens*	262	AACHsa6	HUMTLCA_1
	ATP/ADP Carrier	*H. sapiens*	262	AACHsa7	HUMTLCB_1
	ATP/ADP Carrier	*H. sapiens*	297	AACHsa8	HUMTRL_1
	ATP/ADP Carrier	*Chlamydomonas reinhardi*	308	AACCre	CREANT_1
	ATP/ADP Carrier	*Zea mays*	387	AACZma1	MZEACC_1
	ATP/ADP Carrier	*Z. mays*	318	AACZma2	MZEANT_1
	ATP/ADP Carrier	*Neurospora crassa*	313	AACNcr	NEUATPC_1
	ATP/ADP Carrier	*Solanum tuberosum*	386	AACStu1	POTACC_1
	ATP/ADP Carrier	*S. tuberosum*	386	AACStu2	POTANTG_1
	ATP/ADP Carrier	*Saccharomyces cerevisiae*	318	AACSce1	YSCAAC2_1
	ATP/ADP Carrier	*S. cerevisiae*	307	AACSce2	YSCAAC2A_1
	ATP/ADP Carrier	*S. cerevisiae*	314	AACSce3	YSCAAC3_1
	ATP/ADP Carrier	*S. cerevisiae*	309	AACSce4	YSCPET9_1
		Average length:	310 ± 40		
2	Oxoglutarate/malate carrier	*Bos taurus*	362	KMCBta	BOVOMCPA_1
3	Uncoupling carrier	*Bos taurus*	282	UNCBta	BOVUCPB24
	Uncoupling carrier	*Homo sapiens*	306	UNCHsa	HUMUPCP12
	Uncoupling carrier	*Mus musculus*	307	UNCMmu	MUSUCPM5_1
	Uncoupling carrier	*Oryctolagus cunculus*	306	UNCOcu	RABUCP_1
	Uncoupling carrier	*Rattus norvegicus*	307	UNCRno	RATUCP_1
		Average length:	302 ± 10		
4	Phosphate carrier	*Bos taurus*	314	PICBta	BOVPCR_1
	Phosphate carrier	*Rattus norvegicus*	396	PICRno	RATMPT_1
	Phosphate carrier	*S. cerevisiae*	311	PICSce	YSCMIPHOS
		Average length:	340 ± 40		
5	Yeast MRS proteins	*S. cerevisiae*	318	MRSSce1	YSCMRS3_1
	Yeast MRS proteins	*S. cerevisiae*	314	MRSSce2	YSCMRS3G_1
	Yeast MRS proteins	*Saccharomyces cerevisiae*	296	MRSSce3	YSCMRS3G_2
	Yeast MRS proteins	*S. cerevisiae*	304	MRSSce4	YSCMRS4_2
		Average length:	308 ± 9		
6	Peroxysomal carrier	*Candida boidinii*	401	POXCbo	YSAPMP47_2
7	O. fallax carrier	*Oxytricha fallax*	372	OFXOfa	OFA81MAA_1
8	Yeast solute carrier	*S. cerevisiae*	307	YSCSce	YSCYMC1_1
9	Maize solute carrier	*Zea mays*	436	MSCZma	MZEBT1A_1
10	Thyroid solute carrier	*Rattus norvegicus*	322	TSCRma	RATMSCA
	Thyroid solute carrier	*Homo sapiens*	349	TSCHsa	HUMMSCA_1
		Average length:	336 ± 14		

[a] The proteins are identified by cluster, name, biological source, and accession number. The abbreviation of each protein used in this study (which specifies the protein [first three letters] as well as the organism [second three letters] is provided, as is the number of sequenced residues in the protein. Below the numbers of residues (# residues) for any one cluster of proteins, the average sizes of these proteins are presented ± the variability of the values in standard deviations (SD). The carrier specificities are known for clusters 1 to 4, but those for remaining proteins (clusters 5 to 10) are not. All of the published sequences appear to be complete except for AAC Hsa5 and PIC Bta. Abbrev. = Abbreviations used in this study.

ably predominate as dimeric integral transmembrane polypeptides (subunit mol wt ~30 kDa) that traverse the inner mitochondrial membrane six times with both the N- and C-termini localized to the cytosolic side of the membrane (Klingenberg, 1981; Bogner et al., 1986; Klingenberg and Appel, 1989; Gawaz et al., 1990; Klingenberg, 1990; Capobianco et al., 1991). Higher organisms may possess multiple nuclear genes encoding isozymic forms of any one class of these mitochondrial transporters, and the expression of the different genes encoding the isoforms of a particular exchanger may be differentially regulated in a tissue-specific fashion (Lunardi and Attardi, 1991; Lunardi et al., 1992). The transport mechanisms of these carriers have been characterized extensively in intact mitochondria, in submitochondrial vesicles, and in reconstituted liposomes (Krämer and Klingenberg, 1977; Aquila et al., 1978; Klingenberg and Winkler, 1985; Dierks et al., 1988; Knirsch et al., 1989; Klingenberg, 1990; Winkler and Klingenberg, 1992). It is clear that these proteins normally function in a carrier-type exchange transport mode, but that following treatment with sulfhydryl-specific chemical reagents, some of them can be converted from obligatory exchange antiporters to channel-like uniporters (Dierks et al., 1990a,b). These observations suggest that mitochondrial carriers, and possibly all transmembrane transport carriers, possess a structure that incorporates a preformed channel as a prerequisite for carrier function.

Evidence has been presented suggesting that the four known solute-specific types of mitochondrial porters for which sequence data are available (Table 1) are members of a single superfamily that has been termed the mitochondrial carrier family (MCF) (Klingenberg, 1990). However, limited biochemical evidence suggests that many other solute-specific porters of mitochondria may prove to fall into the MCF. For example, it is known that the apparent subunit molecular weights of many anion carriers are similar. Those for the ATP/ADP exchangers (33 kDa), the inorganic phosphate porters (35 kDa), the uncoupling proteins of mammalian brown adipose tissue (32 kDa), the α-ketoglutarate:malate exchanger (38 kDa), the oxaloacetate carrier (32 kDa), the dicarboxylate exchanger (28 kDa), the pyruvate:H^+ symporter (34 kDa), and the aspartate/glutamate exchanger (33 kDa) are all strikingly similar (LaNoue and Schoolwerth, 1979; Bisaccia et al., 1992; Palmieri et al., 1990, 1992). These observations suggest that mitochondrial MCF proteins, homologous to the ATP/ADP exchanger, may possess diverse solute specificities and thus serve varied functions. Published reports suggest that at least one member of the MCF functions in a nontransport capacity (Wiesenberger et al., 1991), and that another member of the MCF may be present in a distinct eukaryotic organelle, the peroxysome (McCammon et al., 1990). Other mitochondrial porters, specific for both anionic and cationic nutrients as well as inorganic anions and cations, may also prove to be members of this family, although the K^+ carrier and the Na^+/H^+ exchange carrier appear to have different molecular weights (Garlid et al., 1991; Beavis and Vercesi, 1992; Yu and Weiss, 1992).

We and others have noted that many classes of transport proteins of both the carrier and channel types consist of multiple (two to eight) units, each possessing six transmembrane helices (Henderson, 1990; Maloney, 1990; Saier, 1990; Saier et al., 1990; Saier and Reizer, 1991; Nikaido and Saier, 1992; Saier et al., 1992). In recent reports we have summarized evidence suggesting that subunits of the so-called major intrinsic protein (MIP) family of channel proteins consist of units of six transmembrane α-helical spanners that apparently arose by intragenic duplication of a three-spanner-encoding genetic precursor, possibly about 2.5 billion years ago (Pao et al., 1991; Reizer et al., 1993). Moreover, the major facilitator superfamily (MFS) probably arose independently of the MIP family over 3.5 billion years ago by the intragenic duplication of a six-spanner precursor to give proteins containing 12 transmembrane α-helical spanners (Griffith et al., 1992; Allard and Bertrand, 1992; Marger and Saier, 1993). Thus, two families of transport proteins, both consisting of units of six transmembrane α-helices, apparently arose independently, at different times in evolutionary history, and employing different routes.

Wiesenberger et al. (1991) recently sequenced the genes encoding the yeast MRS3 and MRS4 suppressors of mitochondrial RNA splicing defects and noted that these novel members of the MCF, like other sequenced mitochondrial carriers

(Klingenberg, 1990), had a tripartite structure. The six-spanner MCF proteins each consists of three 100 residue repeat sequences with two hydrophobic spanners per repeat. The MRS inner mitochondrial membrane proteins share with the ATP/ADP exchangers and other nuclear-encoded MCF proteins the unusual property that they are imported into mitochondria without proteolytic cleavage of a presequence (Saier et al., 1989; Pratt et al., 1991; Wiesenberger et al., 1991).

The studies summarized here have provided evidence for a multigenic family of mitochondrial (and peroxysomal) solute carriers with distinctive structural features. Following the earlier less ambitious analyses of others (Aquila et al., 1987; Runswick et al., 1987, Klingenberg, 1990; Wiesenberger et al., 1991), we have conducted detailed structural and evolutionary analyses of the protein members of the MCF. We establish a common ancestry for the 37 current members of this family and provide a multiple sequence alignment for all 28 currently recognized, sequence-dissimilar members of this family. This achievement allows us to derive signature sequences for the MCF proteins and to determine the degrees of conservation of residues at each position within the alignment. These multiple alignments allow us to predict the relative structural and functional significance of particular residues. The most highly conserved residues included six glycyl residues and one prolyl residue that presumably play structural roles as well as three strongly hydrophilic residues, lysyl, glutaminyl, and arginyl residues, all of which are found in matrix–localized loops. We also construct a phylogenetic tree for these proteins that shows that with only a couple of exceptions they fall into ten approximately equally distant clusters or subgroups. The three repeat sequences are analyzed in detail, and the statistical analyses allow us to demonstrate their relative degrees of conservation and variability. This information further allows us to predict the relative structural and functional significance of these three repeat sequences and to propose that the MCF arose relatively recently (about 1.5 billion years ago) by triplication of a two-spanner-encoding genetic precursor. This prediction is in agreement with the earlier suggestion of others who have also noted that all mitochondrial carriers are nuclear encoded (Aquila et al., 1987; Klingenberg, 1989). Limited evidence suggests that the two-spanner precursor may have in turn arisen by duplication of a one-spanner-encoding genetic element. The results establish that the MCF arose independently of two ubiquitous families of transporters, the MIP family of channel proteins and the MFS of solute carriers. We estimate the time of emergence of the primordial six transmembrane MCF precursor polypeptide-encoding gene as about 1.5 billion years ago, the time when mitochondria first appeared in eukaryotes. Thus, we predict that these transport proteins were not derived from proteins present in the prokaryotic progenitors of mitochondria.

II. COMPUTER METHODS

The Protein Information Resource (PIR) of the National Biomedical Research Foundation (NBRF) database (version 32) as well as the current GenBank (version 71.0), EMBL (version 24.0), and SwissProt (version 21.0) databases were screened in all homology searches using the FASTA program (Lipman and Pearson, 1985; Pearson and Lipman, 1988). Significance was evaluated by calculating the binary comparison scores, expressed in standard deviations, using the ALIGN program (Dayhoff et al., 1983). Comparison scores are recorded in standard deviations higher than obtained with 200 comparisons of randomized sequences of the two protein segments analyzed. A comparison score of 9 to 10 SD was considered to reflect a sufficient degree of sequence similarity to establish homology, while values of 7 to 8 SD are strongly suggestive of homology (Doolittle, 1981, 1986, 1992; Dayhoff et al., 1983). The RDF2 program (Pearson and Lipman, 1988) generally gave substantially larger scores (by 2 to 4 SD).

Homologous protein sequences were aligned using the Needleman-Wunsch algorithm, and relative evolutionary distances and phylogenetic trees were determined using the progressive alignment or gap minimizing TREE program of Feng and Doolittle (1990) and Doolittle and Feng (1990). All sequence analyses and database searches were performed using the GCG package from the Uni-

versity of Wisconsin (Devereux et al., 1984) and the UCSD VAX/VMS DNASYSTEM package (Smith, 1988). While the figure presenting the multiple sequence alignment (Figure 4) shows the central regions of the homologous proteins that generally exhibit extensive sequence similarity, documenting the relatedness of these proteins, the complete multiple alignments, were generated for all proteins analyzed and are available from MHS upon request. The alignment positions indicated at the top of the multiple alignment shown in Figure 4 are reflective of the complete alignment.

A degenerate signature sequence (Bairoch, 1992) for the available sequenced mitochondrial carriers of the MCF was determined from the most conserved region of the multiply aligned protein sequences. This sequence, presented in the Results section, is general for all current members of the MCF except the peroxysomal protein POX Cbo. It was screened against the protein data banks (PIR, version 32 and SWISSPROT, version 22) and in no case did a protein other than members of the MCF exhibit this identifying sequence. Thus, it is family specific and can assist in the identification of new members of the MCF.

III. RESULTS

A. 38 Sequenced MCF Proteins

Table 1 lists the transport proteins (or presumptive transport proteins) that were discovered during database searches for homologous members of the MCF. The 37 listed proteins fall into ten clusters (1 to 10). The carrier specificities are known for proteins in clusters 1 to 4, but proteins in clusters 5 to 10 have not been functionally characterized. Cluster 5 MRS proteins are suppressors of mitochondrial RNA splicing defects in yeast when made in large amounts (Wiesenberger et al., 1991), but their biochemical nature is not known. The sizes of the sequenced proteins (in numbers of residues) are provided in Table 1 as are their accession numbers and the abbreviations used in this study to designate them.

In order to simplify subsequent analyses and to establish that the proteins listed in Table 1 are all members of a single homologous family, the proteins were initially examined for their percent similarity. Seven of the sequenced ATP/ADP exchangers (AAC) and two of the sequenced yeast MRS proteins were found to be more than 90% identical to another MCF protein. These nine proteins were therefore excluded from further analysis. As will be documented later, the 28 remaining proteins fell into ten subgroups. Intrasubgroup comparisons revealed very high percent identities, but intersubgroup comparisons gave values that were much lower. Consequently, statistical analyses were required to establish homology. One representative member of each subgroup was selected for calculation of binary comparison scores. The results are recorded in Table 2. A binary comparison score of 7 standard deviations (SD) strongly suggests homology (a common evolutionary origin), while a value of 9 SD establishes homology.

The results summarized in Table 2 show that the MSC Zma protein gives higher comparison scores with the other nine proteins than do any of the other proteins (values of 7 to 13 SD). All values are of 9 SD or greater with the sole exception of the comparison score obtained with the OFX Ofa protein, where a value of 7 SD was obtained. However, the OFX Ofa protein gave a score of 13 SD with the KMC Bta protein, establishing that it is a member of the MCF. Thus, all proteins listed in Table 1 are members of a single family.

B. Phylogenetic Tree of the MCF Proteins

Figure 1 presents the phylogenetic tree of 28 of the 37 proteins listed in Table 1. Those not included were proteins that were greater than 90% identical to at least one protein that was included in the study. It can be seen that all 11 of the ATP/ADP exchangers (AAC) clustered together (cluster 1). Three plant AAC proteins clustered tightly together as did the two yeast and the *Neurospora* proteins. The *Chlamydomonas* protein was closer to the yeast and fungal proteins than the plant proteins. Surprisingly, one of the human isoforms (AAC Hsa1) was sandwiched in between the plant proteins and those from lower eukaryotes (yeast,

TABLE 2
Binary Comparison Scores (in Standard Deviations, SD) for the Ten Representative but Dissimilar MCF Proteins Analyzed in this Study[a]

	KMCBta1	UNCOcu	PICBta	MRSSce4	POXCbo	OFXOfa	YSCSce	MSCZma	TSCRma
AACStu1	**8**	**10**	5	**7**	6	4	**10**	**14**	**10**
TSCRma	5	**8**	**8**	**8**	3	4	**8**	**16**	
MSCZma	**10**	**10**	**9**	**13**	**9**	**7**	**10**		
YSCSce	**9**	**11**	5	**9**	**7**	**7**			
OFXOfa	**13**	**8**	5	2	4				
POXCbo	5	4	4	**7**					
MRSSce4	**9**	6	**8**						
PICBta	**9**	**7**							
UNCOcu	**20**								
Average score:	**8.0**								
Variability:	3.5								

[a] Large values (seven standard deviations or greater), suggestive of homology, are presented in bold print. The average comparison score and the variability of this value, both expressed in standard deviations, are presented in the lower left-hand side of the table. Abbreviations are as defined in Table 1.

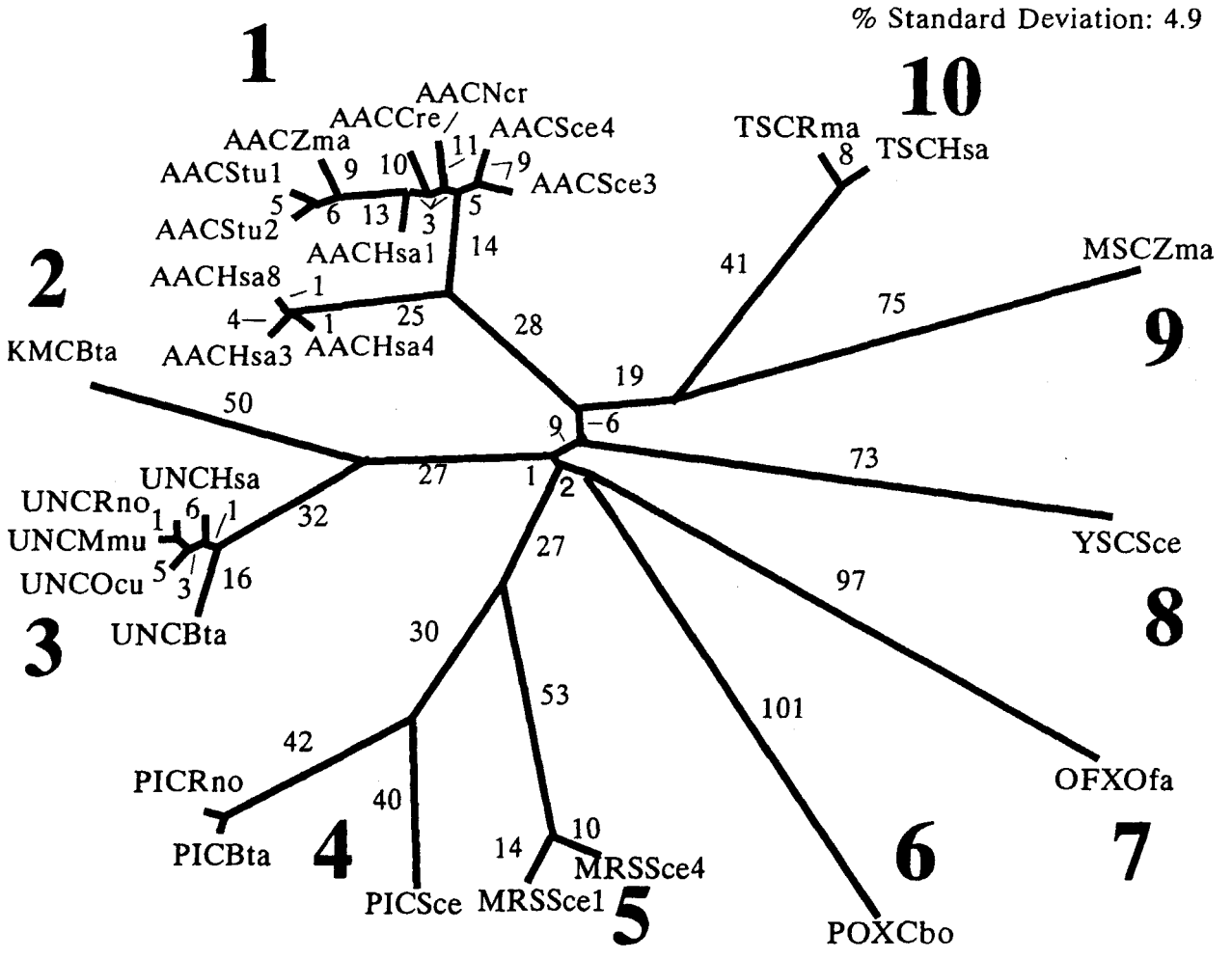

FIGURE 1. Phylogenetic tree of 28 protein members of the MCF. Relative evolutionary distances are provided adjacent to the branches. Methods of tree construction were as described under "Computer Methods." Abbreviations of the proteins are as presented in Table 1. Each of the ten protein clusters or subfamilies are indicated in large letters (1 to 10). Proteins listed in Table 1 that were more than 90% identical to another member of the family were not included when constructing the tree. Relative distances are provided adjacent to the branches.

fungi, and algae), while the other two (AAC Hsa3 and 8) were off on a distinct subbranch.

Cluster 2 (the single sequenced α-ketoglutarate:malate exchange carrier) and cluster 3 (the uncoupling proteins, all of mammals) are off on the second major branch of the tree. The third major branch also has two principal subbranches, the phosphate carriers of mammals and yeast (cluster 4), and the yeast MRS proteins, suppressors of mtRNA splicing defects (cluster 5) (Wiesenberger et al., 1991). Clusters 6 to 9 are each represented by a single protein. These include a putative peroxysomal carrier protein from the yeast, *Candida boidinii* (POX Cbo; McCammon et al., 1990), a putative solute carrier from the protozoan, *Oxytricha fallax* (OFX Ofa; Herrick et al., 1985, 1987), a putative solute carrier from *S. cerevisiae* (YSC Sce; R. Graf, B. Baum, and G. H. Braus, unpublished), and a putative maize carrier protein (MSC Zma; Sullivan et al., 1991). The last cluster, cluster 10, comprises a deep branch off of the maize carrier branch and includes two putative carriers from mammalian thyroid tissue (Zarrilli et al., 1989). As can be seen from Figure 1, the proteins corresponding to the seven major branches probably arose at about the same time in evolutionary history. It is intriguing that so many

dissimilar members of the family have not yet been assigned a function.

C. Hydropathy Profiles of Individual Proteins

One member of each of the ten clusters was selected for initial detailed analyses. The hydropathy plots of these ten proteins are shown in Figure 2. All of these proteins presumably possess six α-helical transmembrane spanners because they are homologous to the ATP/ADP exchangers that have been characterized extensively and are believed to have this topological motif. (See Introduction as well as Kyte and Doolittle [1982], Doolittle [1986], and von Heijne [1992] for hydropathy and topology evaluation for integral membrane proteins.) It can be seen, however, that they differ substantially in their hydrophobicity profiles. As will be documented later, each of these proteins consists of three repeat sequences of about 100 residues, and the boundaries between these three segments are indicated by the light vertical lines in Figure 2. It can be seen that most of the 30 segments exhibit two peaks of hydrophobicity in similar positions, although considerable variation is observed. Based on biochemical analyses conducted primarily with the ATP/ADP exchanger (Klingenberg, 1981; Bogner et al., 1986; Klingenberg and Appel, 1989; Gawaz et al., 1990; Klingenberg, 1990; Capobianco et al., 1991), the six peaks of hydrophobicity observed for most of the proteins probably correspond to the six putative transmembrane helices. It can be seen that putative trans-

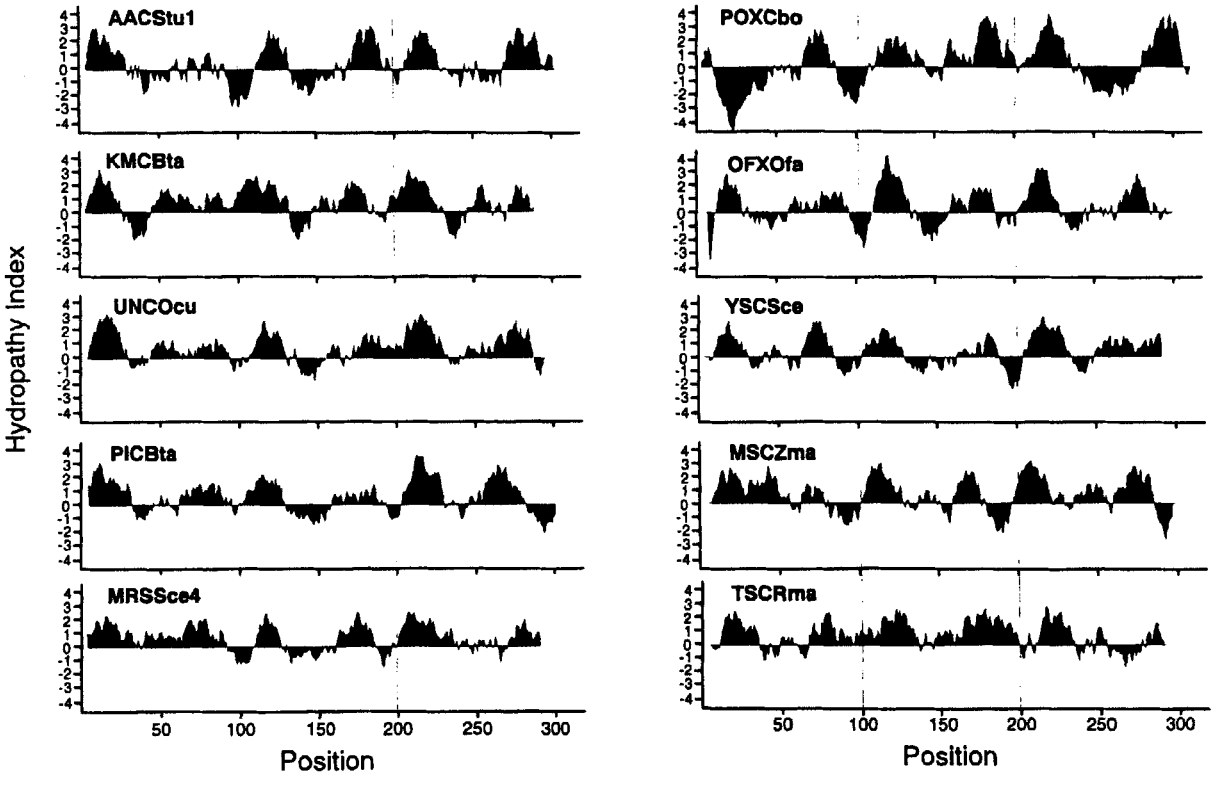

FIGURE 2. Hydropathy analyses of ten representative MCF members, one from each cluster shown in Figure 1. The program of Kyte and Doolittle (1982) was used to generate the hydropathy plots. Vertical lines delineate the boundaries of the two-spanner-containing repeat sequences 1, 2, and 3. Abbreviations for the proteins are as provided in Table 1. The hydropathy plots shown are for the central, homologous regions of the proteins as follows: AAC Stu1: 78–386; KMC Bta: 14–314; UNC Ocu: 6–305; PIC Bta: 1–281 (sequence incomplete at the N-terminus); MRS Sce4: 16-302; POX Cbo, 3–325; OFX Ofa: 7–319; YSC Sce: 18–307; MSC Zma: 41-341; TSC Rma: 28–322.

membrane spanners 1, 3, and 5 are generally more hydrophobic than transmembrane spanners 2, 4, and 6 (Figure 2; see also Aquila et al., 1985, 1987). It is also noteworthy that the latter three peaks of hydrophobicity often appear broader and less well defined than the former peaks. It will be seen later that this is due to the occurrence of hydrophilic residues in the even numbered putative transmembrane spanners.

D. Correlation of Average Hydropathy with Average Similarity

To improve resolution, the hydropathy profiles of the 28 selected protein members of the MCF included in the phylogenetic tree of Figure 1 were averaged (Figure 3A). The six peaks of hydrophobicity that correspond to the putative transmembrane spanners are significantly more clearly delineated in Figure 3A than in most of the profiles for individual proteins shown in Figure 2. The corresponding average similarity plot, based on the same 28 sequences, is depicted in Figure 3B. It is interesting to note that putative transmembrane spanners 1, 3, and 5 are strongly conserved as are the regions of about 20 residues just following these three spanners. By contrast, spanners 2, 4, and 6 are apparently less well conserved and instead of being *followed* by a region of sequence conservation, these three spanners are *preceded* by a conserved region of about 40 residues. The least conserved regions are those regions for which extensive gaps in the multiple alignment occur in several of the proteins. These poorly conserved regions comprise the N-termi-

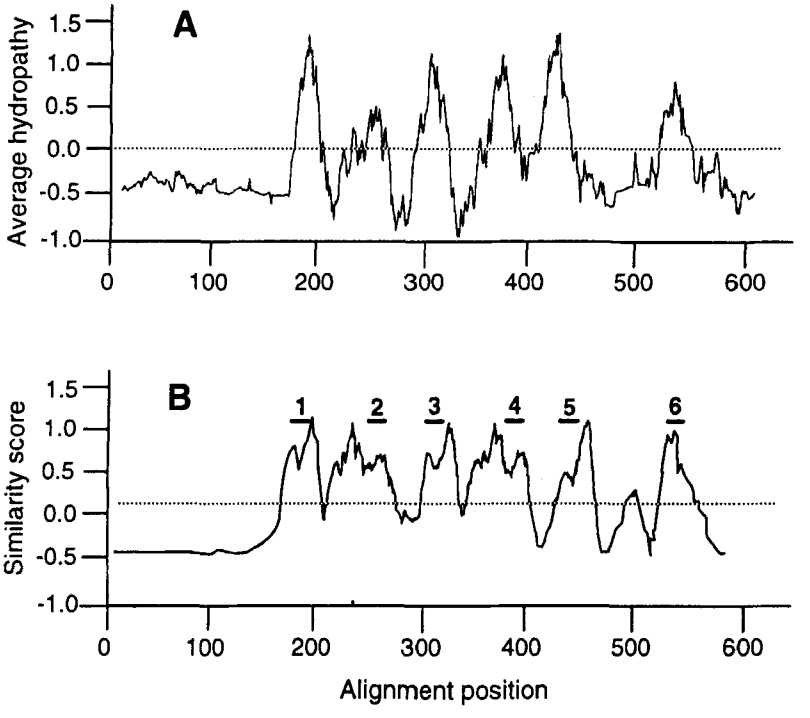

FIGURE 3. Average hydropathy plot (A) and average similarity plot (B) for the ten representative proteins of the MCF depicted in Figure 2. The average hydropathy and average similarity plots were calculated using a sliding window of 20 residues. The average similarity across the entire alignment in (B) is shown as a dotted line. The six bars above the plot in B represent the positions of the six putative transmembrane helical spanners. The plot reveals the degree of coincidence between hydropathy and similarity plots, showing that the regions of greatest similarity include the transmembrane spanners as well as the adjacent regions following spanners 1, 3, and 5, but preceding spanners 2 and 4.

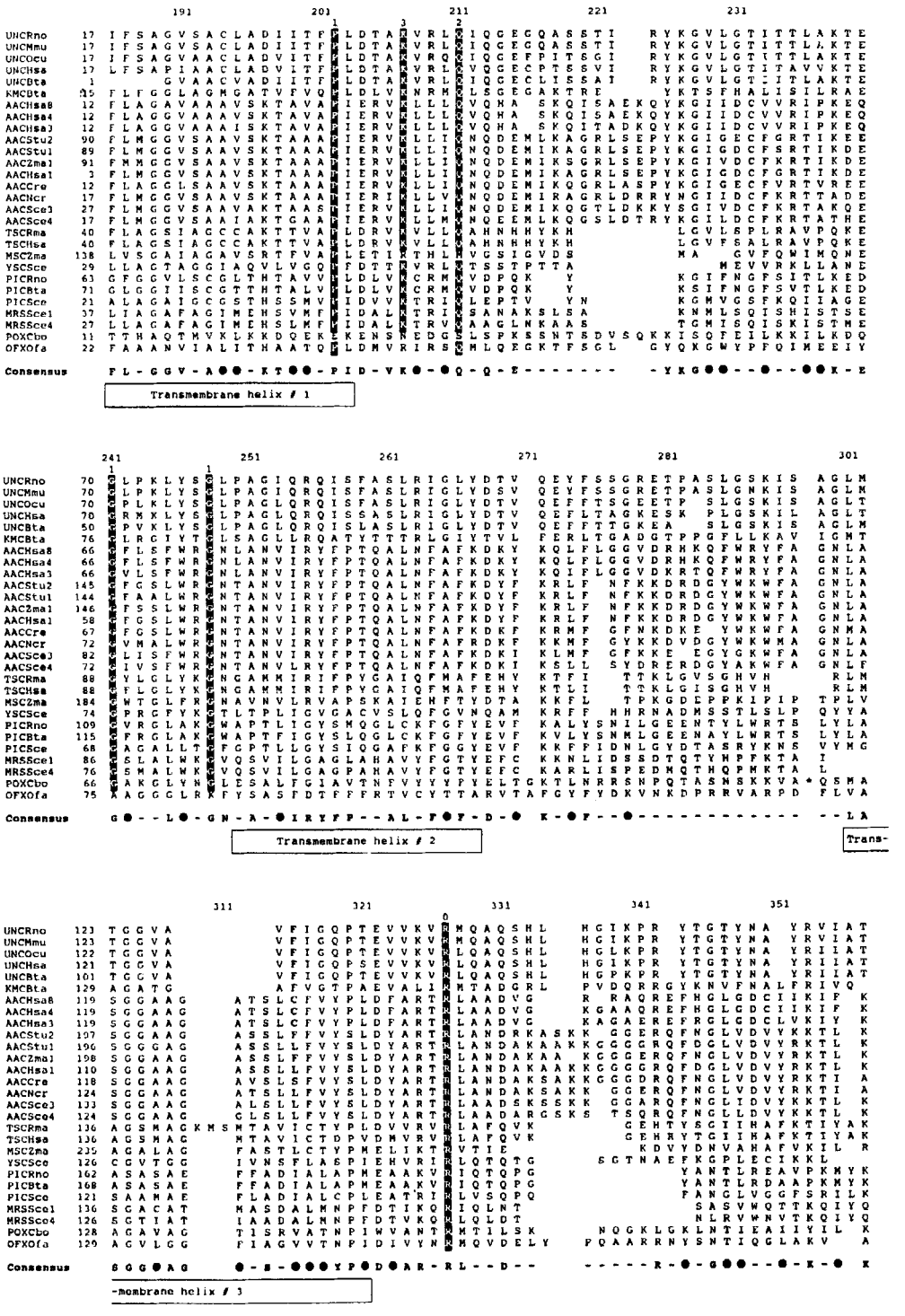

FIGURE 4. Multiple alignment of the 28 proteins of the MCF included in the phylogenetic tree shown in Figure 1. Numbers at the top of the aligned sequences denote the residue positions in the complete multiple alignment. The residue number in each protein is provided at the beginning of each line. Fully conserved residues and residues that are fully conserved with only 1, 2, 3, or 4 exceptions are indicated with a 0, 1, 2, 3, or 4 immediately above the aligned sequences, respectively. The consensus sequence (at least 14 of the 28 residues at any one position conserved) is provided below the multiple alignment in bold print. Positions in which only hydrophobic (nonhydrophilic) residues occur are indicated with a bullet (•). Positions that are not conserved are indicated by a dash (–). A space indicates the lack of a residue at a particular position for a majority of the proteins. The six open bars below the consensus sequence correspond to the six putative transmembrane α-helical spanners (each 18 residues long). An asterisk (*) indicates the presence of a deleted sequence that is not shown in the multiple alignment. The first asterisk (alignment positions 291 to 298) corresponds to the sequence: A L K K G L S V W in the POX Cbo protein. The second asterisk (positions 472 to 508) correspond to the sequence: E D N L K E N S A K S P Y A E T I T K I I S K L P S P I V S M F T L G Y G in the OFX Ofa protein.

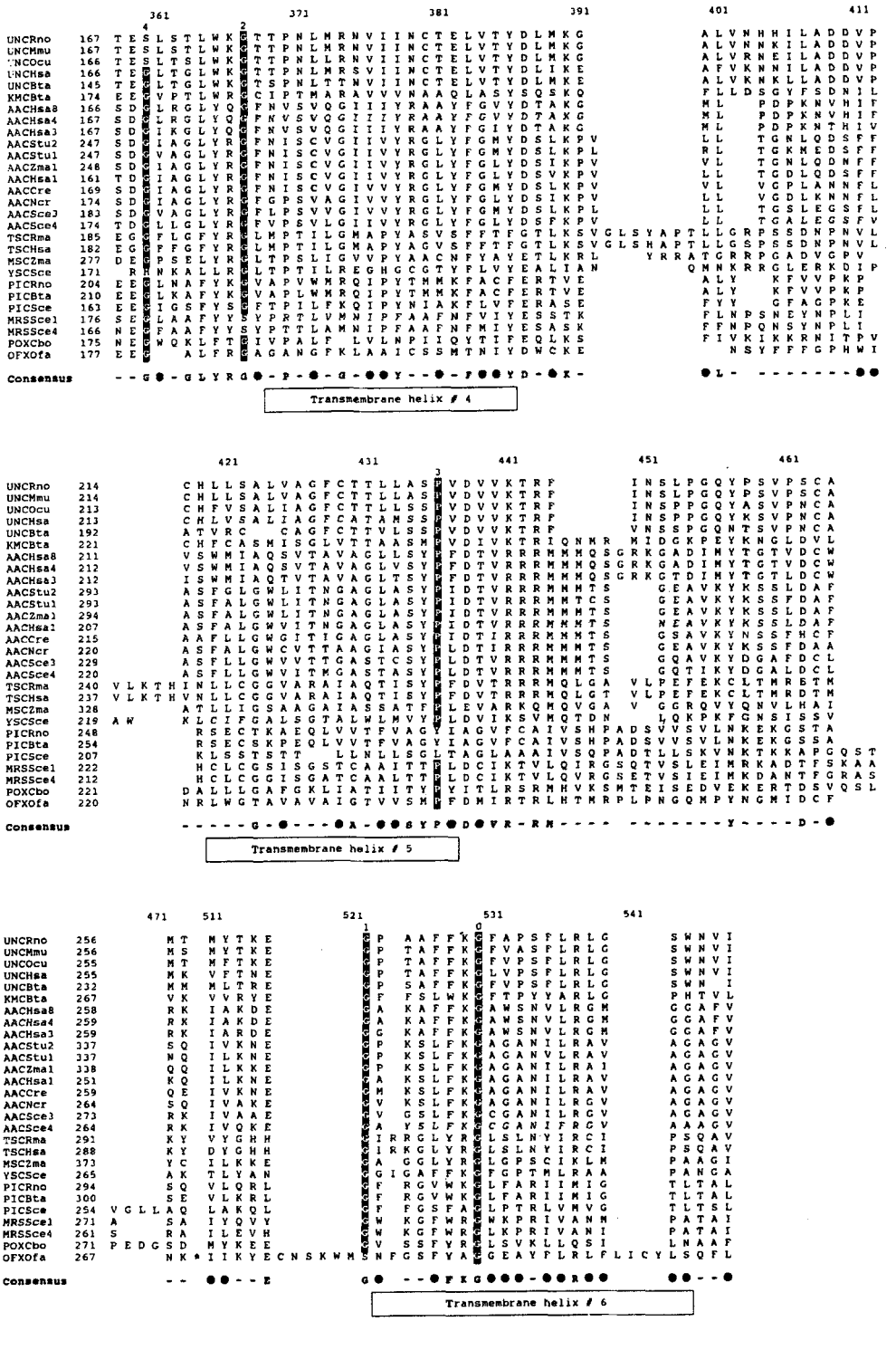

FIGURE 4. (continued)

nal and C-terminal regions of many of the proteins as well as the region between transmembrane spanners 5 and 6 (Figure 3).

E. Multiple Alignment of MCF Protein Sequences

A major portion of the complete multiple alignment of the 28 selected proteins of the MCF is presented in Figure 4. The alignment position is provided at the top of the alignment, and the residue numbers for the various proteins are provided at the beginning of each line. The alignment position does not correspond to the residue number of any one protein. Just above the sequences are indicated those residues that are conserved in all but 0, 1, 2, 3, or 4 (0, 1, 2, 3, 4) positions, and these strongly conserved residues are highlighted with a black background. The consensus sequence is provided below the alignment (see legend to Figure 4).

The portions of the multiple alignment not shown in Figure 4 (preceding alignment position 186 and following alignment position 548) are poorly conserved regions with many gaps in the multiple alignment. In no position within these C- and N-terminal regions of the complete multiple alignment does a residue occur in a majority of the sequences. Thus, there are no notable features in the portions of the complete multiple alignment that are not shown in Figure 4.

A short region in the multiple alignment shown in Figure 4 proved to be adequate for the generation of a degenerate signature sequence (Bairoch, 1992). It is found at positions 198 to 211 in the alignment shown in Figure 4 and is general for all mitochondrial members of the family, but not for the single nonmitochondrial member of the family, the peroxysomal protein POX Cbo. This signature sequence is (LIVSAT) X_3 P (LIVF) (DE) X (LIVTA) (KR) (LIVTCN) (LHR) (LIVMSQ) (QM). (Ambiguous residues at a specific position are given in parentheses, whereas any amino acid at a position in which the residue is not specified is denoted by X.) Although this signature sequence identified all mitochondrial MCF proteins and no other protein in the database, the sequence identified two regions in the KMC Bta protein: one corresponding to alignment positions 198 to 211 as expected; the other corresponding to alignment positions 432 to 445 in Figure 4, corresponding to the sequence: A A S M P V D I V K T R I Q at the end of transmembrane helix #5. This signature sequence occurs at the same position in repeat #3 as does the signature sequence in repeat #1. It may be useful for the identification of new members of the MCF.

F. Analyses of Putative Transmembrane Spanners and Adjacent Loop Regions

The six horizontal bars shown below the consensus sequence in Figure 4 correspond to the positions of the six putative transmembrane α-helical spanners as estimated from the hydropathy plots. Each of these spanners is 18 residues long, and the six 18 residue segments corresponding to these sequences are depicted in helical wheel projections in Figure 5. Residues indicated in Figure 5 refer to the consensus sequence provided at the bottom of Figure 4. A bullet (•) refers to a position in which predominantly hydrophobic residues occur, while an asterisk (*) refers to a position in which predominantly hydrophilic residues occur.

Examination of the helical wheels shown in Figure 5 reveals that helices 1, 3, and 5 have in common a consensus glycyl residue at positions 4 and a consensus prolyl residue at positions 17. All three of these helices have consensus hydrophobic residues at positions 6, 14, and 18. They are all punctuated at their C-terminal ends by a consensus sequence that consists of an aspartyl (D) residue followed by two hydrophobic residues, an arginyl or lysyl (R/K) residue, a variable residue, and an arginyl (R) residue. This last arginyl residue in the sequence following spanner #3 is one of the only two fully conserved residues in the entire multiple alignment shown in Figure 4. The consensus sequence for the C-terminal loop regions following spanners 1, 3, and 5 is thus: D • V R - R.

Spanners 2, 4, and 6 similarly have several features in common (Figure 5). Consensus hydrophobic residues are found at positions 4, 8, 9, 13, 15, 16, and 17. Consensus hydrophilic residues

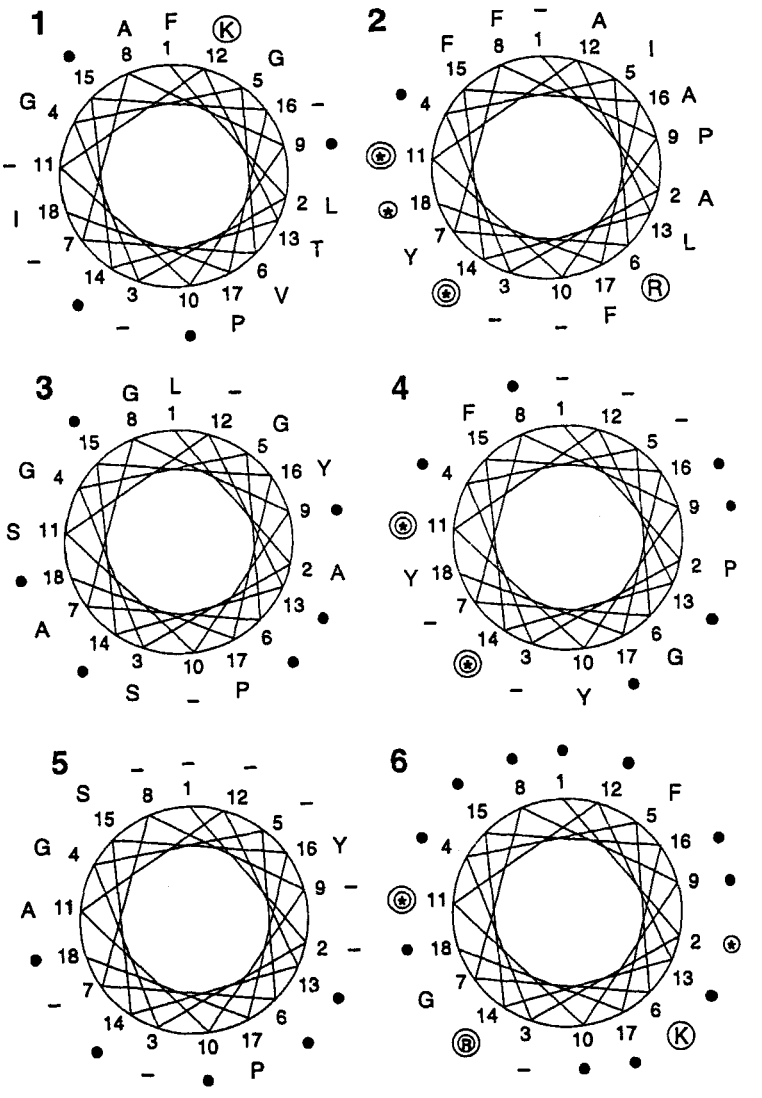

FIGURE 5. Helical wheel depiction of the six (1 to 6) putative transmembrane helices in the proteins of the MCF. The residue numbers (1 to 18) in the putative helices are provided. In general, the conserved residues are as indicated in the consensus sequence shown in Figure 1. A bullet (•) indicates a position in which essentially all residues are hydrophobic. A dash (-) indicates a nonconserved position that, however, is predominantly hydrophobic. A star (*) indicates a nonconserved position that is predominantly hydrophilic. The largely conserved G at position 4 and the largely conserved P at position 17 in helices 1, 3, and 5 as well as the predominantly hydrophilic residues in positions 11 and 14 in helices 2, 4, and 6 (circled asterisks) are particularly worthy of note. The consensus sequences depicted within the six helical wheels correspond to the alignment positions shown in Figure 4 as follows: 1, alignment positions 186 to 203; 2, 250 to 267; 3, 301 to 321; 4, 369 to 386; 5, 420 to 437; 6, 522 to 545.

are found in positions 11 and 14 in all three helices, and basic residues (R/K) occur at position 6 in two of the three helices.

Spanners 2 and 4 (but not spanner 6) are preceded by a long (24 residue), well-conserved, fairly hydrophobic sequence. The following 12

residue consensus sequence, immediately N-terminal to these two spanners is particularly noteworthy: K - - G • - G L Y R G -. C-terminal to spanners 2 and 4 (but not 6) is the short consensus sequence: D - • K. Thus, five of the six transmembrane spanners in the MCP proteins have the consensus sequence, D - • (R/K), punctuating their C-terminal ends. This common motif presumably either has structural/functional significance or reflects a common ancestor of all six transmembrane spanners (see Discussion).

G. Statistical Analyses of Repeat Sequences in MCF Proteins

Members of the MCF exhibit three repeat sequences, each about 100 residues in length, each with two putative transmembrane spanners (Figures 2 and 3; Klingenberg, 1990; Wiesenberger et al., 1991). These repeat sequences were statistically analyzed in order to determine their relative degrees of conservation. Both positionally similar and dissimilar repeats were analyzed. In all of the analyses reported, a single program, the ALIGN program (Dayhoff et al., 1983), was used to calculate comparison scores.

Tables 3A, B, and C present the binary comparison scores obtained for the ten representative, dissimilar, MCF proteins shown in Figure 2 for (A) repeats 1 vs. repeats 1, (B) repeats 2 vs. repeats 2, and (C) repeats 3 vs. repeats 3. Large values, presented in bold type, are suggestive of homology. The results provide evidence that these segments share a common evolutionary origin as expected from the fact that the intact proteins are homologous (Table 2). They also indicate which of these proteins possess strongly conserved segments 1, 2, or 3 (see average values for each protein repeat segment, indicated to the right of the tables).

All comparison scores recorded in Table 3, parts A, B, and C, were averaged. When the scores for repeats 1 were averaged, a value of 5.1 ± 2.2 SD was obtained. The corresponding values for segments 2 and 3 were 5.0 ± 2.4 SD and 3.9 ± 2.4 SD. Thus, repeats 3 appear to be more dissimilar to themselves than are repeats 1 or 2. This fact suggests that residues within segments 1 and 2 may be of greater structural and functional significance than those within segments 3.

A similar analysis of ten AAC proteins is recorded in Tables 4A, B, and C. Because of their striking sequence similarity (these proteins comprise a single cluster in the phylogenetic tree shown in Figure 1), the comparison scores are all relatively high. It can be seen that the values obtained for the ten AAC segments 1 range between 18 and 33 SD with an average value of 24.4 ± 3.7 SD. The values for the ten AAC segments 2 range from 4 and 33 SD with an average value of 20.4 ± 8.6 SD. The values for the 10 AAC segments 3 ranged between 3 and 33 SD with an average value of 17.2 ± 9.3 SD. It is thus apparent that segments 1 exhibit the highest average comparison score and are therefore most similar to each other in sequence. They also show the least sequence variability. By contrast, segments 3 show the lowest average comparison score and the greatest variability. In fact, the average scores for each protein segment when compared with the other nine show a decrease in magnitude with the same order (seg 1 > seg 2 > seg 3). It is therefore clear that segments 1 are most conserved and segments 3 least conserved both within and between clusters of the MCF. The surprisingly low values for the second and third repeat sequences of the ATP/ADP carrier of *Chlamydomonas reinhardi* is striking and suggests that these segments have diverged abnormally rapidly.

In confirmation of the conclusions derived from the data in Tables 3 and 4, the three segments of each of the 28 proteins included in this study were compared (i.e., segments 1 and 2, segments 1 and 3, and segments 2 and 3). The comparison scores obtained are listed in Table 5. When segments 1 and 2 were compared, an average value of 6.1 ± 1.5 SD was obtained. When segments 3 were compared with either segments 1 or 2, the same value of 4.3 ± 2.4 SD was obtained. This result shows that, on the average, repeats 1 and 2 are more similar to each other than either one of these repeats is to segment 3. It confirms the conclusion that segment 3 has diverged more extensively from the primordial sequence than has segment 1 or 2. It is interesting to note that the corn protein, MSC Zma, gives the highest values of all the individual comparison scores. Presumably, this fact reflects a slower rate

TABLE 3
Binary Comparison Scores (in SD) of (A) Repeat Segments 1, (B) Repeat Segments 2, and (C) Repeat Segments 3 for the Ten Dissimilar, Representative Proteins Depicted in Figure 2[a]

A. Segments 1

	KMCBta	UNCOcu	PICBta	MRSSce4	POXCbo	OFXOfa	YSCSce	MSCZma	TSCRma	Averages
AACStu1	6	5	**7**	5	2	2	5	**8**	**9**	5.4
TSCRma	6	**7**	**7**	6	1	2	**7**	**7**		5.6
MSCZma	6	4	6	**7**	4	2	**8**			5.7
YSCSce	**7**	6	5	**7**	4	3				5.5
OFXOfa	6	5	5	1	1					2.8
POXCbo	2	1	4	6						2.7
MRSSce4	5	5	5							5.1
PICBta	**7**	5								5.5
UNCOcu	**9**									5.2
KMCBta										6.0
Average score:	5.1									
Variability:	2.2									

B. Segments 2

	KMCBta1	UNCOcu	PICBta	MRSSce4	POXCbo	OFXOfa	YSCSce	MSCZma	TSCRma	Averages
AACStu1	4	5	4	4	5	2	5	8	7	4.7
TSCRma	2	4	5	5	3	1	3	**12**		4.7
MSCZma	6	5	**7**	5	6	**7**	5			6.7
YSCSce	3	**7**	5	3	3	**7**				4.6
OFXOfa	6	5	5	2	3					4.1
POXCbo	4	3	4	2						3.7
MRSSce4	4	3	**7**							4.0
PICBta	**8**	5								5.5
UNCOcu	**14**									5.7
KMCBta										5.8
Average score:	5.0									
Variability:	2.4									

TABLE 3 (continued)
Binary Comparison Scores (in SD) of (A) Repeat Segments 1, (B) Repeat Segments 2, and (C) Repeat Segments 3 for the Ten Dissimilar, Representative Proteins Depicted in Figure 2[a]

C. Segments 3

	KMCBta	UNCOcu	PICBta	MRSSce4	POXCbo	OFXOfa	YSCSce	MSCZma	TSCRma	Averages
AACStu1	5	6	0	1	5	5	5	**8**	1	4.0
TSCRma	1	1	1	3	4	1	4	6		2.6
MSCZma	6	**8**	2	6	6	5	**7**			5.8
YSCSce	4	**7**	0	**7**	5	3				4.6
OFXOfa	**8**	5	1	2	4					3.7
POXCbo	2	4	0	5						3.9
MRSSce4	5	3	3							3.9
PICBta	2	2								1.2
UNCOcu	**10**									4.9
KMCBta										4.8
Average score:	3.9									
Variability:	2.4									

[a] Large values, suggestive of homology, are presented in bold print. The average value for the comparisons of each protein segment with the other nine listed protein segments is provided on the far right-hand side of the three tables. The average scores and variabilities for all values reported are provided at the lower left-hand sides of the tables.

TABLE 4
Binary Comparison Scores (in SD) of (A) Repeat Segments 1, (B) Repeat Segments 2, and (C) Repeat Segments 3 for Ten Members of the AAC Cluster Depicted in Figure 1[a]

A. Segments 1

	AACHsa1	AACCre	AACHsa3	AACHsa8	AACZma	AACNcr	AACStu1	AACSce3	AACSce4	Averages
AACStu2	28	26	22	19	28	25	32	28	27	26
AACSce4	22	26	21	21	25	25	22	24		24
AACSce3	22	26	23	21	27	27	25			25
AACStu1	27	30	18	19	33	29				26
AACNcr	27	23	22	20	26					25
AACZma	27	27	19	18						26
AACHsa8	19	21	31							21
AACHsa3	20	21								22
AACCre	28									25
AACHsa1										25
Average score:	24.4									
Variability:	3.7									

B. Segments 2

	AACCHsa1	AACCre	AACHsa3	AACHsa8	AACZma	AACNcr	AACStu1	AACSce3	AACSce4	Averages
AACStu2	31	6	19	20	33	28	30	27	27	25
AACSce4	26	5	15	17	25	26	24	27		21
AACSce3	28	5	16	18	26	28	26			22
AACStu1	31	6	18	17	30	26				23
AACNcr	29	6	19	18	28					23
AACZma	32	6	19	18						24
AACHsa8	18	5	29							18
AACHsa3	19	4								18
AACCre	6									5.4
AACHsa1										24
Average Score:	20.4									
Variability:	8.6									

TABLE 4 (continued)
Binary Comparison Scores (in SD) of (A) Repeat Segments 1, (B) Repeat Segments 2, and (C) Repeat Segments 3 for Ten Members of the AAC Cluster Depicted in Figure 1[a]

C. Segments 3

	AACHsa1	AACCre	AACHsa3	AACHsa8	AACZma	AACNcr	AACStu1	AACSce3	AACSce4	Averages
AACStu2	31	3	12	12	30	25	22	29	24	21
AACSce4	21	4	13	11	22	26	23	22		19
AACSce3	24	5	14	13	22	24	32			21
AACStu1	29	3	11	12	30	24				21
AACNcr	23	3	12	12	25					19
AACZma	29	3	13	13						21
AACHsa8	11	5	33							14
AACHsa3	12	5								14
AACCre	3									3.8
AACHsa1										20
Average score:	17.2									
Variability:	9.3									

[a] The average values for the comparisons of each protein with the other nine listed proteins are provided on the right-hand side of the three tables. The average scores and variabilities (in SD) for all values reported are provided at the lower left-hand sides of the tables.

TABLE 5
Binary Comparisons (in SD) of Repeats 1 to 2, 2 to 3, and 1 to 3 of the 28 Proteins Depicted in the Phylogenetic Tree Shown in Figure 2[a]

		Comparison score (SD) when comparing segments		
Cluster	**Protein**	**1 to 2**	**2 to 3**	**1 to 3**
1	AACCre	6	3	5
	AACHsa1	5	4	3
	AACHsa3	6	6	6
	AACHsa8	6	**7**	6
	AACNcr	6	3	4
	AACSce4	**7**	4	**7**
	AACStu1	6	3	4
	AACStu2	5	4	4
	AACSce3	**7**	5	**9**
	AACZma1	6	3	6
2	KMCBta	**7**	6	6
3	UNCBta	5	**7**	4
	UNCHsa	**8**	5	4
	UNCMmu	**8**	5	2
	UNCOcu	**7**	6	3
	UNCRno	**9**	6	3
4	PICBta	5	0	1
	PICRno	5	0	1
	PICSce	**7**	1	1
5	MRSSce1	5	**7**	6
	MRSSce4	5	**8**	**7**
6	POXCbo	3	0	0
7	OFXOfa	4	2	4
8	YSCSce	5	4	5
9	MSCZma	**9**	**10**	**8**
10	TSCHsa	**8**	5	4
	TSCRma	**7**	3	3
	Average for all proteins	6.1 ± 1.5	4.3 ± 2.5	4.3 ± 2.3
	Average for the ten representative proteins	5.7 ± 1.7	4.2 ± 3.2	4.1 ± 2.6
	Average for the ten AAC proteins	5.9 ± 0.7	4.2 ± 1.3	5.5 ± 1.6
	Average for the five UNC proteins	7.4 ± 1.4	5.8 ± 0.7	3.2 ± 7.5
	Average for the three PIC proteins	5.7 ± 0.9	0.3 ± 0.5	1.0 ± 0.0

[a] Values of 7 SD or greater, indicative of homology, are presented in bold print. Average values ± variability in SD for (a) all proteins, (b) the ten representative dissimilar proteins presented in Figure 2, (c) the ten selected ATP/ADP carriers (AAC), (d) the five uncoupling (UNC) proteins, and (e) the three phosphate carriers (PIC) are provided at the bottom of the table.

of evolutionary change in maize when compared with that in the other species examined. By contrast, values obtained for segments 2 and 3 for the phosphate carriers (PIC) and the POX Cbo are very low, presumably reflecting rapid evolutionary divergence (see Discussion).

When individual groups of proteins were statistically analyzed, the same conclusions generally resulted with some variability. Thus, average comparison scores for the segment 1:segment 2 comparisons were always the largest, regardless of whether the ten representative dissimilar MCF proteins or either the AAC or UNC clusters of proteins were examined (see bottom of Table 5). The only anomaly observed was the fact that the average segment 1:segment 3 comparison score for the ten AAC proteins was substantially larger than the average segment 2:segment 3 compari-

son score. Whether this fact reflects distinctive functional differentiation of the three segments in the AAC subcluster is an open question.

IV. DISCUSSION

In this article we have analyzed the 37 sequenced members of the MCF. Construction of a phylogenetic tree (Figure 1) revealed that the proteins fall into ten clusters that, in the cases of the functionally well-characterized carriers, correlate with their solute specificities. For example, all sequenced ATP/ADP exchange carriers (AAC) clustered together as did the uncoupling carriers (UNC) and the inorganic phosphate carriers (PIC). However, proteins presumed to be of dissimilar function were sometimes found to comprise distinct, albeit diffuse, clusters that emerge from single major branches. Three such examples are revealed by the data in Figure 1. The α-ketoglutarate:malate carrier (KMC) and the uncoupling carriers (UNC), clusters 2 and 3 in Figure 1, respectively, emerge from one major branch; the phosphate carriers (PIC) and yeast mitochondrial RNA splicing defect suppressor proteins (MRS), clusters 4 and 5 in Figure 1, respectively, diverge from a second major branch; and the postulated carriers from corn (MSC) and the thyroid gland of mammals (TSC), clusters 9 and 10 in Figure 1, respectively, diverge from a third major branch. In all three cases, these more closely related clusters of proteins presumably diverged from each other more recently in evolutionary history, after duplication of the primordial MCF protein gene gave rise to the seven major branches revealed in Figure 1. In the case of clusters 2 and 3, it is interesting to note that Klingenberg (1990) has suggested that the uncoupling proteins, which are confined to thermogenic brown adipocytes of warm-blooded animals (Nicholls, 1976; Klaus et al., 1991), represent a recent evolutionary development. The immediate precursor of these proteins was the same as that for the α-ketoglutarate:malate exchange carriers (Runswick et al., 1990) and may have had the latter specificity. While the biochemical function of the yeast MRS suppressors of mtRNA splicing defects is unknown, their position in close proximity to the inorganic phosphate carriers (Figure 1) suggests that they function as anion carriers that only secondarily suppress genetic RNA splicing defects when expressed at high levels (Wiesenberger et al., 1991). Similarly, a functional relationship between the corn and thyroid carriers, MSC and TSC, can be contemplated, but in this case no clue as to the biochemical role they play in mitochondria is available.

It has been suggested that protein members of the MCF include many currently unsequenced but physiologically and biochemically characterized mitochondrial carriers. Among these carriers are those specific for oxaloacetate, dicarboxylates, tricarboxylates, aspartate and glutamate, pyruvate, carnitine, amino acids, and inorganic cations and anions (LaNoue and Schoolwerth, 1979; Mitchell, 1979; Palmieri et al., 1990, 1992; Garlid et al., 1991; Beavis and Vercesi, 1992; Yu and Weiss, 1992; see Introduction). It is possible that some of the sequenced proteins of unidentified function that comprise clusters 5 to 10 in Figure 1 will prove to be some of these biochemically characterized, but molecularly unidentified, carriers.

One of the MCF proteins, the putative peroxysomal carrier of *C. boidinii*, POX Cbo, is localized to an organelle other than mitochondria. This fact suggests that additional members of the MCF may be found in other eukaryotic organelles. Targeting signals direct nuclear-encoded proteins to specific organelles (Saier et al., 1989; Dingwall and Laskey, 1991; de Hoop and Ab, 1992). Thus, replacement of one targeting signal on a protein for another as a result of a gene splicing/fusion event during evolution (see Reizer et al., 1991; Vartak et al., 1991) could cause a protein to be localized to more than one organelle. The targeting of an MCF protein to the eukaryotic plasma membrane could similarly result from a change in a targeting signal sequence. It is interesting to note that the best MCF signature sequence, derived from the well-conserved sequences corresponding to alignment positions 198 to 211 in the multiple alignment shown in Figure 4, was not at all conserved in POX Cbo. The basis for this selective divergence has yet to be discovered.

To date, no MCF protein has been found in a prokaryote. We postulate that the primordial MCF protein evolved in eukaryotes shortly after the formation of the endosymbiotic relationship between the progenitor of the mitochondrion and

that of the eukaryotic cell that gave rise to the cell type found in higher eukaryotes (see also Klingenberg, 1989, 1990). This suggestion is substantiated by the large comparison scores obtained when repeat segments 1, 2, and 3 are analyzed (Table 5). It seems clear that the MCF evolved independently, via separate routes, and at a more recent time in evolutionary history than did the MIP family (Pao et al., 1991; Reizer et al., 1993) or the MFS (Henderson, 1990; Griffith et al., 1992; Marger and Saier, 1993). Members of the latter two families of transporters, the former, which functions by a channel-type mechanism, the latter, which functions by a carrier-type mechanism, also probably arose by intragenic duplications, but the duplication events that presumably gave rise to these two families of proteins must have occurred much earlier than those that gave rise to the primordial MCF protein-encoding gene (see Discussion in Reizer et al., 1993). Thus, the degrees of similarity between the repeat sequences in the former two families of transporters are far less than those observed for the repeat sequences in the MCF proteins. The occurrence of members of the latter two families in prokaryotes is in agreement with this conclusion. Although horizontal transmission of genetic material from eukaryotes to prokaryotes has apparently occurred (see Bork and Doolittle, 1992), it seems to have occurred with exceptionally low frequency during evolutionary history. A prokaryotic member of the MCF may, consequently, never be found. The MCF thus represents one of the rare families of transport proteins that is thus far restricted to eukaryotes (Saier and Reizer, 1991).

The statistical analyses reported in Tables 3 to 5 clearly demonstrate that the three repeat segments in the 37 sequenced members of the MCF are all derived from a common, ancestral, genetic element. Intragenic triplication of this genetic element to give the present day tripartite structure of the MCF proteins evidently occurred during the evolution of every one of these proteins. Whether this triplication occurred only once, or several times during evolutionary divergence of the family, however, is not clear. In the case of the MIP family, statistical analyses revealed that the intragenic duplication event giving rise to these channel proteins clearly occurred before the duplication and divergence events giving rise to the different, currently recognized protein members of the family. Thus, repeat segments 1 and 2 in each of the proteins of the MIP family were always more similar to segments 1 and 2, respectively, than to the positionally dissimilar segments. The same was not observed for proteins of the MCF. The average comparison score for the segment 1:segment 1 comparisons for the representative dissimilar MCF proteins was 5.1 ± 2.2 SD, while that for the segment 2:segment 2 comparisons was 5.0 ± 2.4 SD and that for the segment 3:segment 3 comparisons was 3.9 ± 2.4 SD (Table 3). The heterologous comparisons between segments 1, 2, and 3 reported in Table 5 gave values in the same range (4.1 to 5.7 SD). It is therefore not possible from the statistical analyses reported to establish that all of the MCF proteins arose from a single precursor protein containing six transmembrane helices.

Our repeat segment statistical analysis revealed that, in general, segments 1 exhibited the greatest degree of sequence conservation and that segments 3 exhibited the least. This observation was valid for representative divergent members of the MCF (Table 3) as well as for the members of a single cluster of proteins (the AAC or the UNC cluster) (Table 4; see Table 6 for a summary of the averaged data). This observation suggests that the sequences of segments 1 are closest to the primordial repeat sequences and that segments 1 are most important to the structure and function of the MCF proteins. Segments 3 are presumably least important based on the same statistical argument. These results suggest that the C-terminal segments 3 do not play a specialized function as seemed to be the case for the C-terminal repeat sequences of channel-forming members of the MIP protein family (Reizer et al., 1993). Some (but not all) evidence suggests that the second of the two postulated repeat sequences of the MFS carriers may be of greater importance to solute specificity and channel formation than the first of these two repeats (Kaback, 1989; Brooker, 1990; Roepe et al., 1990). In this case, however, the degree of sequence similarity between the two repeat sequences is insufficient to allow statistical analysis of the type reported here or for the MIP family of proteins (Reizer et al., 1993). Because the MCF proteins (and probably other transport-

TABLE 6
Summary of Average Comparison Scores for the Three Repeated Segments of Representative Groups of MCF Proteins[a]

Groups of proteins analyzed	Average comparison scores ± variability					
	1 to 1	2 to 2	3 to 3	1 to 2	1 to 3	2 to 3
All proteins	—	—	—	6.1 ± 1.5	4.3 ± 2.1	4.3 ± 2.5
Ten dissimilar proteins	5.1 ± 2.2	5.0 ± 2.4	3.9 ± 2.4	5.7 ± 1.7	4.1 ± 2.6	4.2 ± 3.2
Ten AAC proteins	24.4 ± 3.7	20.4 ± 8.6	17.2 ± 9.3	5.9 ± 0.7	5.5 ± 1.6	4.2 ± 1.3
Five UNC proteins	—	—	—	7.4 ± 1.4	3.2 ± 1.0	5.8 ± 0.7
Three PIC proteins	—	—	—	5.7 ± 0.9	1.0 ± 0.0	0.3 ± 0.5

[a] The original values reported here are derived from data either included in Tables 3 to 5 or not presented.

ers as well) exhibit characteristics of both carriers and channels (Dierks et al., 1990a,b), it is surprising that MCF proteins that are highly specific for their solutes do not show the property of C-terminal repeat segment specialization noted for the MIP family that are substantially less specific for their solutes. This observation suggests either than the specialization of the C-terminal repeats in the MIP proteins serves a function other than solute specificity or that regional specialization for solute recognition is not a common characteristic of these two families of proteins.

We have noted that five of the six transmembrane α-helical spanners (all except spanner #6) were terminated by a common consensus sequence: (D/E) - • (K/R), where - represents a poorly conserved position and • represents a hydrophobic residue. This common sequence motif could have either evolutionary or functional significance, or both. If the former scenario is correct, this common motif may reflect a common ancestry for the odd- and even-numbered spanners in the MCF proteins. Thus, the primordial two spanner repeat sequence-encoding genetic element may have arisen by intragenic duplication of an even shorter genetic element long before the triplication events that gave rise to the primordial MCF protein gene. Alternatively, the D/E - • R/K sequence may be of functional significance and may therefore have arisen by convergent evolution. Because this consensus sequence terminates both the odd- and even-numbered spanners, it is localized both to the internal and external surfaces of the protein in a semisymmetrical array. This may have functional significance for facilitators such as the MCF proteins that can catalyze transport freely in both directions.

Multiple alignment of 28 sequenced members of the MCF (Figure 4) revealed few fully or largely conserved residues among these proteins. Only two such residues, a G and an R, were fully conserved, and of the remaining largely conserved residues (denoted white on black instead of black on white in Figure 4), almost all were glycyl residues, most likely to be of structural significance. Several aspartate residues were fairly well conserved (see Figure 4), and these tended to terminate the transmembrane α-helical segments (all but helix #6). Similarly, a highly conserved glycyl residue preceded helices 2, 4, and 6. Both glycyl and aspartyl residues occur with exceptionally high frequency in β-turns, although they occur with relatively low frequency in β-strands and α-helices (Saier, 1987). Structural roles for these residues can therefore be postulated. The presence of largely conserved prolyl and glycyl residues at positions 4 and 7 in transmembrane helices 1, 3, and 5 suggests that these helices assume very similar and specific structures. Functional roles in catalysis can be postulated for the conserved hydrophilic residues. Indeed, the consistent presence of hydrophilic residues conserved within putative transmembrane helices 2, 4, and 6 at positions 11 and 14 (Figure 5) suggests that these may be important in the formation of the aqueous pore that is believed to traverse the mem-

brane (Dierks et al., 1990a,b; Klingenberg, 1990). Although little is known regarding the specific functions of these conserved residues, knowledge of their degrees of conservation provide a useful guide for future site-specific mutagenesis studies as well as residue-specific chemical modification studies that can be contemplated for proteins of the MCF (see Trandinh et al., 1992, for a discussion of sequence conservation as an indication of function). Such studies are likely to prove of great importance with respect to the structure, function, and evolution of transmembrane transport proteins in general.

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