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Functional characterization of human *COQ4*, a gene required for Coenzyme Q_{10} biosynthesis

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ABSTRACT

Defects in genes involved in coenzyme Q (CoQ) biosynthesis cause primary CoQ deficiency, a severe multisystem disorders presenting as progressive encephalomyopathy and nephropathy.
The COQ4 gene encodes an essential factor for biosynthesis in *Saccharomyces cerevisiae*. We have identified and cloned its human ortholog, *COQ4*, which is located on chromosome 9q34.13, and is transcribed into a 795 base-pair open reading frame, encoding a 265 amino acid (aa) protein (Isoform 1) with a predicted N-terminal mitochondrial targeting sequence. It shares 39% identity and 55% similarity with the yeast protein. Coq4 protein has no known enzymatic function, but may be a core component of multisubunit complex required for CoQ biosynthesis.
The human transcript is detected in Northern blots as a ~1.4 kb single band and is expressed ubiquitously, but at high levels in liver, lung, and pancreas. Transcription initiates at multiple sites, located 333–23 nucleotides upstream of the ATG. A second group of transcripts originating inside intron 1 of the gene encodes a 241 aa protein, which lacks the mitochondrial targeting sequence (isoform 2). Expression of GFP-fusion proteins in HeLa cells confirmed that only isoform 1 is targeted to mitochondria. The functional significance of the second isoform is unknown. Human *COQ4* isoform 1, expressed from a multicopy plasmid, efficiently restores both growth in glycerol, and CoQ content in $COQ4^{null}$ yeast strains. Human *COQ4* is an interesting candidate gene for patients with isolated CoQ_{10} deficiency.

Coenzyme Q (CoQ) is a small lipophilic molecule that transports electrons from mitochondrial respiratory chain complexes I and II, to complex III [1]. In addition, it functions as a cofactor for uncoupling proteins [2] and other mitochondrial dehydrogenases [1], as an antioxidant stabilizing plasma membrane, and as regulator of the extracellularly induced ceramide-dependent apoptotic pathway [3].

It is composed by a quinone group bound to a polyisoprene tail of variable length in different species: six isoprene units in *Saccharomyces cerevisiae* (CoQ_6), 10 in humans (CoQ_{10}) [4]. The biosynthesis of CoQ involves two different sets of reactions. Synthesis of the isoprenoid tail shares the initial steps with the cholesterol biosynthetic pathway, while the quinone group is synthesized from tyrosine. The terminal steps of CoQ biosynthesis are located in mitochondria, and the products of at least 10 nuclear genes (COQ genes) are involved in this pathway in yeast [4].

Mutations in human COQ genes cause primary CoQ_{10} deficiency (MIM #607426), a disorder characterized by severe progressive encephalomyopathy and nephropathy [5]. Primary CoQ_{10} deficiency is one of the few treatable mitochondrial disorders because oral CoQ10 supplementation can effectively revert the pathological phenotype in affected patients [6,7]. To date, mutations have been identified in four genes: *COQ2* [8], the *PDSS1* [9] and *PDSS2* [10] subunits of *COQ1*, and *COQ8* [11,12]. However many patients with CoQ10 deficiency still lack a genetic diagnosis, and only few of the human COQ genes have been characterized.

Here, we report the cloning and functional characterization of the human ortholog of yeast *COQ4* [13].

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Materials and methods

Identification of human COQ4. The human gene sequences were identified through the "cyber-screening" method described by Petruzzella et al. [14]. In brief, the amino acid sequence of the yeast protein was used as a probe to identify human expressed sequence tags (EST) with significant homology to the yeast gene through the tBLASTn software (www.ncbi.nlm.nih.gov/blast). The predicted cDNA sequence was then aligned to the human genomic DNA sequence, to establish the chromosomal localization, intron/exon organization, and to identify potential pseudogenes.

RNA isolation. Total RNA was extracted and purified from about 10^6 human cultured skin fibroblasts using the RNAzol Kit (Duotech) according to the manufacturer's protocols. Synthesis of total cDNA was carried out using the SuperScript II kit (Invitrogen) and random hexamers provided by the manufacturer.

Amplification and cloning of human COQ4. The COQ4 cDNA was amplified using primers and PCR conditions reported in Supplementary Table 1.

PCR products were cloned in both the pCRIITOPO cloning vector and pYES2.1V5His yeast expression vector (Invitrogen). Individual clones were sequenced.

The coding exons of COQ4 were also amplified from genomic DNA using the primer set and conditions reported in Supplementary Table 1.

Amplification and cloning of S. cerevisiae COQ4. The yeast gene was amplified from total genomic DNA (the yeast gene lacks introns) using the primers reported in Supplementary Table 1 and cloned in the pYES2.1V5His vector.

Northern blot. The probe for Northern blot analysis was obtained by EcoRI digestion of the pCRIITOPO-COQ4 vector and was radiolabeled with α-^{32}P dCTP (Amersham Biosciences) using the Random Primers DNA Labelling System kit (Invitrogen), and successively purified with Quick-Spin columns (Roche) according to the manufacturer's protocol. Radiolabeled probes were hybridized to a commercial preblotted membrane (FirstChoice Human Blot 1 membrane-Ambion) containing 2 μg/lane of poly(A)+ RNA from 10 human tissues. Pre-hybridation and hybridation were both performed in a 50% formamide buffer at 42 °C, with a working concentration of 1.5×10^6 c.p.m. and 0.1 mg of salmon sperm DNA per mL. The excess probe was removed by washing at 65 °C for 15 min using a 0.1% SDS, 0.1 × SSC solution.

Radioactivity was detected with a Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) after an overnight exposure.

RACE analysis. The 5′ and 3′ extremities of COQ4 transcripts were characterized by a RACE (Rapid Amplification of cDNA Ends) protocol using the Gene Racer kit (Invitrogen) according to manufacturer's guidelines. The procedure has been described in detail elsewhere [15]. PCR primers and conditions are reported in Supplementary Table 1. RACE products were cloned in pCR4TOPO and 100 clones were analyzed.

Subcellular localization of protein products. COQ4-GFP-fusion proteins were generated by cloning a HindIII–PstI fragment amplified from pCRIITOPO-COQ4 using primers COQ4_Iso1_HindIII_F or COQ4_Iso2_HindIII_F and COQ4_Full_len_PstI_R, into pEGFPN1.

HeLa cells stably expressing mitochondrially targeted RFP (mtRFP) [15] were grown on coverslips in complete Dulbecco's modified Eagle's media (DMEM) (Sigma) containing 10% fetal bovine serum, until 70% confluent and were transfected with the purified plasmid using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Cells were visualized after 48 h using a Nikon Video Confocal microscope.

Yeast complementation studies. Saccharomyces cerevisiae strain BY4741 COQ4::KanMX6, in which the *COQ4* gene is replaced by a kanamycin resistance cassette, was obtained from the Euroscarf consortium (http://web.uni-frankfurt.de/fb15/mikro/euroscarf).

Transformations were performed with the PEG-Lithium acetate method [16]. Cells were grown in SDc–ura 2% galactose 16 h at 30 °C with shaking to express the recombinant genes. These cultures were used to inoculate liquid YPG media and YPG plates. Mitochondrial purification, lipid extraction, and HPLC separation of quinones were performed as described [17].

Results

Identification and cloning of the human COQ4 gene

The human gene sequence deduced from EST analysis was used to design PCR primers to amplify the human *COQ4* cDNA from total fibroblasts cDNA (Supplementary Table 1). PCR products were cloned in both the pCRIITOPO vector and pYES2.1V5HisTOPO yeast expression vector. Individual clones were sequenced confirming the in-silico predictions. The cDNA sequence was aligned with human genomic DNA to determine the chromosomal localization of the gene. Human *COQ4* spans a region of about 12 kb on chromosome 9q 34.13, and comprises seven exons. Individual exons were amplified from genomic DNA and sequenced. Intron–exon border sequences are reported in Supplementary Table 2. We found two single nucleotide polymorphisms: c.149 G > C (p.50G > A) and c.534 G > C (p.178 G > G). We did not find evidence of *COQ4* pseudogenes. The cDNA contains an open reading frame (ORF) of 795 bp which encodes a predicted 265 amino acid protein, which shows 39% identity and 55% similarity with yeast Coq4p. Fig. 1A shows human Coq4p aligned with the *S. cerevisiae* protein and with proteins of other species identified with a similar procedure. PREDOTAR [18] and MITOPROT [19] software predict a mitochondrial localization for the protein, with a cleavage consensus site at proline 22. The hydropathy profile calculated with the SOSUI software [20] suggests that Coq4p is a soluble protein.

Human COQ4 is ubiquitously expressed

Human *COQ4* expression pattern was assessed by Northern blot analysis. The gene was expressed in all human tissues analyzed, and was detected as a band of approximately 1.3–1.4 kb in the blot (Fig. 1B). Higher expression levels are found in liver, lung, and pancreas (Fig. 1B), whereas beta actin expression was constant in all lanes (data not shown).

Two different COQ4 isoforms exists in human cells

5′ and 3′ Rapid amplification of cDNA ends (RACE) confirmed that the 3′ untranslated region extends for 425 nt after the TGA stop codon, and the poly-A sequence is preceded by a canonical AATAAA consensus signal. 5′ RACE detected multiple transcripts initiating at nucleotide position −333, −251, −85, and −23 in respect to the ATG initiation codon (Fig. 1C). We found also a fifth transcription initiation site located within the short intron 1 of the gene, 141 nucleotides downstream of the isoform 1 ATG (17 nucleotides upstream of the intron1/exon 2 boundary). The resulting transcript is predicted to encode a shorter protein, which initiates at a methionine in exon 2 (isoform 2), and lacks the initial 24 amino acids that specify the predicted mitochondrial targeting sequence. Re-analysis of the human EST database detected several ESTs corresponding to isoform 2. Structure of the 5′ region of the gene is depicted in Fig. 1C. Sequences of the five different *COQ4* transcripts have been deposited in the GenBank database with Accession Nos. EU216419 to EU216423.

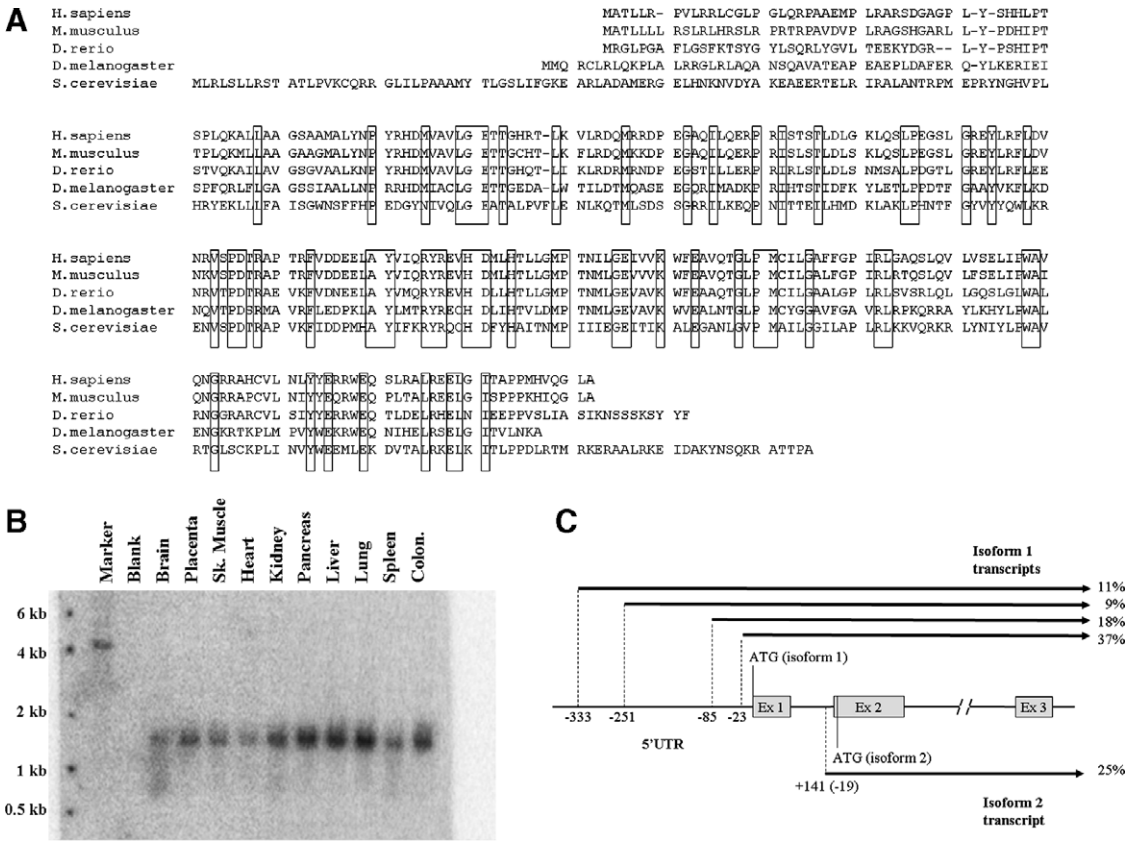

Fig. 1. Structure and expression pattern of human *COQ4*, and alignment of Coq4p of different species. (A) Human Coq4p aligned with Coq4 proteins from different eukaryotic species. Conserved residues are boxed. (B) *COQ4* expression in different tissues. Radiolabeled probes (see text) were hybridized to a commercial preblotted membrane (FirstChoice Human Blot 1 membrane-Ambion Inc., Austin TX, USA) containing 2 μg/lane of poly(A)+ RNA from 10 human tissues. Radioactivity was detected with a Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) after an overnight exposure. (C) Structure of the *COQ4* 5′ region with the different transcription initiation sites detected by RACE experiments. The percentages indicate the relative abundance of each individual transcript (a total of 100 colonies were analyzed).

Bioinformatic analysis of the human *COQ4* promoter region reveals the absence of a classical TATA box, and consensus binding sites for several transcription factors, some ubiquitous, like SP1, and others related to respiratory metabolism such as nuclear respiratory factor 1 (NURF1) and hypoxia inducible factor (Hif-1). Interestingly several consensus elements are located between exon 1 and exon 2 and may account for the transcription of isoform 2 mRNA (Supplementary Fig. 1).

Only COQ4 Isoform 1 is localized to mitochondria

To study the subcellular localization of human Coq4p isoforms, the cDNA, devoid of the stop codon, was cloned into pEGFPN1 to generate COQ4-GFP-fusion proteins which were used to transfect human HeLa cells stably expressing mtRFP [15]. The GFP fluorescence pattern was compared with mtRFP. As shown in Fig. 2, in the case of isoform 1 there is a full overlap between the two patterns, confirming that the isoform 1 is targeted to the mitochondria. Isoform 2 however is localized mainly to the cytosol, and to some extent also to the nucleus, but not to mitochondria. Its pattern resembles that of native GFP, which is localized both to the cytosol and the nucleus (Fig. 2H).

Human COQ4 effectively complements yeast COQ4^{null} mutants

In the absence of a specific functional test, we evaluated whether human *COQ4* was functionally equivalent to the yeast gene using a genetic complementation assay. *COQ4*^{null} *S. cerevisiae* cannot synthesize CoQ_6 and is unable to grow on non-fermentable carbon sources [13]. We therefore transformed a yeast strain harboring a full deletion of the *COQ4* gene with the pYES2.1 V5His vector containing either the yeast or the human *COQ4* cDNA, or with the empty vector.

The growth data in both agar and liquid medium, and analysis of CoQ6 content, indicate that human *COQ4* can restore both growth and CoQ biosynthesis in the yeast mutant (Fig. 3A–C). These data show that human *COQ4* is the ortholog of *S. cerevisiae COQ4*. The high efficiency of complementation is demonstrated by the fact that levels of demethoxy-coenzyme Q (DMQ) detected in complemented strains are similar to the wildtype (Fig. 3C). High levels of DMQ (compared to total CoQ) suggest a poorly functioning biosynthetic pathway [21]. The experiment was repeated using isoform 2 but no complementation was observed (data not shown).

Discussion

The genetic bases of primary CoQ_{10} deficiency have remained elusive for many years and the first mutation in a human COQ gene was reported only in 2006 [8]. Knowledge of the CoQ_{10} biosynthetic pathway in humans is still incomplete, and therefore we have undertaken the task of characterizing the remaining unknown human genes involved in this pathway.

We have identified the human ortholog *COQ4* a gene required for CoQ biosynthesis in yeast, whose precise function is still unclear. Coq4p bears no homology to other classes of proteins and does not seem to possess any enzymatic activity. In yeast it is reported to be associated with other COQ gene products forming a multienzyme complex [22]. It has been hypothesized that Coq4p functions as

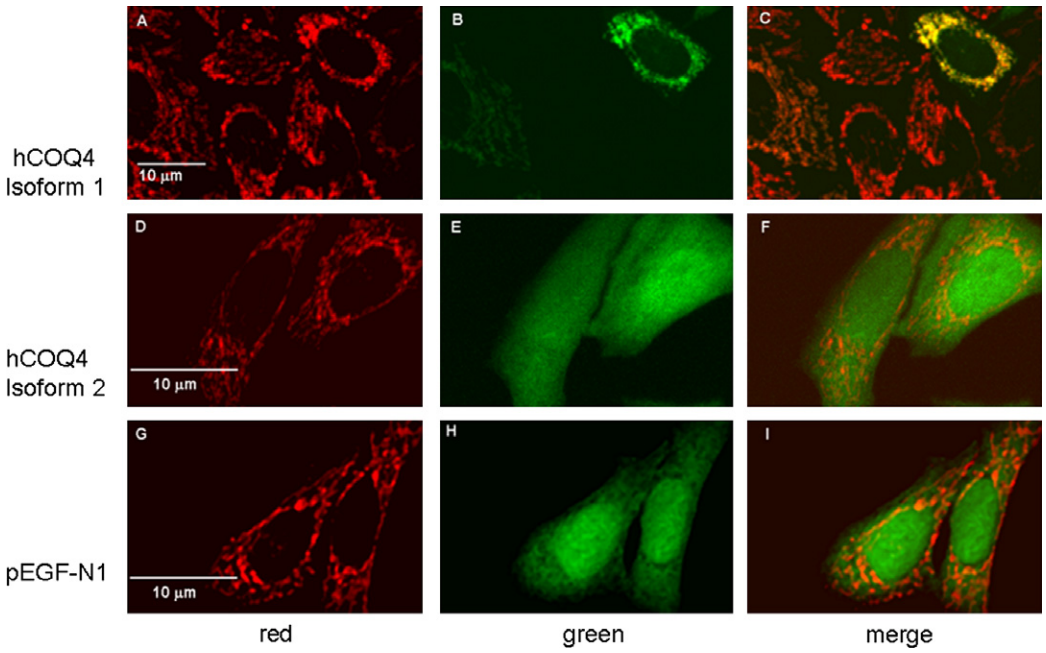

Fig. 2. Subcellular localization of the human *COQ4* gene products. HeLa cells stably expressing mtRFP were transiently transfected with plasmids expressing COQ4-Isoform 1-GFP (A–C), COQ4-Isoform 2-GFP and GFP (D–F), or native GFP (G–I), and visualized using a Nikon Video Confocal Microscope.

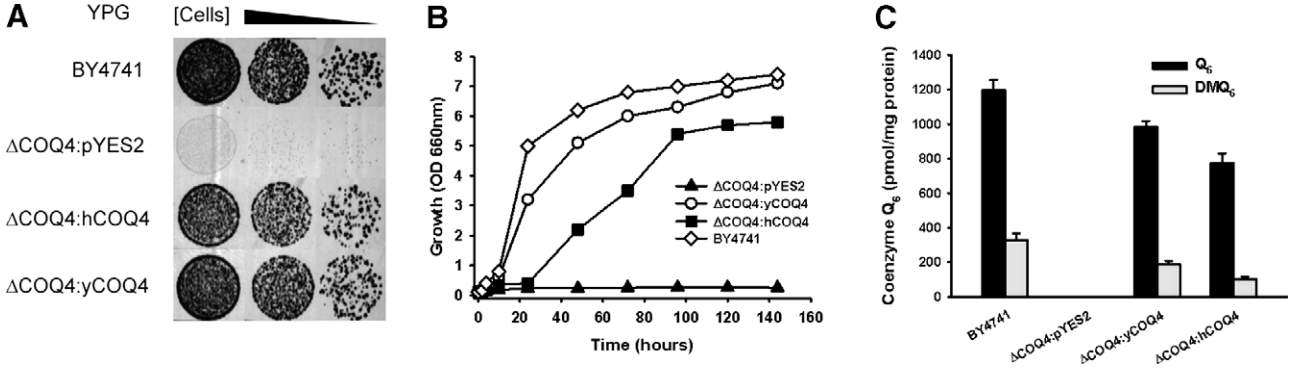

Fig. 3. The human *COQ4* gene restores the growth in non-fermentable substrates and coenzyme Q synthesis in *COQ4*^{null} yeast. (A) Cells grown in SDc–ura 2% galactose were inoculated into YPG agar plates. The initial suspension at 0.5 U OD 660 nm/mL was diluted 1/10 twice. Three microliters of cells solutions was spotted onto YPG plates which were incubated at 30 °C for three days. (B) The same SDc–ura 2% galactose cultures at 0.1 U OD 660 nm/mL were inoculated into liquid YPG medium. Growth was monitored measuring the OD at 660 nm. Data correspond to the average of three measures. The experiment is representative of a set of three. (C) Mitochondrial samples from wild-type cells (BY4741) and *COQ4* mutant cells harboring the human COQ4 gene (ΔCOQ4:hCOQ4) and the yeast *COQ4* gene (ΔCOQ4:yCOQ4) grown in YPG were subjected to lipid extraction and HPLC-ECD quinone separation and quantification as reported in the text. Data corresponds to the average ± SD of at least three determinations.

the core component of this complex, holding together the other COQ gene products [4]. The human gene has probably the same function since it can effectively complement a yeast COQ4^{null} mutant.

The human gene is ubiquitously expressed, with relatively higher expression in liver, lung, and pancreas. Curiously, the pattern of expression is different from *COQ2*, which has relatively higher expression levels in skeletal muscle and in the heart [23] and from *COQ5*, which is present at higher levels in placenta, liver, and brain (L. Salviati, unpublished results). Unfortunately there are no data about tissue expression of other human COQ genes to make further comparisons, however these findings suggest that there is not a common mechanism that regulates expression of COQ genes in humans.

Human *COQ4* is transcribed from multiple transcription initiation sites, a feature common to other genes involved in respiratory chain biogenesis, especially those with a TATA-less promoter [24,25]. However, in this case a subset of transcripts originate within intron 1 of the gene and encode a second isoform of the gene. The role of this isoform is puzzling. Isoform 2 cannot complement COQ4^{null} yeast, the predicted protein lacks a mitochondrial importation sequence, and its localization resembles that of native GFP (Fig. 2H). Other human COQ gene products such as Coq2p [23,24] and Coq5p (L. Salviati, unpublished results) are localized only to mitochondria, therefore the existence of an extra-mitochondrial CoQ biosynthetic pathway seems unlikely. Further experiments are required to clarify the role (if any) of isoform 2. It should be noted that in the mouse, despite a similar genomic organization, we found no evidence of a second *COQ4* transcript, and there is no equivalent ATG initiation codon for a shorter protein isoform.

COQ4 represents an interesting candidate for mutation analysis in CoQ deficient patients, who lack mutations in COQ2, PDSS1 or PDSS2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.172.

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