Characterization of a novel human putative mitochondrial transporter homologous to the yeast mitochondrial RNA splicing proteins 3 and 4

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Abstract We report here a novel human gene, *hMRS3/4*, encoding a putative mitochondrial transporter structurally and functionally homologous to the yeast mitochondrial RNA splicing proteins 3 and 4. These proteins belong to the family of mitochondrial carrier proteins (MCF) and are likely to function as solute carriers. *hMRS3/4* spans ~10 kb of genomic DNA on chromosome 10q24 and consists of four exons that encode a 364-aa protein with six transmembrane domains. A putative splice variant, encoding a 177-aa protein with three transmembrane domains, was also identified. hMRS3/4 has a well-conserved signature sequence of MCF and is targeted into the mitochondria. When expressed in yeast, *hMRS3/4* efficiently restores the mitochondrial functions in *mrs3°mrs4°* knock-out mutants. Ubiquitous expression in human tissues and a well-conserved structure and function suggest an important role for hMRS3/4 in human cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitochondrial carrier; Solute transport; MRS3; MRS4; Chromosome 10

1. Introduction

The mitochondrial proteins MRS3 and MRS4 (yMRS3 and yMRS4) were first identified in *Saccharomyces cerevisiae* in 1991 by their ability to suppress defects in the maturation of mitochondrial group II intron-containing transcripts [1]. Subsequently, they were found to be integral membrane proteins in the inner mitochondrial membrane, belonging to the superfamily of mitochondrial carriers (MCF), and having structural features of solute carriers [2]. This superfamily includes the adenine nucleotide translocator, the phosphate carrier, several mitochondrial metabolite carriers, as well as other proteins of shared structure and unknown function ([3]; Protein family database, Pfam; Prosite accession number PDOC00189).

We have undertaken positional cloning projects to characterize the genes underlying autosomal dominant progressive external ophthalmoplegia with multiple mtDNA deletions (adPEO) and infantile-onset spinocerebellar ataxia (IOSCA). The genes of one form of adPEO and IOSCA have been mapped to chromosome 10q24 between DNA markers D10S198 and D10S1795 [4,5], and D10S192 and D10S1265 [6,7], respectively. Expressed sequence tags (ESTs) weakly homologous to the yMRS3 and yMRS4 had previously been mapped in the immediate vicinity of this region.

Here we present the cloning and characterization of a novel human gene, *hMRS3/4*, encoding a putative mitochondrial transporter with significant structural homology to the closely related yMRS3 and yMRS4, and with ability to replace its homologs in yeast. Sequence analyses were carried out to clarify the possible involvement of *hMRS3/4* in adPEO and IOSCA diseases, sharing the genomic locus with this transporter.

2. Materials and methods

2.1. Construction of the hMRS3/4 cDNA sequence

To construct the *hMRS3/4* cDNA sequence, 104 ESTs homologous with yMRS3 and yMRS4, and some of them previously localized to 10q24, were identified from GenBank. The putative *hMRS3/4* cDNA was constructed by aligning the overlapping ESTs (GenBank accession numbers AA234450, AA361317, AA234031, AA449277, AA298599 and AA298105) and cDNA sequences of corresponding IMAGE cDNA clone inserts (clone numbers 77169, 346560, and 1192817; 3′ cDNA representing exons 2–4). In addition, cDNA sequence information was gained by exon predictions and homology comparisons between yMRS4 and the available genomic sequence of *hMRS3/4* locus (GenBank accession number AC007643). The ESTs represented two different splice forms, and the cDNAs constructed from these had 177-aa and 364-aa open reading frames (ORF). The two forms shared the 3′ UTR and exon 4, but had differential splicing of exons 2 and 3 (Fig. 1). About 13% of the ESTs corresponded to the 177-aa form. The rest were either homologous with the 364-aa form or too short to extend to the variable sequence. The constructed *hMRS3/4* cDNAs and the correct genomic sequence were confirmed by polymerase chain reaction (PCR) amplification of reverse-transcribed muscle, brain or lymphoblast RNA, of genomic DNA, and by subsequent sequencing. Table 1 shows the PCR primers used. PCR amplifications were carried out with either Dynazyme II DNA polymerase (Finnzymes, Finland) or the High Fidelity PCR Master kit (Boehringer-Mannheim-Roche) according to the instructions of the manufacturers and with primer-specific annealing temperatures. BigDye Terminator cycle sequencing protocol (Perkin Elmer) was used for sequencing reactions, and subsequent sequence analyses of both DNA strands were carried out on ABI377 sequencer (Perkin Elmer).

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2.2. Genomic structure of the hMRS3/4 gene

The exact genomic structure of *hMRS3/4* was determined by comparison of the cDNA sequence to the genomic sequence obtained by DNA sequencing or by searching public databases. Recently, the entire genomic sequence of the *hMRS3/4* locus became available in GenBank (accession number AC007643).

2.3. Computer analyses

All sequences were aligned using Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, MI, USA) or GeneJockey II (Biosoft, Cambridge, UK). Nucleotide and protein sequence searches were carried out with BLAST algorithms against public databases. Exon predictions from genomic sequence were carried out using programs EbEST, Genexpress, GeneJockey II, and Genscan. Programs ORF Finder and eukaryotic ORF ID at BCM Search Launcher were used in predicting ORFs from the cDNA sequence. Programs PredictProtein, ProfileScan, ProDom, PSORT II, ScanProsite, and TMpred were used in predicting the potential features of hMRS3/4. (http://web.wi.mit.edu/bio/pub/biopage2.html/).

2.4. Refined chromosomal assignment of hMRS3/4 gene

The presence of *hMRS3/4* sequence in a panel of 83 radiation hybrids (RH, Radiation Hybrid Panel G3, RH01.05, Research Genetics, Huntsville, AL, USA) and in a YAC contig on the 10q24, constructed from the publicly available mega-YACs (CEPH), was analyzed by PCR amplification using primers MRS4-5+MRS4-8 and 7875F+8284R (Table 1). The PCR products were visualized by 1% agarose gel electrophoresis.

2.5. Sequence analyses in patient and control subjects

The 3.2-kb genomic sequence of the 3′-end of *hMRS3/4* was amplified in three overlapping fragments (primer pairs 8284R+10636F, 10634R+1805F2, and 10637R+1805F2; Table 1), and the first exon in a single fragment (primers MRS4ex1F+R; Table 1), from the DNA samples of two adPEO patients, originating from both a Finnish and a Pakistani adPEO family both linked to chromosome 10q24 [4,5], one IOSCA patient from a family also linked to 10q24 [6,7], and two control subjects. Both DNA strands of the PCR fragments were sequenced with PCR and internal primers by ABI377 BigDye Terminator cycle sequencing protocol according to manufacturer's instructions. To enhance detection of a heterozygous mutation, most of the PCR fragments were additionally analyzed by single-strand conformation polymorphism (SSCP) analysis as previously described [8].

2.6. Northern blot analysis

To analyze the expression pattern of *hMRS3/4*, the 1.1-kb insert of IMAGE clone 1192817, PCR-amplified with vector primers and purified with Qiaquick PCR preps (Qiagen, Valencia, CA, USA) according to the instructions of the manufacturer, and the 1.1-kb RT-PCR product amplified with primers MRS4-20+21 from lymphoblast RNA template, were radioactively labeled by standard random-prime methodology (Fig. 1). Additionally, to detect specifically the 364-aa splice form, a 347-bp RT-PCR product amplified with MRS4-34+39 was labeled by integrating ^{32}P during PCR. These probes were hybridized separately to human multiple tissue Northern (MTN™) blot containing mRNAs from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. The 1192817 insert was also hybridized to human brain MTN-blot II containing mRNAs from eight different regions of the brain (Clontech, Palo Alto, CA, USA). Northern hybridizations were carried out in ExpressHyb solution (Clontech, Palo Alto, CA, USA) in accordance with the instructions of the manufacturer. After hybridizations, the membranes were exposed to X-ray film at −70°C overnight or for several days.

2.7. Mitochondrial targeting of hMRS3/4

The *hMRS3/4* cDNA encoding the 364-aa splice form, lacking only the non-conserved 5′-end, and the cDNA encoding the 177-aa variant, were amplified by high-fidelity PCR with primers MRS4-41+21 and MRS4-20 and -21, respectively (Table 1), and cloned into pCR2.1 (Invitrogen, San Diego, CA, USA). They were reamplified with MRS4-207 and -201, MRS4-200 and MRS4-201 to introduce an *Eco*RV and a *Hin*dIII site to facilitate cloning and to eliminate the stop codon. The product was subcloned into pB.RSV.PDX-1∼GFP [9], fusing the hMRS3/4 and EGFP (enhanced green fluorescence protein) cDNAs in-frame and creating pRSV.hMRS3/4-177∼EGFP

Table 1
5′→3′ sequences of primers used in PCR and sequencing

Name	Sequence
7875F	AGCCATCGCATGGTCTGTGT
8284R	GGAGAGAGGTTATCAAAGGTGCTG
10634R	TGAGCAGTGTTTTGCAAACGTCC
10636F	CTCCACTGGTGGCAGGTGATGT
10637R	GGTTCATGGCTGCATCATGAAG
1805F2	CCTGACCCAGCTGCCCGCTAT
MRS4ex1F	CGCGCCTGAGGCGGACACTA
MRS4ex1R	GGTAGTGGCAGGAGCCAGGAA
MRS4-5	GGGGTGCAGGCCAGAGTAAT
MRS4-8	AGGAAAGGTGGTGGCAACAG
MRS4-20	CTTGAGCTGGCCTTCCTATC
MRS4-21	AGGGGAATGAGCTGCTTATC
MRS4-34	CCCCTTCATTTTGCCACACT
MRS4-39	GCCCGCTATCGCAATGTGTT
MRS4-41	GCCCCCGGTACGACAAGATC
MRS4-200	CCGCATGATATCACCATGAACCCTG
MRS4-201	GCTACTAAGCTTGCCAGCCCTCCAC
MRS4-207:	GTCACCGATATCATGGTGGCAGGC
hMRS4-*Sac*I	CACAC<u>GAGCTC</u>ATGCCCCCGGTACGAC
hMRS4-*Hin*dIII:	CACAC<u>AAGCTT</u>TCACTTGCCAGCCCTC

and pRSV.hMRS3/4-364∼EGFP. The constructs were confirmed by DNA sequence analysis. HEK293 cells were grown on 24-mm glass coverslips in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/l glucose and supplemented (DMEM+) with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum at 5% CO$_2$ and 37°C. Transfections of pRSVhMRS3/4-177 and -364∼EGFP by lipofectamine were performed overnight in DMEM. Cells were studied for expression of hMRS3/4∼EGFP 46–72 h post-transfection by confocal laser scanning microscopy (CLSM) as described previously [10]. Prior to analysis the cells were loaded for 20 min with 250 ng/ml MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) in DMEM+ at 37°C, washed with medium and incubated for further 20 min in DMEM+ at 37°C. Cells were monitored for EGFP- and MitoTracker-fluorescence using a Leica CLSM (Leica Lasertechnik, Heidelberg, Germany). The coverslips were placed in a perifusion chamber mounted on a Leica Fluovert FU inverted microscope (Leica Lasertechnik, Heidelberg, Germany), at 37°C. The following settings were used: 100×/1.3 oil Leitz Fluotar objective lens, excitation using a krypton-argon laser (bands 488 and 568 nm), excitation filter KP590, double-dichroic mirror 488/568, and emission filter HQ525/50 for EGFP and a long-pass 580-nm filter for MitoTracker Red, respectively. A beam-splitter at 580 nm was used to separate the EGFP and MitoTracker Red signals. Presentation images were generated using Adobe Photoshop version 4.0.

2.8. Transfection of hMRS3/4 into yeast mrs3°mrs4° and mrs2° knock-out mutants

The *hMRS3/4* cDNA encoding the 364-aa protein and lacking only the non-conserved 5′-end (amplified with primers MRS4-41 and MRS4-21) was cloned into pCR2.1 (Invitrogen, San Diego, CA, USA). The insert was further amplified with hMRS4-*Sac*I and hMRS4-*Hin*dIII (Table 1). The PCR product was digested with restriction enzymes *Sac*I and *Hin*dIII and cloned into the corresponding sites of the vector pVT-U [11] creating plasmid pVT-U-hMRS3/4. The pVT-U-hMRS3/4 plasmid was digested with restriction enzyme *Sph*I and the fragment containing *hMRS3/4* with *ADH1* gene promoter and *ADH1* 3′ untranslated region was cloned into the *Sph*I site of YCplac22 and YEplac112 vectors [12] creating plasmids YCp-lac22-hMRS3/4 and YEplac112-hMRS3/4, respectively. Plasmid pVT-U-hMRS3/4 was transformed into yeast *S. cerevisiae* strain DBY747*mrs2-1* Δ [13] and plasmids YCp-lac22-hMRS3/4 and YEplac112-hMRS3/4 were transformed into yeast *S. cerevisiae* strain DBY747*mrs3°mrs4°* (GW6/gd34) [2].

3. Results

3.1. Genomic structure and cDNA of hMRS3/4 gene

The *hMRS3/4* cDNAs of 1448 bp and 1889 bp were con-

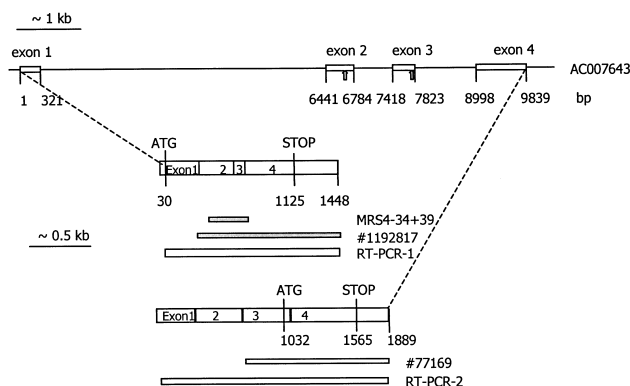Fig. 1. Genomic organization and cDNA composition of hMRS3/4. The gene spans ∼10 kb of genomic DNA and contains four exons transcribed as (i) a 1448-bp splice variant with an estimated coding region of 1095 bp, corresponding to a polypeptide of 364 aa and (ii) a 1889-bp splice variant having an ORF encoding a 177-aa polypeptide. EST, cDNA clone and RT-PCR sequences used in constructing the full-length cDNAs are shown below each splice variant. The cDNA sequence constitutions of the two different probes for Northern analysis are shadowed. Arrows below exons 2 and 3 show alternative splice sites.

structed from overlapping EST sequences available in GenBank, cDNA clone sequences representing these ESTs, and sequencing of RT-PCR products. Comparing the cDNA sequences with the genomic sequence of the locus revealed that the gene consists of four exons spanning about 10 kb of genomic DNA (Fig. 1). Exons 2–4 corresponded to the EST and cDNA clone sequences and consequently also to the transcribed sequences predicted from the genomic sequence with program EbEST. Also the program Genscan at MIT succeeded in correctly predicting the internal and terminal exons of hMRS3/4. The first exon was predicted with program Genexpress from genomic sequence AC007643, and its presence was confirmed by RT-PCR. Additionally, three vertebrate ESTs homologous to yMRS3 and yMRS4 (GenBank accession BE012485, AW326482 and AA104365) were homologous to the predicted human exon 1. The first and the third intron splicing of hMRS3/4 followed the conventional GT-AG rule, whereas the second did not. cDNA- and RT-PCR sequences revealed that the exons 2 and 3 are alternatively spliced with either (i) the 5′-end of exon 2 (228 bp) and 3′-end of exon 3 (58 bp) in the transcript (1448 bp variant) or (ii) full-length exons 2 and 3 in the transcript (1889-bp variant; Fig. 1). Both of these variants are supported by several ESTs from man, mouse, and cow, and by sequencing of RT-PCR products. The 1448-bp splice variant encodes a polypeptide of 364 aa in length. The likely initiator ATG in this cDNA occurs at nucleotide 30, is followed by an ORF of 1095 bp with a stop codon at nt 1125, and a polyA-signal at nt 1431. The 1889-bp splice variant possesses an ORF encoding a 177-aa polypeptide, identical to the C-terminus of the 364-aa protein (Figs. 1 and 2). Fig. 1 schematically shows the genomic structure of hMRS3/4 and the composition of the cDNA splice variants. Exact nucleotide and amino acid sequences of the splice variants are available in public databases (GenBank accession numbers AF327402 and AF327403; EMBL accession numbers AJ303077 and AJ303078).

3.2. Mapping hMRS3/4

To accurately map hMRS3/4, the G3 radiation hybrid panel and a YAC contig on 10q24 were analyzed using hMRS3/4-specific PCR primers. A single product was amplified with MRS4-5 and -8 (Table 1) from total human DNA and from eight samples (no. 1, 44, 51, 57, 60, 64, 79, 82) of the radiation hybrid DNAs, while no PCR product was obtained in the other hybrids, mouse or hamster DNA. Combining Chromosome 10 RH Map, SHGC Chr.10 Radiation Hybrid Map (G3) and RH Consortium Gene Map '98-Chr. 10 (G3 panel; http://www.gdb.org/) data revealed that hMRS3/4 maps close to the DNA marker D10S198, which matches well with the transcript map (http://www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96). This was confirmed by STS content mapping with hMRS3/4-specific primers of CEPH-mega-YAC contig representing the 10q24 region (data not shown).

3.3. Expression pattern of hMRS3/4 in tissues

Northern hybridization analyses utilizing the insert of the IMAGE cDNA clone 1192817 as a probe identified a major hMRS3/4 transcript of approximately 1.8 kb in all tissues examined (placenta, lung, kidney, pancreas, liver, brain, skeletal muscle and heart), with the strongest signals from heart, skeletal muscle, and liver (Fig. 2). A similar expression pattern was seen in the hybridization with MRS4-39+34 RT-PCR product, which was specific to the cDNA of the 364-aa form (results not shown). In all hybridizations, weak signals of approximately 3.2 and 4.4 kb were observed in all tissues, except that the 4.4-kb signal was absent in the brain. These signals most probably result from non-specific hybridization or transcripts with a high sequence homology with hMRS3/4. The 1.8-kb signal corresponds to the length of the cDNA splice variant encoding the 177-aa partial protein. No signal corresponding to the 1448-bp cDNA encoding the 364-aa variant could be detected. However, several other lines of evidence clearly show that the 1448-bp cDNA encodes the functional protein variant. The absence of Northern signal may indicate that we are still lacking some of the 5′ untranslated sequence of the 364-aa cDNA, and that the two cDNA signals overlap with each other in Northerns (Fig. 2).
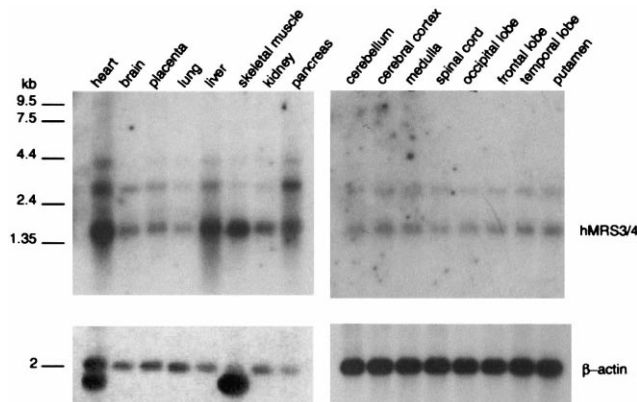

Fig. 2. Northern blot analysis of hMRS3/4. Hybridization with the insert of IMAGE cDNA clone 1192817 as a probe revealed highest expression levels in heart, skeletal muscle, and liver (left: MTN-blot; middle: brain-MTN-blot). The equal loading of RNA on lanes was confirmed by probing the same membrane with β-actin.

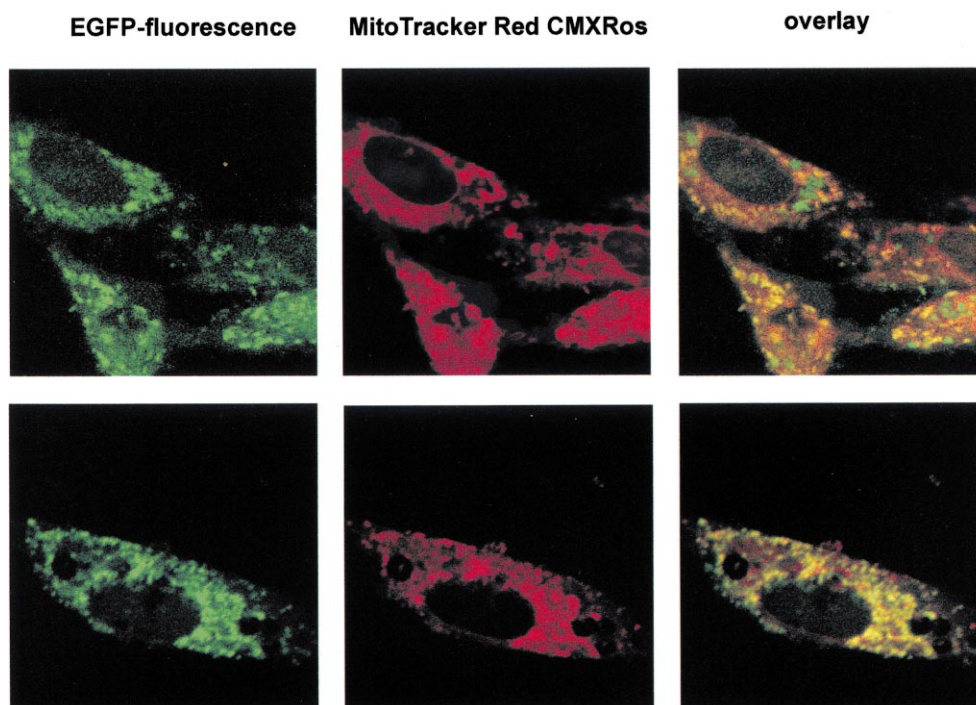**EGFP-fluorescence**   **MitoTracker Red CMXRos**   **overlay**

Fig. 3. Mitochondrial localization of the 177-aa (upper pictures) and 364-aa (lower pictures) hMRS3/4 protein variants. HEK293 cells, transfected with pRSV.hMRS3/4∼EGFP, were loaded with MitoTracker Red CMXRos. Fluorescence images were obtained by CLSM as described in Section 2. The green color is used as the digital pseudocolor for the fluorescence emitted by hMRS3/4-177 or -364∼EGFP, red is the digital pseudocolor for fluorescence emitted by MitoTracker Red, and yellow results from their overlap, indicating the co-localization of hMRS3/4∼EGFP and MitoTracker Red in mitochondria of transfected HEK293 cells.

3.4. Mitochondrial targeting of hMRS3/4

To test the subcellular localization of hMRS3/4, its coding sequence for the 364-aa and the 177-aa protein were tagged with EGFP. MitoTracker Red CMXRos was used as a mitochondrial marker. The signals of MitoTracker and hMRS3/4-177 and -364∼EGFP co-localized in the mitochondria of transfected HEK293 cells, showing that both proteins are targeted into the mitochondria (Fig. 3).

3.5. hMRS3/4 structure and conservation

BLAST searches with the 364-aa hMRS3/4 as a query sequence against SwissProt database showed 38% identity and 55% similarity with the yMRS4 ($E = 7e-53$), and 37% identity and 52% similarity with the yMRS3 ($E = 1e-52$). Programs ProDom, PredictProtein, ProfileScan, and Tmpred suggested that the 364-aa protein contains six transmembrane helical domains conserved in MCF proteins between species (Fig.
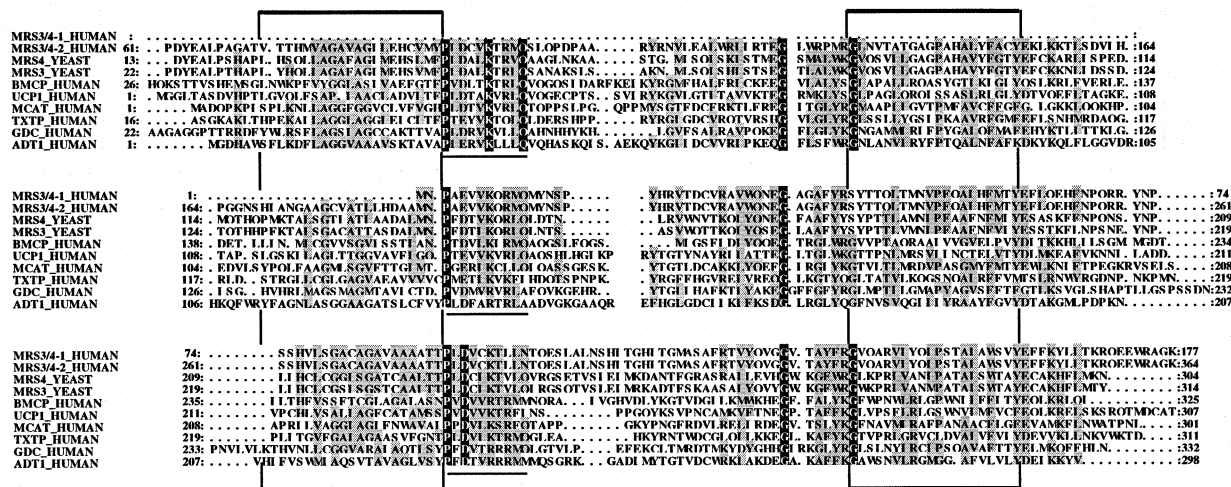

Fig. 4. Multiple-alignment of hMRS3/4 with other members of MCF. The multiple-alignment was performed with ClustalX and improved with MacBoxshade. The boxes indicate the conservative transmembrane domains [2]. The sites of the conservative signature sequence are underlined. MRS: mitochondrial RNA splicing: MRS3/4-1: 177-aa variant; MRS3/4-2: 364-aa variant; BMCP: brain mitochondrial carrier protein; UCP: brown fat uncoupling protein; MCAT: mitochondrial carnitine/acylcarnitine translocase; TXTP: tricarboxylate transport protein; GDC: Grave's disease carrier protein; ADT: ATP/ADP translocase. The first 60, 12, 21, 25, 15, and 21 aa of hMRS3/4, yMRS4, yMRS3, human BMCP, human TXTP and human GDC, respectively, are deleted for convenience of the alignment.
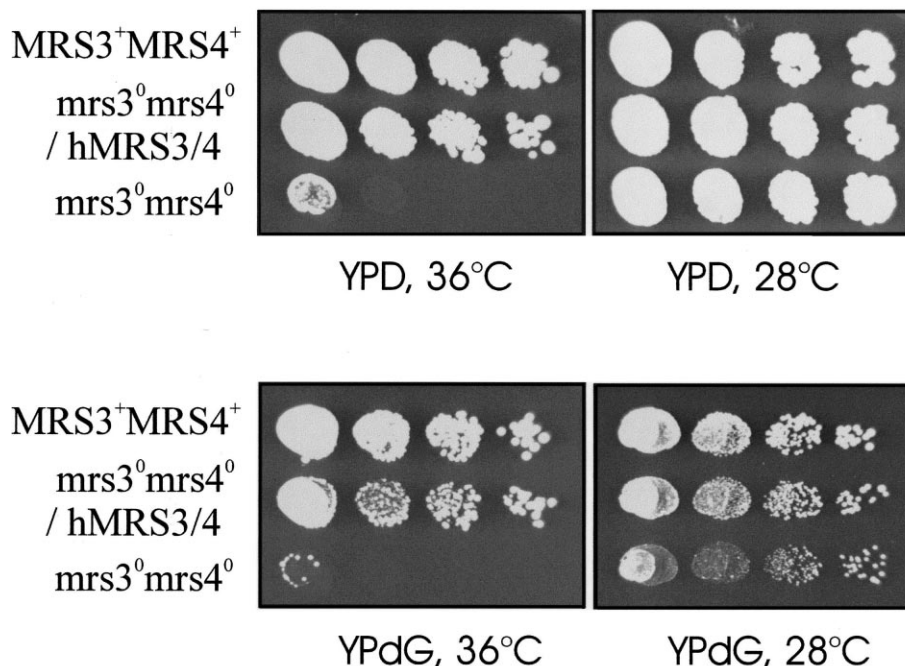
Fig. 5. Functional complementation of the yeast $mrs3^o mrs4^o$ strain. Yeast *S. cerevisiae* strain DBY747$mrs3^o mrs4^o$ was transformed with vector YEplac112-hMRS3/4 containing *hMRS3/4* driven by the yeast constitutive *ADH1* promoter. The transformants ($mrs3^o mrs4^o$/hMRS4), untransformed strain ($mrs3^o mrs4^o$) and isogenic wild type strain DBY747 (MRS3$^+$MRS4$^+$) were suspended in water and 10-fold dilution series were spotted on plates containing glycerol as non-fermentable carbon source supplemented with 0.1% glucose (YPdG) or on glucose medium (YPD). Plates were incubated at 28 or 36°C for 5 days. hMRS3/4 could restore the growth defect of yeast $mrs3^o mrs4^o$ mutants.

4; Pfam accession PF00153). Typical of a MCF protein with internal targeting signals, no N-terminal targeting signal was recognized in the polypeptide. Three mitochondrial energy transfer protein signatures were identified in the protein (Prosite accession number GC0435, PDOC00189, and PS00215) [2,3], and the protein also contained two prokaryotic membrane lipoprotein attachment sites (Prosite accession number PS00013). The N- and C-termini of the 364-aa protein were predicted to face the mitochondrial intermembrane space. The 177-aa splice variant of hMRS3/4 was predicted to contain three complete transmembrane domains, identical to the C-terminus of the 364-aa protein.

3.6. Transfection of hMRS3/4 into a yeast $mrs3^o mrs4^o$ and $mrs2^o$ knock-out mutants

The double knock-out mutations of yeast genes *MRS3* and *MRS4* ($mrs3^o mrs4^o$) caused a temperature-sensitive growth phenotype in yeast cells, i.e. growth inhibition at 36°C both on fermentable (YPD) and non-fermentable (YPdG) substrates, whereas growth at 28°C was only slightly affected (Fig. 5). Single knock-out mutations ($mrs3^o$, $mrs4^o$) did not show any obvious growth defect (not shown). When transfected with multi-copy plasmid YEplac112-hMRS3/4, expressing *hMRS3/4* in yeast under a constitutive promoter, $mrs3^o mrs4^o$ double knock-out mutant regained growth at 36°C both on fermentable and non-fermentable substrates, indicating that hMRS3/4 can substitute its yeast counterparts. Expression of the same *hMRS3/4* construct from a low-copy, centromeric plasmid (YCplac22-hMRS3/4) also restored growth of the $mrs3^o mrs4^o$ mutant, although to a lesser degree (not shown). Overexpression of yMRS3 and yMRS4 have been reported to suppress defects in RNA splicing and cytochrome biogenesis of yeast with *mrs2-1* mutation, a gene involved in mitochondrial Mg^{2+} homeostasis [13,14]. When *hMRS3/4* was expressed in yeast from a multi-copy vector, it also suppressed the growth defect of the *mrs2-1* knock-out mutant, although less efficiently than y*MRS3* and y*MRS4* (data not shown).

3.7. Analysis of patient samples

To study the possible involvement of *hMRS3/4* in the pathogenesis of either adPEO or IOSCA, the exons, including the splice sites, were PCR-amplified and sequenced from the genomic DNA samples of two adPEO patients, one IOSCA patient and two control subjects. No sequence variations between the patients and controls could be identified.

4. Discussion

We characterize here a novel human mitochondrial protein, hMRS3/4. It is structurally and functionally homologous to the yeast mitochondrial proteins MRS3 and MRS4, two members of the mitochondrial carrier protein family, suggested to translocate solutes across the inner mitochondrial membrane [2]. MCF proteins are characterized by three repeats of about 100 aa each, with two highly conserved transmembrane helices per repeat. The human homolog of yMRS3 and yMRS4 consists of 364 aa, contains six putative transmembrane domains, and shares structural features with mitochondrial inner membrane solute carriers. The structure is typical of MCF, harboring no cleavable preproteins for mitochondrial targeting, but possessing internal targeting information [15,16].

Single and double knock-out mutations of y*MRS3* and y*MRS4* have been previously reported not to affect growth of yeast cells at 28°C on fermentable or non-fermentable carbon sources [2]. We show here, however, that the $mrs3^o mrs4^o$

double mutation in yeast causes temperature-sensitive growth inhibition: growth at 28°C was only slightly affected, but at 36°C it was strongly reduced on both substrates. Since single knock-out mutations did not show a growth defect on either temperature, the presence of either yMRS3 or yMRS4 is sufficient to sustain normal growth of yeast cells. The observed phenotype of the double knock-out mutant is remarkable, since most knock-out mutations in nuclear-encoded mitochondrial genes in yeast cause a growth defect on non-fermentable substrates only. The few genes known to be essential for growth on fermentable substrates are all involved in mitochondrial protein or metabolite transport [17]. The fact that the overexpression of yMRS3, yMRS4 and hMRS3/4 can restore growth and mitochondrial metal ion concentrations also in yeast lacking the putative yeast mitochondrial Mg^{2+} transporter MRS2 ([13,14]; Gregan and Schewyen, manuscript in preparation) suggests a role in mitochondrial metal ion transport or homeostasis. The human homolog hMRS3/4 expressed in yeast *mrs3ºmrs4º* mutant cells can substitute for its yeast homologs and restore cell growth, which is consistent with the conservation of the transport function from yeast to man, and in line with observations on some other mitochondrial carriers [18].

hMRS3/4 is ubiquitously expressed showing the highest expression in the heart, skeletal muscle and liver. The lack of the 1.4-kb transcript on Northern hybridization analyses suggests that we may still miss a part of the 5′ untranslated region of the gene. However, homology analyses strongly support the start codon in the 1.4-kb cDNA to be the one suggested, and the yeast complementation assay shows that the expressed protein is functional. The existence of the 1.9-kb splice variant encoding the 177-aa polypeptide is supported by numerous EST sequences in GenBank, and the cDNA is readily amplified by RT-PCR in RNA extracts. The polypeptide is interrupted in the middle of a conserved transmembrane region, when compared with the structures of yMRS3 and yMRS4, lacking 2.5 transmembrane domains of the 364-aa protein. In spite of this, the 177-aa form is effectively targeted into the mitochondria, in which it could possibly serve a transporter role as a dimer. The high homology of both variants to the yeast MRS3 and 4, previously shown to be integral inner membrane proteins of mitochondria [2], together with the targeting of both products into the mitochondria, strongly suggest that hMRS3/4 is a mitochondrial protein.

hMRS3/4 is located on chromosome 10q24, which also harbors disease gene loci for adPEO [4,5] and IOSCA [6,7]. The tissues of adPEO patients accumulate multiple mtDNA deletions, although the primary gene defect is nuclear [4]. A recent report of mutations in the heart- and muscle-specific isoform of adenine nucleotide translocator underlying the chromosome 4-linked adPEO [19] made *hMRS3/4* a good candidate gene for the chromosome 10-linked disease form, since both the proteins belong to the MCF. In addition, the mitochondrial expression pattern of *hMRS3/4*, i.e. ubiquitous expression with increased expression levels in skeletal muscle and heart, suggested a putative role in a mitochondrial disease. The adPEO locus overlaps with that of IOSCA [6,7], an autosomal recessively inherited disease resembling closely another recessive mitochondrial disorder, Friedreich's ataxia (reviewed in [20]), and sharing a spectrum of symptoms with

PEO. We did not detect mutations in *hMRS3/4* in any of our patients, rendering it unlikely as the disease gene underlying these disorders.

The high homology of hMRS3/4 with other MCF members, as well as the common signature sequences, its mitochondrial location and the ability to replace the functions of the homologous yeast proteins, strongly support that it belongs to the MCF and shares the role of its yeast counterparts in human cells i.e. the transport of ions across the inner mitochondrial membrane. Its essential role for growth in yeast cells and conservation across species indicate that the MRS proteins are important for eukaryotes. Further studies on the function of hMRS3/4 may provide new insights in the functions of these MCF proteins in general.

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