Hum Genet (1997) 99 : 329–333

ORIGINAL INVESTIGATION

Ravi Amaravadi · D. Moira Glerum
Alexander Tzagoloff

Isolation of a cDNA encoding the human homolog of *COX17*, a yeast gene essential for mitochondrial copper recruitment

Received: 22 August 1996 / Revised: 31 October 1996

Abstract The *COX17* gene of *Saccharomyces cerevisiae* codes for a cytoplasmic protein essential for the expression of functional cytochrome oxidase. This protein has been implicated in targeting copper to mitochondria. To determine if Cox17p is present in mammalian cells, a yeast strain carrying a null mutation in *COX17* was transformed with a human cDNA expression library. All the respiratory competent clones obtained from the transformations carried a common cDNA sequence with a reading frame predicting a product homologous to yeast Cox17p. The cloning of a mammalian *COX17* homolog suggests that the encoded product is likely to function in copper recruitment in eucaryotic cells in general. Its presence in humans provides a possible target for genetically inherited deficiencies in cytochrome oxidase.

Introduction

Genetic studies have revealed the synthesis of cytochrome oxidase in *Saccharomyces cerevisiae* to depend not only on the expression of the constituent subunit polypeptides but also on a large number of nuclear gene products that intercede at various stages of the assembly pathway (Glerum et al. 1995; McEwen et al. 1993; Schultze and Rodel 1989). The role of these accessory factors in the assembly of mammalian cytochrome oxidase is not clear at present, but is of considerable interest in view of the existence of human pathologies stemming from genetic deficiencies in cytochrome oxidase (DiMauro et al. 1990). In a subset of these myopathies, no mutations have been detected in the subunits of cytochrome oxidase. Instead, the deficiencies most likely result from mutations in genes involved in assembly of the enzyme (Merante et al. 1993). Complementation of yeast mutants with heterologous cDNA libraries can be helpful in assessing whether cytochrome oxidase assembly genes that have been characterized in yeast exist in other eucaryotic or procaryotic organisms. This approach was successfully used to clone human homologs of *COX10* (Glerum and Tzagoloff 1994) and *OXA1* (Bonnefoy et al. 1994b). The product of *COX10* catalyzes farnesylation of protoheme, the first step in heme A biosynthesis (Tzagoloff et al. 1993; Saiki et al. 1993). The function of *OXA1* is still not understood (Bonnefoy et al. 1994a).

This communication describes the use of functional complementation to clone the human homolog of *COX17*. Yeast *COX17* codes for a 69 amino acid cytosolic protein involved in recruitment of copper to mitochondria (Glerum et al. 1996). Null mutations in *COX17* elicit a loss of cytochrome oxidase due to the failure of the mutants to complete assembly of the complex. Transformation of a *cox17* mutant with a human cDNA expression library enabled us to isolate a complementing plasmid whose insert encodes a protein with primary sequence similarity to yeast Cox17p. The presence of human *COX17* implies the widespread requirement of this gene for eucaryotic respiratory chain biogenesis.

Materials and methods

Strains, transformation conditions, and screen for functional complementation

The method of Schiestl and Gietz (1989) was used to transform the *cox17* null mutant, W303ΔCOX17 (MATα ade-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1) (Glerum et al. 1996), with a human cDNA expression library. This library, a generous gift from Dr. Leonard Guarente (Department of Biology, Massachusetts Institute of Technology), consists of HeLa cell cDNA cloned into the *ADH1* expression cassette of the *Escherichia coli*/yeast shuttle vector pDB20 (Becker et al. 1991). This vector also carries the *URA3* gene for selection of transformants. Transformation mixtures were plated on minimal glucose medium containing all auxotrophic requirements of W303ΔCOX17 except uracil. Plates generally containing 1.0×10^4–1.5×10^4 uracil-independent transformants were replicated onto YEPG media (3% glycerol, 2% ethanol, 1% yeast extract) and incubated at 30° C for up to 1 week.

R. Amaravadi · D. M. Glerum · A. Tzagoloff (✉)
Department of Biological Sciences, Columbia University, New York, NY 10027, USA
Tel.: +1-212-854-2920; Fax: +1-212-865-8246

Miscellaneous methods

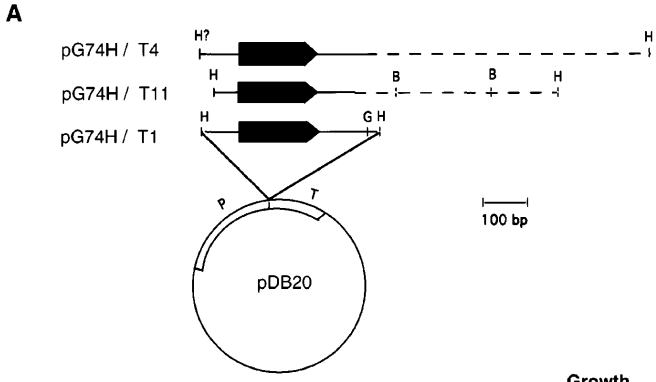
Isolation of plasmid DNA from yeast, transformation of *E. coli* with recombinant plasmids, restriction enzyme mapping, and isolation and ligation of restriction fragments were all performed by standard methods (Maniatis et al. 1982). DNA sequencing was done by the method of Maxam and Gilbert (1977), using 5′ end-labeled single-stranded restriction fragments.

Results

Complementation of a *cox17* null mutant by recombinant plasmids with human cDNAs

W303DCOX17 is a respiratory deficient mutant carrying a disrupted allele of *COX17* (Glerum et al. 1996). Several large-scale transformations of this mutant with a yeast expression library constructed from HeLa cell cDNA (Becker et al. 1991) yielded approximately 8×10^5 uracil-independent clones. Further screens revealed that 40 transformants had acquired the ability to grow on the nonfermentable substrate glycerol. In each case, the respiratory competent phenotype cosegregated with the uracil prototrophy, confirming that the transformed phenotype was plasmid-dependent.

Plasmid DNA was isolated from 20 independent respiratory competent clones and was amplified in *E. coli*. Most of the complementing plasmids had cDNA inserts of a size (450 bp) identical to that of pG74H/T1 (Fig. 1A). Two plasmids, pG74H/T4 and pG74H/T11, had larger inserts of 1.1 kb and 0.85 kb, respectively. As discussed in a subsequent section, the latter two contain chimeric cDNAs, only part of which are related to the insert of pG74H/T1.



B

Plasmid	Insert	Vector	Growth on Glycerol day1	day3
pG74 / ST8	H … H	YE p352	+	++
pG74H / T1	H … G H	pDB20	+/-	++
pG74H / ST5	H … B	YE p352	+/-	++
pG74H / ST6	H … BH	pDB20	+	++
pG74H / ST7	H … G H	YI p351	-	-
pG74H / ST11	H … BH	pDB20	+/-	++

Fig. 1A,B Restriction maps of human cDNA clones and of derivative plasmids. **A** The locations of the *Hind*III (*H*), *BamH*1 (*B*), and *Bgl*II (*G*) sites in the cDNA inserts of pG74H/T1, T4, and T11 are shown above the pDB20 vector. The *dashed lines* in the inserts of pG74H/T4 and T11 correspond to sequences derived from other cDNAs. The *question mark* denotes an approximate location of the upstream *Hind*III site in pG74H/T4. The reading frame coding for the human Cox17p is indicated by the *solid arrow*. *Open arcs* in pDB20 represent the promoter (*P*) and terminator (*T*) regions of *ADH1*. **B** Complementation of the *cox17* null mutant by different constructs containing human and yeast sequences. YEp352 is a multicopy plasmid with *URA3* as the selective marker and YIp351 is an integrative plasmid with the yeast *LEU2* gene (Hill et al. 1986). *Minus signs* indicate absence of visible growth on YEPG, +/- indicate poor growth, and ++ indicate good growth. The *open bar* and *arrow* denote yeast coding and flanking regions, and the *solid line* and *arrow* represent the corresponding human sequences

The presence of a common sequence in plasmids capable of complementing the yeast *cox17* mutant

Based on its sequence, the cDNA insert recovered from pG74H/T1 has a length of 443 nucleotides (nt; Fig. 2). Analysis of the DNA sequence revealed a single open reading frame starting with a methionine codon at nt +1. The predicted protein consists of 62 amino acids with a calculated M_r of 6,916. The primary structure of the protein predicted from the DNA sequence is homologous to yeast Cox17p (see discussion below), suggesting that the cloned cDNA codes for the human homolog. The 3′ untranslated sequence contains a characteristic mammalian

```
-86                5'-CCGGAAGTGACTGCGGACGAATCGGCGTTTGCCGAG
-50   GCTGGCATAGATTTGGCTGTCTCCGCTCATAGCTGCTTTTGGCGCGAAAG
        Met Pro Gly Leu Val Asp Ser Asn Pro Ala Pro Pro
 +1     ATG CCG GGT CTG GTT GAC TCA AAC CCT GCC CCG CCT
        Glu Ser Gln Glu Lys Lys Pro Leu Lys Pro Cys Cys
+37     GAG TCT CAG GAG AAG AAG CCG CTG AAG CCC TGC TGC
        Ala Cys Pro Glu Thr Lys Lys Ala Arg Asp Ala Cys
+73     GCT TGC CCG GAG ACC AAG AAG GCG CGC GAT GCG TGT
        Ile Ile Glu Lys Gly Glu Glu His Cys Gly His Leu
+109    ATC ATC GAG AAA GGA GAA GAA CAC TGT GGA CAT CTA
        Ile Glu Ala His Lys Glu Cys Met Arg Ala Leu Gly
+145    ATT GAG GCC CAC AAG GAA TGC ATG AGA GCC CTA GGA
        Phe Lys Ile OP
+181    TTT AAA ATA TGA AATGGTGGTCTGCTGTGTGAATAAATAATTCCT
+226   GAAGAATGAAGAAGATTAATTTTGGGAGTTCTTTGACGAACTTTGATATG
+276   TGGAAAAAGTATTTATAATTTATTGTAAGAAGAAAGTAAAATATTACTAG
+326   TGGAAGATCTTCAAAAAAAAAAAAAAAAAAAA
```

Fig. 2 Nucleotide sequence of the cDNA insert of pG74H/T1. Only the sequence of the coding strand is shown. The open reading frame proposed to code for human Cox17p is translated and is shown above the DNA sequence. A possible polyadenylation signal has been *underlined*. The sequence has been deposited in GenBank under accession number L77701

polyadenylation signal at nt 212 and includes a polyA sequence of 19 nucleotides.

Partial sequence analysis of the 850-bp fragment cloned in pG74H/T11 indicated the presence of the sequence from nt –53 to 292 reported in Fig. 2, and another fragment with part of the cDNA for human reticulocalbin (Ozawa 1995). The fusion of the two different sequences occurred at nt 292 of the insert in pG74H/T1. The insert in pG74H/T4 is somewhat more complex, having been produced by the fusion of the cDNA present in pG74H/T1 to two other fragments, one originating from human mitochondrial DNA and the other coding for the ribosomal protein L32 (Young and Trowsdale 1985).

In the three plasmids studied, the presence of an identical reading frame whose encoded product is homologous to Cox17p, provides strong evidence that the restoration of respiration in the *cox17* mutant is due to complementation by the human equivalent of the yeast protein.

Growth properties of the *cox17* null mutant transformed with yeast and human *COX17*

The *cox17* mutant transformed with pG74H/T1 has a slower growth rate on YEPG than either wild-type yeast or the mutant transformed with the yeast *COX17* gene (Fig. 3). The failure of the cDNA fragment in pG74H/T1, removed from the *ADH1* cassette and integrated in single copy (pG74H/ST7 in Fig. 1B) into the chromosomal DNA of the mutant, to confer growth on the respiratory substrate also argues that the human cDNA only partially complements the mutant. This could be due to less efficient expression of the human gene or because the human protein is only partially functional in yeast. To distinguish between these two possibilities, a new plasmid was constructed in which the regions 5′ and 3′ of the coding sequence in pG74H/T1 were replaced by the sequences flanking the yeast gene (pG74H/ST5 in Fig. 1B). This construct is identical to pG74/ST8 (see Fig. 1B), except for the presence of the human coding sequence. Introduction of this plasmid into the *cox17* null mutant did not result in any noticeable improvement in growth on YEPG over that conferred by pG74H/T1 (not shown). The lack of effect of the yeast flanking sequences on complementation suggests that the explanation for the partial restoration of respiration lies in the properties of the protein itself. This is born out by failure of the human cDNA (pG74H/T1) to complement the *cox17* mutant when integrated in single copy at the *LEU2* locus.

In contrast to pG74H/T1 and pG74H/T4, growth on YEPG of the transformant harboring pG74H/T11 was almost as good as wild type (Fig. 3). Since the inserts in pG74H/T1 and pG74H/T11 have different 5′ and 3′ flanking sequences, either sequence could affect expression of the human *COX17* gene. The 3′ flanking region in pG74H/T11 containing the recombinant sequence coding for part of reticulocalbin was excluded from being important since its removal (pG74H/ST6 in Fig. 1B) did not alter the growth properties of the transformant. Replacement of the 3′ flanking region of pG74H/T1 with the 3′ region of pG74H/ST6 also did not affect growth of the transformant on glycerol (see pG74H/ST11 in Fig. 1B). To exclude an effect of plasmid copy number, pG74H/T11 was integrated at the *URA3* locus. The transformant harboring the human gene with the shorter 5′ leader in single copy was complemented for its respiratory defect, but grew more slowly than wild type or transformants containing the plasmid in multicopy. These results indicate that the absence of 33 nucleotides in the upstream region of pG74H/T11 is responsible for the improved growth on glycerol, probably as a result of more efficient expression of the protein. The notion that heterologous 5′ flanking regions may adversely affect expression driven by the yeast *ADH1* promoter has been previously suggested by Ammerer (1983).

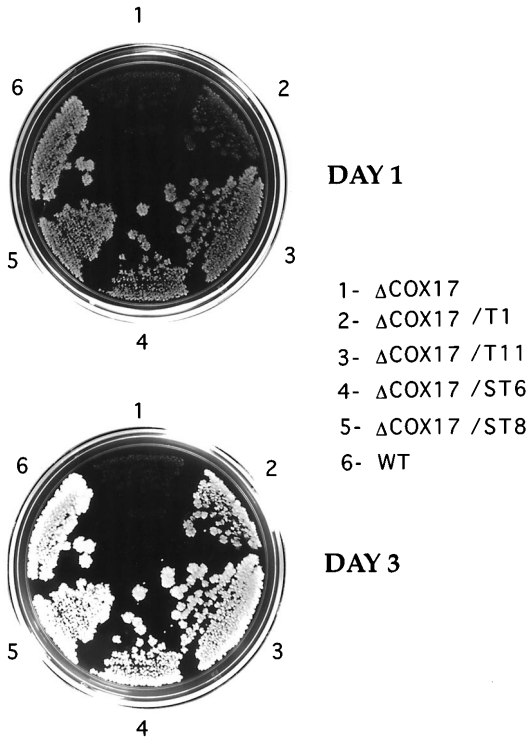


Fig. 3 Growth of the *cox17* null mutant harboring plasmids coding for the yeast and human Cox17p. The mutant, wild-type, and transformant strains were replica-plated from rich glucose medium (YPD) to rich glycerol medium (YEPG). Growth on the YEPG plate was scored after incubation at 30° C for 1 day (*upper*) and 3 days (*lower*). The following strains were streaked on: *sectors 1* the *cox17* null mutant W303DCOX17, *2* mutant transformed with pG74H/T1; *3* mutant transformed with pG74H/T11; *4* mutant transformed with pG74H/ST6; *5* mutant transformed with pG74/ST8; and *6* wild-type W303-1B

Human and yeast Cox17p are highly homologous and contain a cysteine-rich domain also present in metallothioneins

Human and yeast Cox17p share 22 identities and 8 conservative substitutions (Fig. 4). A comparison of the

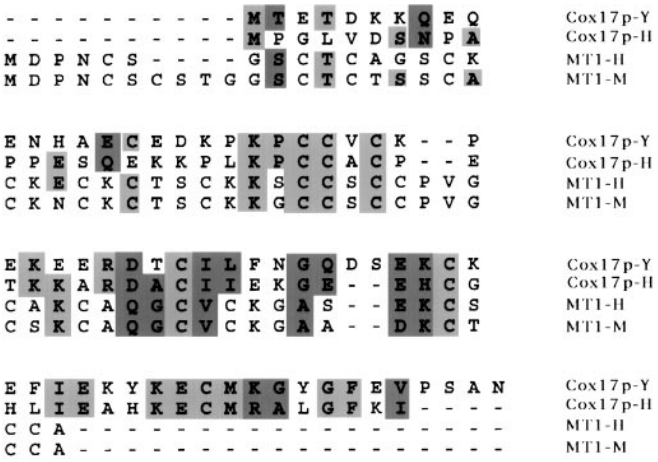


Fig. 4 Homology of human and yeast Cox17p. Human (*H*) and yeast (*Y;* Glerum et al. 1996) Cox17p's, and the human (MT1-H; Schmidt et al. 1985) and mouse (MT1-M; Glanville et al. 1981) metallothionein sequences were aligned by the motif recognition algorithm of Vingron and Argos (1991). Identical residues and conserved substitutions present only in the two Cox17p sequences are highlighted by the *lighter* and *darker boxes*, respectively. Also highlighted are identical and conserved residues present in at least one Cox17p and one of the metallothionein sequences. Wherever possible, identical or conserved residues across all four species have been marked

Cox17p to human (Schmidt et al. 1985) and mouse (Glanville et al. 1981) metallothionein reveals a short common domain with the sequence KxCCxC. Two other conserved cysteines in Cox17p can be aligned with cysteines in the mammalian metallothioneins if two short gaps are allowed in the sequences. All five of the cysteines that are conserved between the metallothioneins and the Cox17ps are involved in binding copper (Hamer 1986).

Discussion

A large number of nuclear genes of *S. cerevisiae* have been implicated in the assembly of cytochrome oxidase. Of these, only two human homologs have thus far been identified (Glerum et al. 1994; Bonnefoy et al. 1994b). Here we present evidence for the existence of another human gene whose encoded product functions in cytochrome oxidase assembly. This protein is homologous to a cytoplasmic protein of yeast previously proposed to function in delivery of copper to mitochondria (Glerum et al. 1996). A HeLa cell cDNA encoding the human Cox17p homolog was selected from an expression library by functional complementation of a yeast *cox17* null mutant. The primary structure of the protein, deduced from the nucleotide sequence of the cDNA clone, displays a high degree of sequence similarity to yeast Cox17p (35% identities). The existence of a human Cox17p homolog, and its ability to substitute in vivo for the yeast protein, strongly supports the notion that Cox17p is likely to be present in all mammalian cells in which cytochrome oxidase is the terminal oxidase of the respiratory chain.

Even though human Cox17p is capable of complementing the defect in the yeast *cox17* null mutant, the growth properties of transformants harboring different constructs suggest that the human Cox17p is less efficient than the yeast protein in the heterologous environment. This is supported by 1) the slow growth rate on glycerol of transformants harboring pG74H/T1 (single or multicopy) in which transcription is driven by the moderately strong *ADH1* promoter (Ammerer 1983), and 2) a similar slow growth on glycerol of the *cox17* mutant transformed with a plasmid containing the human coding sequence flanked by the yeast 5′ and 3′ UTRs. The same plasmid with the yeast coding sequence elicits wild-type growth on glycerol. In addition, growth differences of transformants harboring two complementing plasmids with the same coding region but different flanking regions (pG74H/T1 and pG74H/ST6) demonstrate that noncoding human sequences, particularly in the 5′UTR, can affect expression of the human protein, and hence may also contribute to the observed growth phenotypes.

The human and yeast Cox17p's share some features with metallothioneins (Hamer 1986). Both Cox17p and metallothionein are low-molecular-weight proteins with a high molar content of cysteine residues. The similarity is particularly marked by the presence, in both Cox17p and in the metallothioneins, of a short conserved domain with the sequence KxCCxC. This motif is also conserved in the yeast metallothionein (Butt et al. 1984), which otherwise shows little conservation in terms of primary structure with the mammalian proteins. Structural studies of mammalian metallothioneins indicate that the 12 copper ions are trigonally coordinated by 18 cysteines (George et al. 1986). Assuming a similar coordination in Cox17p, the six conserved cysteine residues would maximally bind four coppers. The difference in metal binding potential of the two proteins is consistent with their postulated roles. Metallothioneins are believed to protect cells against toxic metals by virtue of their high binding capacity for copper, cadmium, and other heavy metals (Hamer 1986). The ability of high exogenous copper concentrations to rescue *cox17* null mutants argues against a metal-scavenging role for Cox17p, but rather favors a role in delivery of copper to mitochondria, as proposed earlier (Glerum et al. 1996). The possibility that Cox17p may also be involved in the delivery of other heavy metals to the mitochondria cannot be excluded.

Acknowledgements This research was supported by research grant GM50187 from the National Institutes of Health, U.S. Public Health Service. D. M. G. is the recipient of a Medical Research Council of Canada postdoctoral fellowship.

References

Ammerer G (1983) Expression of genes in yeast using the *ADC1* promoter. Methods Enzymol 101:192–201

Becker DN, Fikes JD, Guarente L (1991) A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. Proc Natl Acad Sci USA 88:1968–1972

Bonnefoy N, Chalvet F, Hamel P, Slonimski PP, Dujardin G (1994a) *OXA1*, a *Saccharomyces cerevisiae* nuclear gene whose sequence is conserved from prokaryotes to eukaryotes controls cytochrome oxidase biogenesis. J Mol Biol 239:201–212

Bonnefoy N, Kermorgant M, Groudinsky O, Minet M, Slonimski PP, Dujardin G (1994b) Cloning of a human gene involved in cytochrome oxidase assembly by functional complementation of an *oxa1*$^-$ mutation in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 91:11978–1982

Butt TR, Sternberg EJ, Gorman JA, Clark P, Hamer D, Rosenberg M, Crooke ST (1984) Copper metallothionein of yeast, structure of the gene, and regulation of expression. Proc Natl Acad Sci USA 81:3332–3336

DiMauro S, Lombes A, Nakase H, Mita S, Fabrizi GM, Tritschler H-J, Bonilla E, Miranda AF, DeVivo DC, Schon E (1990) Cytochrome *c* oxidase deficiency. Pediatr Res 28:536–541

George GN, Winge D, Stout CD, Cramer SP (1986) X-ray absorption studies of the copper-beta domain of rat liver metallothionein. J Inorg Biochem 27:213–220

Glanville N, Durnam DM, Palmiter RD (1981) Structure of mouse metallothionein-I gene and its mRNA. Nature 292:267–269

Glerum DM, Tzagoloff A (1994) Isolation of a human cDNA for heme A;farnesyltransferase by functional complementation of a yeast *cox10* mutant. Proc Natl Acad Sci USA 91:8452–8456

Glerum DM, Koerner TJ, Tzagoloff A (1995) Cloning and characterization of *COX14*, whose product is required for assembly of yeast cytochrome oxidase. J Biol Chem 270:15585–155901

Glerum DM, Shtanko A, Tzagoloff A (1996) Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J Biol Chem 271:14504–14509

Hamer DH (1986) Metallothionein. Annu Rev Biochem 55:913–951

Hill JE, Myers AM, Koerner TJ, Tzagoloff A (1986) Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast 2:163–167

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Maxam AM, Gilbert W (1977) A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560–564

McEwen JE, Hong KH, Park S, Preciado GT (1993) Sequence and chromosomal localization of two *PET* genes required for cytochrome *c* oxidase assembly in *Saccharomyces cerevisiae*. Curr Genet 23:9–14

Merante F, Petrova-Benedict R, McKay N, Mitchell G, Lambert M, Morin C, DeBraekeleer M, Laframboise R, Gagne R, Robinson BH (1993) A biochemically distinct form of cytochrome oxidase (COX) deficiency in the Saguenay-Lac-Saint-Jean region of Quebec. Am J Hum Genet 53:481–487

Ozawa M (1995) Cloning of a human homologue of mouse reticulocalbin reveals conservation of structural domains in the novel endoplasmic reticulum resident Ca_{2+}-binding protein with multiple EF-hand motifs. J Biochem 117:1113–1119

Saiki K, Mogi T, Ogura K, Anraku Y (1993) *In vitro* heme O synthesis by the *cyoE* gene product from *Escherichia coli*. J Biol Chem 268:26041–26044

Schiestl RH, Gietz RD (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as carriers. Curr Genet 16:339–346

Schmidt CJ, Jubier MF, Hamer DH (1985) Structure and expression of two human metallothionein-I isoform genes and a related pseudogene. J Biol Chem 260:7731–7737

Schulze M, Rodel G (1989) Accumulation of the cytochrome *c* oxidase subunits I and II in yeast requires a mitochondrial membrane-associated protein, encoded by the nuclear *SCO1* gene. Mol Gen Genet 216:37–43

Tzagoloff A, Nobrega MP, Gorman N, Sinclair P (1993) On the functions of the COX10 and COX11 gene products. Biochem Mol Biol 31:593–598

Vingron M, Argos P (1991) Motif recognition and alignment for many sequences by comparison of dot-matrices. J Mol Biol 218:33–43

Young JAT, Trowsdale J (1985) A processed pseudogene in an intron of the HLA-DP beta 1 chain gene is a member of the ribosomal protein L32 gene family. Nucleic Acids Res 13:8883–8891