

maf1 Mutation alters the subcellular localization of the Mod5 protein in yeast*

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Two forms of Mod5p, a tRNA modification enzyme, are found in three intracellular compartments, mitochondria, cytoplasm and nucleus, but are encoded by a single *MOD5* gene. The two forms of the enzyme, Mod5p-I and Mod5p-II differ at the N-termini and are produced by initiation of translation at different start codons. Mod5p-I does contain a mitochondrial targeting signal and is distributed between mitochondria and cytoplasm, whereas Mod5p-II is found in the cytosol and nucleus (Boguta, M., *et al.* 1994, *Mol. Cell. Biol.* 14, 2298-2306).

In the present work mutants which mislocalize the Mod5p-I enzyme were isolated. The screen was based on a correlation between the amount of cytosolic protein and the efficiency of tRNA mediated suppression. Identification of mutants is possible because a red pigment accumulates in the cells unable to suppress an *ade2-1* nonsense allele. The *maf1* mutant, with an altered intracellular localization of the Mod5p-I protein, was isolated. Immunofluorescence data suggest that the mutation causes mislocalization of the Mod5p-I to the nucleus.

The targeting of protein molecules to specific subcellular compartments is an important aspect of eukaryotic cellular biology. Effective management of intracellular trafficking requires signals on the proteins to be localized, receptors on their target organelles and other proteins with which they interact on their journey.

There are many examples of eukaryotic genes coding for proteins found in more than one

subcellular location (Martin, N.C. & Hopper, A.K. in preparation for *International Review of Cytology*). As was shown recently, the *MOD5* gene products in *S. cerevisiae* are found in three cellular compartments: mitochondria, cytoplasm and nucleus [1]. The *MOD5* gene codes for two different forms of the enzyme, Δ^2 -isopentenyl pyrophosphate: tRNA isopentenyl transferase (Mod5p-I and Mod5p-II) responsible for the modification of both mitochondrial

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and cytoplasmic tRNAs. Mod5p-I and Mod5p-II differ at their amino termini and are produced by initiation of translation at two alternative in-frame AUGs located at codons 1 and 12, respectively [2]. Initiation at AUG₁ generates N-terminally extended Mod5p-I which is imported to mitochondria, but a fraction of the protein is also located in the cytosol [3]. Mod5p-II, initiated from the AUG₁₂, does not localize to mitochondria, but is found in cytosol and nuclei [1].

The N-terminal signal responsible for mitochondrial localization of Mod5p-I protein has been identified [2]. The nuclear targeting sequence has not yet been identified, however the MOD5 ORF does contain a sequence in its C-terminal region that matches the bipartite nuclear targeting sequence consensus [4]. This sequence is sufficient to direct a surrogate protein to nuclei (Hunter, L.A. & Hopper, A.K., unpublished results).

We are interested in the identification of organellar receptors or of other proteins involved in Mod5p intracellular transport. Our approach is to search for mutants which mislocalize the Mod5p. This selection is possible since the activity of Mod5p in the cytosol is necessary for the efficient functioning of nuclear-encoded tRNA suppressors. The efficiency of tRNA mediated suppression reflects the amount of cytosolic Mod5p protein activity [2].

Cells unable to suppress the *ade2-1* nonsense allele accumulate a red pigment, causing red colony color on rich media. Disruption of MOD5 or the *mod5-1* mutation cause reduced efficiency of SUP7 and SUP11 suppressor activity because these tRNAs lack i⁶A modification. Cells harbouring SUP7 *mod5-1 ade2-1* are red in contrast to the white SUP7 *ade2-1 MOD5* cells.

mod5-M2, a mutant form of the MOD5 gene was constructed *in vitro* by changing AUG₁₂ to a noninitiating codon. The YCfmod5-M2 plasmid generates Mod5p-I, the longer form of Mod5p protein in roughly endogenous quantities. The residual Mod5p-I in the cytoplasm results in about 55% cytoplasmic tRNA i⁶A modification. This is sufficient for partial suppression of *ade2-1*. SUP7 *mod5-1 ade2-1* cells, harboring the YCfmod5-M2 plasmid are pink rather than white like cells harbouring the wild type allele [2].

Partial suppression, dependent on the distribution of Mod5p between different cellular

compartments, allows the selection of mutants with an altered amount of enzyme in the cytoplasm. Here we report the isolation and characterization of the *maf1* mutant. The *maf1* mutation causes mislocalization of Mod5p-I to the nucleus.

METHODS

Strains, media and growth conditions. The *Saccharomyces cerevisiae* strain used in the screen for mutants was T8-1D (*MAT α SUP11 mod5-1 ade2-1 lys2-1 can1-100 his4-519 leu2-3,112 ura3-1*). For the control experiments MB105-6A (*MAT α SUP11 mod5-1 ade2-1 trp5-2 lys2-1 can1-100 leu1 ura3-1*), MB103-9B (*MAT α SUP7 mod5-1 ade2-1 lys2-1 trp5-2 leu2-3,112 ura3-1*) and MB117-1A (*MAT α ade2-1 lys2-1 leu1 ura3-1*) were used. All the strains were constructed by genetic crosses, sporulation and tetrad analysis. The parental strains were MD14A (*MAT α SUP7 mod5-1 ade2-1 can1-100 lys2-1 his5-2 trp1 leu2-3,112 ura3-1*), [2] MD1B (*MAT α ade2-1 lys1-1 met4 his5-2 trp1 leu2-3,112*) and J20-11B (*MAT α SUP11 ade2-1 lys2-1 trp5-2 leu1 ura3-1*) (A.K.H. laboratory).

Yeast media YPG, N3, WO and SP1 were described by Boguta *et al.* [5]. WO minimal glucose medium was supplemented with the appropriate amino acids. The growth of cells was tested by replica-plating of the yeast colonies on appropriate media followed by 2 days incubation at 28, 34 or 37°C.

Mutagenesis. Cells were grown to early stationary phase. For mutagenesis cells were exposed to 0.5% ethyl methanesulphonate (EMS) for 2 hours. Mutagenized cells were plated on nonselective YPG medium.

Plasmids. Centromeric plasmids YCfmod5-M2 and YCfmod5-M2KR6 and multicopy plasmids YEpmmod5-M2KR6 and pJDBmod5-M2KR6, coding for Mod5p-I were used ([2] and unpublished). KR6 has a mutation of the N-terminal sequence of Mod5p-I (unpublished). Centromeric plasmid YCfMOD5 [3], carrying the wild type copy of MOD5 was used as the control.

Indirect immunofluorescence. Cells were grown on glucose selective medium at 23°C. Indirect immunofluorescence experiments were carried out by the modification of the procedures of Kilmartin & Adams [6] and

Pringle *et al.* [7]. The Mod5p antigen was detected by an affinity-purified Mod5p specific antibody [2] at a 1/10 dilution. A 1/300 dilution of a FITC-conjugated goat-antirabbit antibody was used as a secondary antibody. Cells were viewed using a Nikon Microphoto Fluorescence Microscope and a Nikon Plan Apo 60x objective.

RESULTS

Suppression efficiency in the presence of Mod5p-I

We tested the efficiency of Mod5p-I mediated suppression in *SUP11* and *SUP7 mod5-1* strains harbouring *ade2-1*, *trp5-2* and *lys2-1* nonsense alleles and the YCfmod5-M2 plasmid. MB103-9B and MB105-6A strains (see Methods) were transformed with YCfmod5-M2. The efficiency of *ade2-1* suppression was assessed by colony color; the efficiency of suppression of *lys2-1* and *trp5-2* alleles was tested by growth on media lacking lysine or tryptophan. The results are summarized in Table 1.

SUP7 and *SUP11* are both tyrosine-inserting tRNA suppressors, but *SUP11* suppression is less efficient. There is no suppression of the nonsense alleles studied in the presence of the *mod5-1* mutation in MB103-9B and MB105-6A. The lack of suppression was complemented by the YCfMOD5 plasmid carrying wild type MOD5, resulting in white colony color and wild-type growth on media lacking lysine or tryptophan. Partial suppression was obtained in the presence of YCfmod5-M2. This effect is more pronounced with the less efficient *SUP11* suppressor. The weak growth on media lacking

lysine or tryptophan however prevented a direct positive colony selection. Therefore to obtain mutants with altered levels of Mod5p-I in the cytosol we used the difference in efficiency of *ade2-1* suppression.

Selection of mutants with decreased Mod5p-I activity in the cytoplasm

Since the Mod5p-I protein is dually localized in yeast, one might expect that the cytosolic pool of the enzyme would be decreased if the mitochondrial delivery were increased. Mislocalization of cytoplasmic Mod5p-I to other cellular locations could also lead to decrease of cytosolic activity and decrease of suppression efficiency in the presence of YCfmod5-M2. Decreased expression or increased turnover of Mod5p-I would be the alternative reason of the lower suppression.

T8-1D, the strain bearing chromosomal *SUP11 mod5-1* mutations and YCfmod5-M2 plasmid coding for Mod5p-I was mutagenized. The colonies were screened for decrease of suppression efficiency. With the color screening procedure, we selected 15000 red colonies among 160000 pink colonies. The majority of colonies were red due to plasmid loss, but some retained the plasmid as assessed by uracil prototrophy. Only 8 temperature-sensitive red Ura⁺ mutants were analysed further. We focused on the termosensitive (ts) mutants as mutations in genes important to protein sorting would be expected to be conditionally lethal. Also a ts phenotype is convenient for cloning the wild type allele of the mutant gene.

Back reversion or inactivation of the dominant *SUP11* mutation were excluded by cross with MB117-1A. If a strain has the dominant

Table 1
Suppression efficiency in the presence of Mod5p-I

<i>mod5-1</i> Strain	Suppressor mutation	Plasmid	Colony color	Suppression ability		
				<i>ade2-1</i>	<i>trp5-2</i>	<i>lys2-1</i>
MB103-9B	<i>SUP7</i>	-	red	-	-	-
		YCf-MOD5	white	+	+	+
		YCf-mod5M2	pink	±	+	+
MB105-6A	<i>SUP11</i>	-	red	-	-	-
		YCf-MOD5	white	+	+	+
		YCf-mod5M2	pink	±	±	±

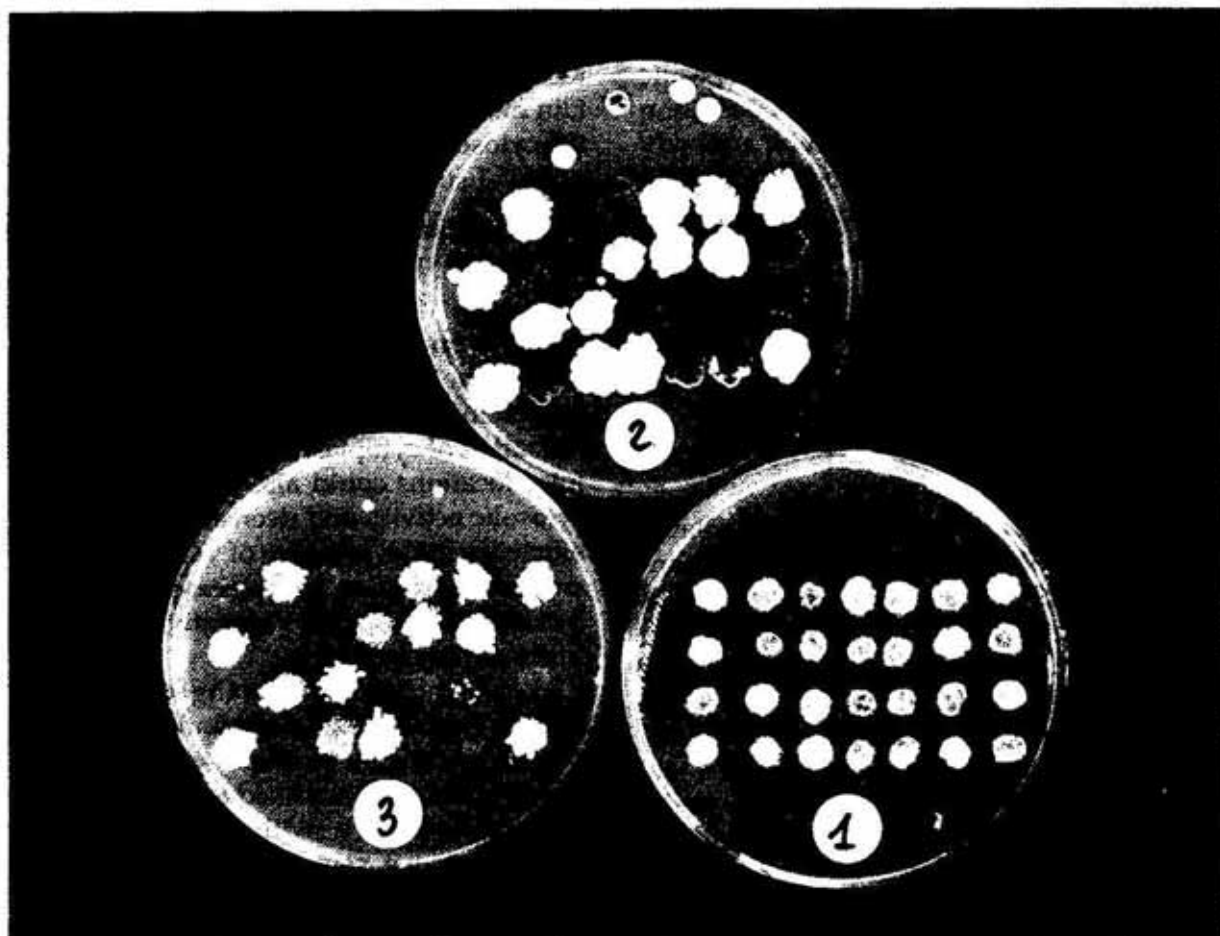


Fig. 1. Meiotic segregation of the *maf1* mutation in the *SUP11 mod5-1* chromosomal mutations background and in the presence of the *YCfmod5-M2* (1) growth on WO-ura plate. All spores harbor the plasmid. (2) Colony color on YPG medium (3) growth on N3 glycerol medium at 37°C. *maf1* is responsible for the *ts* phenotype. The red color is linked to the *ts* phenotype.

SUP11 suppressor gene a diploid *SUP11 ade2-1/ade2-1* will be white. Diploids, selected on WO medium lacking leucine, were white. This suggested that decreased *ade2-1* suppression in our mutants is connected with lower level of Mod5p-I in cytosol.

We also assessed whether loss of *ade2-1* suppression and temperature sensitivity co-segregate by backcrossing 8 mutants to MB105-6A followed by tetrad dissection and subsequent analysis of haploid progeny. The relevant genotype of all spores was *SUP11 mod5-1 ade2-1*. Only in one mutant were the temperature-sensitive phenotype and decreased suppression of *ade2-1* in the presence of *YCfmod5-M2* linked. We have named this mutant *maf1*. The result of segregation analysis is presented in Fig. 1 and the *ts* phenotype of *maf1* is described in Table 2. All the spores have *YCfmod5-M2* and can grow on WO plates lacking uracil (Fig. 1, plate 1). Spores are red on non-selective YPG me-

dium (plate 2) because *ade2-1* suppression is inefficient due to decreased Mod5p-I in the cytoplasm. No red spores grow at 37°C (plate 3). Therefore the temperature sensitive phenotype and the red colony color result from the same defect. The 2:2 segregation shows that these two phenotypes are caused by a single chromosomal mutation.

Indirect immunofluorescence

Loss of Mod5p-I cytoplasmic activity could be caused by many different types of events. To assess whether the phenotype of *maf1* was due to an altered subcellular location of Mod5p-I, we employed indirect immunofluorescence. The subcellular location of Mod5p-I in the mutant and the parental strain were compared. The *maf1* mutant and parental strain T8-1D were transformed with the multicopy plasmids *YEpmod5-M2KR6* and *pJDBmod5-M2KR6* coding for Mod5p-I, modified for

Table 2
Growth of *maf1* mutant at different temperatures on glucose (YPG) and glycerol (N3) plates

Strain	28°C		34°C		37°C	
	YPG	N3	YPG	N3	YPG	N3
T8-1D	+	+	+	+	+	+
<i>maf1</i>	+	+	+	±	+	-

increased mitochondrial delivery. Overexpression of Mod5p is necessary to detect the subcellular location of that antigen by microscopy [1]. Additionally the cells were stained with 4,6-diamino-2-phenylindole (DAPI) to show the position of nuclear and mitochondrial DNA (Fig. 2c, 2d).

Figure 2b shows representative photographs of *maf1* containing pJDBmod5-M2KR6. Surprisingly the *maf1* cells have substantial Mod5p signal in the nucleus but not in the cytoplasm. A nuclear location for Mod5p-I is consistent with the genetic selection if *SUP11* is isopentenylated in the cytosol but not in the nucleus.

DISCUSSION

Previous work with genes coding for enzymes with functions in multiple compartments has focused on using mutagenesis to define *cis* acting sequences important to their location. Mutations in targeting signal sequences or mutations forcing the use of alternative AUGs to make different forms of the proteins both lead to changes in the balance in the cellular distribution of sorting enzymes ([2, 3, 8] and Martin, N.C. & Hopper, A.K., in preparation). We have now initiated a search for genes which when altered, cause analogous phenotypes, namely altered distribution of Mod5p. We report here the identification of a mutation, *maf1*, which we know to alter the normal distribution of one form of Mod5p, Mod5p-I.

Mod5p, like two other tRNA modification enzymes, Trm1p and Cca1p, is a sorting isoenzyme, coded by a single gene but shared between different cellular compartments (Martin, N.C. & Hopper, A.K., in preparation). Each is made by a gene with more than one in frame ATG such that one protein is extended on the amino terminus relative to the other. *MOD5* is one of few genes reported to encode an activity

found in three subcellular compartments: cytosol, mitochondria and nucleus [1]. For Mod5p, the 11 amino-acid extension found on Mod5p-I is necessary but not sufficient for mitochondrial targeting. Amino acids 1 through 21 are sufficient for targeting a passenger protein to mitochondria and these whole sequence is only found in the long form of the Mod5p, Mod5p-I.

Mod5p-II, in contrast, is missing the mitochondrial targeting information and it is normally found in the cytosol and the nucleus. One puzzling feature of the wild type distribution of Mod5p is that form I is found in the mitochondria, form II is found in the nucleus but both forms commingle in the cytosol [1].

Despite the fact that amino terminal sequences from Mod5p are necessary and sufficient for targeting the protein to mitochondria, Mod5p-I is unusual in that there is a functional pool of the amino terminally extended mitochondrial form in the cytosol. Either the targeting of the protein to mitochondria is not efficient or there are positive interactions that maintain a portion of the protein in the cytosol. If targeting were not efficient one could imagine that the portion of Mod5p-I not imported could take on a conformation which would prevent its subsequent import into the organelle. If the presence of the amino terminal sequence obscured a nuclear targeting sequence (NTS) as suggested for *UNG* [9] (see below), a cytosolic pool would result. Alternatively, there may be mechanisms that lead to the positive retention of Mod5p-I in the cytosol. A mutation that alters a putative cytosolic "anchor" could allow normally cytosolic protein to locate to mitochondria or to nuclei.

We report here a genetic selection which can be used to identify mutants defective in the distribution of Mod5p, an enzyme shared between mitochondria, the cytosol and the nucleus in yeast. Our selection is based on the fact that suppression of nonsense mutations is im-

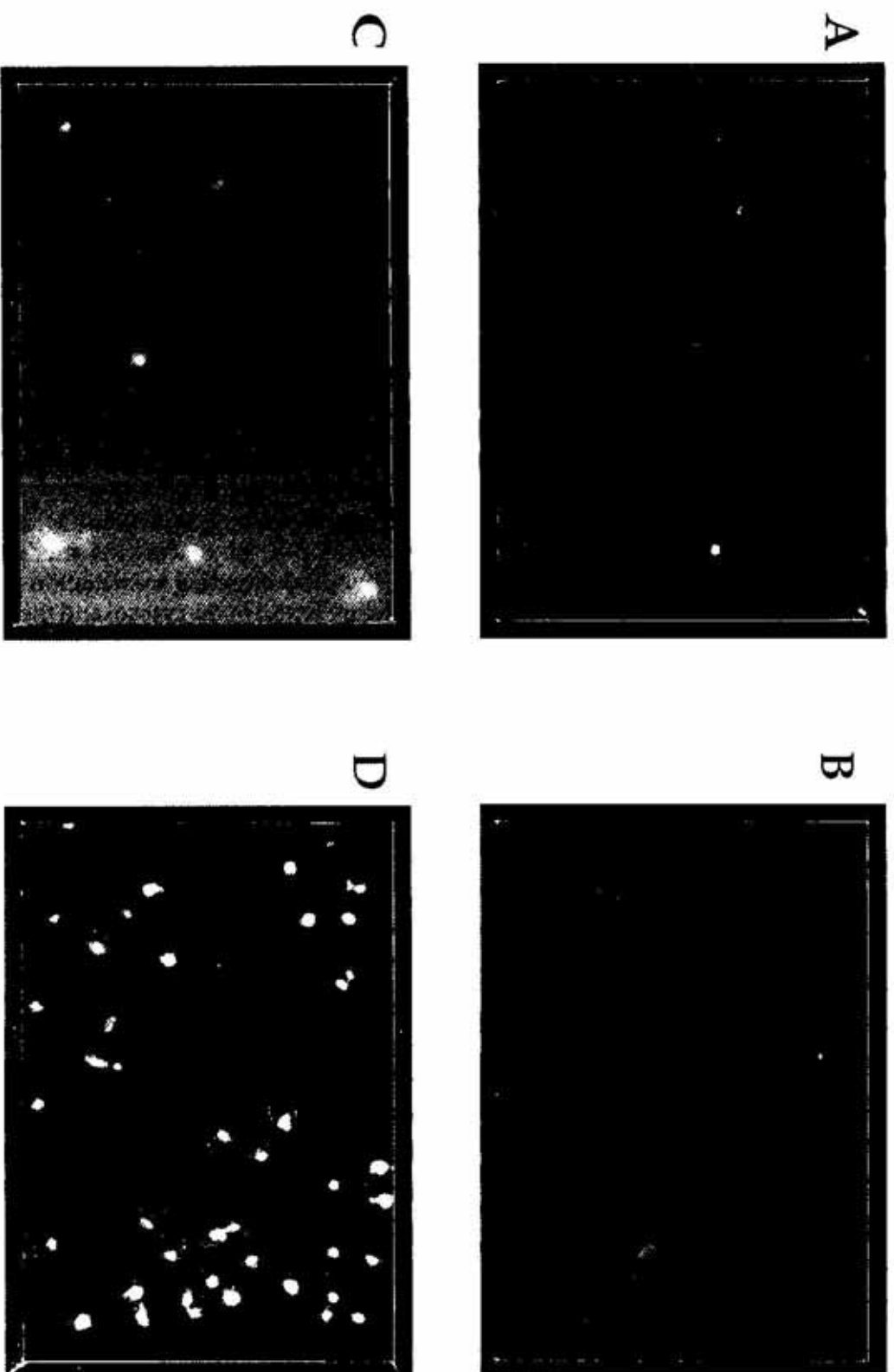


Fig. 2. The localization of Mod5p-1 protein in mutant cells.
a) Parental strain with pJDBmod5-M2KR6 plasmid; b) *maf1* strain with pJDBmod5-M2KR6; c, d) DAPI stained cells of wild type and mutant strain, respectively.

paired if the cytosolic level of Mod5p protein falls. The first mutant selected using this screen, *maf1*, was found because it causes a decrease in Mod5p activity in the cytosol. There are two obvious models which can explain the phenotype of *maf1* mutations in the context of protein targeting. Either there is less activity in the cytosol because more protein is imported into mitochondria or there is less cytosolic activity because more protein is imported into nuclei.

Indirect immunofluorescence experiments demonstrate that in *maf1* cells, the normal location of Mod5p-I in the mitochondria and the cytosol is altered. Mod5p-I in *maf1* cells is in the mitochondria and the nucleus. Thus *maf1* cells seem to have reduced cytosolic activity because the enzyme is now found in the nucleus instead of the cytosol. The putative product of *MAF1* could be involved in achieving the balance of intracellular distribution of Mod5p. For example, a mutation in this gene could interfere with a process that anchors a portion of Mod5p-I in the cytosol. Alternatively, it could facilitate specific recognition of the Mod5p-I nuclear signal and this activity could be improved in *maf1* cells. Proteins binding the nuclear localization sequences are known in yeast [10]. In either case, the putative NTS and the putative cytosolic anchor should be present in both Mod5p-I and in Mod5p-II. Experiments to determine the consequence of *maf1* on the distribution of Mod5p-II will be done.

Because of its phenotype under conditions that require mitochondrial function, *maf1* is not likely to be specific for only Mod5p-I. Mod5p is not an essential gene for mitochondrial function. One hypothesis to explain the thermosensitive growth only under conditions requiring mitochondrial function is that other proteins, essential to mitochondrial biogenesis, are affected. F1- β ATPase distribution is not altered in *maf1* cells (unpublished). However F1 β -ATPase is a typical mitochondrial enzyme and *in vivo* is found only in mitochondria. It could be that only sorting isoenzymes are affected by *MAF1* and we plan to test the localization of other sorting enzymes in *maf1* cells. *CCA1* and *PIF1* both code for sorting isoenzymes. The lack of Cca1p in mitochondria would lead to the thermosensitive phenotype we observe [9]. So would a lack of the *PIF1* gene product, a DNA helicase essential to the maintenance of mitochondrial DNA [11].

The mechanisms that lead to balanced distribution of sorting isoenzymes are not known. In the case of Bcl-2, competition for binding to different membranes could dictate distribution [12, 13]. Experiments with Trm1p, a protein found in nuclei and mitochondria, do not support a model stating that there is simple competition between nuclear and mitochondrial targeting signals. Destroying the Trm1p NTS does not lead to increased mitochondrial concentrations, rather the protein accumulates in the cytosol (Rose, A.M. *et al.*, submitted). Removal of the N-terminal presequence carrying the mitochondrial targeting signal of human UNG facilitates the nuclear import of this enzyme. In this case, it has been suggested that the NTS becomes accessible only after removal of the N-terminus which masks it [9]. Mod5p-I and Mod5p-II each are normally located in two compartments. Such a model could account for the non-nuclear location of Mod5p-I as well.

We are cloning *MAF1* by complementation of the *maf1* phenotype to determine whether it is similar to any known gene products. We are also interested in finding suppressors of the *maf1* phenotype. Through suppressor analysis, we expect to identify genes with products which may be directly or indirectly involved in recognition of *cis* acting sequences important to the distribution of Mod5p, and possibly other sorting isoenzymes.

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