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Mutations Altering the Mitochondrial-Cytoplasmic Distribution of Mod5p Implicate the Actin Cytoskeleton and mRNA 3′ Ends and/or Protein Synthesis in Mitochondrial Delivery

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The *Saccharomyces cerevisiae MOD5* gene encodes proteins that function in three subcellular locations: mitochondria, the cytoplasm, and nuclei (M. Boguta, L. A. Hunter, W.-C. Shen, E. C. Gillman, N. C. Martin, and A. K. Hopper, Mol. Cell. Biol. 14:2298–2306, 1994; E. C. Gillman, L. B. Slusher, N. C. Martin, and A. K. Hopper, Mol. Cell. Biol. 11:2382–2390, 1991). A mutant allele of *MOD5* encoding a protein (Mod5p-I,KR6) located predominantly in mitochondria was constructed. Mutants defective in delivering Mod5p-I,KR6 to mitochondria were sought by selecting cells with increased cytosolic activity of this protein. Twenty-five mutants defining four complementation groups, *mdp1*, *mdp2*, *mdp3*, and *mdp4*, were found. They are unable to respire at 34°C or to grow on glucose medium at 38°C. Cell fractionation studies showed that *mdp1*, *mdp2*, and *mdp3* mutants have an altered mitochondrial-cytoplasmic distribution of Mod5p. *mdp2* can be suppressed by *ACT1*, the actin-encoding gene. The actin cytoskeleton organization is also aberrant in *mdp2* cells. *MDP2* is the same as *VRP1* (S. F. H. Donnelly, M. J. Picklington, D. Pallotta, and E. Orr, Mol. Microbiol. 10:585–596, 1993). *MDP3* is identical to *PAN1*, which encodes a protein that interacts with mRNA 3′ ends and affects initiation of protein synthesis (A. B. Sachs and J. A. Deardoff, Cell 70:961–973, 1992). These results implicate the actin cytoskeleton and mRNA 3′ ends and/or protein synthesis as being as important for protein distribution in *S. cerevisiae* as they are for distribution of cytosolic proteins in higher eukaryotes. This provides the potential to apply genetic and molecular approaches to study gene products and mechanisms involved in this type of protein distribution. The selection strategy also offers a new approach for identifying gene products involved in the distribution of proteins to their subcellular destinations.

Eukaryotic cells have organelles separated physically from one another by lipid bilayers and the cytosol. Most proteins have a single destination such as the nucleus, mitochondria, peroxisomes, lysosomes, or the cell surface. However, we and others have discovered a class of genes that encode sorting isozymes, enzymes found in more than one cellular compartment (references 9, 10, 13, 18, 22, 34, 49, and 50; see also references cited in references 28 and 80).

The *Saccharomyces cerevisiae MOD5* and *CCA1* genes that encode the tRNA processing enzymes N^6-(Δ^2)-isopentenyl PP_i: tRNA isopentenyltransferase (IPP transferase) and ATP (CTP):tRNA nucleotidyltransferase, respectively, are unique in that the sorting isozymes they encode are found in two organelles (mitochondria and nuclei) as well as the cytosol (10, 79, 80). *MOD5* codes for two proteins that differ from each other by the presence or absence of an amino-terminal extension (28, 69). Translation initiation at the first AUG of the open reading frame (ORF) produces Mod5p-I, which is located in mitochondria and the cytosol; translation initiation at the second AUG at codon 12 gives rise to Mod5p-II, located in the cytosol and nucleus (10).

The mechanisms that locate proteins to single subcellular destinations have been intensively investigated. The importance of *cis*-acting sequences on the proteins is clearly established. Inroads are being made in identifying components on the organelles that serve as receptors and import channels. Cytosolic factors play critical roles (for an example, see review in reference 72), and recent observations suggest that the site of a protein's synthesis can also be an important factor in dictating its final destination (reviewed in reference 77). Despite this general outline of protein location, there is a very incomplete understanding of how the cell manages intracellular traffic. The natural distribution and function of sorting isozymes in multiple cellular compartments should provide a powerful tool to apply new genetic approaches to studies of protein delivery. This is because the distribution of sorting isozymes is balanced between multiple destinations, and the balance can be altered by *cis*-acting mutations as shown by the phenotypes caused by changing ATGs of *MOD5* and *CCA1* (10, 28, 80). The balance should also be altered by mutations in *trans*-acting genes required for the appropriate protein distribution.

To test whether indeed sorting isozymes can be used as a new genetic tool to study protein distribution, we initiated studies to identify mutants affecting gene products involved in the distribution of Mod5p-I between the mitochondria and cytosol. We reasoned that some of these gene products might also affect delivery of other proteins uniquely targeted to mitochondria. We report that selection for yeast mutants with an altered distribution of Modp5-I has identified new roles for

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype	Source or reference
MT-8	*MAT*α *SUP7 ade2-1 mod5*::*TRP1 ura3-1 lys 2-1 lys1-1 leu2-3,112 trp1 can1-100*	28
MB105-6A	*MAT***a** *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4*	This laboratory
T8-1D	*MAT*α *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 his4-519*	This laboratory
T7-7B	*MAT*α *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 his4-519 mdp1-1*	MB105-6A, spontaneous mutation; backcrossed with T8-1D
TZ33	*MAT*α *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 his4-519 mdp2-1/vrp1*	T8-1D, spontaneous mutation
T27-1D	*MAT***a** *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp2-1/vrp1*	TZ33 crossed to MB105-6A segregant
TZ34	*MAT*α *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 his4-519 mdp3-8/pan1-8* [*rho*$^-$]	T8-1D, spontaneous mutation
T37-2A	*MAT***a** *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu1 trp5 met4 mdp3-8/pan1-8* [*rho*$^+$]	Segregant of TZ-34 mated to MB105-6A
TZ81	*MAT*α *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 his4-519 mdp3-9/pan1-9*	T8-1D, ethyl methanesulfonate mutagenesis
LRA26	*MAT*α *leu2 ura3-52 his4-539 cdc25-5*	K. Tatchell
Sc467	*MAT***a**/*MAT*α *leu2-3,112/leu2-3,112 ade1/ade1 ura3-52/ura3-52 ile/ile MEL1/MEL1*	J. E. Hopper
T65-1D	*MAT*α *leu2-3,112 ade1 ura3-52 ile MEL1 mdp2*::*LEU2*	Segregant from Sc467 after disruption of *MDP2*

known genes that implicate the actin cytoskeleton and mRNA 3′ ends and/or protein synthesis in the distribution of proteins to mitochondria.

MATERIALS AND METHODS

Strains, media, growth conditions, and transformation. *Escherichia coli* RR1 and DH5α were used for propagation of recombinant DNA constructs. Strain RZ1032 (42) was used for propagation of M13 phage, and JM109 was the recipient for mutagenesis reactions. HB101 F′ *lac pro* and HB101 Tn*5*::PyrA(F$^-$) were used for γδ mutagenesis. Standard methods for growth and transformation of *E. coli* were employed (64).

The yeast strains used are listed in Table 1. Media were prepared and standard genetic manipulations were performed as described by Sherman (67). Yeast strains were transformed by the lithium acetate method of Ito et al. (38) as modified by Chen et al. (12). Osmotic sensitivity was tested on yeast extract-peptone-dextrose agar plates containing 0.9 M NaCl (NaCl plates), 1.2 M KCl (KCl plates), or 1.8 M sorbitol (sorbitol plates) by a drop test (14). To test for growth by the drop test method, cells were grown to log phase, the cell density was adjusted to 3×10^7 cells per ml, and 10 μl of an appropriate dilution of the suspension was spotted on a plate.

Plasmid constructs. (i) *mod5-M2,KR6*. *MOD5* and *mod5-M2,KR6* alleles differ at three codons. At codon 12, *MOD5* has the translation initiation ATG codon, whereas the mutant allele codon is ATT. At codons 14 and 15, the wild type specifies two Lys residues, whereas the mutant specifies two Arg residues. All three changes were generated by a single round of mutagenesis as described previously (28) with the oligonucleotide 5′AAATATTTCTAGAAGAGTTAT AGTGA3′ and the plasmid pUCmod5-E (28). DNA sequencing was used to confirm appropriate mutagenesis. Digestion with *Eco*RI released a 1,498-bp DNA fragment containing the phage f1 origin of replication and the 5′-proximal one-half of the mutant *MOD5* gene, and this was used to replace the comparable fragment of YCfMOD5 (28) and generated YCfmod5-M2,KR6.

To construct YEpmod5-M2,KR6, a 1.8-kbp *Bst*YI fragment from YCfmod5-M2,KR6 that encodes the entire *MOD5* gene and regulatory regions was transferred into the *Bam*HI site of plasmid YEp24.

To construct an epitope-tagged version of the *mod5-M2,KR6* allele, the 1.8-kbp *Bst*YI fragment of YCfmod5-M2,KR6 was transferred to the *Bam*HI site of Bluescribe vector (Stratagene). With the oligonucleotide 5′AAGGAGACTGTG GGCGGCCGCTGAAAATATTAG3′, a *Not*I site was introduced at codon 428, just before the stop codon. Appropriate mutagenesis was confirmed by DNA sequencing. A *Not*I fragment containing three adjacent hemagglutinin (HA) epitopes from plasmid pBF30 (73) was transferred to the newly created *Not*I site of *mod5-M2,KR6*. The *Bst*YI fragment containing the tagged *mod5-M2,KR6* gene was transferred into the *Bam*HI site of YCp50, generating YCpmod5-M2,KR6-ET.

(ii) *ACT1* constructs. The 4-kbp *Eco*RI fragment of pT3 containing the *ACT1* gene, which complemented *mdp2*, was inserted into the *Eco*RI site of YCp50 and YIp5 vectors, yielding YCpT3 and YIpT3, respectively. Resulting YCpT3 was used for transformation of the *mdp2* mutant. YIpT3 was digested with *Xho*I and used for integrative transformation of the T8-1D yeast strain. Plasmid YIpT3 was digested with *Mlu*I and *Bgl*II, and ends were filled by using Klenow fragment of DNA polymerase I and ligated. The *Eco*RI fragment of YIp-T3ΔMluBgl was transferred into YCp50 vector, and resulting YCpT3ΔMluBgl was used for testing complementation of the *mdp2* mutation.

(iii) *MDP2/VRP1* constructs. A 12-kbp *Not*I-*Nru*I fragment was deleted from pT6 plasmid containing the *MDP2/VRP1* gene, and resulting pT6ΔNotNru was digested with *Kpn*I. Two *Kpn*I fragments (1,582 and 3,566 bp) were removed, and the plasmid was religated and used for transformation.

The *mdp2*::*LEU2* disruption was obtained as follows. A 4-kbp *Hin*dIII fragment containing the *MDP2* gene was inserted into pUC19. A ~1.5-kbp *Not*I-*Eag*I fragment containing the 5′ half of *MDP2* was removed, and the ends were blunted with Klenow fragment and ligated to a 2.2-kbp *LEU2*-containing DNA, generating pUCHL. pUCHL was digested with *Hin*dIII and used to transform the diploid strain Sc467 (Table 1).

(iv) *MDP3/PAN1* constructs. The 4-kbp *Eco*RI fragment of pT4 containing *PAN1* was transferred into the *Eco*RI sites of YIp5 and YCp50 vectors, yielding YIpT4 and YCpT4, respectively. Resulting YCpT4 was tested for complementation of *mdp3*, and YIpT4 was digested with *Xho*I for linearization and used for integrative transformation of the yeast strain T8-1D.

(v) γδ mutagenesis. γδ mutagenesis of pT3 and pT6 was performed by the procedure of Guyer (30). *E. coli* HB101 F′ *lac pro* was transformed with respective plasmids, and ampicillin-resistant colonies were selected. Cells from a single colony were incubated with HB101 F$^-$ Tn::*pyrA*, and kanamycin-ampicillin-resistant transconjugates were selected. Plasmid DNA was extracted and screened by restriction analysis.

DNA hybridization. A 2.5-kbp *Eco*RI fragment of pT3, a 0.7-kbp *Hin*dIII fragment of pT6, and a 2-kbp *Eco*RI fragment of pT4 were radiolabeled by a random primer DNA labeling system (Gibco BRL) following the manufacturer's instructions. The labeled DNAs were used to hybridize a strip of nitrocellulose containing yeast chromosomes separated by pulsed-field gel electrophoresis and a filter set containing the *S. cerevisiae* genome in cosmid and λ clones (61); (membranes were purchased from the American Type Culture Collection).

Sequence analysis. A combination of three strategies was employed to determine the *MDP2* sequence: (i) the 3.5-kbp *Sma*I-*Eag*I, 1,580-bp *Not*I-*Eag*I, 1,436-bp *Kpn*I, 356-bp *Kpn*I, and 453-bp *Eco*RI fragments of plasmid pT6 were subcloned in Bluescript (KS$^+$), and the DNA sequence was obtained by using T3 and T7 primers; (ii) deletions of subclones in Bluescript (KS$^+$) were constructed and sequenced by using T3 and T7 primers; and (iii) *MDP2*-specific oligonucleotides were used to fill the gaps that could not be completed by the previous two methods. The site of γδ insertion was defined precisely by determining the surrounding sequences with forward and reverse primers complementary to γδ terminal sequences (66). For *PAN1*, partial sequence of the 4-kbp *Eco*RI fragment of pT4 subcloned into Bluescript (KS$^+$) was obtained by using T3 and T7 primers. All nucleotide sequences were determined by using the Sequenase 2.0 DNA sequencing kit (U.S. Biochemical Corp.). Sequences were searched for identity or similarity with other genes at the National Center for Biotechnology Information with the BLAST network service (6).

Radioimmunoassay for i^{6}A tRNA and pulse-labeling. Radioimmunoassay for i^{6}A tRNA was performed as described previously (28). Pulse-labeling experiments on the β subunit of F1 ATPase import were carried out according to the method of Brandt (11).

Cell fractionation. Cell fractionation was performed by a modification of procedures described previously (17). Two hundred milliliters of wild-type and *mdp* mutant strains harboring the plasmid YEpmod5-M2,KR6 or YCpmod5-M2,KR6-ET was grown on synthetic medium lacking uracil (SC-URA) and containing 2% lactate and 0.05% glucose at 23°C to mid-log phase (optical density, ~1.0), shifted for 2 h to 37°C, and harvested. Proteinase inhibitors were added to all buffers to the final concentrations of 1 mM phenylmethylsulfonyl fluoride, 1 mg of leupeptin per ml, 5 mg of pepstatin per ml, and 1 mM ε-aminocaproic acid. Oxalyticase (Enzogenetics, Inc.) was used instead of zymolyase to generate spheroplasts. Where indicated, mitochondria were treated for 30 min at 4°C with 4 μl (per 100-μl mitochondrial fraction) of proteinase K to remove cofractionating proteins. Proteinase K was inactivated by addition of 2.5 μl of 0.1 M phenylmethylsulfonyl fluoride.

Immunoblot analysis. Protein concentration was determined by the method of Peterson (56) with bovine serum albumin as a standard. Proteins were resolved on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels and transferred to

TABLE 2. Effects of N-terminal mutations on nonsense suppression and i^6A modification of tRNA

Plasmid[a]	% tRNA with i^6A modification		Suppression	
	Mitochondrial	Cytoplasmic	*ade2-1* (color)	*lys2-1*
None	0	0	− (red)	−
YCfMOD5	100	100	+ (white)	+
YCfmod5-M2	100	53	+/− (pink)	+
YCfmod5-M2,KR6	103	11	+/− (dark pink)	−

[a] YCfMOD5 produces both Mod5p-I and Mod5p-II, YCfmod5-M2 produces Mod5p-I, and YCfmod5-M2,KR6 produces Mod5p-I,KR6 that contains lysines instead of arginines at amino acids 14 and 15.

nitrocellulose paper. Rabbit anti-Mod5 antibody (28) was used in a 1:2,500 dilution. Rabbit anti-F1-ATPase β subunit antibody was a gift from M. Yaffe and was used in a 1:800,000 dilution. A polyclonal antibody against Rna1p (35) was used in a 1:200,000 dilution. A mouse antiactin monoclonal antibody was purchased (Boehringer Mannheim) and was used in a 1:30,000 dilution. Mouse 12CA5 monoclonal anti-HA antibody was purchased from Berkeley Antibody Company and used in a 1:10,000 dilution. Horseradish peroxidase-conjugated donkey anti-rabbit or monkey anti-mouse antibody was used as a secondary antibody at a 1:7,500 dilution. Blots were incubated overnight at room temperature with primary antibody and for ~1.5 h with secondary antibody. Antibodies were detected with the enhanced chemiluminescence system (Amersham). Quantitation of protein identified by antibody was performed by scanning short exposures of X-ray film with a Molecular Dynamics laser scanner and Quantity One software.

Indirect immunofluorescence and staining of DNA, actin, and chitin. For immunofluorescence, cells were grown on glucose selective medium and processed as described previously (10). For DNA staining of live cells, ~10 μl of log-phase cells were applied to glass slides, the liquid medium was aspirated, and 10 μl of 0.5-μg/ml DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) was used to stain DNA. After ~1 min, the DAPI was aspirated, the cells were washed one time with H_2O, and the cells were viewed by fluorescence microscopy. For actin staining, cells were grown on SC–2% lactate medium at 23°C and incubated for 2 h at 37°C. Actin was stained with rhodamine-phalloidin (Molecular Probes) by a previously described procedure (3). Chitin was stained with Calcofluor (Sigma) (59).

RESULTS

Increasing the predicted hydrophobic moment of the mitochondrial targeting sequence decreases cytoplasmic Mod5p-I. The amino terminus of Mod5p-I is different from other mitochondrial targeting signals in that the sequence of the first 18 residues has a predicted hydrophobic moment (21) of 5.22 μH rather than the usual ~8 to 10 μH (76). In an effort to determine whether an inefficient targeting signal of Mod5p-I accounts for the fact that some of it is cytoplasmic, we generated a mutant allele, *mod5-M2,KR6*. The three changes made result in mutation of the second ATG codon to ATT and conversion of lysine codons 14 and 15 to arginine codons. This allele produces Mod5p-I,KR6, whose mitochondrial targeting sequence has a predicted hydrophobic moment of 7.28 μH. The wild-type allele, the allele containing just the ATG-to-ATT change (*mod5-M2* [28]), and the *mod5-M2,KR6* allele were introduced on centromere-based plasmids (YCfMOD5, YCfmod5-M2, and YCfmod5-M2,KR6, respectively) into the yeast strain MT-8, which has a *mod5* disruption allele. The effect of the Lys-to-Arg mutations on the levels of cytoplasmic and mitochondrial Mod5p-I was assessed by monitoring the modification of tRNAs, by nonsense suppression, and by subcellular fractionation.

Modification of cytoplasmic and mitochondrial tRNAs was determined by a radioimmunoassay (28) (Table 2). Mitochondrial tRNAs from cells containing the wild-type, *mod5-M2*, or *mod5-M2,KR6* allele were completely modified. Thus, the protein encoded by *mod5-M2,KR6* is catalytically active. Cytoplasmic tRNA modification was lower when cells contained YCfmod5-M2,KR6 (11%) than when the same strain contained the YCfmod5-M2 plasmid (53%) or the wild-type allele on YCfMOD5 (100%). Therefore, increasing the hydrophobic moment of Mod5p-I causes a decrease in the levels or activity of the cytosolic Mod5p-I. Consistent with this result, suppression of the *ade2-1* and *lys2-1* nonsense alleles was affected (Table 2). While cells containing *MOD5* or *mod5-M2* are able to suppress *lys2-1* and can grow on media lacking lysine, cells containing the *mod5-M2,KR6* mutant allele cannot.

The distribution of Mod5p-I in the cytoplasm and mitochondria of MT-8 cells containing genes encoding Mod5p-I and -II (YEpMOD5), Mod5p-I (YEpmod5-M2), or Mod5p-I,KR6 (YEpmod5-M2,KR6) was determined by immunoblotting (Fig. 1). Multicopy plasmids encoding Mod5p rather than single-copy plasmids were used because the existing Mod5p antibody is not of sufficient quality to monitor endogenous quantities of Mod5p-I in subcellular fractions. The quantities of cytoplasmic and mitochondrial proteins from each strain were monitored by probing the blots with anti-Rna1p and anti-F1-β-ATPase, respectively (Fig. 1B). Appropriate cellular fractionation was confirmed by the lack of cytoplasmic Rna1p in the mitochondrial fractions and the lack of F1-β-ATPase in the cytoplasmic fractions (not shown). Cells possessing the *mod5-M2,KR6* al-

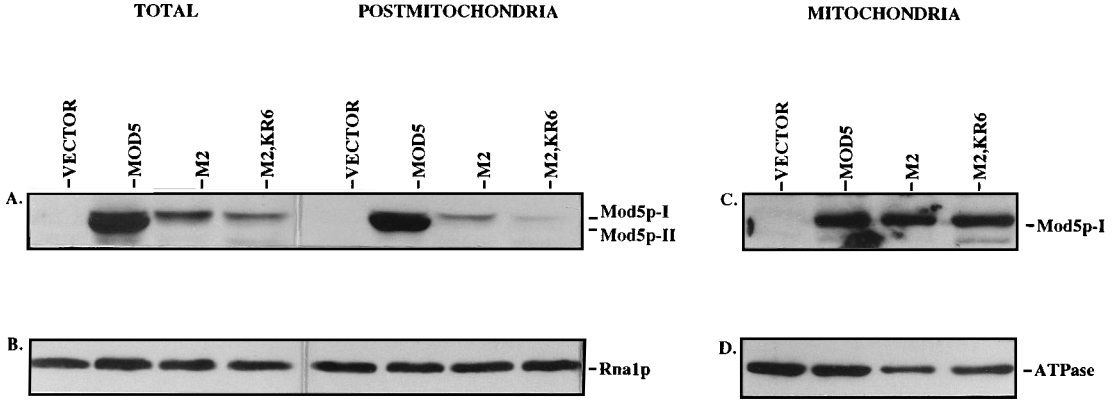


FIG. 1. Subcellular distribution of Mod5p-I,KR6. Distribution of Mod5p was analyzed in MT-8 cells harboring YEp24 (vector), YEpMOD5 (MOD5), YEpmod5-M2 (M2), or YEpmod5-M2,KR6 (M2,KR6) by cell fractionation and immunoblotting. Blots A and C were probed with affinity-purified anti-Mod5p antibody. Blots A and C were probed a second time with either anti-Rnalp (B) or anti-F1-β-ATPase (D), respectively. Total and postmitochondrial supernatant fractions have ~30 μg of protein per lane. Mitochondrial fractions have ~10 μg of protein per lane.

lele have less Mod5p-I in the cytoplasm relative to Rna1p, a cytoplasmic marker (35), than do cells possessing the *mod5-M2* allele (Fig. 1A and B). About one-half of Mod5p-I is mitochondrial, and one-half is in the cytosol (28). Therefore, the maximum possible increase in mitochondrial Mod5p due to the KR6 mutation is twofold. Measurements in multiple experiments of Mod5p-I relative to F1-β-ATPase indicate that there is a marginally increased level (1.2- to 2.0-fold) of Mod5p-I in the mitochondria from cells containing the *mod5-M2,KR6* allele compared with cells with the *mod5-M2* or *MOD5* wild-type allele (Fig. 1C and D).

Collectively, the levels of i^6A on cytosolic and mitochondrial tRNA, the efficiency of nonsense suppression, and the subcellular fractionation studies support the hypothesis that protein coded by *mod5-M2,KR6* is more efficiently imported into mitochondria than protein coded by *mod5-M2*. However, we cannot entirely exclude the possibility that Mod5p-I,KR6 has a shorter half-life in the cytoplasm than does Mod5p-I.

Selection for mutants with altered mitochondrial-cytoplasmic distribution of Mod5p-I,KR6. We have initiated a genetic study to identify the proteins that are involved in distribution of sorting isozymes to the appropriate subcellular destinations. Cells possessing the *mod5-M2,KR6* allele have the vast majority of Mod5p sequestered in mitochondria, and hence, cytosolic tRNA is depleted of the i^6A modification. Since suppression by cytoplasmic $tRNA^{Tyr}_{UAA}$ is exquisitely sensitive to the levels of i^6A at position 37, we reasoned that we could obtain increased cytoplasmic Mod5p by selecting for mutations of *SUP11 mod5-1 lys2-1* cells harboring the YCfmod5-M2,KR6 plasmid that restore *lys2-1* nonsense suppression in a Mod5p-I-dependent fashion. Two types of mutations were anticipated, those which cause increased levels of *MOD5* expression and those which cause increased cytosolic Mod5p-I activity. Increased cytosolic activity could result from a posttranslational modification, increased retention or stability of the protein in the cytosol, or decreased delivery to mitochondria. We anticipated that the latter class of mutations might affect the distribution of proteins other than Mod5p and hence would affect mitochondrial function and/or biogenesis. Therefore, to enrich for this type of lesion, we screened among mutants which were able to suppress *lys2-1* and grow on media lacking lysine at 23°C for those which also displayed mitochondrial defects at elevated temperatures.

Spontaneous UV- or ethyl methanesulfonate-induced prototrophic mutants were selected on media lacking lysine in strains MB105-6A and T8-1D (relevant genotypes, *SUP11 mod5-1 lys2-1*) harboring the low-copy plasmid YCfmod5-M2,KR6. The rate of appearance of spontaneous Lys^+ cells was $\sim 10^{-4}$. Lysine prototrophs were screened for those which failed to grow at 34 and 38°C on rich media containing glycerol and
rich media containing glucose as carbon sources, respectively. The Lys^+ temperature-sensitive candidates were tested for dependence of nonsense suppression on the presence of the YCfmod5-M2,KR6 plasmid. Candidates which fulfilled these criteria were subjected to tetrad analysis, and those which showed cosegregation of plasmid-dependent nonsense suppression and temperature-sensitive growth were analyzed further.

Twenty-five mutants were identified. All of the mutations were recessive and segregated as single genes. The mutations formed four complementation groups, *mdp1* (mitochondrial down protein import mutants), *mdp2*, *mdp3*, and *mdp4*. The *mdp1* group consists of 17 members, *mdp2* consists of 2, *mdp3* consists of 4, and *mdp4* consists of 2. Subsequent genetic analysis showed mutations within complementation groups to be allelic. For all members of the *mdp2*, *mdp3*, and *mdp4* complementation groups and most members of *mdp1* complementation groups, the mutants show preferential growth defects on nonfermentable carbon sources. That is, these mutants are able to grow on glucose-containing media, but not on glycerol-containing media at 34°C. These mutants therefore appear to be defective in mitochondrial function. The *mdp3-8* mutant (strain TZ-34) was found as a [rho^-] mitochondrial mutant, and a tendency to accumulate [rho^-] mutations was observed for *mdp3-8* [rho^+] segregants obtained from crossing the *mdp3-8* mutant with the wild-type strain (MB105-6A [Table 1]) and sporulating the diploids.

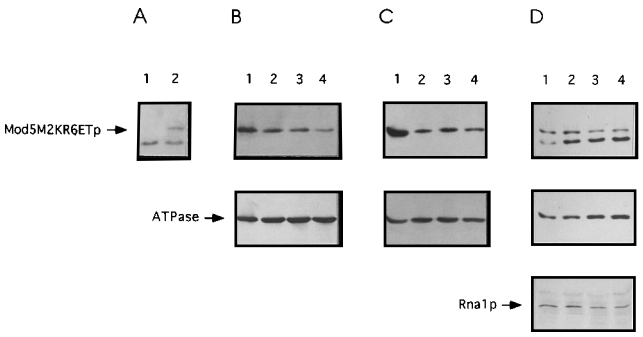


FIG. 2. Steady-state levels of Mod5p-I,KR6-ET in subcellular fractions of parent and *mdp* mutants. Cells were grown to early log phase in lactate-containing media at 23°C and then transferred to 37°C at 2 h prior to harvesting. (A) Total cell extracts from parent strain, T8-1D, containing either YCpmod5-M2,KR6 (lane 1) or the tagged construct, YCpmod5-M2,KR6-ET (lane 2), probed with mouse monoclonal antibody (12CA5) specific for the influenza virus HA epitope. (B) Mitochondrial fractions from strains T8-1D (parent, lane 1), T7-7B (*mdp1-1*, lane 2), TZ33 (*mdp2-1*, lane 3), and TZ81 (*mdp3-9*, lane 4). All strains contained the plasmid YCpmod5-M2,KR6-ET, which codes for the epitope-tagged Mod5p-I,KR6-ET protein. The top row shows the Mod5p antigen. The blot was stained a second time with anti-F1-β-ATPase (second row). (C) Same as panel B except that mitochondrial fractions were treated with protease K. (D) Total cell extracts from the same cells as in panels B and C. Top row, blot probed with 12CA5; second row, same blot probed with anti-F1-β-ATPase; bottom row, blot probed with anti-Rna1p. Arrows indicate the relevant proteins.

Mitochondrial Mod5p-I,KR6 is decreased in *mdp* mutants. If the *mdp* mutants have increased Mod5p-I function in the cytosol because they are impaired in delivery of this protein to mitochondria, then the steady-state levels of mitochondrial Mod5p should be decreased in the mutant cells. As the available antibody to Mod5p (28) is not of sufficient quality to monitor endogenous levels of Mod5p in subcellular fractions and there was concern that the *mdp* mutations might not affect overproduced levels of Mod5p in the same way as endogenous levels, we assessed mitochondrial Mod5p in cells possessing an epitope-tagged version of *mod5-M2,KR6*. Mod5p-I, KR6, rather than Mod5p-I, was monitored because the mutants were selected for altered activity of Mod5p-I,KR6. We have not yet assessed the subcellular location of Mod5p-I. The plasmid YCpmod5-M2,KR6-ET complements the *mod5-1* mutation, and the phenotypes of *mdp* cells possessing this plasmid are identical to cells possessing YCfmod5-M2,KR6. Mod5p encoded by YCpmod5-M2,KR6-ET can be detected in endogenous quantities with a monoclonal antibody specific to the HA epitope (Fig. 2A). As seen previously (66), the anti-HA also cross-reacts with another cytoplasmic protein of unknown identity.

Parent and mutant cells containing plasmid YCpmod5-M2, KR6-ET were grown on lactate media (to increase the yield of mitochondria) at a permissive temperature (23°C) and then incubated for 2 h at the restrictive temperature (37°C). The

levels of Mod5p in total cell extracts and mitochondria purified from these extracts were determined by protein blot analysis. Antibodies specific to Rna1p (35) (Fig. 2) and Act1p (not shown), two cytoplasmic proteins, were used as internal standards to assess the relative levels of Mod5p in the total extracts of each mutant (Fig. 2D) and to confirm that mitochondrial fractions were devoid of cytoplasmic contamination (not shown). These studies showed that the steady-state levels of Mod5p in *mdp2* and *mdp3* mutants are indistinguishable from the levels in the parent strain. *mdp1* cells may have marginally (1.5 to 2.0×) increased cellular levels of Mod5p.

In contrast, the *mdp* mutants have less mitochondrial Mod5p than does the parental strain when indexed against F1-β-ATPase. F1-β-ATPase was used as a tool for assessing the relative levels of Mod5p in mitochondrial fractions because pulse-labeling followed by immunoprecipitation of F1-β-ATPase showed no accumulation of the F1-β-ATPase precursor for *mdp1* mutants and only marginal accumulation of this precursor for *mdp2* mutants (not shown). Figures 2B and C show mitochondrial fractions untreated or treated, respectively, with proteinase K. For untreated mitochondrial preparations, *mdp1*, *mdp2*, and *mdp3* mutants have reduced amounts of Mod5p relative to F1-β-ATPase compared with the parental strain. The effect appears more dramatic for mitochondrial fractions treated with proteinase K presumably because cytosolic proteins including Mod5p associate with the outer surface of the semipurified mitochondria either in vivo or in vitro during the course of organelle purification. Calculation of the relative quantity of Mod5p/ATPase in mitochondria to Mod5p/Rna1p in total extracts for each mutant compared with the parent strain shows that the mutants have about 20% of the wild-type level of Mod5p in mitochondria. Because F1-β-ATPase mitochondrial levels could be affected to some extent by the *mdp* mutations, our calculations might underestimate the defect of Mod5p distribution to mitochondria.

Given that the *mdp* mutants were identified on the basis of increased cytosolic Mod5p activity and that they have reduced mitochondrial Mod5 antigen but equivalent levels of this antigen in total extracts, we expected that Mod5p would be increased in mitochondria-depleted cellular supernatants. However, significant increases of Mod5p were not detected in the postmitochondrial supernatant (not shown). A likely explanation for this result rests in the way the subcellular fractionation is done. The initial mitochondrial pellet contains unbroken cells as well as other cellular components which sediment or which are associated with components which sediment. Perhaps Mod5p associates with these other components, or perhaps Mod5p is loosely associated with the outer mitochondrial surface and is removed during the subsequent washes necessary to yield purified mitochondria.

We also attempted to locate Mod5p in wild-type and mutant strains by indirect immunofluorescence. However, neither the untagged version nor the epitope-tagged version of Mod5p-I can be viewed in low copy. Mod5p-I can be viewed when expressed from the multicopy pJDB207 vector (10), but its antigenicity is destroyed when cells are fixed at high temperatures. Therefore, we located overproduced levels of Mod5p-I, KR6 in cells incubated at 23°C. Even under these nonoptimum conditions, the *mdp* mutants demonstrated more intense cytoplasmic staining and less intense mitochondrial staining than did the parent (not shown).

Since the mutants demonstrate preferential growth defects on nonfermentable carbon sources and have reduced mitochondrial levels of Mod5p, we investigated whether there was an unusual distribution or quantity of mitochondria in these cells. Wild-type and mutant cells were grown on lactate media at a permissive temperature and then incubated for 2.5 h at the nonpermissive temperature. Mitochondria were located by staining cellular DNA with DAPI. By this method, the quantity of mitochondrial genomes and their distribution do not appear different among wild type and *mdp1*, *mdp2*, and *mdp3* mutants (Fig. 3). Similar experiments conducted with different carbon sources, different temperatures, and the strains harboring the *mdp2*::*LEU2* allele also provided no evidence for mitochondrial morphological defects in the *mdp* mutants (not shown). Collectively, the data showing equivalent total Mod5p levels but increased cytosolic activity of IPP transferase and decreased mitochondrial pools of Mod5p support the hypothesis that the *mdp* mutants are defective in distributing Mod5p-I to mitochondria. The data indicating that mitochondria-impaired function in the absence of obvious mitochondrial morphological defects support the notion that defective mitochondrial protein distribution is not an indirect consequence of a morphological defect. To understand how the *MDP* gene products might be involved with subcellular distribution, we cloned and characterized two of these genes, *MDP2* and *MDP3.*

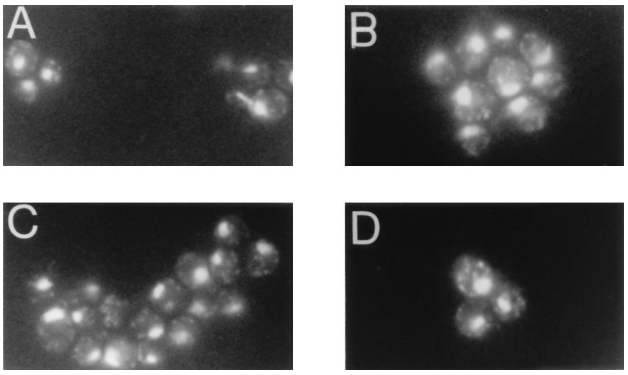


FIG. 3. DAPI staining of mitochondrial DNA in *mdp* mutant and wild-type strains. Cells were grown as for Fig. 2, and DNA was stained with DAPI. The location of nuclear and mitochondrial DNA was viewed by fluorescence microscopy. (A) T8-1D (parent); (B) T7-7B (*mdp1-1*); (C) TZ33 (*mdp2-1); (D) TZ81 (mdp3-9*).

The *mdp3* mutations are in the *PAN1* gene. The wild-type *MDP3* gene was cloned from a YCp50 library (43) by complementation of the temperature-sensitive growth phenotype. Two plasmids containing overlapping inserts allowed the cells to grow at 37°C. Plasmid pT4, containing a 10-kbp insert, was analyzed further. A ~4-kbp *Eco*RI fragment of pT4 capable of complementation was transferred to the integrating vector, YIp5, and this complementing DNA was introduced into the genome of the parental strain. The resulting integrant was crossed to the *mdp3-8* mutant strain (T37-2A [Table 1]), and the diploid cells were sporulated. Seventeen tetrads analyzed were all parental ditype (i.e., two $Ura3^+$ Mdp^+ and two $Ura3^-$ Mdp^- progeny), indicating that the cloned sequence integrated into the chromosome at a position very close (≤3 centimorgans) to the *mdp3-8* mutation. Therefore, the cloned DNA most likely contains the authentic *MDP3* gene. We mapped the *MDP3* gene to chromosome IX, close to *bar1*, by hybridization of an *Eco*RI fragment to filters containing the yeast genome in lambda and cosmid clones (61) and to a chromosome blot. The same *Eco*RI fragment complemented the *mdp3-8* mutation when subcloned, but complementation was less efficient than for the original plasmid. A partial DNA sequence of the *Eco*RI fragment was obtained and found to be identical with *PAN1*, an essential gene affecting translation

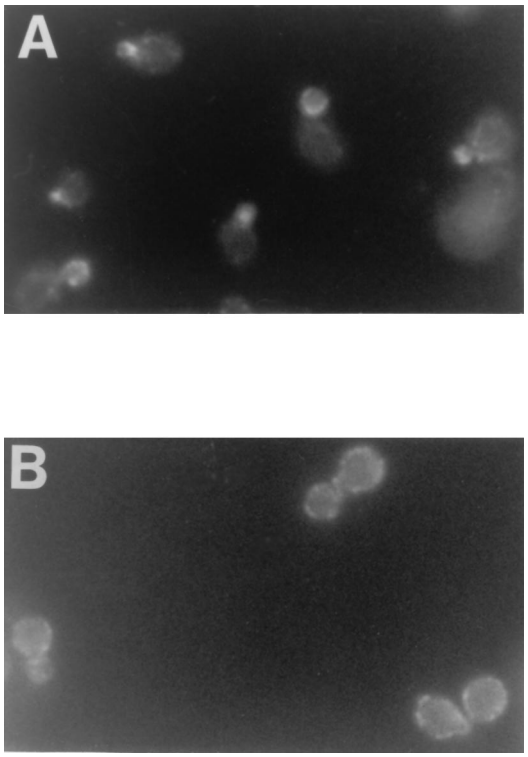


FIG. 4. F-actin detection by rhodamine-conjugated phalloidin. (A) Parent strain, T8-1D; (B) *mdp2-1* mutant strain, TZ33.

(63a). Thus, the *mdp3-8*-complementing gene is *PAN1*. The *PAN1* gene spans most of the *Eco*RI 4-kbp fragment. Complementation of *mdp3-8* by an incomplete *PAN1* ORF is consistent with the previous finding that only part of the gene is necessary for function (63a). The previously isolated *pan1* mutants also showed defects in growth on nonfermentable carbon sources (63). Hereafter, *MDP3* will be referred to as *PAN1*.

***ACT1* is a suppressor of the *mdp2-1* mutation.** Two plasmids, pT3 and pT7, that complemented the temperature-sensitive growth phenotype of *mdp2-1* were found by screening a single-copy YCp50 library (43). The plasmids contained overlapping 6- and 4-kbp inserts which hybridized to chromosome VI and the λ clone containing the *ACT1* gene which codes for actin (27, 52). *ACT1* was responsible for complementation of *mdp2-1* as determined by transposon mutagenesis of the pT3 plasmid and restriction analysis of mutant clones. When the γδ transposon was inserted into the *ACT1* intron, the cloned sequence no longer complemented the *mdp2-1* mutation. Deletion of a *Mlu*I-*Bgl*II fragment containing most of the *ACT1* gene also destroyed complementation of the *mdp2-1* mutation. To determine whether *ACT1* is the *MDP2* gene, we tested whether the *mdp2-1* mutation is located in the *ACT1* locus. The *Eco*RI 4-kbp actin gene-containing fragment was transferred into an integrating vector, digested with *Xho*I, and transformed for integration into the parental strain. The integrant was crossed to the *mdp2-1* mutant, and diploid cells were sporulated. Analysis of 34 resulting tetrads (PD-4, NPD-4, and T-26) demonstrated that *ACT1* and *MDP2* segregate independently. Therefore, *ACT1* is a suppressor of the *mdp2-1* mutation when on the YCp50 low-copy vector or when integrated into the genome.

The actin cytoskeleton is aberrant in *mdp2-1* mutant cells. Since one additional copy of wild-type *ACT1* suppresses the *mdp2-1* mutation, we examined the actin cytoskeleton organization in *mdp2-1* mutant cells. Rhodamine-conjugated phalloidin was used to stain F-actin. We found that the actin cytoskeleton of *mdp2-1* cells is aberrant when cells are grown on lactate medium at 23°C. The cellular distribution of actin in wild-type yeast cells is asymmetric and changes during the cell cycle (2). Early in the cell cycle, patches of actin localize to the site where the bud will emerge; later, these patches are localized toward the tip of the growing bud. In contrast to the bud, the mother cell contains relatively few patches but has instead actin cables. Shortly before the cells divide, the cables disappear. Cells of the parental strain have an actin distribution consistent with previous findings (Fig. 4A). For *mdp2-1* budding cells, the staining is more diffuse, actin patches are randomly distributed, and cables are not visible in the mother cell (Fig. 4B). This delocalized distribution of actin is characteristic of yeast cells defective in cytoskeletal proteins: actin (53), fimbrin (1), actin-capping proteins (7), tropomyosin (46), and proteins whose functions are specifically associated with the actin cytoskeleton: Sac1p (15), Sac20p, Sac30p (54), Sla1p, and Sla2p (33). The actin cytoskeletons of *mdp1* and *pan1* cells were the same as for parental cells (not shown).

In *S. cerevisiae*, the actin cytoskeleton reassembles in response to osmotic stress (14). Sensitivity to osmotic pressure is one of the characteristics of yeast actin mutants (53). The *mdp2-1* mutant cells showed increased osmosensitivity compared with the parental strain grown at 28°C on NaCl or KCl plates. Addition of sorbitol had no effect (Fig. 5). Chitin deposition is also directed by the actin cytoskeleton, and *mdp2-1* cells have aberrant chitin deposition (not shown). Taken together, suppression of *mdp2-1* by actin and the defects in F-actin staining, osmoregulation, and chitin deposition show that the actin cytoskeleton of *mdp2* cells does not function appropriately.

The *mdp2* mutations are in the *VRP1* gene. Actin overproduction is lethal in *S. cerevisiae* (37, 48). Therefore, to avoid isolating the *ACT1* gene again, we used a multicopy library to clone the authentic *MDP2* gene. One clone, pT6, containing a 16-kbp insert, was able to complement the *mdp2-1* mutation. Hybridization of a 0.7-kbp *Hin*dIII fragment of pT6 to the set of filters with genomic lambda clones showed that this clone contains a fragment of chromosome XII located in the region of *CDC25*, *CDC3*, and *ILV5* loci. To determine whether *mdp2-1* maps to this region of chromosome XII, the *mdp2-1* mutant strain (T27-1D [Table 1]) was mated to a *cdc25-5* mutant (strain LRA26 [57]). Eleven tetrads from the resulting diploid were analyzed. The segregation of markers (9 PD:0 NPD-2 T) showed that the *mdp2-1* mutation is located about 9.1 centimorgans from *cdc25-5*. Therefore, pT6 contains sequences located in the genome at a position genetically linked to *MDP2*.

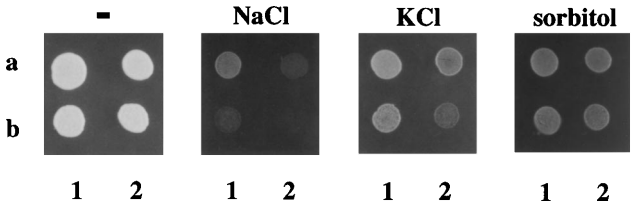


FIG. 5. Osmosensitivity of *mdp2-1*. Parental (lanes 1) and *mdp2-1* (lanes 2) strains were incubated at 28°C on yeast extract-peptone-dextrose plates (−), NaCl plates (NaCl), KCl plates (KCl), and sorbitol plates (sorbitol). A total of 3×10^5 cells (a) or 3×10^4 cells (b) were spotted onto the plates.

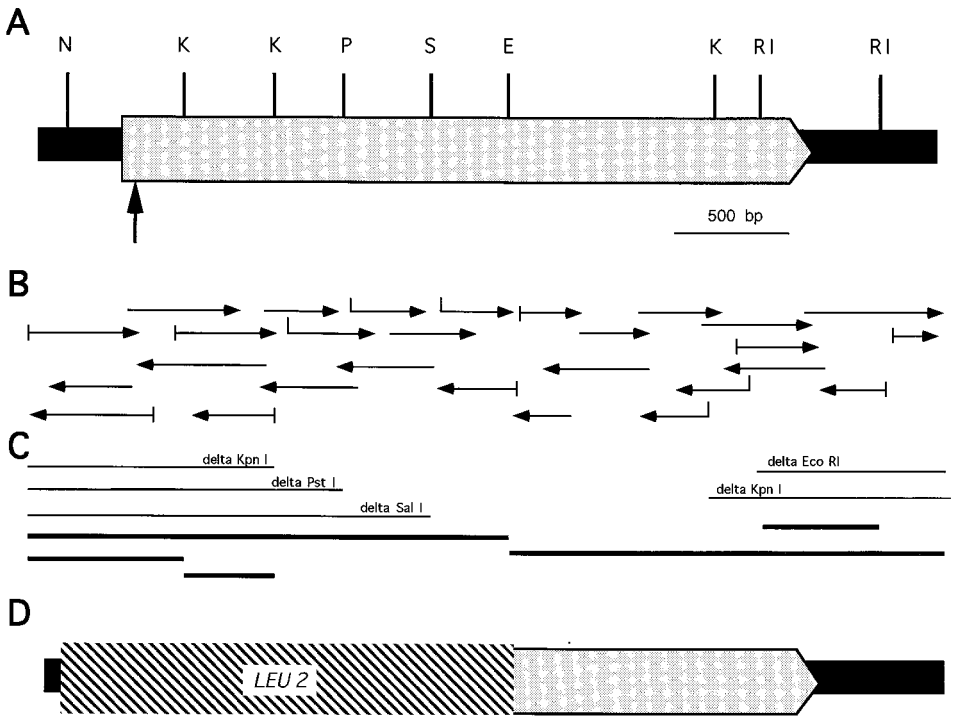


FIG. 6. Restriction map and sequencing strategy of the *MDP2* gene. (A) Restriction map of the *MDP2* gene. The heavy line represents genomic DNA, and the box represents the ORF and its direction of translation. The upright arrow indicates the localization of a γδ insertion that destroys *MDP2* activity. Restriction sites: N, *Not*I; K, *Kpn*I; P, *Pst*I; S, *Sal*I; E, *Eag*I; RI, *Eco*RI. (B) Strategy for sequencing the *MDP2* gene. Plain arrows indicate sequence obtained with oligonucleotide primers; arrows starting at vertical bars indicate sequence obtained from subclones; otherwise, the sequence was obtained from deletions. (C) Heavy lines represent fragments of *MDP2* subcloned into Bluescript (KS^+); thin lines represent deleted fragments from subclones. (D) Diagram of the *mdp2*::*EU2* allele.

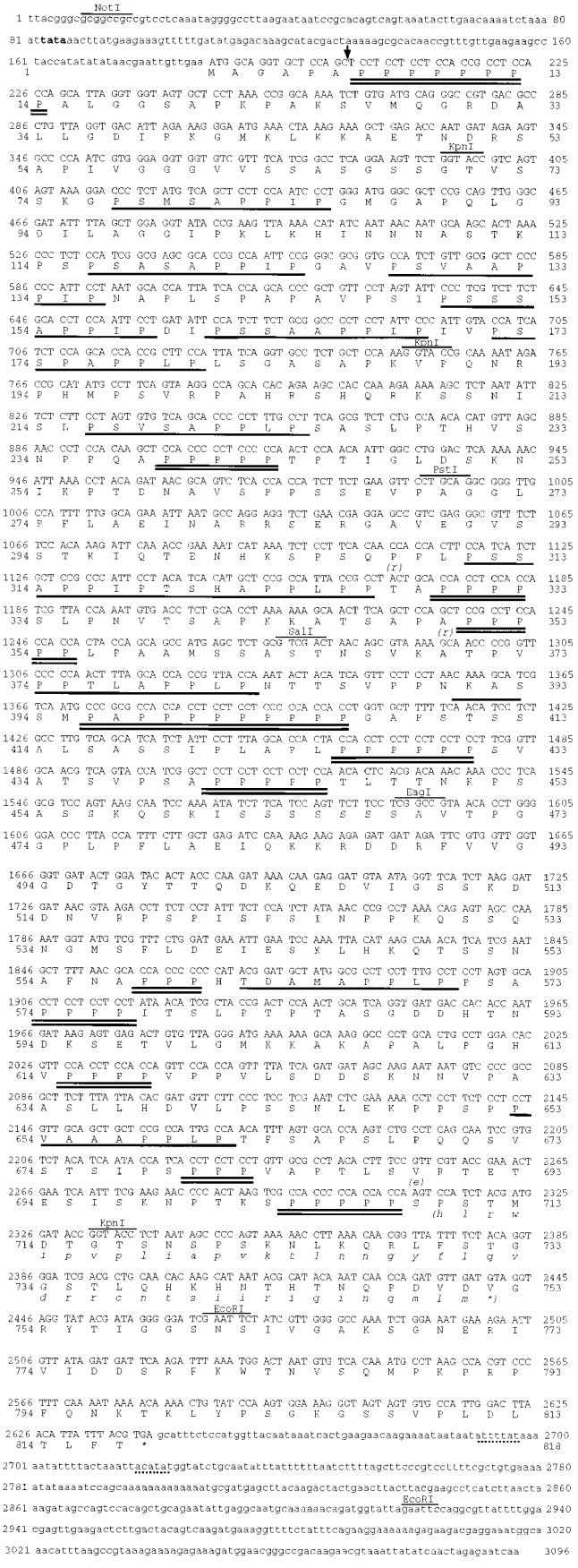
γδ transposon mutagenesis was used to localize the gene responsible for complementation on plasmid pT6. Determining the DNA sequence surrounding the site of one γδ insertion that destroyed complementation revealed that the transposon was inserted into an ORF, next to the L10e ribosomal protein-encoding gene (51). Deletion of 1,582- and 356-bp *Kpn*I fragments from pT6ΔNotNru within this ORF (Fig. 6) resulted in loss of complementation.

A disruption allele of the ORF, *mdp2*::*LEU2*, was generated and was used to replace the homologous region in the diploid strain Sc467 (Table 1). Leu^+ transformants were sporulated, and the resulting tetrads were dissected. Four viable progeny resulted. Leu^+ progeny grew slowly, but otherwise their phenotypes resembled strains containing the *mdp2-1* allele. That is, *mdp2*::*LEU2* strains grow on both glycerol and glucose-containing media at 23°C, fail to grow on a nonfermentable carbon source at 28°C, and fail to grow on all media at temperatures above 30°C. The actin cytoskeleton of a *mdp2*::*LEU2* strain (T65-1D [Table 1]) is disorganized even when the cells are grown on glucose-containing media at 23°C (not shown). Unlike *mdp2-1*, the disruption allele is not suppressed by an additional copy of *ACT1*.

pT6 contains a gene that complements *mdp2-1*, and this gene is located in the genome very close to the *CDC25* locus, which is genetically linked to *MDP2*. Disruption of this gene causes defects in the organization of the actin cytoskeleton, as do spontaneous *mdp2* lesions. We conclude that the authentic *MDP2* is encoded by this gene.

The DNA sequence of both strands of the *MDP2* ORF was determined (Fig. 6 and 7). It was found to be a very close match to the yeast *VRP1* gene (19). The *MDP2* sequence differs from *VRP1* in six nucleotide positions and extends about 500 bp further in the 3′ direction. Five different nucleotides give three amino acid substitutions and may represent strain-dependent heterogeneity. More important, one nucleotide addition at position 2315 changes the reading frame and creates a different sequence of 42 amino acids starting from position 710. The predicted Mdp2p is 66 amino acids longer than the previously reported Vrp1p sequence (Fig. 7). We verified that Mdp2p contains the carboxy-terminal extension since cells containing a plasmid with an insertion of the HA epitope at the last codon of the predicted ORF generate protein which migrates appropriately on SDS-polyacrylamide gels and that cross-reacts with the HA antibody (not shown). A recent entry of the *S. cerevisiae* genome sequence effort (GenBank accession number U19028) verifies our DNA sequence. We believe that, despite the differences in DNA sequence, *MDP2* and *VRP1* are the same gene since disruption of *VRP1* causes similar distortions of the actin cytoskeleton as do *mdp2-1* and *mdp2*::*LEU2* mutations (19). Hereafter, *MDP2* will be referred to as *MDP2/VRP1*.

MDP2/VRP1 encodes an extremely proline-rich protein (22%) that also has a high serine (15%) and alanine (9%) content. Other than the stretches of prolines, the amino acid sequence does not show significant homology to other proteins. Some stretches consist of three to nine prolines, while the other proline-rich regions are organized in 12 nine-amino-acid repeats with the consensus sequences PXXXAPPI/LP, where X is most often S or A (Fig. 7). This consensus sequence resembles the peptide sequence for SH3 domain ligands (19, 24, 60). Interestingly, *PAN1* is also somewhat proline rich (8.1%) and possesses proline-containing motifs such as PXP (where X is I, V, or T) and PXQP (where X is T or V). Pan1p



also possesses a copy of the Mdp2p/Vrp1p consensus, PXXX APPLP, and another sequence resembling this, PPIP.

DISCUSSION

The location of Mod5p-I in mitochondria and the cytoplasm raises the question of what factors influence the cytoplasmic-mitochondrial equilibrium of Mod5p-I. To address this problem, we searched for mutations in genes whose products are involved in the cellular distribution of Mod5p-I. Starting with a *mod5* allele, *mod5-M2,KR6*, that codes for a protein restricted nearly exclusively to mitochondria, we selected for mutants that increase the cytoplasmic pool of this protein. Although we attempted to devise the selection procedure to enrich for mutants with decreased mitochondrial pools of Mod5p, theoretically mutations which result in the sorting defect as well as mutations which cause enhanced activity, synthesis, or stability of Mod5p-I,KR6 could have been uncovered. As assessed by the ability to suppress nonsense alleles efficiently, *mdp* mutants have more Mod5p in the cytosol than does the parental strain. This apparently is not the result of increased transcription of *MOD5* or increased stability of Mod5p since the amounts of total cellular Mod5p relative to Rna1p (Fig. 2) and to actin (not shown) are not detectably different for the *mdp2/vrp1* and *pan1* mutants than for the wild-type strain. Rather, the data support the notion that the *mdp* mutants have mitochondrial sorting defects because mitochondria isolated from the mutants have depleted pools of Mod5p compared with the wild-type strain.

Several lines of evidence argue that mutant hunts based upon subcellular redistributions of sorting isozymes may provide a powerful new genetic means to identify gene products involved in the sorting process. First, it does not appear that the genetic selection enriches for mutations that affect multiple cellular processes. Finding multiple lesions in each complementation group means that alteration of the mitochondrial-cytoplasmic distribution of Mod5p-I is caused by mutations in a limited numbers of genes. If the phenotype could have been achieved by mutations slowing cell metabolism, macromolecular synthesis, or growth, many more genes would have been identified. Second, our selection for increased cytoplasmic Mod5p-I is similar to that described by Maarse et al. (47), who selected for cytoplasmic accumulation of a fusion protein with a mitochondrial targeting sequence. Among the isolates were mutants of the inner membrane mitochondrial import apparatus. The significant difference in our selection is in the use of an enzyme naturally distributed to more than one location. Finally, the *mdp* mutants show defects in mitochondrial function, as evidenced by growth defects on nonfermentable carbon sources. Since *MOD5* is an unessential gene (28) that is not required for growth on nonfermentable carbon sources, the mitochondrial defects of *mdp* mutants may result from altered subcellular distribution of other proteins. However, our studies of the processing of the precursor to F1-β-ATPase provided no evidence for defects in delivery of this protein to mitochondria

FIG. 7. DNA sequence of the *MDP2* gene and predicted amino acid sequence of the protein product. Upper numbering corresponds to nucleotide sequence. Lower numbering corresponds to amino acid sequence. The position of the γδ insertion is indicated (↓). The TATA-like box is in boldface. Sequences matching upstream and downstream elements involved in 3′ terminus formation are underlined with a dotted line. Proline-rich sequences resembling SH3 binding motifs are underlined once; homopolymeric proline sequences are underlined twice. Restriction sites shown in Fig. 5 are indicated. A partial published amino acid sequence of Vrp1p that differs from our sequence is in italics below the Mdp2p sequence.

in *mdp* cells. It will be interesting to determine which other mitochondrial proteins are affected by the *MDP* gene products.

Why search for additional gene products that affect delivery of proteins to mitochondria? Many approaches have been used to identify genes encoding proteins involved in mitochondrial import. Beginning with mitochondrial membranes and/or contact sites, reverse genetics has been used to identify genes encoding receptors and the translocation channel (8, 31, 36, 40, 55, 70, 71). Other components of the yeast mitochondrial import apparatus have been identified via multicopy suppressors of mutations of genes encoding components of the contact sites (39), by selecting for mutants not able to import particular gene fusion proteins to mitochondria (36, 47), or by screening temperature-sensitive mutants for defects in mitochondrial import (23, 62). Surprisingly, besides components of the heat shock response (reviewed in reference 72) the genetic screens and selections have failed to identify cytoplasmic proteins that play a role in mitochondrial import. New genetic approaches, such as ours, have the potential to identify novel cytoplasmic proteins important to appropriate delivery of proteins to mitochondria.

Mdp2p/Vrp1p affects the actin cytoskeleton. Pan1p is a protein that copurifies with the poly(A)-binding protein-dependent poly(A) RNase and affects initiation of protein synthesis, but its enzymatic function is not known at this time (63, 63a). Our findings implicate a role of the actin cytoskeleton and the 3′ ends of mRNA and/or initiation of protein synthesis in delivery of proteins to mitochondria. How might these gene products influence the distribution of Mod5p to mitochondria? It has been documented that the yeast cytoskeleton interacts with mitochondria. Movement of mitochondria during meiosis has been shown to be actin dependent (44). Furthermore, in mitotic cells there is a high frequency of colocalization of mitochondria with actin cables, and many, but not all, mutations of *ACT1* that affect the cytoskeleton also affect mitochondrial organization (20). We have not detected the condensed and/or clumped altered mitochondria evident for some *act1* mutants in cells containing *mdp1*, *mdp2*, or *mdp3* mutations (Fig. 3). Presumably, *mdp2* affects the actin cytoskeleton and distribution of protein to mitochondria without affecting mitochondrial organization.

Studies of *S. cerevisiae* and other organisms show that the actin cytoskeleton plays a major role in cellular delivery processes. For example, it is implicated in vesicular transport of protein products to growing yeast daughter cells (53), in yeast endocytosis (41), in mobility of the intracellular pathogen *Listeria monocytogenes* throughout the host cell (58), and in delivery of mRNAs to appropriate cellular sites for protein synthesis (reviewed in references 16 and 68). This latter delivery process is mediated by 3′ untranslated regions of mRNA and mRNA-binding proteins associated with the cytoskeletal framework (reviewed in references 32, 65, and 77).

Protein synthesis has also been implicated in mitochondrial protein delivery. Although mitochondrial protein import is generally considered to be a posttranslational process (reviewed in reference 45), experiments showing location of cytoplasmic ribosomes translating mitochondrial proteins at the mitochondrial outer surface (4, 5) and more recent studies employing a yeast homologous in vitro system (25) and in vivo experiments (26) provide evidence that import in vivo may occur closely coupled to translation.

In consideration of the close coupling of translation and mitochondrial import in *S. cerevisiae* in vivo (75) and the documented roles of 3′ mRNA sequences and the actin cytoskeleton in establishing protein location in other systems, a testable model emerges, one in which Mdp2p/Vrp1p and Pan1p could play roles in mRNA localization and/or localized translation with subsequent effects on protein import into mitochondria. Variations upon this theme include models whereby the actin cytoskeleton might be involved directly in posttranslational import of proteins to mitochondria.

Finally, it is possible that Mdp proteins are involved in signal transduction processes that ultimately impact upon mitochondrial protein delivery. The existence of sequences similar to SH3-binding motifs (60) in Mdp2p/Vrp1p (19) and Pan1p indicates that they could bind to proteins that contain SH3 domains. There is precedence for roles of SH3-binding proteins in protein delivery. For example, the SH3-binding protein dynamin is involved in vesicle secretion and recycling (reviewed in reference 78). The dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic has the sequence PGGGAPPLP, similar to the motifs present in Mdp2p/Vrp1p and Pan1p (74). There is an indication that another dynamin-like yeast protein, Mgm1p, might be involved in protein import into mitochondria (29). Information regarding the intracellular locations of Mdp2p/Vrp1p and Pan1p should help distinguish among the possible models for how these proteins could have an effect in the subcellular distribution of Mod5p.

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