The Single Amino Acid Changes in the Yeast Mitochondrial S4 Ribosomal Protein Cause Temperature-Sensitive Defect in the Accumulation of Mitochondrial 15S rRNA†

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ABSTRACT: Four different mutant alleles of a nuclear gene (*MNA6*), which lose mt 15S rRNA at nonpermissive temperature (36 °C), were previously generated by EMS mutagenesis of *Saccharomyces cerevisiae*. To understand the biochemical basis for the loss of 15S rRNA in these mutants, the wild-type and mutant alleles of the *MNA6* gene were isolated and characterized. The DNA sequencing of the cloned *MNA6* gene revealed that it has an open reading frame specifying a 486 amino acid polypeptide, which appears to be a yeast mt homologue of the S4 r-protein family. The large size of this yeast S4 homologue is due to a nonhomologous long C-terminal extension. The *MNA6* gene also appeared to be identical to the previously isolated yeast *NAM9* gene. The in vitro expression under coupled transcription−translation reaction conditions followed by mt import demonstrated that *MNA6* indeed encodes a ∼56 kDa protein targeted to the mitochondria. We have also demonstrated by Western blot analysis using anti-Mna6p antibody that Mna6p is associated with the small subunit of mitoribosomes. The sequence analysis of the four mutant *mna6* alleles revealed that Leu^{109} → Phe, Arg^{111} → Lys, Pro^{424} → Leu, or Pro^{438} → Leu amino acid substitution in Mna6p causes temperature-dependent loss of the 15S rRNA. These mutations do not affect the mitochondrial import or accumulation of Mna6p. Rather the evidence points to an inability of mutant Mna6p to be assembled into the mitoribosomes of cells grown at 36 °C.

The mitochondrion, a key organelle in energy metabolism of eukaryotic cells, is responsible for most of the energy generation necessary for biochemical and mechanical functions of the cells. It is an organelle whose generation and function depend on the coordinated expression and interaction of nuclear genes and genes carried within the organellar genome itself. The mitochondrial (mt)[1] genetic system is required for the synthesis of a limited number of mt proteins (i.e., eight proteins in yeast), mostly the subunits of the energy-transducing enzyme complexes imbedded in the inner membrane (*1*−*5*). The enzyme complexes of the inner membrane are involved in respiration and biosynthesis of most of the cellular ATP. Additional single mt genes encode components of the mt translation system (i.e., 2 rRNAs and 24 tRNAs in yeast). These mt genes were expressed by nuclearly-encoded mt transcription and translational machineries which are synthesized in the cytoplasm and then imported into mitochondria (*3*−*5*). For example, like other ribosomes, mitoribosomes are composed of RNAs which are encoded in the mt DNA and of many protein molecules, almost all of which are supplied by the nuclear genome (*6*, *7*). This nuclear−mt interaction raises several interesting questions: how these two physically separated genomes are coregulated and interact with each other to achieve the biosynthesis of mitoribosomes; how proteins encoded by the nuclear genes are correctly transported into mitochondria after translation in the cytosol; and what are the features of these r-proteins that are essential for their interaction with each other or with rRNAs.

The endosymbiotic hypothesis for the bacterial origin of mitochondria (*8*) as well as the prokaryotic-like antibiotic sensitivity of mitoribosomes (chloramphenicol sensitive and cycloheximide resistant) (*9*) suggest that mitoribosomes might be structurally and functionally analogous to bacterial ribosomes. However, in comparison to the bacterial (*10*, *11*) and cytoplasmic ribosomes (*12*−*14*) which have already been extensively investigated, relatively little is known about the structure, function, and biosynthesis of mitoribosomes. In the interest of characterizing the nuclear genes of yeast that are involved in mt gene expression, 1300 temperature-sensitive (ts) nuclear *pet* mutants of *Saccharomyces cerevisiae* were previously generated by EMS mutagenesis. These mutants function normally at 23 °C but are respiratory-deficient at 36 °C. Thirteen of them in eight complementation groups (*mna1* to *mna8*) are defective in mt RNA accumulation (*15*). The initial characterization of these *pet* mutants of yeast suggested that mutation in at least three nuclear

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[1] Abbreviations: mt, mitochondrial; wt, wild type; r-protein, ribosomal protein; mitoribosome(s), mitochondrial ribosome(s); ts, temperature sensitive; EMS, ethyl methane sulfonate; SD, synthetic dextrose medium; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; nt, nucleotide(s); CTE, C-terminal extension.

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genes causes the instability of 15S rRNA at 36 °C. Since petite yeast strains unable to carry out mt protein synthesis are nevertheless able to generate a normal level of 15S rRNA (*16*), the specific loss of 15S rRNA in these nuclear *pet* mutants may not be due simply to the formation of petite mt genome at nonpermissive temperature or to a defect in the general mt transcription/translation apparatus.

To explore how this selective set of nuclear gene products is involved in the stability of 15S rRNA, an emphasis has been placed on the *mna6* mutants. The wild type (wt) and four mutant alleles of the *MNA6* gene have been cloned and sequenced. The cloned gene appears to be identical to the previously isolated *NAM9* gene (*17*). For simplicity, the *MNA6/NAM9* gene will be referred to as *MNA6* throughout this paper. We have demonstrated by in vitro expression followed by mt import as well as Western blot analysis that the *MNA6* gene product (Mna6p) is a 56 kDa ribosomal protein (r-protein) of the small subunit of mitoribosome. Furthermore, amino acid sequence homology searches revealed that Mna6p is related to the S4 small subunit r-protein of bacteria, chloroplasts, and some eukaryotic cells. However, Mna6p is a much bigger protein than other S4 homologues due to the presence of a large C-terminal extension (CTE). The DNA sequencing of four mutant alleles of *MNA6* also disclosed that a single amino acid substitution either in the N-terminal S4 domain or in the CTE domain of Mna6p is responsible for the loss of 15S rRNA in these yeast mutants at 36 °C. The instability of mutant protein does not appear to fully account for this phenotype; rather, the evidence points to a defect of an early rRNA−protein interaction in the process of mitoribosome assembly.

MATERIALS AND METHODS

Yeast Strains and Media. The *Saccharomyces cerevisiae* strain 124 (*MAT*$_a$ *can*1 *his*1 *trp*1 *ino*1-13 *ino*4-8) was previously used for EMS mutagenesis (*15*). All other yeast strains used were isogenic or congenic to the D273-10B or W303 genetic background of *Saccharomyces cerevisiae*.

Several complete media were used: YPD (1% yeast extract, 2% bactopeptone, 2% glucose), YPG (1% yeast extract, 2% bactopeptone, 3% glycerol), and YPGal (1% yeast, 2% bactopeptone, 2% galactose, 0.2% glucose). The minimal SD medium was 0.67% yeast nitrogen base without amino acids plus 2% glucose. The sporulation medium contained 1% potassium acetate, 0.1% yeast extract, 0.05% glucose plus essential amino acids.

Yeast Transformation and Nucleic Acid Manipulation. Yeast transformation with plasmid DNA was carried out by electroporation. Yeast was grown exponentially in 100 mL of YPD medium at 23 °C with vigorous shaking (i.e., 250 rpm). When the cell density reached ~10^8 cells/mL, yeast was harvested by centrifugation, washed with sterile distilled water, and then resuspended in 10 mL of pretreatment buffer (20 mM Tris-HCl, pH 8.0, plus 25 mM DTT). After incubation at 23 °C with gentle agitation (i.e., 75 rpm) for 30 min, cells were collected by centrifugation and washed 3−4 times with 100 mL of ice-cold sterile distilled water and then once with 10 mL of ice-cold 1.2 M sorbitol. Yeast cells were resuspended in 0.2 mL of ice-cold 1.2 M sorbitol, and then 50 μL of cell suspension was taken into a 1.5 mL eppendorf centrifuge tube. After mixing with 0.5−1.0 μg of yeast plasmid DNA (i.e., a yeast−*E. coli URA3* shuttle vector) and 2 μg of carrier salmon sperm DNA, the cell suspension was transferred into an ice-cold 0.2 cm electroporation cuvette. The cuvette was introduced into the chamber of a BIO-RAD gene pulser followed by an electric pulse at 1.5 kV, 200 Ω, and 25 μFD for 4−5 ms. After addition of 200 μL of recovery solution (1.2 M sorbitol, 0.7% nitrogen base without uracil but containing essential amino acids), cells were spread onto uracil-less SD plates and allowed to grow for 3−4 days at 23 °C. To examine their respiratory competence at both permissive and nonpermissive temperatures, the transformants were replica-plated onto two sets of glycerol plates and then incubated, one set at 23 °C and the other set at 36 °C.

Standard techniques were employed for gel electrophoresis, restriction endonuclease mapping, and other molecular analyses of the cloned gene. DNA sequencing was carried out by the dideoxy chain termination sequencing method.

In Vitro Expression of the Cloned MNA6 Gene. The cloned *MNA6* gene was expressed in vitro using a coupled transcription/translation reaction system (TNT kit, Promega Corp.). The *MNA6* gene in the pRS316 vector (*18*) was transcribed in vitro by the T7 RNA polymerase using a T7 promoter. These in vitro synthesized capped transcripts were translated in the same reaction mixture containing nuclease-treated reticulocyte lysate in the presence of [^{35}S]methionine. The labeled protein was visualized by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and fluorography.

In Vitro Import of Mna6p into Isolated Mitochondria. Import of the in vitro synthesized protein into isolated mitochondria was carried out as described by Sanyal and Getz (*19*). In a 100 μL import reaction [0.6 M sorbitol, 3% bovine serum albumin (fat free), 10 mM MOPS, pH 7.2, 80 mM KCl, 2 mM ATP, and 1 mM NADH], the ^{35}S-labeled protein was incubated with 50 μg of the isolated mitochondria at 30 °C for 30 min. After the import reaction, 40 μg of proteinase K was added to the reaction mixture in one of the duplicate experiments and incubated on ice for 30 min to remove the extramitochondrially located Mna6p. Mitochondria were reisolated, washed with the import buffer, and resuspended in Laemmli buffer (*20*). The protease-resistant band of Mna6p was visualized by SDS−PAGE and fluorography.

Production of Antibody against Mna6p. Polyclonal anti-Mna6p antibody was generated by injecting a rabbit with Mna6p expressed in *E. coli*. In brief, a 1.0 kb fragment of the *MNA6* gene corresponding to amino acids 138−486 of Mna6p was isolated from the cloned *MNA6* gene by PCR using a 5′ primer (5′-GGGGAATTCATCCAAGCTATACTT-TAAAGCCTGGA) and a 3′ primer (5′-CGAGAATTC-GAGAGGCTGAAGATCAGTCAA). The PCR product was ligated into the *Eco*RI site of the pATH1 vector (*21*) to create an in-frame *trpE−MNA6* gene fusion. The correct orientation of this insert was determined by restriction site analysis. The recombinant plasmid carrying the *trpE−MNA6* gene was transformed into *E. coli* HB101, and the fusion protein was expressed by tryptophan starvation in the presence of 3-β-indoleacrylic acid (*21*). The 77 kDa trpE−Mna6p fusion protein containing 325 amino acids of the *E. coli* anthranilate synthetase (trpE) followed by 349 amino acids of Mna6p was resolved by SDS−PAGE and then electroeluted and lyophilized. A rabbit was immunized by subcutaneous injection of 200 μg of purified trpE−Mna6p fusion protein

in 500 μL of distilled water plus an equal volume of complete Freund's adjuvant. A total of 100 μg of trpE−Mna6p in incomplete Freund's adjuvant was used for three subsequent subcutaneous injections. One week after the third boost, the rabbit was bled to obtain serum with strong reactivity to Mna6p on Western blot. The antisera were aliquoted into different tubes and stored at −20 °C.

Isolation of Mitoribosomes. The mitoribosomes and their subunits were isolated according to the procedure of Partaledis and Mason (*22*). In brief, cells from an exponentially growing culture were collected by centrifugation, and spheroplasts were prepared by zymolyase treatment at 30 °C for 1−2 h. After resuspension in 3 volumes of 0.6 M sorbitol−1 mM EDTA solution containing 1 mM PMSF, cells were disrupted by hand homogenizer (10 strokes). The cell debris was removed by two consecutive centrifugations at 3500 rpm for 10 min in a SS-34 rotor. The supernatant was recentrifuged at 10 000 rpm for 15 min to collect the mt pellet. The isolated mitochondria were washed twice by suspension in a 0.6 M sorbitol−1 mM EDTA buffer followed by centrifugation at 3500 rpm for 10 min and then at 10 000 rpm for 15 min of the supernatant. The mt pellet was resuspended in TMA 50 buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM ammonium sulfate) containing 6 mM β-mercaptoethanol and 1 mM PMSF. Sodium deoxycholate (1%, final concentration) was added, and then the pellet was solubilized by hand homogenizer. The lysate was clarified by centrifugation at 15 000 rpm for 20 min in a Beckman Ti 70.1 rotor. To separate the 50S and 37S subunits of mitoribosomes, the supernatant carrying 10 mg of total mt protein was layered onto 12 mL of an isokinetic sucrose density gradient (15−27%) in TMA 500 buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 500 mM ammonium sulfate). After centrifugation at 24 000 rpm for 16 h in a Beckman SW41 Ti rotor, fractions of 0.6 mL were collected by a fractionator and the locations of the subunits were detected by A_{280} absorption. The ribosomal subunits in these fractions were precipitated by adding PEG 8000 (0.1 g/mL, final concentration), and then collected by centrifugation in 1.5 mL eppendorf tubes.

Western Blot Analysis. The mt samples were run onto 10−20% gradient polyacrylamide−SDS gels, transferred to an Immobilon-P transfer membrane (Millipore), and probed with primary antisera [i.e., rabbit polyclonal anti-Mna6p (1:5000 dilution) or mouse monoclonal antibody (hybridoma supernatant) against the 35 kDa r-protein (Mrp13) of the mt small subunit (1:10 dilution) or against the 16 kDa r-protein (Mrp49) of the mt large subunit (1:10 dilution)]. These monoclonal antibodies were provided by Dr. T. Mason and M. Sanchirico (University of Massachusetts, Amherst). Secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti-rabbit (BRL) or HRP-conjugated sheep anti-mouse (Sigma)] was used at 1:1000 dilution. ECL chemiluminescence detection reagents (Amersham Corp.) were used as described in the manufacturer's instructions to visualize antibody cross-reacting protein(s) on Kodak XAR-5 film.

Cloning of the Mutant mna6 Alleles by PCR. To define the nature of Mna6p mutations, the *MNA6* gene was cloned from each of the mutants and the wt parent strain 124 (*15*). The yeast chromosomal DNA containing the *MNA6* gene was isolated by the polymerase chain reaction (PCR) using a 5′ primer (ATATGTCGACATTCAGATGCCCATCTAAGAAGTCG) and a 3′ primer (CGAGAATTCGAGAGGCTGAGGATCAGTCAA) corresponding to the *MNA6* gene sequences 338 nt upstream and 29 nt downstream of the AUG and UGA codons, respectively. The PCR DNA product was cloned into the *Sal*I/*Eco*RI sites of pUC19. The identity of the *MNA6* gene in the pUC19 vector was initially determined by restriction endonuclease mapping followed by DNA sequencing.

Primer Extension Analysis. Primer extension reaction was carried out with radiolabeled primers and the isolated mt RNAs. Two oligonucleotides (ACGTATGACTCGTATGCGTCATGTCCTTA for the 15S rRNA and TATAAAGGTTTACCCTTAGGTT for the 21S rRNA), complementary to the sequence of the mt 15S rRNA or 21S rRNA starting from nt positions 129 or 145 downstream of the transcriptional start site of the respective gene, were synthesized for this experiment. The mt RNA was isolated from cells grown at 36 °C and then annealed to the ^{32}P-end-labeled primer. The reverse transcription was performed with Superscript (BRL) at 37 °C for 45 min. The primer-extended (i.e., cDNA) products were precipitated with ethanol, and analyzed by 6% polyacrylamide−8 M urea gel electrophoresis and autoradiography.

RESULTS

Several years ago we generated ts nuclear mutants of *Saccharomyces cerevisae* which lose mt RNAs at 36 °C. These mutants fall into eight complementation groups (i.e., *mna1* to *mna8*). The mutants belonging to *mna6*, *mna7*, and *mna8* complementation groups lose the 15S rRNA of the small subunit of mitoribosomes at 36 °C but not the 21S rRNA of the large mitoribosomal subunit (*15*). This finding suggested that these nuclear genes of yeast might code for r-proteins of the small subunit of mitoribosomes. In this study, we have characterized the *mna6* mutant alleles.

Cloning and Identification of the MNA6 Gene. To isolate the wt gene complementing the *mna6* mutation, a wt yeast genomic library was used for yeast transformation. The genomic library was made by insertion of 10−20 kb *Sau*3A partially digested chromosomal DNA fragments of *Saccharomyces cerevisiae* (strain GRF88) into the YCP50 yeast shuttle vector carrying a yeast selectable *URA3* marker (*23*). Since the original *mna6* mutants had poor transformability and no *ura3* nuclear marker, a new strain carrying both the ts phenotype and the *ura3-52* marker was generated by crossing the original *mna6-1* mutant (*MAT*$_a$ *his1 trp1* ts respiratory defect) with a wt haploid yeast CG379 (*MAT*$_\alpha$ *ura3-52 trp1-289 leu2-3 leu2-112 his7-2 ade5*) followed by meiotic segregation of the resulting diploid. This new *mna6* strain (*MAT*α *ura3-52 trp1-289 his1*, ts respiratory defect) was transformed with the yeast genomic library. Ura$^+$ transformants were selected and then tested for their ability to grow on complete glycerol medium at 36 °C. Of 5000 independent Ura$^+$ transformants, 4 were able to grow on glycerol plates at 36 °C. Moreover, cosegregation analysis also showed that the ability to grow on glycerol medium at 36 °C was strictly linked to the episomal Ura$^+$ prototrophy. The plasmid carrying the *MNA6* gene was recovered separately from four yeast transformants into *E. coli*, and then restriction endonuclease mapping was performed. Sub-

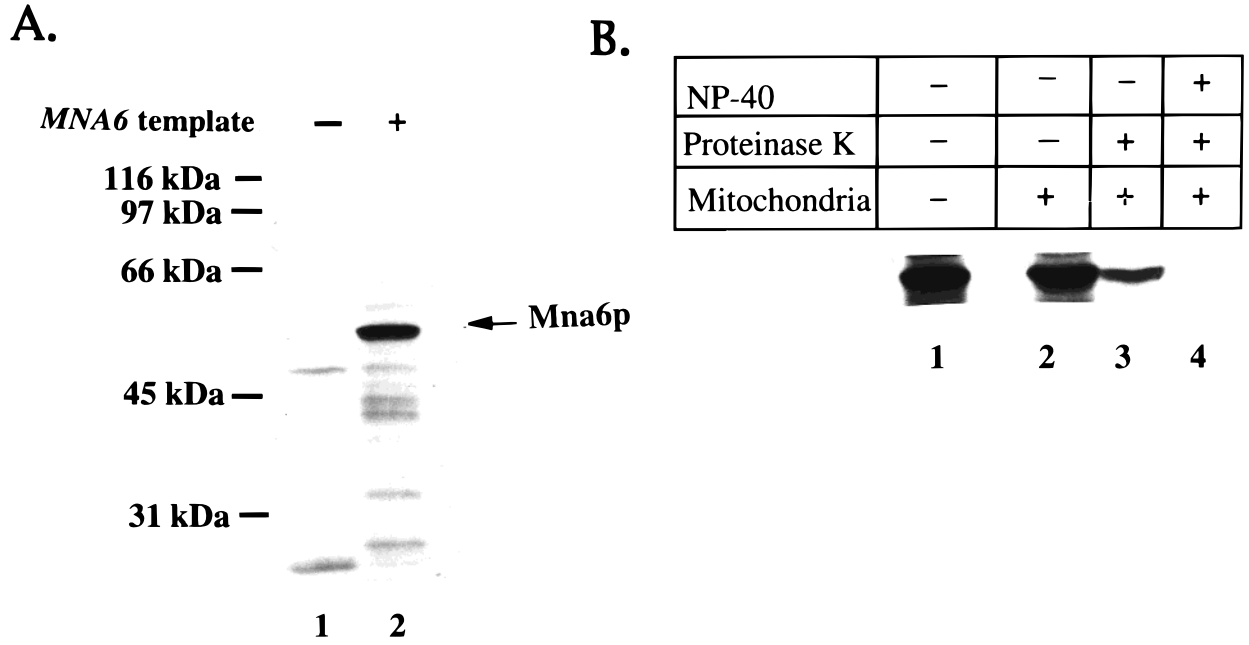


FIGURE 1: In vitro expression and import of Mna6p into isolated mitochondria. (A) In vitro expression of the cloned *MNA6* gene by the coupled transcription/translation systems using the TNT kit from Promega Corp. The protein was labeled with [^{35}S]methionine in the absence (lane 1) or in the presence (lane 2) of exogenous *MNA6* template. (B) In vitro import of Mna6p into isolated mitochondria. The ^{35}S-labeled protein was incubated with isolated mitochondria at 30 °C for 30 min. After incubation for another 30 min on ice with or without proteinase K, mitochondria were reisolated and then analyzed by SDS−PAGE/fluorography. Lane 1, Mna6p itself; lane 2, mt-associated Mna6p; lane 3, protease-resistant mt-associated Mna6p; and lane 4, Mna6p degradation after lysis of mitochondria with NP40 followed by proteinase K digestion.

cloning of well-defined restriction fragments determined the presence of the *MNA6* gene on an *Eco*RI fragment. A 2.2 kb *Eco*RI fragment, which was present in all four plasmids recovered from different transformants, suppressed the ts phenotype of the *mna6* mutant, suggesting that the entire *MNA6* gene is present in this *Eco*RI insert.

DNA sequencing was carried out with various oligonucleotides homologous to different regions of the gene as internal primers. The cloned *MNA6* gene carries a 1458 bp open reading frame that codes for a basic protein of estimated molecular mass 56 kDa. The protein sequence homology searches of the GeneBanks revealed that the *MNA6* encodes an S4-like r-protein (see below) and is also identical to the previously isolated *NAM9* gene (GeneBank Accession number M60730) (*17*). To confirm the genetic identity of the *MNA6* and *NAM9* genes, the yeast strain with the disrupted *nam9:URA3* gene (*MAT*α *his*3 *leu*2 *ura*3 *nam9:URA3*) (provided by Dr. Magdalena Boguta, Warsaw, Poland) was crossed with the ts *mna6* mutant (*MATa his*1 *trp*1). Diploids were selected by combination of the complementary auxotrophic markers (i.e., histidine-less SD-plate), and their respiratory function was examined on glycerol plates at both permissive (23 °C) and nonpermissive (36 °C) temperatures. The *nam9:URA3* disrupted strain itself did not grow on glycerol plates, as expected; both the *mna6* mutant and the *mna6*/Δ*nam9* diploid showed a ts respiratory-deficient phenotype (data not shown). This finding confirmed the earlier observation.

In Vitro Expression and mt Import of Mna6p. To demonstrate that the *MNA6* gene encodes a 56 kDa mt protein, the cloned *MNA6* gene was expressed under the coupled transcription−translation reaction conditions, and then used for in vitro mt import study. For this expression, the coding sequence of the *MNA6* gene was ligated to the 3′ end of a T7 promoter in the pRS316 vector (*18*). Mna6p was expressed in vitro from the cloned gene in the presence of [^{35}S]methionine. To determine the size of the in vitro product, the labeled protein was visualized by SDS−PAGE and fluorography (Figure 1). No protein in the size range of 56 kDa was detected in the absence of exogenous template (Figure 1, lane 1) whereas a ∼56 kDa protein was predominantly expressed in the presence of *MNA6* template (Figure 1A, lane 2). This result is consistent with the 486 amino acid open reading frame in the cloned *MNA6* gene.

To determine whether the in vitro synthesized protein is targeted to mitochondria, an import assay was performed as described under Materials and Methods. A major portion of the incubated protein was associated with the mitochondria (Figure 1B, lane 2), and a significant proportion of Mna6p was resistant to added proteinase K (lane 3). However, when mitochondria were lysed with the addition of Nonidet P-40 after the import assay, the Mna6p became sensitive to proteinase K (lane 4). This finding is indicative of the mt import of Mna6p.

Generation of Anti-Mna6p Antibody and Subcellular Localization of Mna6p. The in vitro import study demonstrated that Mna6p is capable of being targeted to mitochondria. To prove directly that Mna6p is indeed a mt protein, we have generated a polyclonal anti-Mna6p antibody and then used it for Western blot analysis. To examine the immunoreactivity of the rabbit anti -Mna6p antibody preparation, the yeast mt proteins were separated by SDS−PAGE, transferred to an Immobilon-P membrane, and then incubated with either preimmune serum or immune serum (Figure 2A). The resulting immunoblot revealed that a major 56 kDa yeast protein was decorated by anti-Mna6p antibody but not by preimmune serum. However, this protein band was not detected in the *mna6:URA3* disrupted strain, confirming its

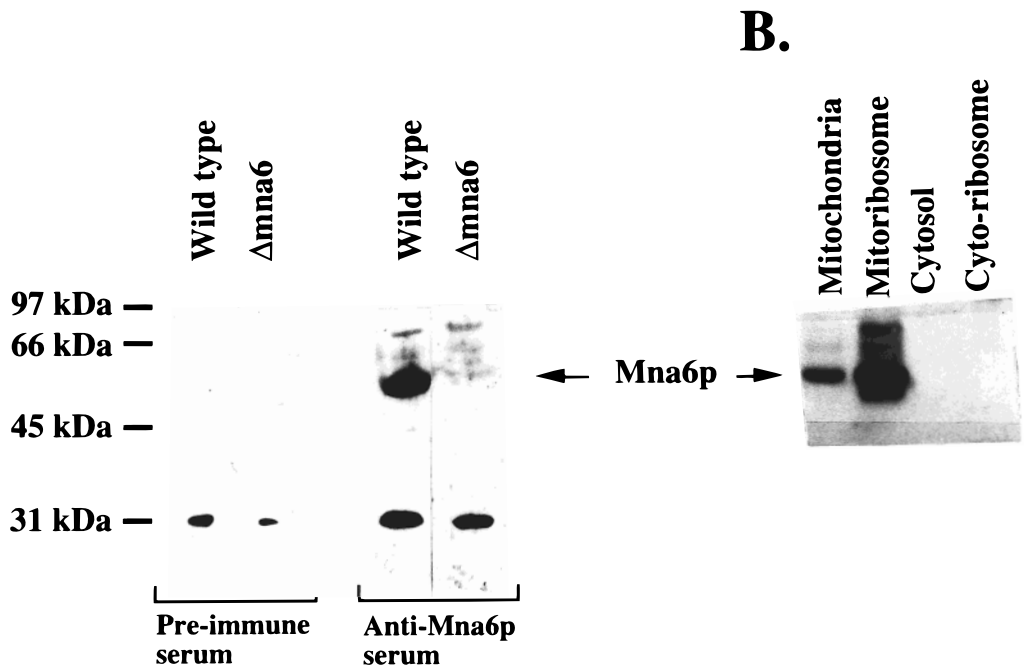


FIGURE 2: Generation of anti-Mna6p antibody and subcellular localization of Mna6p by Western blot analysis. (A) The anti-Mna6p antibody was prepared as described under Materials and Methods. Ten micrograms of mt protein was resolved by SDS−PAGE and then transferred to a nitrocellulose membrane. The membrane was then probed with either preimmune serum or anti-Mna6p antibody. (B) Subcellular localization of Mna6p. The yeast lysate was fractionated into mitochondria, mitoribosomes, cytosol, and cytosolic ribosomes, and then characterized by Western blot analysis using the anti-Mna6p antibody.

origin from the *MNA6* gene. A 31 kDa nonspecific band was also seen with both preimmune serum and anti-Mna6p antibody, and was probably a cross-contaminant. Since the sequence homology searches predicted that Mna6p is a r-protein, the presence of Mna6p in the mitoribosomes was further examined (Figure 2B). In a negative control experiment, the cytoplasmic ribosomes were also examined. It has been found that the 56 kDa Mna6p was predominantly present in the mt fractions, especially in the mitoribosomal pellet. This result corroborates the earlier suggestion that Mna6p is a mt r-protein.

Mna6p Localization on the Small Subunit of Mitoribosome. To determine whether Mna6p is a protein of the small or large subunit of mitoribosome, the 50S and 37S subunits of mitoribosome were separated by sucrose gradient centrifugation as illustrated in Figure 3. The identity of the small and large subunit peaks was confirmed by detecting the subunit-specific mt r-proteins (i.e., Mrp13, a 35 kDa protein of the small subunit; Mrp49, a 16 kDa protein of the large subunit). Both Mna6p and the small subunit r-protein Mrp13 were detected in the 37S peak fractions of the sucrose gradient, demonstrating that Mna6p is a r-protein of the small subunit of mitoribosomes.

Expression of Mna6p in Different Petite Strains. Since Mna6p is associated with the mitoribosomes, its accumulation might be influenced by the presence or absence of mt rRNA genes as is the case with some other mt r-proteins (*22*, *24*−*26*). This possibility was tested using different petite strains of yeast lacking various segments of the mt genome. This experiment is also useful to explore the dose effect of mt rRNAs on the accumulation of Mna6p since the copy number of mt rDNA in different petite strains varies due to deletion and amplification with circular redundancy of the remaining mt DNA to the size of the normal mt genome (*27*). By selecting several petites (i.e., CEP2, F11, P2, ND157, O_1P2, and rho°) and a wt strain (D273-10B), the accumulation of Mna6p under different mt genetic backgrounds was examined. The wt mt DNA of *Saccharomyces cerevisiae* carries 1 copy each of 35 genes including 2 rRNAs (21S and 15S). The CEP2 petite strain carries both the 21S rRNA and 15S rRNA genes, the F11 strain carries ∼7 copies of the mt 21S rRNA gene but no 15S rRNA gene, the P2 strain carries ∼10 copies of the mt 15S rRNA gene but no 21S rRNA gene, the ND157 carries neither of these 2 mt rRNA genes, the O_1P2 strain carries the 15S rRNA but no 21S rRNA gene, and the rho° strain carries no mt DNA (Figure 4A). Mitochondria were isolated from each strain grown in galactose medium, and Western blot analysis was carried out with either anti-Mna6p or anti-Mtf1 antibody. The mt transcription factor (Mtf1) was assessed as an internal standard. The highest level of Mna6p was found with the wt and CEP2 strains whereas strains lacking the 15S rDNA (F11, ND157, rho°) nevertheless contain easily detectable levels of Mna6p (Figure 4B). Mna6p was also detected in other petite strains albeit at much lower levels compared to the wt strain. This observation suggests that the 15S rRNA is not required for the stable expression of Mna6p though the steady-state level of Mna6p is highest in the presence of both 15S and 21S rRNAs.

Sequencing of the Mutant mna6 Alleles. Since the *mna6* mutants (*mna6-1*, *mna6-2*, *mna6-3*, and *mna6-4*) belonging to the same complementation group were generated from four independent mutagenesis experiments, they are expected to carry different mutations. To identify these mutations, each mutant allele of *MNA6* was cloned and sequenced. DNA sequencing was performed more than once using different batches of PCR products to avoid any sequence artifact attributable to misincorporation of nucleotide (nt) in the PCR procedure. By comparing the wt and mutant sequences, a single nt substitution was found in each mutant allele. A

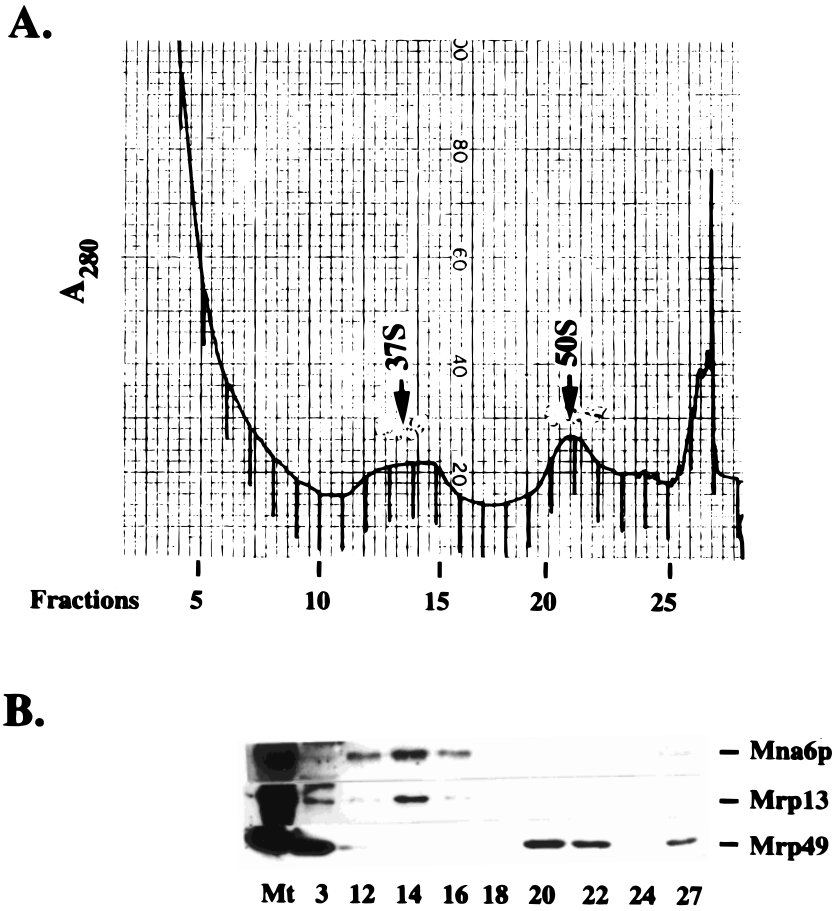


FIGURE 3: Submitochondrial localization of Mna6p. The large and small subunits of yeast mitoribosomes were separated from the wt strain by centrifuging the mt extract over a 15−27% sucrose gradient in a Beckman SW41 rotor at 24 000 rpm for 16 h. (A) Fractions (600 μL) of this gradient were collected from the top, and the protein peaks were detected by A_{280}. (B) The presence of Mna6p, Mrp13 (r-protein of the small subunit), and Mrp49 (r-protein of the large subunit) in these fractions was determined by Western blot analysis using cognate antibodies.

mismatch at nt position 332 in the case of *mna6-1* was found where an adenosine replaced a guanosine. Both the wt and other mutant alleles contained a guanosine nt at that location. Similarly, by comparing DNA sequences of other mutant alleles, a single C → T change at position 1313 of *mna6-2*, a single C → T change at position 325 of *mna6-3*, and a single C → T change at position 1271 of *mna6-4* were identified. All these nt changes are expected from the EMS mutagenesis which specifically modifies the guanosine residue. These nt changes created new codons with single amino acid substitutions of Arg^{111} → Lys in *mna6-1*, Pro^{438} → Leu in *mna6-2*, Leu^{109} → Phe in *mna6-3*, and Pro^{424} → Leu in *mna6-4*. The results are illustrated in Figure 5. Another single amino acid substitution (Ser^{82} → Leu) in the S4 domain of MNA9/Mna6p was also identified by Boguta et al. (*17, 29*) using a ts respiratory defect yeast mutant harboring the mutation. This mutation suppresses the ochre mutation in the mt genome by increasing the translational ambiguity of mitoribosome (*17*).

Is the Mutant Mna6p Stable at 36 °C? The ts phenotype and the loss of 15S rRNA in the mna6 mutants are probably due to a structural alteration of the mutant Mna6p. Since each Mna6p mutant contains a single amino acid substitution at a different location of the protein (see Figure 5), the molecular and biochemical consequences due to these mutations might be different. For example, the loss of 15S rRNA at nonpermissive temperature might be due to either inefficient binding of the mutant Mna6p to the 15S rRNA, the instability of mutant Mna6p itself, or an indirect role requiring Mna6p for proper mitoribosomal assembly. To gain insight into these possibilities, the presence of Mna6p in the mt subfractions of cells grown at permissive or nonpermissive temperature was also examined. The wt and mutant strains were grown in galactose medium over 15 h at 23 or 36 °C, and then mitochondria or mitoribosomes were isolated as described earlier. The presence of Mna6p in these mt fractions was tested by Western blot analysis (Figure 6). Irrespective of the growth temperatures, all mutant and wt strains have significant levels of Mna6p in their mitochondria. In contrast, the Mna6p levels in the mitoribosomal fractions of mutant strains but not in the wt strain vary significantly depending on growth temperature. This result suggests that these mutations in Mna6p did not block the mt import or mt accumulation of Mna6p, but probably affect the incorporation of Mna6p into the mitoribosomes.

To further examine the state of assembly of the small and large subunits of the mitoribosomes in these strains, we analyzed in the same preparations the distribution of other markers of the small (Mrp13) and large (Mrp49) mitoribosomal subunits. This was done by Western blot analysis using monoclonal anti-Mrp13 or anti-Mrp49 antibody. The level of the small subunit r-protein Mrp13 was substantially reduced in all four *mna6* mutants grown at 36 °C, and the most dramatic change was noticed with the *mna6-3* (Figure

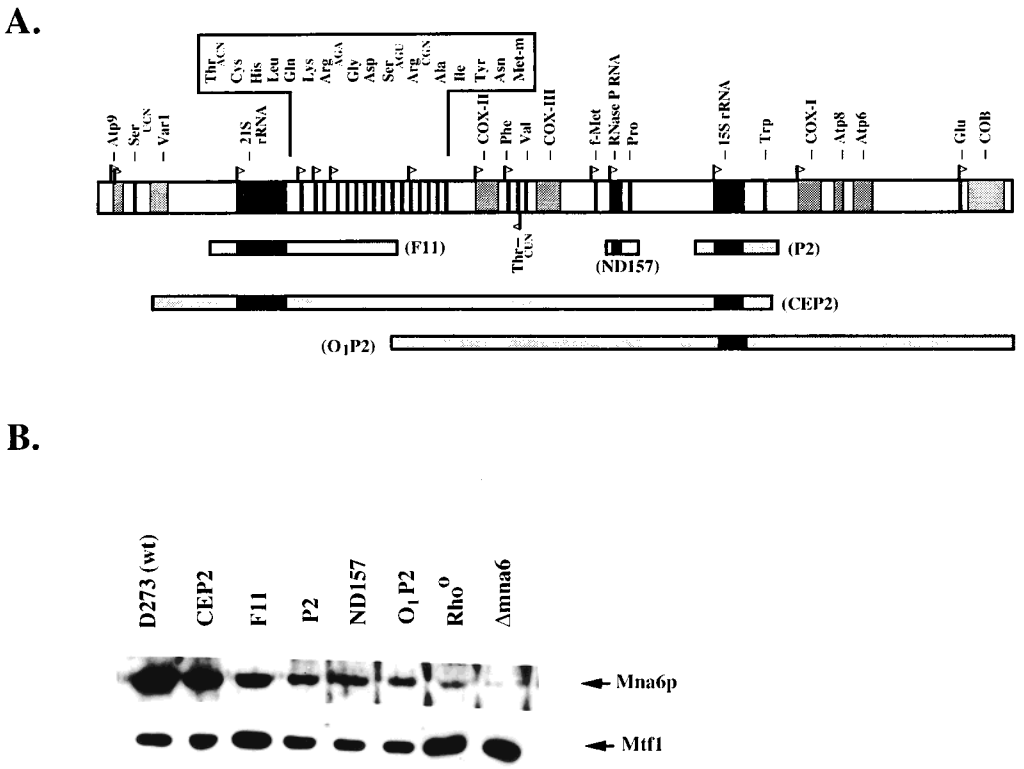


FIGURE 4: Levels of Mna6p in different petite strains. (A) The wt mt DNA of *Saccharomyces cerevisiae* carries 1 copy each of 35 genes including 2 rRNAs (21S and 15S) whereas petite strains carry multiple copies of the same gene due to a large deletion of the mt genome followed by amplification of the remaining mt DNA. (B) Yeast was grown in galactose medium, and mitochondria were isolated. Fifteen micrograms of mt protein was loaded onto an SDS−polyacrylamide gel, and then following electrophoresis, Western blot analysis was performed with the anti-Mna6p or anti-Mtf1 antibody.

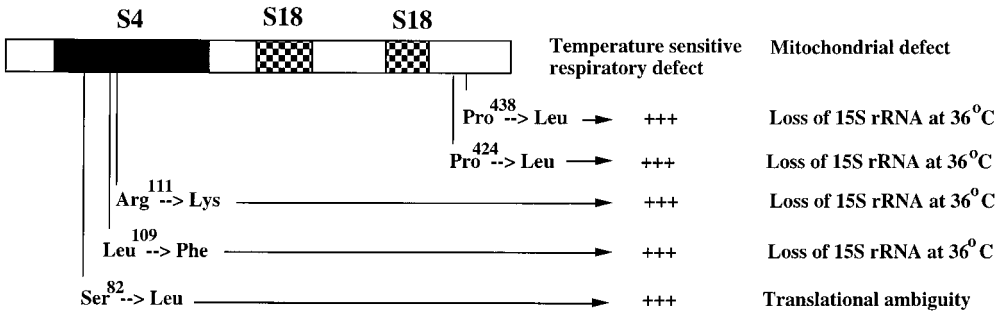


FIGURE 5: Schematic presentation of the location of Mna6p mutations and their consequences in mt function. The filled and checkered areas represent the conserved domains of S4 and S18 r-proteins, respectively (see legends of Figures 8 and 9 for details). The $Ser^{82} \rightarrow Leu$ change in the S4 domain of NAM9/Mna6p was previously isolated by Boguta et al. (*17*).

6). However, at 36 °C the level of the large subunit r-protein Mrp49 in most mitoribosomal preparations changed only modestly in three of the four mutants. Again, the only exception was the *mna6-3* mutant, whose Mrp49 concentration dropped significantly at nonpermissive growth conditions. Since a delicate regulation of the levels of individual mt RNA species operates at the level of differential transcription and transcript degradation (*28*), the degree of instability of mitoribosomes and/or its components in these yeast mutants may also vary depending on the growth conditions (e.g., time and/or temperature of cell growth).

Analysis of the Quality and Content of mt rRNAs by Primer Extension. To assess the biosynthesis and stability of mt rRNA in the *mna6* mutants, we have performed primer extension analysis with the wt and two *mna6* mutants (i.e., *mna6-1* and *mna6-3*) which showed the most profound loss of r-proteins in the mitoribosomal fractions. The mt RNA was isolated from cells grown at 36 °C for different time periods (i.e., 8, 12, or 24 h) and then annealed to the end-labeled primers as described under Materials and Methods. The 157 and 77 nt cDNAs were expected from the precursor and mature forms of the 15S rRNA, respectively, whereas a 166 nt cDNA would be made on the 21S rRNA template. The primer extension result is shown in Figure 7. As predicted, the levels of the precursor and mature 15S rRNA transcripts as well as the 21S rRNA in the wt strain did not change over 24 h of growth at 36 °C (Figure 7, lanes 1−3). In contrast, both mutant strains exhibited a gradual decrease in the 15S rRNA levels with time of cell growth at 36 °C (Figure 7, lanes 4−9). Mutant *mna6-3* had completely lost the 15S and 15.5S rRNA by 24 h at 36 °C. It was also apparent that the ratio of the precursor to the mature form of the 15S rRNA transcript is significantly higher in the mutants compared to the wt strain. This could be due to the slow processing of the 80 nt presequence from the precursor 15.5S rRNA in the mutant strains. However, these mutants

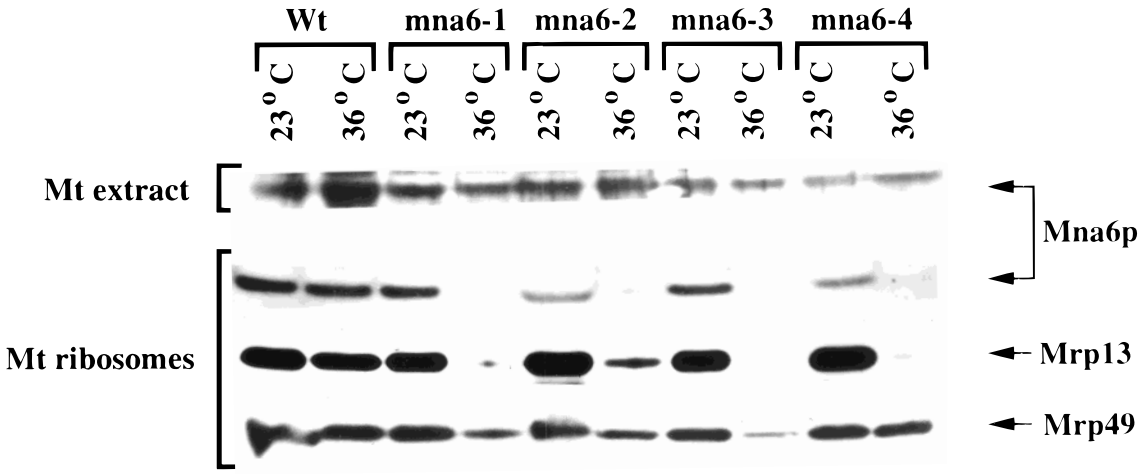


FIGURE 6: Mna6p in the mitochondria of yeast mutants grown at permissive or nonpermissive temperature. The wt and mutant strains were grown in galactose medium at permissive (23 °C) or nonpermissive (36 °C) temperature, and then mitochondria and mitoribosomes were isolated. Proteins from the total mt extract or isolated mitoribosomes were analyzed by SDS−PAGE followed by Western blot analysis using antibody against Mna6p, Mrp13, or Mrp49.

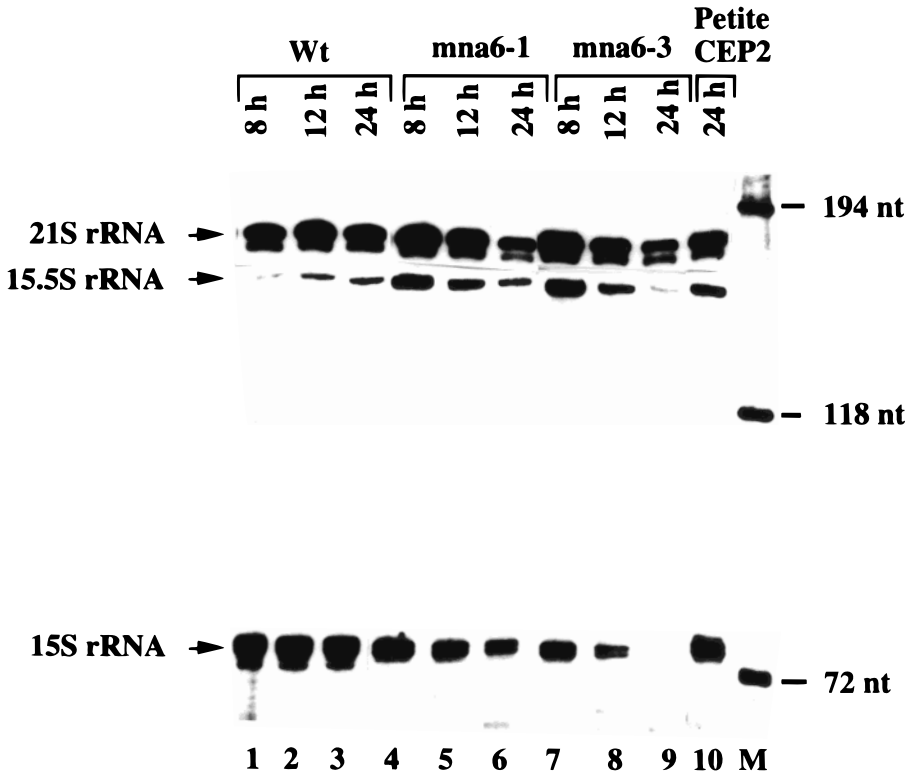


FIGURE 7: Primer extension on mt RNA. The end-labeled primer complementary to the sequence of the mt 15S or 21S rRNA, 129 or 145 nt downstream from the transcriptional start site, was used in this experiment. Five micrograms of mt RNAs isolated from cells grown at 36 °C was annealed to 10 pmol of an end-labeled primer by heating at 70 °C for 5 min and cooling slowly to room temperature. The reaction was started by adding 200 units of reverse transcriptase (Superscript, BRL) and then incubated at 37 °C for 45 min. The cDNA products were precipitated with ethanol, and then visualized by 6% polyacrylamide−8 M urea gel electrophoresis and autoradiography. The 157 and 77 nt long cDNAs were from the precursor and mature forms of the 15S rRNA, respectively, whereas a 166 nt cDNA was made on the 21S rRNA template.

did not exhibit any significant changes of the 21S rRNA levels when grown up to 12 h at 36 °C; but thereafter the 21S rRNA levels decreased. It is possible that the partial loss of the 21S rRNA with extended growth at 36 °C could have been due to the reduced levels of the large subunit of mitoribosome in the absence of its partner small subunit. Interestingly, a petite yeast strain (i.e., CEP2) carrying both the 15S rRNA and 21S rDNA but lacking the mt protein synthesis was able to generate the normal levels of both rRNAs (lane 10). This suggests that the disappearance of rRNAs in the *mna6* mutants is not simply attributable to a defect of mt protein synthesis.

DISCUSSION

Eukaryotes have two distinct translation systems, one in the cytoplasm and one in the mitochondria. The cytoplasmic and mt ribosomes are structurally and functionally different, and their r-proteins are encoded by separate sets of nuclear genes. In *Saccharomyces cerevisiae*, as many as 200 of the estimated 6000 total nuclear genes might specify cytoplasmic

and mt r-proteins. Isolation and characterization of these genes will provide valuable information about the structure, function, and evolution of these two different sets of r-proteins in the same eukaryotic cell. For analysis of nuclear−mitochondrial interaction, yeast has been a good model system not only because of the genetic power but also because yeast is a facultative anaerobe capable of surviving in the absence of mt function, a characteristic not shared with other eukaryotes.

We have previously generated a series of ts nuclear mutations of *Saccharomyces cerevisiae* which are defective in mt nucleic acid biosynthesis (i.e., *mna* mutants) (*15*). In this work, the yeast mutants belonging to the *mna6* complementation group were investigated by cloning and sequencing the wt and mutant alleles of the *MNA6* gene, and characterization of the *MNA6* gene product, especially its stability and potential role in mitoribosomal assembly. Based on its sequence, Mna6p is clearly a mt homologue of the S4 r-protein family (Figure 8). However, Mna6p is a much bigger protein than other S4 homologues due to the presence of an unusual CTE (Figure 9). The Western blot analysis revealed that Mna6p is indeed a r-protein of the small subunit of mitoribosomes so that its altered structure could account for the loss of mt 15S rRNA in the *mna6* mutants at nonpermissive temperature.

DNA sequencing of mutant alleles of *MNA6* revealed that each carries a single amino acid change in the Mna6p. Each resulted from a single nt change consistent with the EMS mutagenesis used to derive these mutants. The amino acid changes in *mna6-1* and *mna6-3* are conservative, the cationic lysine substituting for arginine at position 111 and the hydrophobic phenylalanine substituting for leucine at position 109 (Figure 5). Arg^{111} is highly conserved though some S4 homologous r-proteins do have a lysine residue in this position (Figure 8). However, all organellar (mitochondria and chloroplasts) S4 homologues sequenced as well as *E. coli* S4 have arginine conserved in this position. Similarly, the Leu^{109} is conserved in organellar S4 homologues. Phenylalanine is not found in this position of any of the normal sequences examined. It is noteworthy that both of these mutated residues are the first and third residues of a very highly conserved tripeptide (hydrophobic−hydrophobic−cationic) in these S4 homologues. The fact that the mutations in *mna6-1* and *mna6-3* are themselves very conservative suggests that normal functions and interactions of Mna6p require precision in the spatial relationship around this tripeptide. On the other hand, the mutations involved in alleles *mna6-2* and *mna6-4* are both proline to leucine changes within the CTE. As proline is a helix breaker, the conformation of the mutant protein is probably disrupted in this region at the nonpermissive temperature. Previously, Dmochowska et al. (*29*) reported that the $Ser^{82} \rightarrow$ Leu change in the S4 domain of Mna6p suppresses an ochre mutation in the yeast mt genome, and also causes the ts respiratory defect of yeast.

Based on information from studies of other S4 r-proteins, the S4 domain of Mna6p might directly interact with the 15S rRNA. It has been found that the Lys^{82} of *E. coli* S4 r-protein, an equivalent to Lys^{89} in Mna6p, has been cross-linked to the *E. coli* 16S rRNA (*30*). The crystal structure analysis of *Bacillus stearothermophilus* S4 r-protein revealed an extensive rRNA-binding surface of this protein (*31*). Interestingly, several of these potential rRNA-binding amino acid residues of *B. stearothermophilus* S4 are highly conserved, and are also present in Mna6p. For example, the Arg^{93}, Asp^{95}, Tyr^{99}, Arg^{100}, Arg^{108}, Arg^{111}, Gln^{112}, His^{116}, Arg^{125}, Ser^{130}, Tyr^{131}, and Arg^{142} residues of *B. stearothermophilus* S4 correspond to the Arg^{104}, Asp^{106}, Phe^{110}, Arg^{111}, Arg^{119}, Arg^{122}, Gln^{123}, His^{127}, Lys^{136}, Ser^{141}, Tyr^{142}, and Lys^{153} residues in the S4 domain of Mna6p, respectively (see Figure 8). All these data suggest that Mna6p, like S4 homologues in bacteria, might be one of the first r-proteins to bind to rRNA during the early assembly of the 37S subunit. A defect of altered Mna6p in binding to the 15S rRNA may destabilize the latter. On the other hand, the presence of steady-state levels of Mna6p in the mitochondria does not require the availability of its partner 15S rRNA. Previously, other laboratories demonstrated that the accumulation of some mt r-proteins is tightly linked to their assembly onto the mitoribosomes (*22*, *24*−*26*) while others are virtually unaffected by the presence or absence of some mt ribosomal components (*32*). The differences in the behavior of individual proteins may relate to their position in the pathway for the assembly of mitoribosomes.

Clearly, conditional mutations in this small r-protein Mna6p influence the assembly of the small ribosome under nonpermissive conditions. We presume that the loss of 15S rRNA is the result of its accelerated degradation in the absence of a stable small mitoribosomal subunit. This phenotype is not attributable to a dramatic decline of the Mna6p. Indeed, in each of the mutants there are only modest differences in the amounts of Mna6p expressed in the mitochondria regardless of the growth temperature. Though present in the mitochondria, little Mna6p was found in the mitoribosome when mutant yeast was grown at nonpermissive temperature with the precise amount of Mna6p varying with the nature and site of the mutations. The assembly of the small mitoribosome is assessed by the colocalization of 15S rRNA, Mna6p, and a small r-protein marker, Mrp13. The two mutations in the CTE (i.e., *mna6-2* and *mna6-4*) have recognizable Mna6p in the mitoribosomal fraction. *Mna6-2*, the yeast strain that has a mutation closest to the C-terminus, has the least profound loss of the Mna6p in the mitoribosome. As expected, if Mna6p is a primary 15S rRNA interacting protein, other small mitoribosomal proteins would not be expected in the mitoribosomal particle. This was indeed the case for Mrp13, another small mitoribosomal protein. Here too the lesion is least dramatic for *mna6-2*. It is the mutations in the S4 homology domain (i.e., especially *mna6-3*) that exhibit the most profound deficiency in mitoribosomal assembly in yeast grown at nonpermissive temperature. However, using a large r-protein Mrp49 as a marker of the large ribosomal assembly, it is clear that variations in Mna6p and its consequent impact on small mitoribosomal assembly and stability have an additional impact on the biosynthesis and/or stability of the large ribosomal subunit. Only *mna6-4* showed no difference in the amount of Mrp49 recovered in the mitoribosomal fraction. The other three mutants all exhibit lower amounts of Mrp49 in the mitoribosomal fraction when cells are grown at nonpermissive temperature. This is particularly evident in *mna6-3*, one of the S4 homology domain mutants. The basis for the stable interaction between small and large ribosomal subunits is not clear.

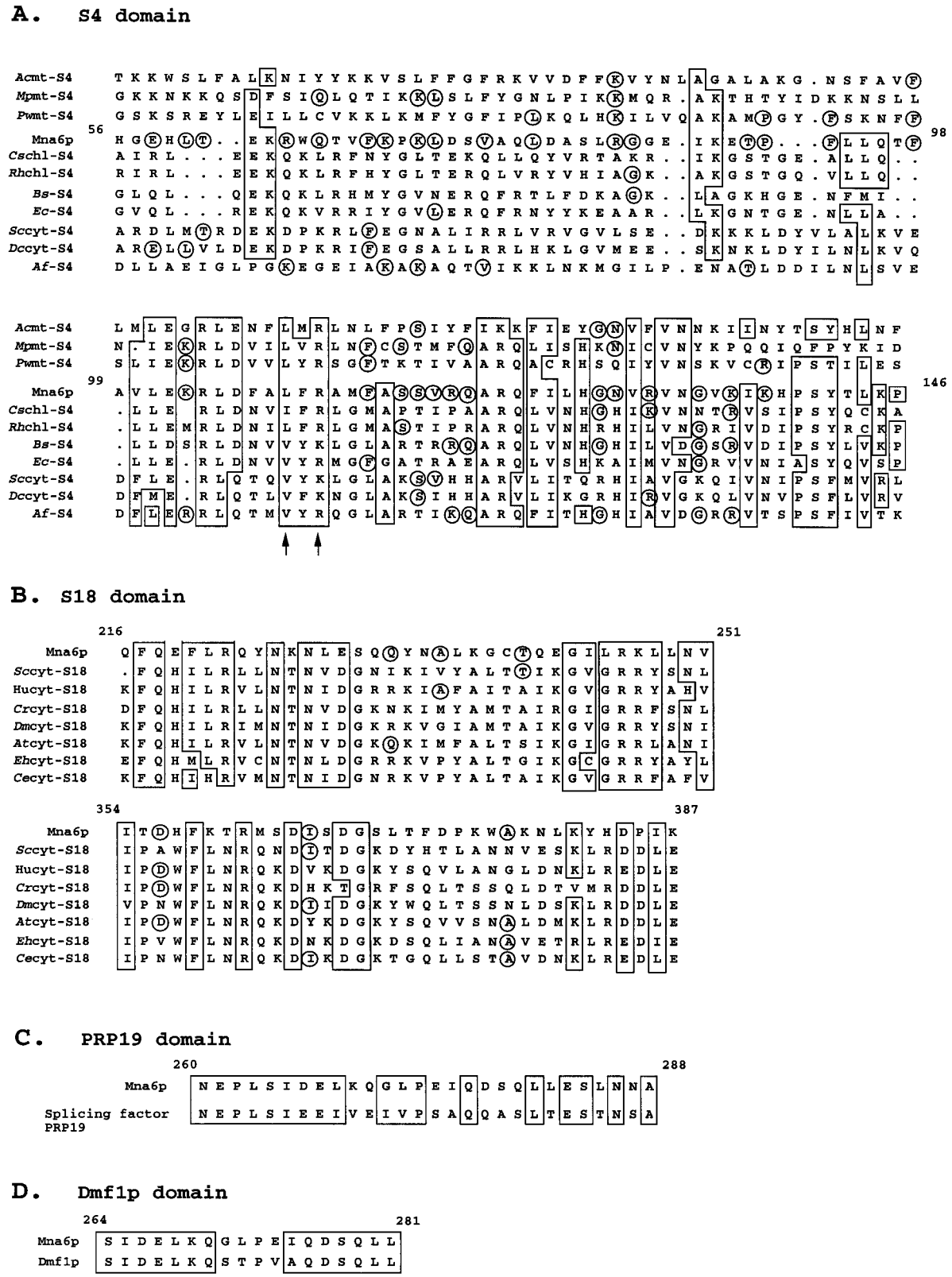
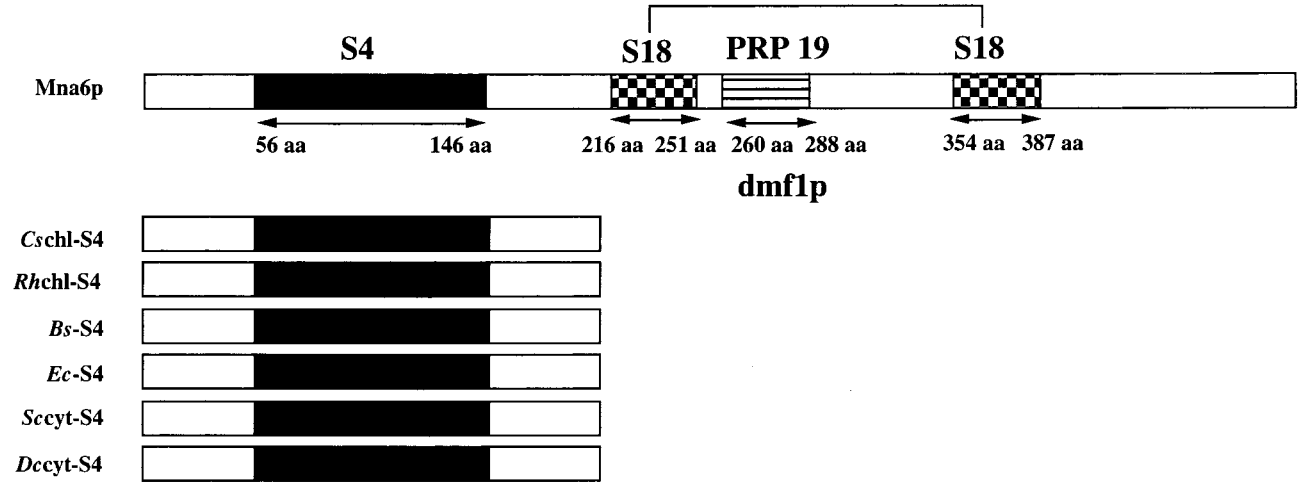


FIGURE 8: (A) Amino acid sequence conservation between the S4 r-protein homologues from 11 different species. The highly conserved amino acid residues are boxed whereas the less conserved amino acids are circled. The locations of Mna6p mutations in the S4 domain (i.e., Leu[109]→Phe and Arg[111]→Lys) are indicated by the vertical arrowheaded lines. *Ac*mt-S4, mt S4 r-protein of *Acanthamoeba castellanii*; *Mp*mt-S4, mt S4 r-protein of *Marchantia polymorpha*; *Pw*mt-S4, mt S4 r-protein of *Prototheca wickerhammi*; *Cs*chl-S4, chloroplast S4 r-protein of *Cryptomonas sp.*; *Rh*chl-S4, chloroplast S4 r-protein of *Rhapis humilis*; *Bs*-S4, S4 r-protein of *B. subtilis*; *Ec*-S4, S4 r-protein of *E. coli*; *Sc*cyt-S4, cytoplasmic S4 r-protein of *Saccharomyces cerevisiae*; *Dc*cyt, cytoplasmic S4 r-protein of *Dictyostelium*; *Af*-S4, S4 r-protein of *Archaeoglobus fulgidus*. (B) Sequence homology between Mna6p and the S18 r-protein family. *Sc*cyt-S18, cytoplasmic S18 r-protein of *Saccharomyces cerevisiae*; Hucyt-S18, cytoplasmic S18 r-protein of human; *Cr*cyt-S18, cytoplasmic S18 r-protein of *Chlamydomonas reinhardtii* (alga); *Dm*cyt-S18, cytoplasmic S18 r-protein of *Drosophila melanogaster*; *At*cyt-S18, cytoplasmic S18 r-protein of *Arabidopsis thaliana* (plant); *Eh*cyt-S18, cytoplasmic S18 r-protein of *Entamoeba histolytica* (amoeba); *Ce*cyt-S18, cytoplasmic S18 r-protein of *C. elegans*. (C) Sequence homology between Mna6p and the yeast nuclear splicing factor PRP19. (D) Sequence homology between Mna6p and the dmf1p cell division protein of *Schizosaccharomyces pombe*.

A.



B.

Amino acid residues in the mt S4 r-protein		"A+T" nt-content in the mt small rRNA
Yeast mitochondria	486 aa	77%
P. wickerhamii mitochondria	511 aa	66%
A. castellanii mitochondria	374 aa	67%
A. thaliana mitochondria	362 aa	54%
M. polymorpha mitochondria	194 aa	48%
E. coli	203 aa	45%
Sunflower chloroplast	201 aa	46%

FIGURE 9: (A) Locations of the conserved sequence domains in Mna6p and in other S4 r-proteins. (B) Co-relation between the large-size polymorphism of the mt S4 protein and the high "A+T" nt-content in their small rRNAs.

Our observations on the assembly or stability of the small mitoribosomal subunit as a consequence of a defect in the function of a small mitoribosomal protein are similar to those made for yeast cytoplasmic ribosome assembly (*33*, *34*). In this latter case, depletion of a r-protein (i.e., L16) results in impaired processing or increased degradation of the rRNA or its precursor (*34*). Also the synthesis of other subunit protein components may be affected. However, in regard to the interaction between the cytoplasmic subunits, no effect of a depletion of a protein of one subunit on the assembly of the other subunit has hitherto been noted. This is unlike the apparent interaction of subunit assembly observed in this study. In this case, a defect of the small mitoribosomal component Mna6p influences the concentration of the large subunit protein Mrp49.

GeneBank searches for Mna6p homologues revealed that the N-terminal half of Mna6p carries a highly conserved domain of *E. coli* S4 r-protein. Figure 8A shows the predicted Mna6p sequence aligned with 10 representatives selected from the over 50 known members of the S4 r-protein superfamily including 34 chloroplast S4 proteins. Of the 10 members in the alignment, 3 were mt S4 r-proteins from the predicted gene sequences of bryophyte plant *Marchantia polymorpha*, of chlorophyte alga *Prototheca wickerhammi*, and of amoeboid protozoan *Acanthamoeba castellanii*. Mna6p (i.e., its S4 homology domain) shows similarity to the *E. coli* S4 r-protein in a number of ways: (i) both proteins are basic in nature and contain a well-conserved S4 domain; (ii) they play a critical role in the assembly and translational fidelity of the ribosomes with which they are associated; and (iii) both proteins appear to be the primary rRNA binding proteins. The *E. coli* S4 r-protein has been extensively studied in different laboratories, and there is a wealth of information regarding the role of *E. coli* S4 r-protein in the assembly and function of *E. coli* ribosomes (*10*, *35*, *36*). These include tRNA and 16S rRNA binding sites, and a site for the late subunit assembly reactions with S1, S2, S10, S18, and S21 r-proteins. The mutations in Mna6p (i.e., positions 109 and 111) would be encompassed within the S4 domain, and so may be inferred to affect the interaction with the 15S rRNA. The *E. coli* S4 is known to bind directly to the highly conserved "530-loop" of 16S rRNA (*36*) which has an

important role in codon−anticodon recognition and in the maintenance of translational accuracy (*37*). Interestingly, a mutation (i.e., $G_{589} \rightarrow A$) in the similar stem−loop structure of the mt 15S rRNA also causes translational ambiguity of the yeast mitoribosomes (*38*). The S4 domain and its RNA binding capacity may have other effects than the organization of the small ribosomal subunit. In *E. coli*, S4 has been shown to regulate r-protein gene expression (*39*). This could possibly relate to effects on the accumulation of other r-proteins, perhaps of both ribosomal subunits, thereby coordinating the synthesis of both subunits.

The recent Yeast Protein Data (*7, 40*) lists 18 other confirmed or probable yeast mt r-proteins showing a high degree of sequence homology to the known *E. coli* r-proteins. For example, the small subunit r-proteins MrpS2, MrpS4/MrpS8, MrpS5, MrpS9, MrpS21, and MrpS28 of yeast mitochondria are homologous to *E. coli* S14, S2, S5, S9, S21, and S15, respectively; whereas the large subunit r-proteins of yeast mitochondria, MrpL2, MrpL6, MrpL7, MrpL8, MrpL9, MrpL10, MrpL14, MrpL16, MrpL20, MrpL33, MrpL38, and MrpL39, carry conserved domains of *E. coli* L27, L4, L5, L17, L3, L15, L10, L16, L23, L30, L14, and L33, respectively. The possibility of the existence of other mt homologues cannot be ruled out since the protein data information from the yeast genome sequence has not been completed yet. Surprisingly, seven of these conserved mt r-proteins are much larger than their *E. coli* counterparts. The mt r-proteins MrpL7 and MrpL20 carry a large N-terminal extension whereas the other five mt r-proteins, MrpS4, MrpS9, MrpS28, Mna6p, and MrpL9, have a long CTE. Mna6p itself is 2.5 times larger than *E. coli* or chloroplast S4 r-protein (i.e., 486 amino acids of Mna6p versus ∼200 amino acids of bacterial or chloroplast S4 protein) (Figure 9A). However, this increment in size is not distributed along the whole linear sequence of the protein; rather, there is a highly homologous S4 domain flanked by a nonhomologous extension at the C-terminal end of the molecule. A recent analysis of yeast mt r-protein MrpS28 demonstrated that an amino-terminal extension of 117 amino acids and the S15 r-protein-like domain at the C-terminus are both essential for MrpS28 activity but these 2 domains can also function in trans (*41*).

The significance of a long CTE of Mna6p is not clear, although there are three other conserved domains in the CTE. Our two mutants in the CTE do not directly affect any of these homology domains. The alignment in Figure 8B includes seven selected cytoplasmic S18 r-proteins from lower and higher eukaryotes. Amino acids 216−251 and 354−387 of Mna6p are homologous to the eukaryotic sequences of cytoplasmic S18 r-proteins (*42, 43*), which are in the size range of 150 amino acids and are mainly involved in the initiation of translation. The role of Mna6p in translational initiation in yeast mitochondria has not been explored. Interestingly, a reduced dosage of cytoplasmic r-protein S18 suppresses a mt initiation codon mutation in *Saccharomyces cerevisiae* (*44*) although the mechanism is unknown. Residues 260−288 and 264−281 closely resemble the precursor RNA processing factor (PRP19) of *Saccharomyces cerevisiae* (*45, 46*) and the cell division protein dmf1p of *Schizosaccharomyces pombe* (*47*), respectively (Figure 8C,D). Unlike the mammalian mt genome, the yeast mt DNA carries several intervening sequences in three different genes (i.e., *COB*, *COX*1, and *21S rRNA* genes) (*48*). These mt introns are classified as group I and group II according to their self-splicing mechanism and by the similarity of their predicted secondary structure to other self-splicing introns. Genetic and biochemical analyses of yeast demonstrated that some mt introns undergo self-splicing in vitro, whereas splicing of other introns requires protein factor(s) encoded either by the introns themselves (i.e., maturase), by nuclear genes (i.e., mt tRNA synthetase), or by both (*4, 49−51*). Furthermore, the yeast mt group I intron cytochrome b_1-5 exhibits autosplicing in vitro at high Mg^{2+} concentrations, but requires the splicing factor CBP2 for reaction under physiological conditions (*52*). It is possible that the in vivo splicing of mt introns might be catalyzed by a protein complex (i.e., spliceosome), and that the PRP19 domain of Mna6p could be a part of this spliceosome protein complex. Dmf1p of fission yeast participates in cell division (*45*). Since the sporulation of bakers' yeast requires functional mitochondria (*27*), the Dmf1p domain of Mna6p might be important for this purpose. In addition, the CTE also shows some minor sequence homologies with membrane proteins of *Mycoplasma hominis*, yeast, *B. burgdorferi*, and T-cell, with *Porphyra* chloroplast histidyl-tRNA synthetase, with oligopeptide-binding protein of *B. subtilis*, and with *S. pombe* helicase. It is noteworthy that a few yeast proteins appear to have more than one function. For example, the cytoplasmic r-protein L32 of *S. cerevisiae* is involved both in translation and in RNA processing (*53*) whereas the yeast *MMS19* gene product participates in DNA repair and RNA polymerase II transcription (*54*). Two other yeast mt r-proteins (i.e., MrpL8, MrpL33), like Mna6p, also carry more than one sequence homology domain (*38*). According to the yeast protein data bank information, MrpL8 has two homology domains: *E. coli* r-protein L17 and S13 sequences at the N-terminus and C-terminus, respectively. MrpL33 carries an *E. coli* L30 r-protein domain at the N-terminus and a yeast cytoplasmic L16 r-protein domain at the C-terminus. There is also evidence for bifunctionality for a number of r-proteins that are involved in either replication, transcription, RNA processing, DNA repair, or autogenous regulation of translation in *E. coli*, yeast, human, *Xenopus laevis*, and *Drosophila* (*55*).

Several possibilities regarding the function and evolution of these extension sequences can be entertained. (i) Since the increment in size is not distributed throughout the whole sequence of the mt r-proteins, these sequence extensions could have been created by gene fusion. (ii) The additional protein sequence at the C- or N-terminal extension of some mt r-proteins may compensate for the absence of 5S rRNA in yeast mitochondria. (iii) Are these sequence extensions related to the fact that *S. cerevisiae* is such an adaptable facultative anaerobe, requiring its function under glucose-repressed, glucose-derepressed, or anaerobic circumstances? It is noteworthy that the yeast cytoplasmic homologue of S4 r-protein does not carry the CTE sequence. (iv) Since yeast mt DNA has a very high A+T base composition, this is true also of the rRNAs. So one possibility is that the novel sequence extension of these mt r-proteins is required to bind rRNA of unusual base composition. In fact, there is a good correlation between the large size polymorphism of mt S4 r-protein and the high "A+T" content of mt genome in some other species also (Figure 9B).

In conclusion, Mna6p is a mt homologue of the S4 r-protein family carrying an unusual CTE with some other conserved sequence domains. We are currently exploring how the Mna6p mutations affect the biosynthesis of 15S rRNA and the mitoribosome.

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