Human members of the *SCO1* gene family: complementation analysis in yeast and intracellular localization

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Abstract **Cytochrome *c* oxidase is a multiprotein complex in the mitochondrial membrane whose biogenesis requires a number of proteins besides the structural subunits. Several yeast proteins as well as a human disease-related protein have been reported which are involved in cytochrome *c* oxidase assembly. The *S. cerevisiae* Sco1p protein has been implicated in the transfer of copper to cytochrome *c* oxidase subunits Cox1p and/or Cox2p. Here we report on the complementation behavior in yeast of two recently identified ScSco1p homologs of chromosome 17 and chromosome 22 from human. When allotropically expressed in yeast, both genes fail to complement the lack of the *ScSCO1* gene. However, a chimera of the N-terminal half of ScSco1p and the C-terminal half of the chromosome 17 homolog does substitute for the ScSco1p function. Interestingly, the respective chimera with the human homolog of chromosome 22 is not able to complement. Expression of EGFP fusions in HeLa cells shows that both human ScSco1p homologs are located in the mitochondria of human cells.**

Key words: Sco1p; Copper; COX; Mitochondrium; EGFP fusion; Human

1. Introduction

Cytochrome *c* oxidase (COX) is an essential constituent of the mitochondrial respiratory chain. In yeast, mutations affecting COX lead to respiratory deficiency. In human, genetic deficiencies of COX are often associated with human pathologies [1]. Recently a human gene (*SURF1*) has been identified which, if mutated, causes Leigh syndrome [2]. The *SURF1* gene product, which is not a COX subunit, is the first example of a human protein involved in the COX assembly.

In yeast, a number of proteins involved in the COX biogenesis have been identified [3]. One of these proteins is Sco1p. This nuclear encoded protein is anchored in the inner mitochondrial membrane by a single transmembrane segment [4]. The major carboxy-terminal part of ScSco1p protrudes into the mitochondrial intermembrane space (IMS) [5,6]. Strains lacking *SCO1* are respiratory deficient because newly synthesized Cox1p and Cox2p are rapidly degraded [7,8]. Based on the findings that overexpression of Sco1p, even better in combination with slightly elevated concentrations of copper in the growth medium, can partially suppress the deficiency of Cox17p, a copper-binding protein in the cytosol and the IMS, it was suggested that ScSco1p might be involved in the attachment of copper to COX [6,9]. In support of this view is the presence of a potential metal-binding motif CxxxC, which is essential for the ScSco1p function [10]. Two cysteine residues separated by three amino acids have been shown to contribute to a copper-binding site in Cox2p [11], but are also characteristic for ferredoxins [12].

Many aspects of copper homeostasis seem to be evolutionary conserved from yeast to human [13]. During the last years, human homologs to ScAtx1p (Hah1p, [14]), ScCcc2p (Wilson disease protein, [15]), ScCtr1p (hsCtr1p, [16]) and ScCox17p (hsCox17p, [17]) have been identified. In all cases, the human homologs were able to substitute for the authentic protein in yeast.

In the course of the systematic sequencing projects, a number of DNA sequences with a high degree of homology to the *S. cerevisiae ScSCO1* gene were detected. Among these are homologs on chromosome 2 of *S. cerevisae* (*ScSCO2*) [18] and on chromosome 22 (*HsSCO1* (chr. 22)) as well as of chromosome 17 (*HsSCO1* (chr. 17)) [19] in human. We here report that the two human homologs by themselves fail to functionally complement the respiratory deficiency of a *sco1* null mutant. A chimeric protein with the C-terminal half derived from HsSco1p (chr. 17) and the N-terminal half from ScSco1p, however, can substitute for ScSco1p, while a similar construct with HsSco1p (chr. 22) is not functional in yeast. Mitochondrial localization of the human homologs in HeLa cells is demonstrated by use of fusions of the *HsSco1* reading frames to EGFP.

2. Materials and methods

2.1. Strains and media

Escherichia coli strain DH5α (BRL) was used in this work. *Saccharomyces cerevisiae* strain GR20 (*MAT*α, *leu2-3*, *leu2-112*, *his3-11*, *his3-15*, *ura3-228*, *ura3-251*, *ura3-372*, *sco1::URA3*) [20] was employed for the complementation analysis. Media were as described [21].

2.2. Plasmids

The oligonucleotide primers used for PCRs are listed in Table 1. The construct YEp351/ScSco1 contains the promoter, the encoding region and the terminator of *ScSCO1* [10]. A cDNA clone containing the *HsSCO1* gene (chr. 22) was obtained from Luc Smink (Sanger Centre, Hinxton, UK) and a cDNA clone containing the *HsSCO1* gene (chr. 17) was provided by Prof. Massimo Zeviani (National Neurological Institute 'C. Besta', Milano, Italy). Construct p415 ADH-HsSco1 (chr. 22) was obtained by cloning a PCR product (primers 1 and 2) into the *Bam*HI-*Sal*I sites of the vector p415 ADH [22]. A similar strategy was used for construct p415 ADH-HsSco1 (chr. 17) (primers 3 and 4). ScSco1p(1–158) and ScSco1p-(1–258) were obtained by PCR using primers 5, 6 and 5, 7, respectively, and YEp351/ScSco1 as template. The PCR products were cloned in the *Sac*I, *Sal*I sites of plasmid YEp351 [23].

Chimeras under the control of the *ScSCO1* promoter were obtained by sequential PCR and cloned into the vector YEp351. For ScSco1p(1–95)/HsSco1p (chr. 22)(83–266), the initial two PCRs (a and b) were performed with primers 5, 9 (*ScSCO1* from the position −317 to +285) and 8, 2 (*HsSCO1* from the position +247 to +801), respectively. The complementary sequences of the primers 8 and 9 are

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                        MLKLSRSANLRLVQLPAARLSGNGAKLLTQRGFFTVTRLWQSNGKKPLSRVPVGGTPIK   ScSco1p
                    MLNSSRKYACRSLFRQANVSIKGLFYNGGAYRRGFSTGCCLRSDNKESPSARQPLDRLQLG   ScSco2p
                        MLLLTRSPTAWHRLSQLKPPVFPGT---LGGQALHLRSWLLSRQ-G--PAETGGQGQ-PQG   HsSco1p (chr.22)
MAMLVLVPGRVMRPLGGQLWRFLPRGLEFWGPAEGTARVLLRQFCARQAEAWRASGRPGYCLGTRPLSTARPPPPWSQKG   HsSco1p (chr.17)

DNGKVREGSIE-FSTGKAI-ALF-LAVGGALSYFFNREKRRLE-TQKEAEANRGYGKPSLGGPFHLEDMYGNEFTEKNLL   ScSco1p
DEINEPEPIRTRFFQFSRWKATIALLLLSGGTYAYLSRKRRLLETEKEADANRAYGSVALGGPFNLTDFNGKPFTEENLK   ScSco2p
PGLRTRLL-I----TGLFG-AG--L--GGAWLALR-AEKERLQ-QQKRTEALRQAAVG--QGDFHLLDHRGRARCKADFR   HsSco1p (chr.22)
PGDSTRPSKPG-PVSWKSL-AIT-FAIGGALLAGMKHVKKEKA-EKLEKERQRHIGKPLLGGPFSLTTHTGERKTDKDYL   HsSco1p (chr.17)

GKFSIIYFGFSNCPDICPDELDKLGLWLNTLSSKYGI-T-LQPLFITCDPARDSPAVLKEYLSDFHPSILGLTGTFDEVK   ScSco1p
GKFSILYFGFSHCPDICPEELDRLTYWISELDDKDHI-K-IQPLFISCDPARDTPDVLKEYLSDFHPAIIGLTGTYDQVK   ScSco2p
GQWVLMYFGFTHCPDICPDELEKLVQVVRQLEAEPGL-PPVQPVFITVDPERDDVEAMARYVQDFHPRLLGLTGSTKQVA   HsSco1p (chr.22)
GQWLLIYFGFTHCPDVCPEELEKMIQVVDEIDSITTLPD-LTPLFISIDPERDTKEAIANYVKEFSPKLVGLTGTREEVD   HsSco1p (chr.17)

NACKKYRVYFSTPPNVKPGQDYLVDHSIFFYLMDPEGQFVDALGRNYDEKTGVDKIVEHVKSYVPAEQRAKQKEAWYSFL   ScSco1p
SVCKKYKVYFSTPRDVKPNQDYLVDHSIFFYLIDPEGQFIDALGRNYDEQSGLEKIREQIQAYVPKEERERRSKKWYSFI   ScSco2p
QASHSYRVYYNAGPKDE-DQDYIVDHSIAIYLLNPDGLFTDYYGRSRSAEQISDSVRRHMAAFRSVLS               HsSco1p (chr.22)
QVARAYRVYYSPGPKDED-EDYIVDHTIIMYLIGPDGEFLDYFGQNKRKGEIAASIATHMRPYRKKS                HsSco1p (chr.17)

FK                                                                    (295 aa)   ScSco1p
FN                                                                    (301 aa)   ScSco2p
                                                                      (266 aa)   HsSco1p (chr.22)
                                                                      (301 aa)   HsSco1p (chr.17)
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Fig. 1. Multiple alignment of homologs of ScSco1p. Identical amino acids are shown in bold letters. Identities to ScSco1p are: *Saccharomyces cerevisiae* Sco2p (ScSco2p) 51.01%; *Homo sapiens* Sco1p on chromosome 22 (HsSco1p (chr. 22)) 30.36% and on chromosome 17 (HsSco1p (chr. 17)) 23.06%. Potential TM regions are underlined.

underlined (see Table 1). The switch between *ScSCO1* and *HsSCO1* is indicated (/). The two PCR products, which have a common 18 bp overlap were used as templates in a third PCR (c) with primers 5 and 2 to create the chimeric gene. After cleavage with *Sac*I and *Sal*I, the fragment was cloned into YEp351 which was cut with the same restriction enzymes.

The same strategy was used to construct the other chimeras with the primers listed in Table 2.

The fusion construct between the HsSco1p (chr. 22) and the EGFP protein was obtained by cloning a PCR product (primers 25, 26) into the *Hin*dIII-*Sac*II sites of the pEGFP-N1 vector (Clontech). A similar strategy was used for the fusion construct between HsSco1p (chr. 17) and EGFP (primers 27, 28).

The nucleotide sequence of all constructs was confirmed by sequencing.

2.3. Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (complete medium). Cultures were maintained at 37°C in a humidified atmosphere containing

Table 1
Primers for PCR

Primer	Sequence (5′→3′)	Position
1	TATAGGATCCAT**ATGCTGCTGCTGACTCGGAGCCC**	*HsSCO1* (chr. 22) +1
2	TATAGGATCCGTCGAC**TCAAGACAGGACACTGCGG**	*HsSCO1* (chr. 22) +801
3	TATATAGGATCC**ATGGCGATGCTGGTCCTAGTACCC**	*HsSCO1* (chr. 17) +1
4	TATATATAGTCGAC**CTAGCTCTTTTTTCTGTATGGCCT**	*HsSCO1* (chr. 17) +906
5	GTCCTCGAGCTC**CCAATTGAAACTAAATTG**	*ScSCO1* −317
6	TATATATAGTCGACTTATTA**CTTATCCAGTTCATCAGGACAGAT**	*ScSCO1* +474
7	ATATATAAGCTTCTATCA**TCTACCCAAAGCATCAACA**	*ScSCO1* +774
8	AGGGAGAAA/**GAGAGGCTGCAGCAGCAAAAG**	*HsSCO1* (chr. 22) +247
9	CAGCCTCTC/**TTTCTCCCTGTTGAAGAAATA**	*ScSCO1* +285
10	CTGGATAAG/**CTGGTGCAGGTGGTGCGGCAG**	*HsSCO1* (chr. 22) +430
11	CTGCACCAG/**CTTATCCAGTTCATCAGGACA**	*ScSCO1* +474
12	CAGCCATTA/**TTCATCACTGTGGACCCCGAGCGG**	*HsSCO1* (chr. 22) +490
13	AGTGATGAA/**TAATGGCTGCAGAGTAATACCATA**	*ScSCO1* +531
14	CCAGCAAGA/**GACGACGTTGAAGCCATGGCCCGC**	*HsSCO1* (chr. 22) +514
15	AACGTCGTC/**TCTTGCTGGATCACAAGTTATAAA**	*ScSCO1* +555
16	AGAGTATAC/**TACAATGCCGGCCCCAAGGATGAG**	*HsSCO1* (chr. 22) +625
17	GGCATTGTA/**GTATACTCTGTACTTCTTGCATGC**	*ScSCO1* +666
18	CGTAGAAAA/**GTACACGCGGTAACTGTGACTAGC**	*HsSCO1* +624
19	CGCGTGTAC/**TTTTCTACGCCTCCAAACGTCAAA**	*ScSCO1* +667
20	TATATATGTCGAC**CTTCCCACGTTCACATAG**	*ScSCO1* +1043
21	AGGGAGAAG/**AAGGAAAAGGCAGAGAAGTTAGAGAAG**	*HsSCO1* (chr. 17) +349
22	CTTTTCCTT/**CTTCTCCCTGTTGAAGAAATAAGAAAG**	*ScSCO1* +285
23	CTGGACAAG/**ATGATCCAAGTCGTGGATGAAATAGATAGC**	*HsSCO1* (chr. 17) +538
24	TTGGATCAT/**CTTGTCCAGTTCATCAGGACAGAT**	*ScSCO1* +474
25	AGTATAAAGCTT**ATGCTGCTGCTGACTCGGAGCCCC**	*HsSCO1* (chr. 22) +1
26	AGTATACCGCGG**AGACAGGACACTGCGGAAAGCCGC**	*HsSCO1* (chr. 22) +798
27	TATATAAAGCTT**ATGGCGATGCTGGTCCTAGTACCC**	*HsSCO1* (chr. 17) +1
28	TATATATACCGCGG**GCTCTTTTTTCTGTATGGCCTCAT**	*HsSCO1* (chr. 17) +903

Note: primer annealing regions are shown in bold letters. Numbers on the right indicate the 5′ positions of the annealing regions in the respective genes. Overlapping sequences are underlined, (/) indicates the switch between two genes.

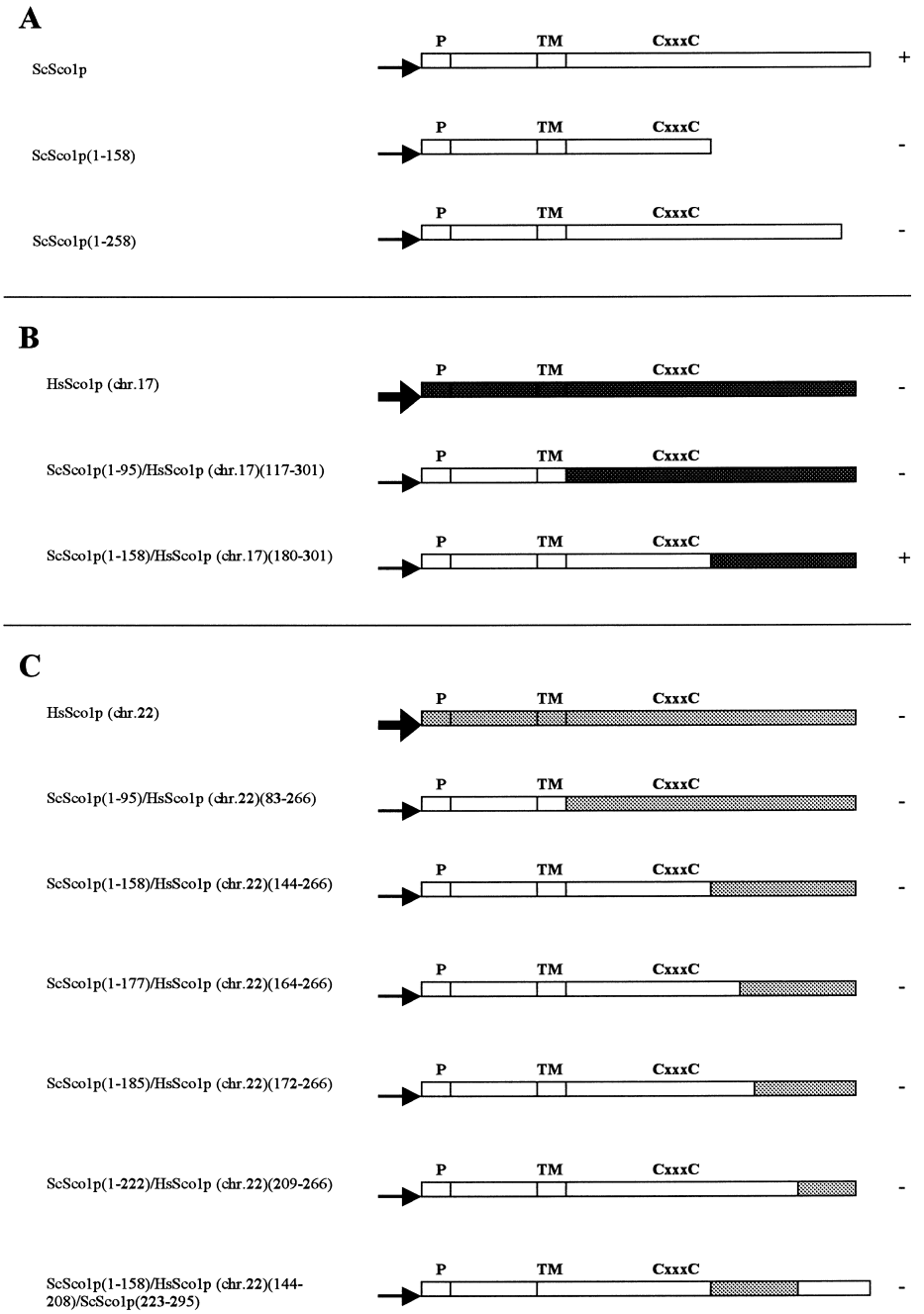

Fig. 2. Overview of Sco1p constructs tested. A: Schematic presentation of ScSco1p and ScSco1p C-terminally truncated versions. B: Schematic presentation of HsSco1p (chr. 17) and ScSco1p/HsSco1p (chr. 17) chimeras. C: Schematic presentation of HsSco1p (chr. 22) and ScSco1p/HsSco1p (chr. 22) chimeras. P indicates the putative mitochondrial pre-sequence, TM the putative transmembrane domain and CxxxC the potential metal-binding site. The thin arrow indicates the *ScSCO1* promoter and the fat arrow the *ADH1* promoter. Sequences derived from ScSco1p are in white, from HsSco1p (chr. 17) in dark grey and from HsSco1p (chr. 22) in light grey. Growth of GR20 transformants on YPGly are indicated on the right side: + = growth, − = no growth.

5% CO_2. For transient expression, cells were subcultured in TC Chamber Slides (Nunc Lab-Tek) and transformed using liposome-mediated transfection (Tfx-20, Promega) as described by the manufacturer. Transfectants were visualized with a fluorescence microscope (JenaLumar) 24 h after transfection. For staining of mitochondria, cells were incubated for 30 min in the presence of 50 nM Mito Tracker Red CMXros (Molecular Probes).

2.4. Miscellaneous procedures

Standard techniques were used for restriction endonuclease analysis of DNA, purification and ligation of DNA fragments, transformations and the recovery of plasmid DNA from *E. coli* [24]. Yeast transformations employed a LiAc procedure [25]. DNA sequences were determined by the dideoxy chain termination method of Sanger [26] using 5′ IRD800-labelled primers (MWG-BIOTECH) and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and the LI-COR DNA sequencer, model 4000 (MWG-BIOTECH).

3. Results and discussion

The homologs of ScSco1p share a high degree of similarity with respect to the primary sequence (see Fig. 1) and to their

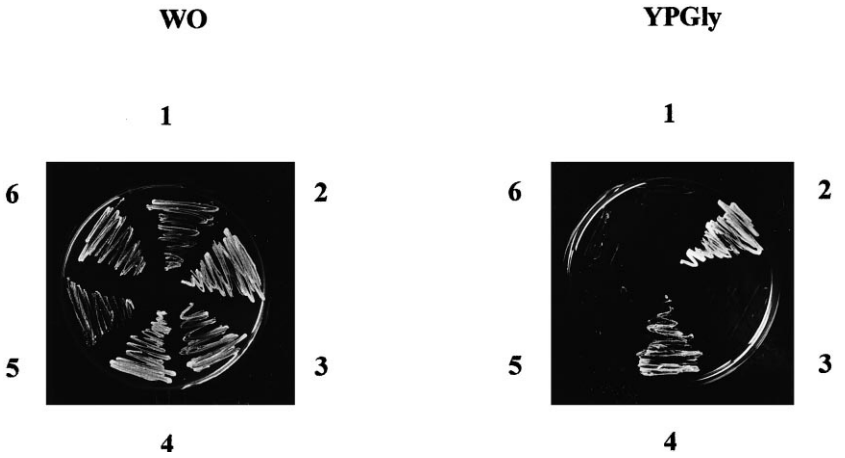

Fig. 3. Complementation behaviour of Sco1p derivates in strain GR20. ΔSco1 strain GR20 was transformed with YEp 351 (control (1)) or plasmids encoding: (2) Sco1p, (3) ScSco1p(1–158)/HsSco1p (chr. 22)(144–266), (4) ScSco1p(1–158)/HsSco1p (chr. 17)(180–301), (5) HsSco1p (chr. 22), (6) HsSco1p (chr. 17). Growth on minimal medium (WO) and glycerol medium (YPGly) was monitored after 3 days incubation at 30°C.

overall structure. All contain a hydrophobic stretch in the amino-terminal third with the potential to act as a transmembrane (TM) region. Their N-termini are reminiscent of mitochondrial pre-sequences in that they lack negatively charged amino acids while they contain positively charged and hydroxylated amino acid residues [27]. The putative metal-binding motif CxxxC is conserved from yeast to human. Although the high degree of homology suggests that the proteins might be functional homologs of ScSco1p, it was reported that the *ScSCO2* gene is not a functional homolog of *ScSCO1*, neither does it restore the respiratory competence in a *Scsco1* null mutant nor does deletion of *ScSCO2* result in a respiratory deficient phenotype [9].

In order to determine whether the homologous human genes of chromosome 17 (sequence ID: AF026852) and chromosome 22 (sequence ID: AL021683) are functional homologs on *ScSCO1*, we tested them for their ability to substitute for *ScSCO1*.

The respective cDNAs of the homologs were cloned behind the strong *S. cerevisiae ADH1* promoter (see Section 2) and transformed into strain GR20, which is deleted for *ScSCO1*. Both constructs fail to restore respiratory competence (see Figs. 2 and 3), i.e. the genes are not per se functional homologs of *ScSCO1*. The N-terminal part of ScSco1p contains a mitochondrial import sequence and the predicted TM region which direct the protein to mitochondria and anchor it in the inner mitochondrial membrane [4]. To exclude the possibility that the import signal and/or the TM region of the human proteins are not functional in *S. cerevisiae*, we constructed chimeras consisting of the N-terminal 95 amino acids of ScSco1p (including the import signal and TM region) and the C-terminus of the human homologs of chromosome 17 (ScSco1p(1–95)/HsSco1p (chr. 17)(117–301)) and chromosome 22 (ScSco1p(1–95)/HsSco1p (chr. 22)(83–266)), respectively. These constructs, too, proved not to be able to complement the respiratory deficiency of strain GR20 (see Fig. 2).

In a second set of chimeras, the N-terminal 158 amino acids of ScSco1p up to the CxxxC motif were fused to the C-termini of the respective human homologs of chromosome 22 (ScSco1p (1–158)/HsSco1p (chr. 22)(144–266)) and chromosome 17 (ScSco1p (1–158)/HsSco1p (chr. 17)(180–301)). In a control experiment, we showed that the amino-terminal 158 amino acids of ScSco1p (ScSco1p(1–158)) alone do not restore the respiratory competence in the Δ*sco1* strain GR20. Interestingly, the ScSco1p(1–158)/HsSco1p (chr. 17)(180–301) chimera restored the respiratory competence in GR20, while the chimeric protein ScSco1p(1–158)/HsSco1p (chr. 22)(144–266) did not (see Figs. 2 and 3). Transformants bearing ScSco1p-(1–158)/HsSco1p (chr. 17)(180–301) show a slight cold sensitive phenotype, i.e. a somewhat reduced growth rate at 23°C as compared to 30°C or 37°C (data not shown). The difference in complementation behavior is not due to a proteolytic sensitivity of ScSco1p(1–158)/HsSco1p (chr. 22)(144–266), as a protein of the expected molecular mass can be detected with ScSco1p antibodies in Western analysis (data not shown).

Table 2
Primers used in the construction of the chimeras

Construct	PCR a	PCR b	PCR c
ScSco1p(1–95)/HsSco1p (chr. 22)(83–266)	5, 9[a]	8, 2[b]	5, 2
ScSco1p(1–158)/HsSco1p (chr. 22)(144–266)	5, 11[a]	10, 2[b]	5, 2
ScSco1p(1–177)/HsSco1p (chr. 22)(164–266)	5, 13[a]	12, 2[b]	5, 2
ScSco1p(1–185)/HsSco1p (chr. 22)(172–266)	5, 15[a]	14, 2[b]	5, 2
ScSco1p(1–222)/HsSco1p (chr. 22)(209–266)	5, 17[a]	16, 2[b]	5, 2
ScSco1p(1–158)/HsSco1p (chr. 22)(144–208)/ScSco1p(223–295)	5, 18[c]	19, 20[a]	5, 20
ScSco1p(1–95)/HsSco1p (chr. 17)(117–301)	5, 22[a]	21, 4[d]	5, 4
ScSco1p(1–158)/HsSco1p (chr. 17)(180–301)	5, 24[a]	23, 4[d]	5, 4

Note: the plasmids used as template encode: [a]ScSco1p, [b]HsSco1p (chr. 22), [c]ScSco1p(1–158)/HsSco1p (chr. 22)(144–266) [d]HsSco1p (chr. 17).

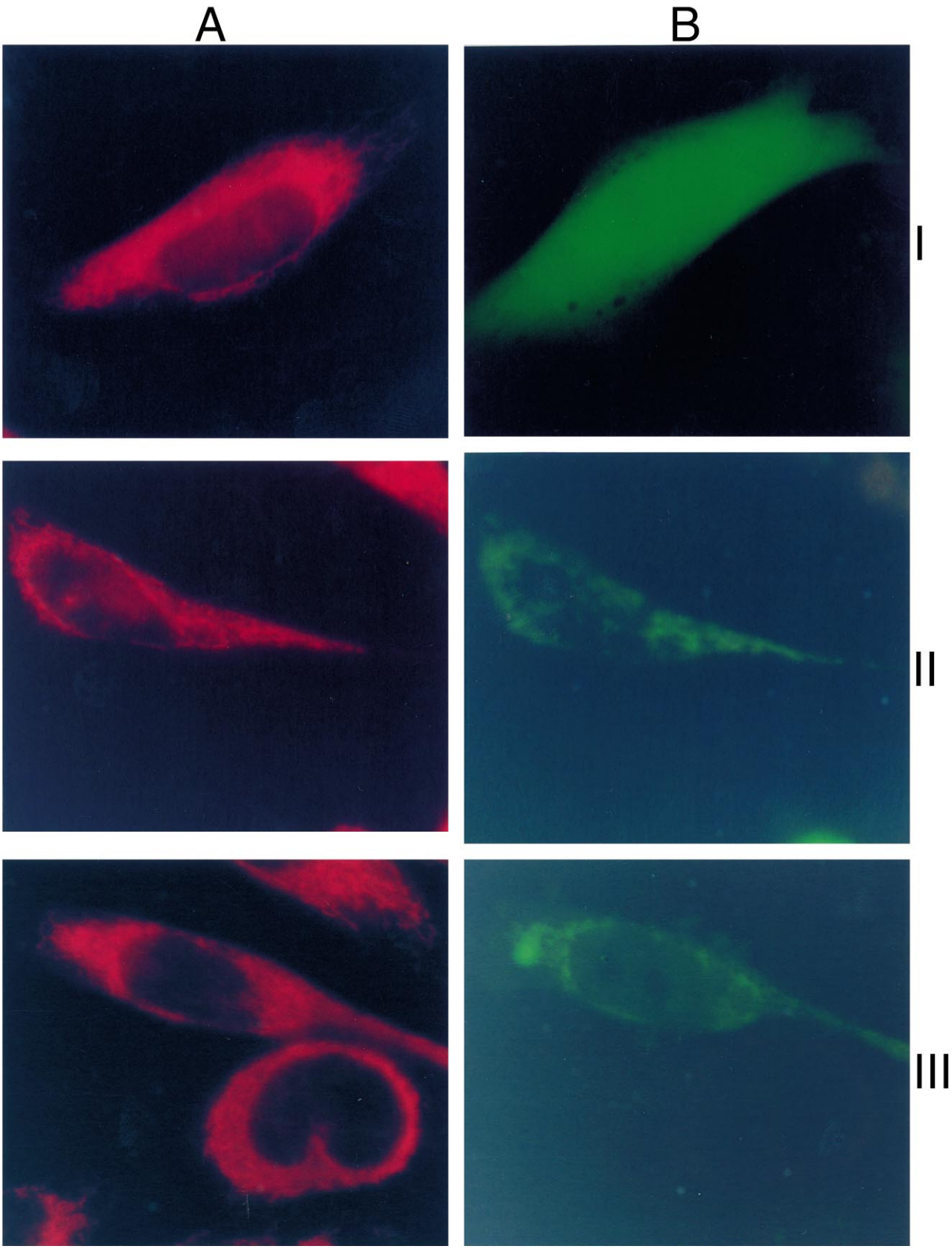

Fig. 4. Fluorescence microscopic examination of HeLa cells transfected with EGFP-bearing plasmids. HeLa cells were transfected with the indicated EGFP constructs. After 24 h, cells were counterstained with the mitochondria-specific dye Mito Tracker Red CMXros. A: Red fluorescence by Mito Tracker Red CMXros. B: Green fluorescence by EGFP. HeLa cells were transfected with plasmids encoding EGFP (I), HsSco1p (chr. 17)-EGFP (II) and HsSco1p (chr. 22)-EGFP (III).

We next tested whether parts of the C-terminal region of the human chromosome 22 homolog may replace the respective yeast sequences. To this end, we progressively shortened the C-terminal portion derived from the human homolog as outlined in Fig. 2: ScSco1p(1–177)/HsSco1p (chr. 22)(164–266), ScSco1p(1–185)/HsSco1p (chr. 22)(172–266) and ScSco1p(1–222)/HsSco1p (chr. 22)(209–266). All constructs were transformed into strain GR20 and tested for their ability to restore the respiratory competence. None of the chimeras was able to substitute for the ScSco1p function.

In yeast, a truncated version of ScSco1p (ScSco1p(1–258)), lacking the C-terminal 35 amino acids, is not functional (see Fig. 2). Because this region is one of the most divergent between the homologs, we wondered whether the authentic ScSco1p C-terminus might be necessary for the function of a chimeric protein. To this end we constructed the chimera ScSco1p(1–158)/HsSco1p (chr. 22)(144–208)/ScSco1(223–295). In this protein, the amino-terminal 158 amino acids as well as the carboxy-terminal 73 amino acids are derived from ScSco1p, while the internal segment corresponds to amino acids 144–208 of HsSco1p (chr. 22). This construct too proved not to be functional in yeast.

Although we cannot exclude the possibility that improper folding of the chimeras interferes with their function in yeast, we favor the idea that despite of the lesser degree of homology (23% HsSco1p (chr. 17) versus 30% HsSco1p (chr. 22)), the chromosome 17 homolog represents a functional ScSco1p homolog. HsSco1p (chr. 22) may serve a distinct as yet unknown function, which still has to be defined.

The finding that both human Sco1p proteins by themselves fail to substitute the ScSco1p function in yeast raised the question whether the human homologs are located in mitochondria. To examine the subcellular distribution, the proteins were fused to EGFP and placed behind the CMV promoter (see Section 2). HeLa cells were transfected with an EGFP-bearing plasmid (control), with the *HsSCO1* (chr. 22)-EGFP-bearing plasmid and with the *HsSCO1* (chr. 17)-EGFP-bearing plasmid, respectively. Transformants expressing the HsSco1p (chr. 17)-EGFP clearly exhibited staining in punctuated structures (see Fig. 4). Evidence that these structures represent mitochondria was obtained by staining with the mitochondria-specific dye Mito Tracker Red CMXRos, which resulted in an identical staining pattern. In the case of HsSco1p (chr. 22)-EGFP, we also observed an identical staining pattern (see Fig. 4). In contrast to HsSco1p (chr. 17)-EGFP, however, occasionally a slight green fluorescence was also detected in the cytosol, possibly reflecting a higher concentration of the EGFP fusion protein or a less efficient import. HeLa cells transfected with the EGFP-bearing control plasmid showed, as expected, an homogeneous green fluorescence which did not correspond to the labelling pattern with Mito Tracker Red CMXRos (see Fig. 4). Our data demonstrate that both chimeric HsSco1p-EGFP proteins are localized in mitochondria and that mitochondrial localization is mediated by the HsSco1p portions of the fusion proteins. We conclude that both of the human homologs to ScSco1p are mitochondrial proteins.

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