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A functional dominant mutation in *Schizosaccharomyces pombe* RNase MRP RNA affects nuclear RNA processing and requires the mitochondrial-associated nuclear mutation *ptp1-1* for viability

Janet L.Paluh[1] and David A.Clayton[2]

Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305-5427, USA

[1]Present address: Department of Cell and Molecular Biology, 345, Life Sciences Addition, University of California, Berkeley, CA 94720-3200, USA

[2]Corresponding author

The essential gene for RNase MRP RNA, *mrp1*, was identified previously in *Schizosaccharomyces pombe* by homology to mammalian RNase MRP RNAs. Here we describe distinct site-specific mutations in RNase MRP RNA that support a conserved role for this ribonucleoprotein in nucleolar 5.8S rRNA processing. One characterized mutation, *mrp1-ND90*, displays dominance and results in accumulation of unspliced precursor RNAs of dimeric $tRNA^{Ser}$-$tRNA^{Met}i$, suggesting a novel nuclear role for RNase MRP in tRNA processing. Cells carrying the *mrp1-ND90* mutation, in the absence of a wild-type copy of *mrp1*, additionally require the mitochondrially associated nuclear mutation *ptp1-1* for viability. Analysis of this *mrp1* mutation reinforces previous biochemical evidence suggesting a role for RNase MRP in mitochondrial DNA replication. Several mutations in *mrp1* result in unusual cellular morphology, including alterated nuclear organization, and are consistent with a broader nuclear role for RNase MRP in regulating a nuclear signal for septation; these results are a further indication of the multifunctional nature of this ribonucleoprotein.

Keywords: cell morphology/RNase MRP RNA/rRNA processing/*Schizosaccharomyces pombe*/tRNA processing

Introduction

The endoribonuclease ribonucleoprotein (RNP) RNase MRP is localized to both mitochondria (Li *et al.*, 1994; Davis *et al.*, 1995; or cytoplasm, in a pattern that is consistent with mitochondrial localization, Matera *et al.*, 1995) and the nucleolus (Reimer *et al.*, 1988; Gold *et al.*, 1989; Jacobson *et al.*, 1995). RNase MRP initially was characterized biochemically from human, mouse, cow, frog and yeast (Chang and Clayton, 1987; Topper and Clayton, 1990; Bennett *et al.*, 1992; Schmitt and Clayton, 1992; Dairaghi and Clayton, 1993; Paluh and Clayton, 1995) for its ability to process *in vitro* synthesized RNAs corresponding to either the leading strand origin of mitochondrial DNA (mtDNA) replication in vertebrates or the *Saccharomyces cerevisiae ori5* putative origin sequence. In the nucleolus, RNase MRP RNA is believed to play a role in rRNA maturation since mutations in *S.cerevisiae* RNase MRP RNA are associated with altered 5.8S rRNA processing (Schmitt and Clayton, 1993; Chu *et al.*, 1994) and *in vitro* RNase MRP accurately processes pre-rRNA substrates (Lygerou *et al.*, 1996).

RNase MRP RNP has been identified solely in eukaryotes and contains a single RNA component (for review, see Schmitt *et al.*, 1993) that shows considerable structural similarity to that of the related RNP RNase P (Forster and Altman, 1990). RNase P is ubiquitous and best known for its role in processing the 5′ end of precursor tRNAs (for reviews, see Darr *et al.*, 1992; Altman *et al.*, 1993). However, RNase P also processes the precursor to 4.5S RNA in *Escherichia coli* (Bothwell *et al.*, 1976) and is able to process previously characterized RNase MRP mitochondrial substrates *in vitro* at a separate unique site (Potuschak *et al.*, 1993). Stoichiometric co-immunoprecipitation of both RNase MRP and RNase P RNPs using antibodies from patients having certain autoimmune diseases (Hashimoto and Steitz, 1983; Reddy *et al.*, 1983; Gold *et al.*, 1988, 1989) suggests that these RNPs share a common 40 kDa protein component referred to as the Th/To antigen (Reimer *et al.*, 1988; Yuan *et al.*, 1991). A single shared protein component of RNase MRP and RNase P, POP1p, has been identified in *S.cerevisiae* (Lygerou *et al.*, 1994) that may be related to the Th/To antigen. A temperature-sensitive lethal mutation, *pop1-1*, in the gene encoding this protein results in altered 5.8S rRNA processing as well as defects in tRNA processing (Lygerou *et al.*, 1994).

The genes encoding the RNA component of RNase MRP from *S.cerevisiae* and *Schizosaccharomyces pombe* are essential (Schmitt and Clayton, 1993; Paluh and Clayton, 1995). However, the precise function of RNase MRP required to maintain cell viability has not been determined. Although altered 5.8S rRNA processing is observed in *S.cerevisiae*, it is unlikely that this defect alone results in cell death. This interpretation rests on the observation that multiple forms of 5′ processed 5.8S rRNAs are found assembled into ribosomes (Rubin, 1974) and the ratio between use of alternative pathways for production of 5.8S rRNA in *S.cerevisiae* can be altered without resulting in loss of viability (Henry *et al.*, 1994).

To understand better both the nuclear and mitochondrial functions of RNase MRP, previously we generated site-specific mutations in *S.pombe mrp1* at sites conserved between other RNase MRP RNAs and shared with the cage region of RNase P RNAs (Paluh and Clayton, 1996). We established a system of plasmid shuffle for fission yeast to analyze these mutations and screened plasmid-borne mutations for their ability to complement a chromosomal deletion of *mrp1*. This technique is exploited in the present work to include a strain carrying the nuclear mutation *ptp1-1*. In *S.pombe*, *ptp1-1* is one of two loci

identified to be required for loss of mtDNA (rho°) following prolonged exposure to ethidium bromide (Haffter and Fox, 1992). Here we describe several mutations in the gene for *S.pombe* RNase MRP RNA that confirm a conserved nucleolar role for this RNP in 5.8S rRNA processing and provide the first genetic evidence linking RNase MRP to mitochondrial biogenesis. Additionally, we present novel phenotypes associated with altered RNase MRP function that significantly extend our current understanding of the nuclear capabilities of this multifunctional RNP.

Results

Altered 5.8S rRNA processing is associated with distinct site-specific mutations in the gene for RNase MRP RNA, mrp1

In *S.cerevisiae*, a single temperature-sensitive mutation in the gene for the RNA component of RNase MRP results in defective 5.8S rRNA processing that is characterized by a shift in the ratio of small 5.8S (S) to large 5.8S (L) rRNA forms (Schmitt and Clayton, 1993; Chu *et al.*, 1994). *S.pombe mrp1* mutations have been constructed (Materials and methods, Figure 1A) that are temperature-sensitive for growth on plates relative to wild-type (Figure 2A). Strains and plasmids used for analysis of these mutations are described in Tables I and II. Analysis of low molecular weight total RNA extracted from *S.pombe* cells deleted for the chromosomal copy of *mrp1* and carrying complementing plasmids containing various *mrp1* mutations was performed. Cells were grown at either the permissive temperature, 30°C, or shifted to the restrictive temperature, 36°C, and total RNAs were extracted and resolved on denaturing gels before staining with ethidium bromide. RNA species corresponding to 5.8S rRNA, 5S rRNA and tRNAs from cells harboring the various mutations are indicated (Figure 2B). An altered ratio of 5.8S (S) to 5.8S (L) forms, consistent with a nucleolar defect in 5.8S rRNA processing, was observed for a subset of the *mrp1* mutations, including *mrp1-TOs* and *mrp1-RR90*. Interestingly, 5.8S rRNA processing was similar to wild-type for both *mrp1-B7* and *mrp1-E4* mutations. Microheterogeneity of 5.8S (S) and 5.8S (L) rRNA forms in *S.pombe* was revealed by primer extension analysis of 5.8S rRNA (Figure 2C) and confirmed the shift toward 5.8S (L) forms observed by ethidium bromide staining (Figure 2B). The ratio of 5.8S (S) to 5.8S (L) forms is relatively constant for wild-type and cells carrying the mutation *mrp1-RD5*, while cells carrying mutations *mrp1-RR90* and *mrp1-TOs* showed a greater shift toward the 5.8S (L) rRNA with longer incubation at the restrictive temperature (Figure 2D). Interestingly, the loss of a larger form of 5S rRNA correlated with mutations that affect 5.8S rRNA processing (Figure 2B, lanes 2 and 3; Figure 2D, *mrp1-RR90* and *mrp1-TOs*).

Functional complementation of a chromosomal deletion of mrp1 by mrp1-ND90 requires the presence of the nuclear mutation ptp1-1

Plasmid-borne mutations in *mrp1* were analyzed by a plasmid shuffling procedure for *S.pombe* that utilizes counterselection in the presence of canavanine (Paluh and Clayton, 1996). With ammonia as the source of nitrogen, canavanine is imported into *can1*$^+$ cells resulting in lethality. Counterselection utilizes the ability of the *S.cerevisiae CAN1* gene to complement *S.pombe can1-1* mutants (Ekwall and Ruusala, 1991). Strain JLP206 (Table I) contains a chromosomal deletion of *mrp1* that is complemented by plasmid pJPps2b (Table II), carrying wild-type *mrp1* along with the counterselectable *CAN1* gene. Mutations in *mrp1* are introduced into JLP206 on a *ura4*$^+$ plasmid and the strain grown in the presence of canavanine to test for complementation and examine phenotypes of the various *mrp1* mutations. Plasmids used for counterselection are listed in Table II, and a diagram describing the plasmid shuffle system is shown in Figure 3A.

In the petite-negative yeast *S.pombe*, the ability to survive loss of mtDNA (*rho*$^-$ and *rho*°) requires the presence of specific nuclear mutations (Haffter and Fox, 1992; Massardo *et al.*, 1994). One of these mutations, *ptp1-1*, allows growth of *rho*° cells that are induced after prolonged incubation in ethidium bromide. These *ptp1-1 rho*° cells grow substantially more slowly than *ptp1-1*, *rho*$^+$ cells and are easily recognized on plates (Figure 4A). In order to identify mutations in the *mrp1* RNA that correspond to mitochondrial phenotypes, we introduced the *ptp1-1* nuclear mutation into strain JLP206 to generate strain JLP207 (Table I). Plasmids containing various *mrp1* mutations (Table II) were transformed into strain JLP207 (also carrying pJPps2b), and *ura*$^+$ transformants were selected on MSA supplemented with adenine and histidine (MSA + AH). Transformants were selected twice on this medium and then tested by plasmid shuffle on canavanine-containing plates. Plates were incubated for an extended period of time to accommodate the expected slower growth phenotype of *ptp1-1 rho*° cells. The *mrp1-ND90* mutation was able to complement following plasmid shuffle in JLP207 (Figure 3B). This mutation was unable to complement a chromosomal deletion of *mrp1* in a background that is wild-type for *ptp1-1*, suggesting that the defect is mitochondrial in nature (Paluh and Clayton, 1996).

The ability of such a large deletion in *mrp1* to produce a functional RNA component for RNase MRP was unexpected. Two colony sizes were observed after plasmid shuffle (Figure 4B). By testing for the presence of plasmid markers (*his*$^+$ for pJPps2b, wild-type *mrp1*; or *ura*$^+$ for pJPurND90, *mrp1-ND90*), we were able to determine that the larger colonies contained the wild-type *mrp1* plasmid, while the smaller colonies were *his*$^-$, and only contained pJPurND90 (Figure 4, panel 1). Picking individual smaller colonies and streaking again to selective plates confirmed this observation (Figure 4C, panel 2). No other mutations in *mrp1* were found to require *ptp1-1* for viability. However, mutations TOs, B7 and E4 were no longer temperature-sensitive when expressed in strain JLP207, suggesting that the phenotypes for these mutations may also have a mitochondrial nature (data not shown).

RNase MRP has been shown *in vitro* to be capable of primer RNA metabolism for mtDNA synthesis such that impairment of this function *in vivo* would be expected to lead to eventual loss of mtDNA (Clayton, 1992). To determine if mtDNA loss was occurring in the population of JLP207 *mrp1-ND90* cells, these cells were transferred to supplemented non-fermentable ethanol/glycerol medium (ET/Gly+AHU; Figure 4C). Although the density of the streak on ethanol/glycerol plates was less than comparable streaks on dextrose medium (compare Figure 4B with C, panel 1),

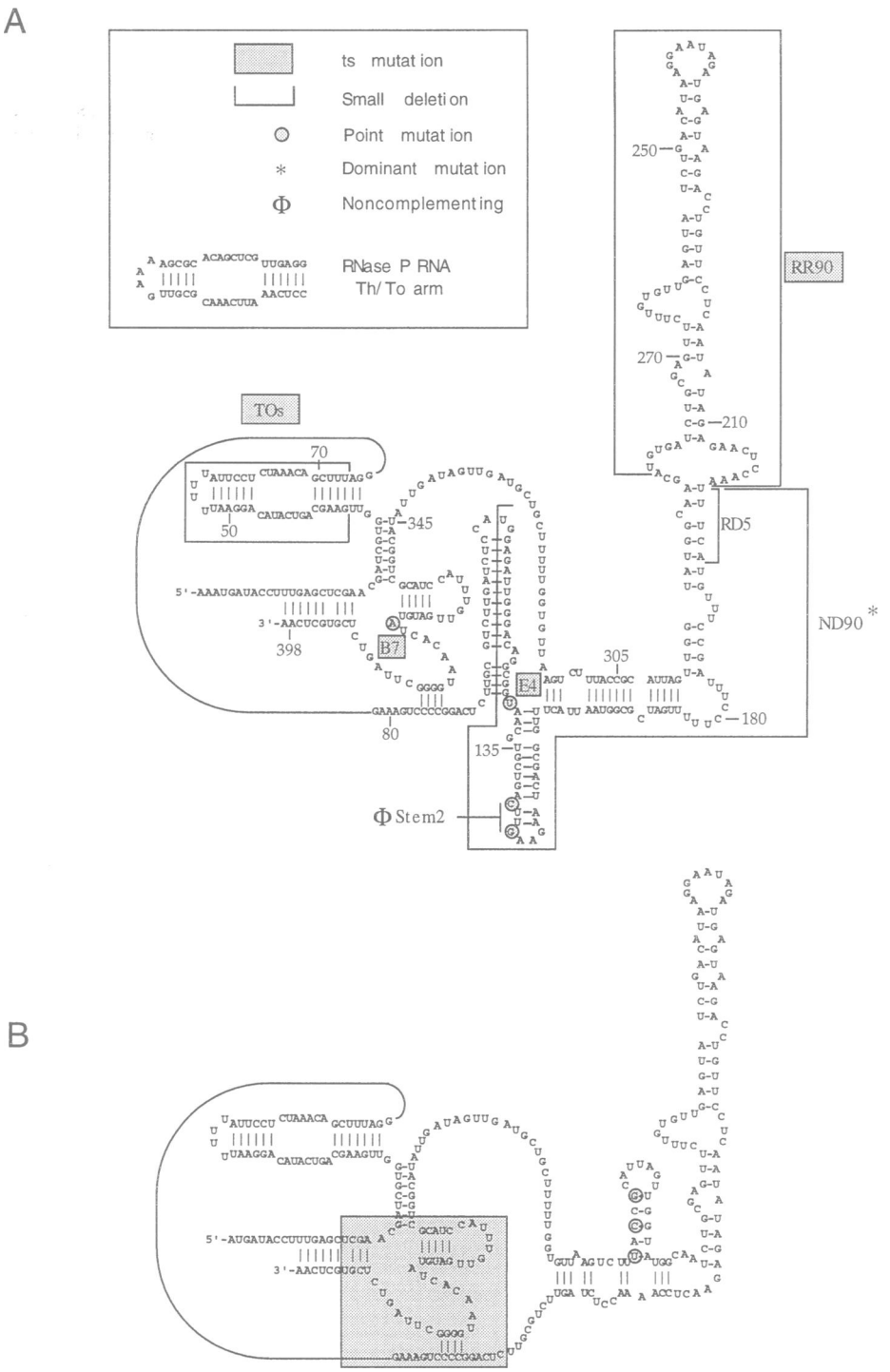

Fig. 1. Predicted secondary structure of *S.pombe* RNase MRP RNA. (**A**) *mrp1* RNA mutations discussed in the text. (**B**) Possible structure of the RNA encoded by *mrp1-ND90*. Circled nucleotides represent available compensating sites for RNA–RNA or RNA–protein interactions mentioned in the Discussion. The region of the RNA enclosed within the shaded box shares >60% identity with *S.pombe* RNase P RNA (Paluh and Clayton, 1995).

we were unable to isolate single colonies that grew on dextrose but failed to grow on Et/Gly+AHU plates (Figure 4C, panel 2). However, microscopic examination of JLP207 *mrp1-ND90* cells for nuclear and mtDNA did suggest that loss of mtDNA was occurring in 2% of the population (Figure 4D). We note that, unlike in *S.cerevisiae*, in which the spontaneous generation of petites readily occurs at this high frequency (Fox *et al.*, 1991), in wild-type *S.pombe*, or cells carrying *ptp1-1*, any spontaneous generation of petites has not been observed. Therefore, any clearly detectable loss of *S.pombe* mtDNA is probably significant.

mrp1-ND90 displays dominance that results in altered 5.8S rRNA and tRNA processing

When total RNA was extracted from strains JLP206 and JLP207, each containing plasmid-borne copies of both

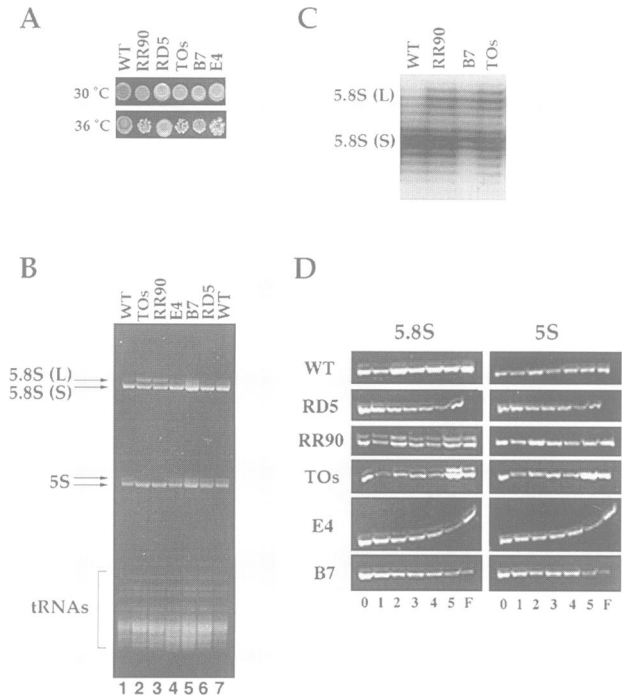

Fig. 2. Distinct site-specific mutations in *S.pombe mrp1* result in an altered ratio of the 5.8S (S):5.8S (L) rRNA forms. The strains used are listed in Table I and their construction is described in Materials and methods. Wild-type (WT, JLP101), RD5 (JLP105), RR90 (JLP106), TOs (JLP107), B7 (JLP112) and E4 (JLP114). (**A**) Temperature sensitivity is associated with *mrp1* mutations RR90, TOs, B7 and E4. A single dilution plating for each strain is shown for cells grown at 30 and 36°C. (**B**) Low molecular weight RNAs stained with ethidium bromide following denaturing electrophoresis on a 10% polyacrylamide–7 M urea gel. Cells were grown at 30°C to mid-log before shifting to 36°C for 5 h. (**C**) Primer extension analysis of 5.8S rRNA using the oligonucleotide sp5.8S and total RNA samples used in (B). (**D**) 5.8S rRNA and 5S rRNA profiles for JLP101 wild-type (WT) cells and cells carrying *mrp1* mutations grown at 30°C (0) or shifted to 36°C for 1 h (1), 3 h (2), 6 h (3), 9 h (4), 18 h (5) or 24 h (F).

wild-type *mrp1* (pJPps2b) and pJPurND90, and resolved on a denaturing polyacrylamide gel (Figure 5A), a shift towards the 5.8S (L) rRNA form was seen. Loss of the larger form of 5S rRNA, as noted previously with temperature-sensitive mutations in *mrp1* affecting 5.8S rRNA processing, was also observed. In addition, an altered pattern of low molecular weight RNA species migrating in the range of 50–120 nucleotides was present that was independent of gel electrophoretic conditions. Northern analysis was performed using oligonucleotide probes for either 5.8S or 5S rRNAs (Figure 5A, lanes 4–6 and lanes 10–12). Enhanced degradation of 5.8S rRNA was observed only in cells carrying both wild-type *mrp1* and the *mrp1-ND90* mutation. No degradation of 5S rRNA was detected in these same cells.

To examine if tRNA processing was affected in cells carrying *mrp1-ND90*, total RNA was extracted and analyzed from multiple isolates of JLP206 and JLP207 cells carrying both pJPps2b and pJPurND90 or JLP207 cells carrying only *mrp1-ND90* (Figure 5B, upper panel). An oligonucleotide corresponding to the intron present in $tRNA^{Ser}$ precursors, sup9t, has been used previously to identify accumulated precursor tRNAs (Potashkin and Frendewey, 1990). Northern analysis using sup9t (Figure 5B, lower panel) detected $tRNA^{Ser}$ processing intermediates for all isolates of JLP206 and JLP207 cells carrying both wild-type *mrp1* and *mrp1-ND90* mutations, but not for JLP207 cells carrying only *mrp1-ND90* or for strain JLP101. Since such processing intermediates were undetectable when tRNA processing proceeded normally, a dominant effect of *mrp1-ND90* on tRNA processing in these cells is probably occurring.

Mutations in mrp1 result in altered cellular morphology

Cells carrying temperature-sensitive *mrp1* mutations were examined by microscopy for gross cellular alterations in

Table I. *S.pombe* strains used in this study

Strain		Genotype	Source
JLP50	h+	*ade6-M216 ptp1-1 can1-1*	this work
JLP54	h–	*ade6-M216 leu1-32 ptp1-1 can1-1*	this work
JLP55	h–	*ade6-M216 leu1-32 ura4-D18 ptp1-1 can1-1*	this work
JLP59	h–	*ade6-M216 leu1-32 ura4-D18 his3-D1 ptp1-1 can1-1*	this work
JLP99	h+/h+	*ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 mrp+/mrp1::LEU2*	Paluh and Clayton (1995)
JLP100	h90	*ade6-M210 leu1-32 ura4-D18 mrp1::LEU2* [pJPurWT, *mrp1+, ura4+*]	Paluh and Clayton (1996)
JLP101	h+	*ade6-M210 leu1-32 ura4-D18 mrp1::LEU2* [pJPurWT, *mrp1+, ura4+*]	Paluh and Clayton (1996)
JLP105	h90	*ade6-M210 leu1-32 ura4-D18 mrp1::LEU2* [pJPurRD5, *mrp1-RD5, ura4+*]	this work
JLP106	h90	*ade6-M210 leu1-32 ura4-D18 mrp1::LEU2* [pJPurRR90, *mrp1-RR90, ura4+*]	Paluh and Clayton (1996)
JLP107	h90	*ade6-M210 leu1-32 ura4-D18 mrp1::LEU2* [pJPurTOs, *mrp1-TOs, ura4+*]	this work
JLP112	h90	*ade6-M210 leu1-32 ura4-D18 mrp1::LEU2* [pJPurPtB7, *mrp1-B7, ura4+*]	this work
JLP114	h90	*ade6-M210 leu1-32 ura4-D18 mrp1::LEU2* [pJPurPtE4, *mrp1-E4, ura4+*]	this work
PHP14	h–	*ade6-M216 leu1-32 ptp1-1 [rho°]*	Hafffter and Fox (1992)
PHP25	h+	*ade6-M216 ura1-161 ptp1-1*	Haffter and Fox (1992)
FY254	h–	*ade6-M210 leu1-32 ura4-D18 can1-1*	S.L.Forsburg
FY392	h–	*ade6-M210 leu1-32 ura4-D18 his3-D1*	S.L.Forsburg
JLP200	h90	*ade6-M210 leu1-32 ura4-D18 his3-D1 can1-1*	Paluh and Clayton (1996)
JLP201	h+	*ade6-M210 leu1-32 ura4-D18 his3-D1 can1-1*	Paluh and Clayton (1996)
JLP204	h+	*ade6-M210 leu1-32 ura4-D18 his3-D1 can1-1 mrp1::LEU2* [pJPurWT, *mrp1+, ura4+*]	Paluh and Clayton (1996)
JLP205	h+	*ade6-M210 leu1-32 ura4-D18 his3-D1 can1-1 mrp1::LEU2* [pJPurWT, *mrp1+, ura4+*; pJPps2b, *mrp1+, CAN1, his3+*]	Paluh and Clayton (1996)
JLP206	h+	*ade6-M210 leu1-32 ura4-D18 his3-D1 can1-1 mrp1::LEU2* [pJPps2b, *mrp1+, CAN1, his3+*]	Paluh and Clayton (1996)
JLP207	h+	*ade6-M210 leu1-32 ura4-D18 his3-D1 can1-1 mrp1::LEU2*, ptp1-1 [pJPps2b, *mrp1+, CAN1, his3+*]	this work

Table II. *S.pombe* plasmids used in this study

Mutation	Plasmid	Relevant genotype	*mrp1* fragment	Base plasmid	Reference
WT	pJPurWT	*mrp1+, ura4+, ars1*	1.1 kbp *Pst*I–*Xba*I	pURN18	Barbet *et al.* (1992)
WT	pJPps2b	*his3+, CAN1, mrp1+, ars1*	1.1 kbp *Pst*I–*Xba*I	pBG-1-his3+, puc19-CAN	Paluh and Clayton (1996)
ND90	pJPurND90	*ura4+, ars1*	(Del 111–198)	pURN18	Paluh and Clayton (1996)
RR90	pJPurRR90	*ura4+, ars1*	(Del 199–282)	pURN18	Paluh and Clayton (1996)
RD5	pJPurRD5	*ura4+, ars1*	(Del 194–199)	pURN18	this work
B7	pJPurPtB7	*ura4+, ars1*	[A→G (370)]	pURN18	this work
E4	pJPurPtE4	*ura4+, ars1*	[Del U (130)]	pURN18	this work
TOs	pJPurTOs	*ura4+, ars1*	(Del 34–74, insert RNase P RNA sequence, Figure 1)	pURN18	this work
Stem2	pJPurStem2	*ura4+, ars1*	[C→A (141), G→A (144)]	pURN18	Paluh and Clayton (1996)

morphology. Two mutations, *mrp1-RR90* and *mrp1-B7*, displayed different morphological phenotypes. Cells carrying the *mrp1-RR90* mutation grew more slowly than wild-type cells at 30°C and were generally elongated to maximum normal cell length (Figure 6c). In contrast, cells carrying *mrp1-B7* (Figure 6b) appear similar to wild-type at 30°C (Figure 6a). When shifted to 36°C, cells carrying mutation *mrp1-B7* became predominatly rounded (Figure 6d–f) and filled with calcofluor-positive staining material (data not shown), while *mrp1-RR90*-containing cells continue to elongate at 36°C without dividing (Figure 6g). The cells carrying mutation *mrp1-RR90* become banana-shaped when cell length exceeds normal boundaries (Figure 6g) and a fraction of the population is multiseptate (Figure 7a) and branched (data not shown) after prolonged incubation at the restrictive temperature. Calcofluor staining of multiseptate *mrp1-RR90* cells that also display aberrant septal deposition is shown (Figure 7a).

Nuclear DNA in cells carrying mutations *mrp1-RR90* and *mrp1-B7* was examined using DAPI and bisbenzamide staining. The nuclear DNA in *mrp1-RR90* cells is disorganized at 30°C (Figure 7b) as well as at 36°C (data not shown) and these cells display long interphase spindles (Figure 7c, α-tubulin). In contrast, the DNA in cells carrying mutation *mrp1-B7* at 36°C appear uniformly 'C-shaped' with a deep groove (Figure 7d and e). Occasionally two less-dense staining C-shaped DNA forms are seen within a single cell (Figure 7e), which may indicate that nuclear division has occurred in the absence of cell division.

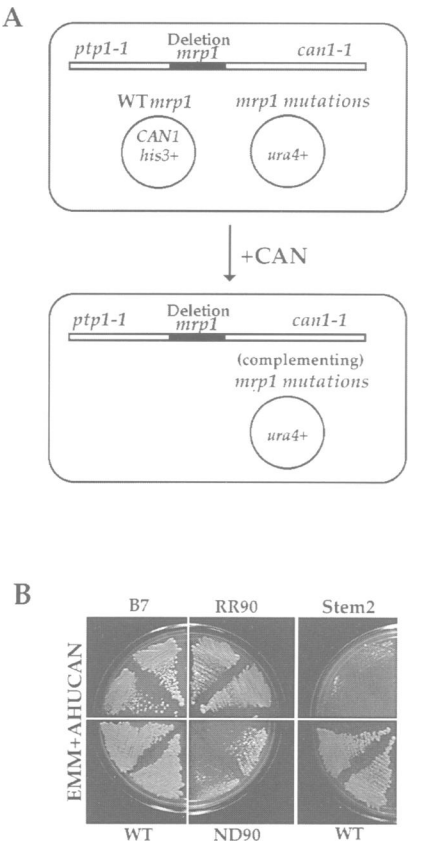

Fig. 3. Counterselection scheme to test plasmid-borne *mrp1* mutations for mitochondrially associated phenotypes. (**A**) Diagram of plasmid shuffle using strain JLP207 transformed with a plasmid-borne *mrp1* mutation to be tested. Growth of cells on canavanine-containing medium (+CAN) requires complementation in the absence of wild-type (WT) *mrp1*. (**B**) JLP207 cells transformed with individual plasmid-borne mutations in *mrp1* (Table II) were streaked to canavanine-containing plates to test for complementation.

Discussion

RNase MRP has been identified solely in eukaryotes, and the RNA component of this RNP is present within two subcellular compartments, the nucleolus (Reimer *et al.*, 1988) and mitochondria (Li *et al.*, 1994; Davis *et al.*, 1995) or cytoplasm (Matera *et al.*, 1995). While the genes encod-ing the RNA component of RNase MRP from both *S.cerevisiae* and *S.pombe* have been shown to be essential (Schmitt and Clayton, 1993; Paluh and Clayton, 1995) and this RNP is required for appropriate nucleolar 5.8S rRNA processing in both yeasts (for reviews, see Clayton, 1994; Morrissey and Tollervey, 1995; Lygerou *et al.*, 1996; this work), the essential phenotype is unlikely to involve this process alone (Henry *et al.*, 1994), implying that this RNP must participate in additional nuclear functions. Mutations in *mrp1* reported here suggest previously unknown nuclear roles for RNase MRP in tRNA maturation and nuclear control of septation, while reinforcing previous extensive biochemical evidence for a role for this RNP in mtDNA replication. We suggest that the macromolecular assemblies involving RNase MRP, with or without RNase P, are more extensive than previously imagined.

A requirement for RNase MRP in correct processing of 5.8S rRNA is conserved and may also involve maturation of 5S rRNA

Two pathways exist for maturation of rRNA precursors in eukaryotes to ultimately generate 5.8S rRNAs having

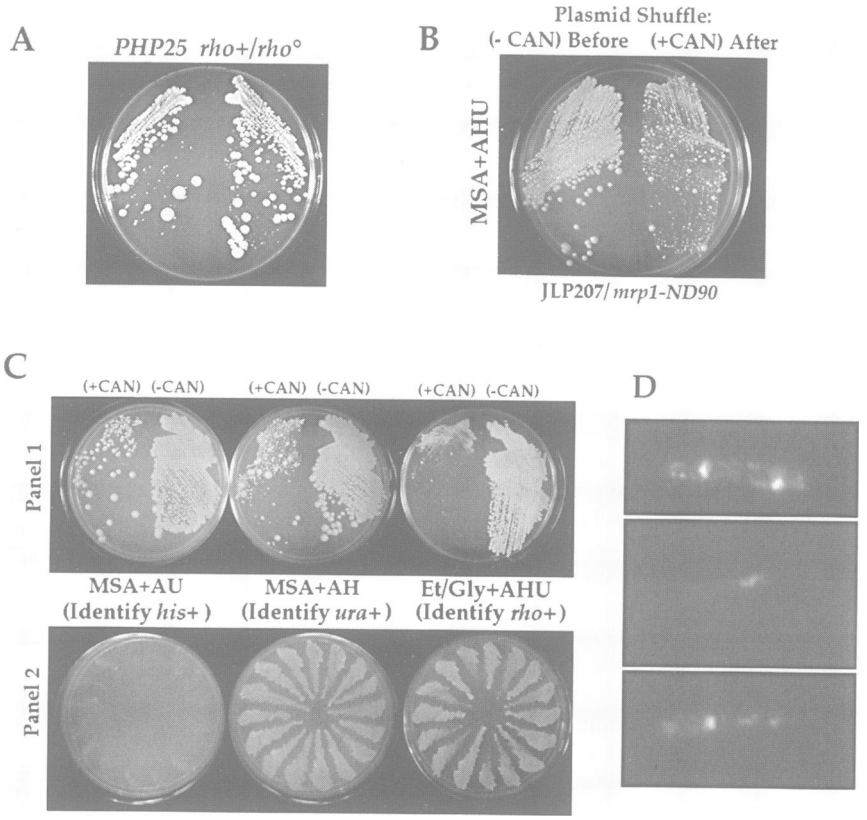

Fig. 4. JLP207 cells carrying *mrp1-ND90* are viable in the absence of *mrp1* and contain a mixture of rho^+ and *rho°* cells. (**A**) Original *ptp1-1* strain, PHP25, after treatment with ethidium bromide to induce loss of mtDNA (*rho°*). The tiny colonies are *rho°*. (**B**) JLP207 cells transformed with pJPurND90 were restreaked to MSA+AUH plates either before (–CAN, control) or following (+CAN) plasmid shuffle to recheck viability of canavanine-resistant cells. (**C**) Streaks of JLP207 cells transformed with pJPurND90. The types of plates used are described between panels 1 and 2 and apply to both panels. In panel 1, cells were streaked either before or following plasmid shuffle to identify his^-, ura^+ colonies. In panel 2, individual slower growing his^-, ura^+ colonies were picked from plates in panel 1 and retested for his^- auxotrophy and ura^+ prototrophy before checking growth on Et/Gly+AHU plates. (**D**) Slower growing colonies, obtained from MSA+AHU plates (B, +CAN), were stained with a solution of 1 μg/ml DAPI + 1 μg/ml bisbenzamide.

heterogeneous 5′ termini (Henry *et al.*, 1994; Morrissey and Tollervey, 1995). In *S.cerevisiae*, RNase MRP plays a direct role in one of these pathways (Henry *et al.*, 1994; Lygerou *et al.*, 1996) and mutations in the gene encoding the RNA component of RNase MRP as well as a mutation in a shared protein component of RNase MRP and RNase P RNPs result in an altered ratio of 5.8S rRNA forms (Schmitt and Clayton, 1993; Chu *et al.*, 1994; Lygerou *et al.*, 1994). We observed a similar shift from 5.8S (S) to 5.8S (L) rRNA forms with distinct site-specific temperature-sensitive mutations in the gene for *S.pombe* RNase MRP RNA, *mrp1*, and with the dominant mutation, *mrp1-ND90*. In addition, loss of a larger form of 5S rRNA consistently was associated with an altered ratio of 5.8S (S) to 5.8S (L) rRNA in *S.pombe*, independent of electrophoresis conditions.

In prokaryotes, 5S rRNA precursors require both 5′ and 3′ end processing events (for review, see Erdmann, 1976), while in eukaryotes processing of precursor 5S rRNAs is restricted to the 3′ end (O'Brien and Wolin, 1994, and references therein). In *S.pombe*, as in vertebrates, the gene for 5S rRNA is separate from the rDNA cluster (Mao *et al.*, 1982). However, assembly of 5S rRNA as an integral component of the pre-ribosomal 60S subunit, along with 5.8S rRNA, occurs in the nucleolus (Erdmann, 1976). We suggest that genetic reconfiguration of the rDNA operon from prokaryotes to eukaryotes, including formation of a separate 5.8S rRNA and relocation of the 5S rRNA, may have necessitated multiple nucleolar roles for RNase MRP in maturation of these two low molecular weight rRNAs.

mrp1-ND90 requires ptp1-1 for viability and genetically links RNase MRP to mitochondrial biogenesis

The predicted *in vivo* role of RNase MRP in mtDNA replication is based on previous extensive biochemical evidence of site-specific processing of *in vitro* synthesized RNA substrates corresponding to the leading strand origin of replication in vertebrates (for reviews, see Topper *et al.*, 1988; Clayton, 1991). RNase MRP is able to process heterologous mitochondrial RNA substrates from metazoans to yeast, suggesting that the role played by this ribonucleoprotein in primer RNA metabolism during mtDNA replication is conserved. Although the biochemical data are convincing, the ability of fungi to use alternative modes of mtDNA propagation that are independent of wild-type transcription (Fangman *et al.*, 1989; Maleszka *et al.*, 1991; Maleszka, 1992; Han and Stachow, 1994) has left the importance of RNase MRP in mitochondrial biogenesis in simpler eukaryotes ambiguous.

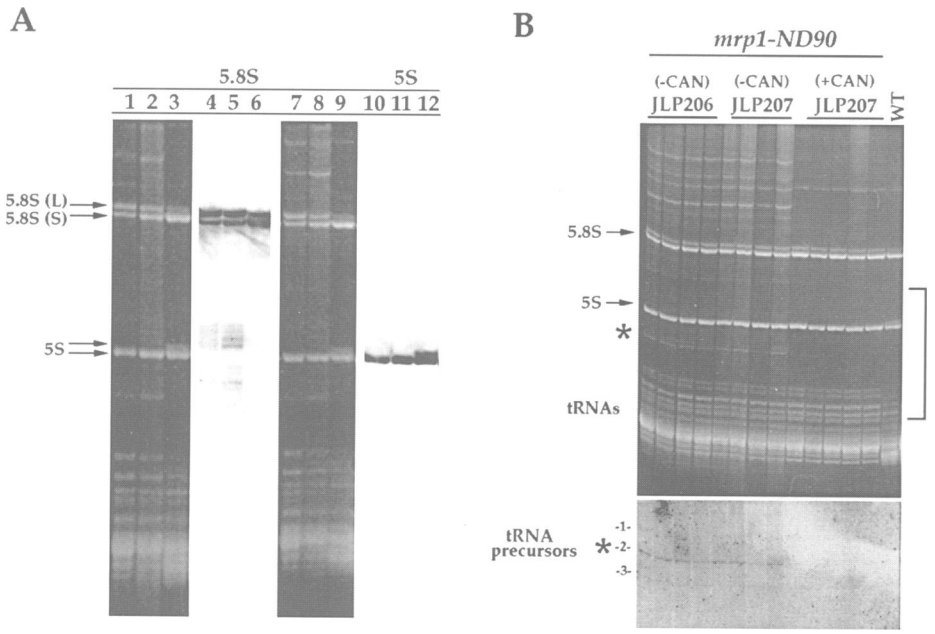

Fig. 5. The presence of *mrp1-ND90* results in a dominant phenotype that includes defects in both 5.8S rRNA and tRNA processing in the presence of wild-type *mrp1*. (**A**) Duplicate runs of low molecular weight RNAs resolved on denaturing 10% polyacrylamide–7 M urea gels and stained with ethidium bromide before probing for either 5.8S rRNA (lanes 4, 5 and 6) or 5S rRNA (lanes 10, 11 and 12). Oligonucleotides used for Northern analysis are described in Materials and methods. Lanes 1, 4, 7 and 10, JLP206 + pJPurND90 (–CAN); lanes 2, 5, 8 and 11, JLP207 + pJPurND90 (–CAN); lanes 3, 6, 9 and 12, JLP101 (pJPurWT, wild-type *mrp1*). (**B**) Total RNA was resolved on a denaturing 10% polyacrylamide–7 M urea gel and transferred to nytran before probing with oligonucleotide sup9t, specific to the intron of $tRNA^{ser}$. Lanes 1–4, JLP206 + pJPurND90 (–CAN, multiple isolates); lanes 5–8, JLP207 + pJPurND90 (–CAN, multiple isolates); lanes 9–12, JLP207 + pJPurND90 (+CAN; his^-, ura^+ multiple isolates); lane 13, JLP101 (pJPurWT, wild-type); lane 14, JLP107 (pJPurWtoP); and lane 15, JLP106 (pJPurRR90).

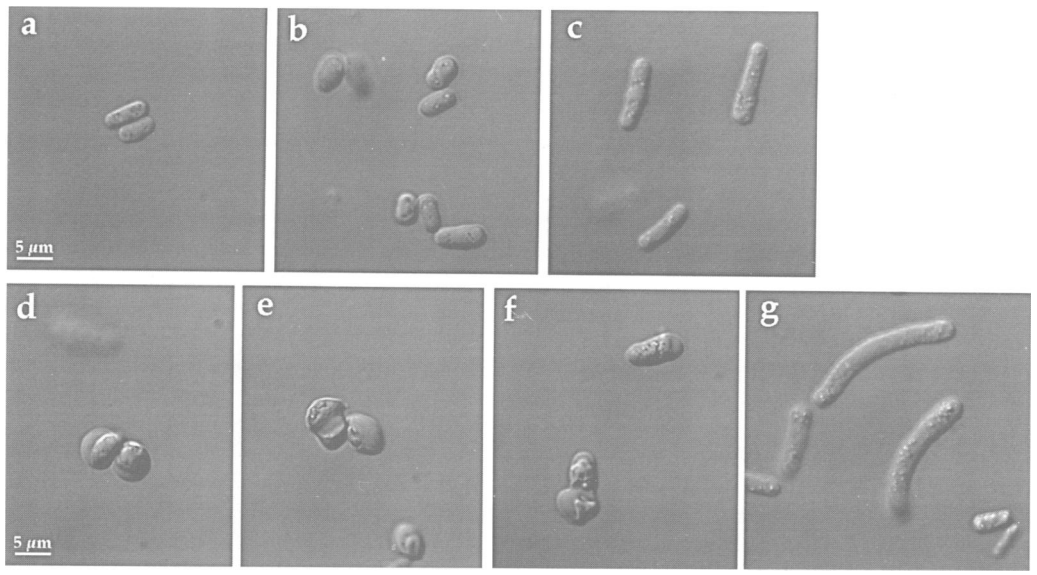

Fig. 6. DIC images showing altered cellular morphologies associated with mutations *mrp1-B7* and *mrp1-RR90*. (**a**) JLP101 (wild-type *mrp1*) 30°C; (**b**) JLP112 (*mrp1-B7*) 30°C; and (**c**) JLP106 (*mrp1-RR90*) 30°C. (**d**), (**e**) and (**f**) JLP112 36°C; (**g**) JLP106 36°C.

Schizosaccharomyces pombe is termed a petite-negative yeast since large deletions of mtDNA (*rho*$^-$) or total loss of mtDNA (*rho*°) result in cell death, and any spontaneous generation of petites is not observed. This is similar to the situation in mammalian cells in which loss of mtDNA is a rare event which may require, in concert, some appropriate compensating nuclear mutations that permit cell viability. In the absence of a wild-type copy of *mrp1*, cells harboring *mrp1-ND90* require the presence of *ptp1-1* for viability. Although the identification of *ptp1-1* is not yet known, this nuclear gene is likely to encode a mitochondrial component since the *ptp1-1* allele permits viability after induction of *rho*° cells following ethidium bromide treatment (Haffter and Fox, 1992). This dependence on *ptp1-1*, along with the detectable loss of mtDNA in a fraction of the cell population carrying the *mrp1-ND90* mutation, are together consistent with a role for RNase MRP in the maintenance of mtDNA, presumably involving mtDNA replication. It is interesting that the majority of the *mrp1-ND90, ptp1-1* cells are able to grow on non-fermentable carbon sources, suggesting that *ptp1-1* may be partially relieving the *mrp1-ND90* defect either in function or transport of the RNA.

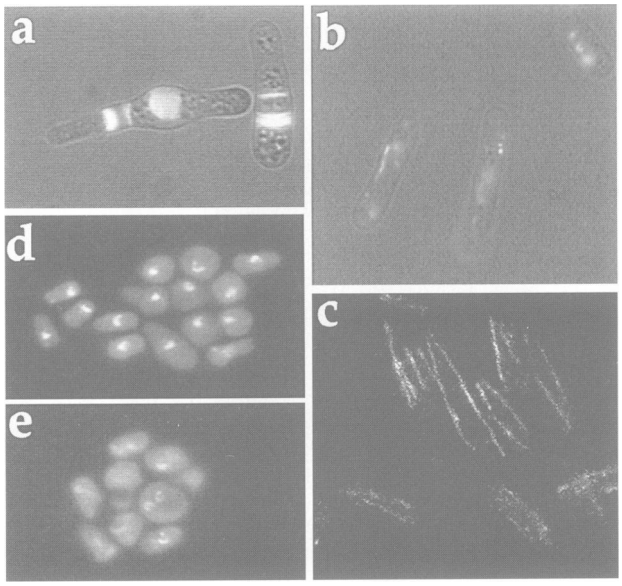

Fig. 7. Aberrant septum deposition and changes in nuclear DNA are associated with mutations in *mrp1*. (**a**) Calcofluor staining of JLP106 cells (*mrp1-RR90*); (**b**) DAPI staining of JLP106 cells (*mrp1-RR90*); (**c**) α-tubulin pattern of JLP106 cells at 30°C; and (**d**) and (**e**), JLP112 (*mrp1-B7*) cells stained with DAPI.

A mutation in RNase MRP displays dominance and results in accumulation of unspliced tRNA^{Ser} precursors

Unspliced tRNA^{Ser} precursors were readily detected in cells carrying both wild-type *mrp1* and *mrp1-ND90*, along with alterred 5.8S rRNA processing, but were absent from cells containing only wild-type *mrp1*. Interestingly, cells carrying only the mutation *mrp1-ND90* appeared more similar to wild-type in their pattern of low molecular weight RNAs and did not accumulate detectable precursor tRNAs. End processing of the unusual dimeric precursor tRNA^{Ser}-tRNA^{Met}i is believed to precede intron removal (Willis *et al.*, 1986, and references therein). It is unclear whether RNase MRP plays a direct role in processing the dimeric tRNA^{Ser}-tRNA^{Met}i precursor tRNA or whether accumulation of precursor tRNAs is an indirect result of hindrance of RNase P activity, perhaps by titrating out a shared protein component. Although *in vitro* evidence suggests that RNase MRP does not process a monomeric precursor tRNA substrate (Lygerou *et al.*, 1996), processing of the dimeric precursor tRNA was not examined, and *in vivo* depletion of *S.cerevisiae* RNase MRP RNA appears to enhance monomeric precursor tRNA processing by RNase P (Lygerou *et al.*, 1996, Figure 3). Alternatively, RNase MRP may be required in some way for splicing of the intron. Since tRNA^{Ser} precursors are not evident in cells carrying either wild-type *mrp1* or *mrp1-ND90* alone, an additional interaction between these two *mrp1* molecules must also be required.

The mutation *mrp1-ND90* removes an internal segment of the RNase MRP RNA (Figure 1A), including two predicted stem–loop structures that are conserved between all RNase MRP RNAs as well as nucleotides that have been shown to be important for function in both *S.cerevisiae* (Schmitt and Clayton, 1994) and *S.pombe* (Stem2 mutation). Although the functionality of this RNA component is surprising, a possible structure for the *mrp1-ND90* RNase MRP RNA that may resolve this paradox is shown (Figure 1B). An alternative stem–loop structure is possible that retains key nucleotides important for RNA–RNA or RNA–protein interactions (compare Figure 1A with B). While maintaining critical enzymatic parameters, including the unaltered cage region of the RNA, such an RNA component might result in sacrificed enzyme specificity.

Defects in cellular morphology suggest a role for RNase MRP in regulating nuclear control of cell division

Distinct morphological phenotypes are observed in mitotically dividing cells carrying plasmid-borne temperature-sensitive mutations in *mrp1*. However, rarely is incomplete segregation of nuclear DNA observed with any of these mutations. A model has been proposed (Chang and Nurse, 1996) for the appropriate positioning of the division site for medial fission in which a nuclear signal is required in addition to appropriate cell cycle regulation. It would be predicted from this model that disorganization of the nuclear DNA, as occurs with mutation *mrp1-RR90*, or restriction of the nuclear DNA to a fixed configuration, as in cells carrying mutation *mrp1-B7*, would result in cells that are either elongated or are continually supplied with divisional material, respectively. No changes in cell morphology have been observed with mutations affecting RNase MRP in *S.cerevisiae* that uses a cortical marker for placement of the septum (Chant, 1996).

It is possible that the altered 5.8S rRNA processing associated with mutations in *mrp1* leads directly to changes in cell morphology. This seems unlikely, however, since cells carrying the mutation *mrp1-E4*, that display no shift in 5.8S rRNA species at 36°C similar to mutation *mrp1-B7*, are elongated and branched at the restrictive temperature (data not shown). Slight changes in cellular morphology have been observed with certain debilitating mutations in the gene for 5.8S rRNA in *S.pombe* affecting 5.8S rRNA processing (S.Abou Elela, personal communication). Morphological phenotypes, similar to those associated with mutations in *mrp1*, have not been reported for loss of ribosomal proteins in fission yeast and are not observed by growing cells in the presence of cycloheximide (data not shown).

A model for a multilevel nuclear role for RNase MRP

RNase MRP is known to associate with higher molecular weight complexes within cells (Kiss *et al.*, 1992; D.Lee and D.A.Clayton, unpublished results), although the exact macromolecular nature of this situation remains to be determined. Only two protein components associated with RNase MRP have been identified (Lygerou *et al.*, 1994; Schmitt and Clayton, 1994) and one of these, encoded by *POP1*, is shared with RNase P. This work suggests that RNase MRP may play a much broader role in processing low molecular weight nuclear RNAs crucial for ribosomal assembly and function, including tRNAs. If this is the case, it is reasonable to assume that the combined effect of altered processing of these RNAs may interfere with nucleolar structure and cell division. A model is presented (Figure 8) that encompasses the observations presented here and which provides a framework for testing this

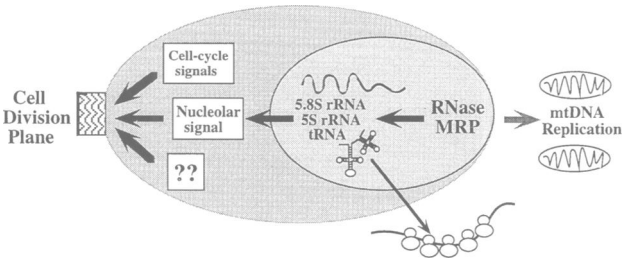

Fig. 8. A model combining the multifunctional cellular roles of RNase MRP. Protein synthesis and mitochondrial biogenesis supply the necessary critical machinery and metabolic energy for normal cell growth and division. A model is proposed in which RNase MRP regulates cell division by providing the appropriate nucleolar signal for this process. This is accomplished by both efficient processing of low molecular weight RNAs important for ribosomal assembly and function and regulation of mitochondrial biogenesis, presumably through the replication of mtDNA and maintenance of the mitochondrial genome.

hypothesis. Further studies in *S.pombe* will provide the opportunity to decipher genetically and biochemically the full extent of nuclear and mitochondrial roles for RNase MRP in a cell that reflects vertebrate features of rRNA processing, compactness of its mitochondrial genome and its mechanism of cell division.

Materials and methods

Yeast strains, media and genetic methods

Standard genetic procedures, lithium acetate transformation, YE and EMM media have been described (Moreno *et al.*, 1991). YEAC contained 100 μg/ml adenine and 2 g/l casamino acids. YEEG contained 2% ethanol and glycerol in place of dextrose. MSA medium was prepared as described (Egel *et al.*, 1994). EMM or MSA minimal media were supplemented with 75 μg/ml histidine, adenine or leucine and 100 μg/ml adenine as needed, and when used for counterselection contained 75 μg/ml canavanine.

The *S.pombe* strains used in this study are listed in Table I. Strains JLP101, JLP105, JLP106, JLP107, JLP112 and JLP114 were generated by transformation of the stable diploid JLP99 with the appropriate plasmid-borne mutations in *mrp1* and subsequent tetrad dissection as described previously (Paluh and Clayton, 1995). Strain FY254 was a generous gift from Dr Susan L.Forsburg (Salk Institute, San Diego). Construction of strain JLP206 and details of plasmid shuffle by counterselection on canavanine will be published elsewhere (Paluh and Clayton, 1996).

Strains PHP14 and PHP25 were a generous gift from Dr Tom Fox (Cornell University, Ithaca). In constructing strain JLP207, the presence of *ptp1-1* was monitored by the ability to induce *ptp1-1 rho°* cells (described in detail in Haffter and Fox, 1992). Cells were grown in the presence of 12.5 μg/ml ethidium bromide and 2% potassium acetate for 7 days. The cells were then harvested and plated on YEAC media. Slower growing *ptp1-1 rho°* candidates were tested for their inability to grow on YEEG plates containing a non-fermentable carbon source. Construction of strain JLP207 for plasmid shuffle was as follows (refer to Table I for full genotypes): JLP200 (h90) was crossed to PHP25 and random spores were plated to select for canavanine resistance and his^+ prototrophy ($can1\text{-}1$, his^+). These colonies were isolated and replica plated to select ura^-, leu^-. None of these was also *ptp1-1*; however, two isolates were obtained that combined *ade6-M216*, *can1-1* and *ptp1-1* markers (JLP50). JLP50 (h+) was crossed with PHP14 (h–) and tetrads dissected to obtain JLP54 (h+). This strain was crossed with FY254 (h–) to obtain strain JLP55. Strain JLP55 (h–) was crossed with JLP201 (h+) to obtain JLP59 (h–). This strain was crossed to the original plasmid shuffle strain JLP206 to obtain JLP207.

Construction of mutations in mrp1 and sequencing

Mutations generated in the gene for RNase MRP RNA *mrp1* are shown in Figure 1A and described in Table II. Two mutations, *mrp1-ND90* and *mrp1-RD5*, were constructed using restriction sites present within the *mrp1* gene. The mutation *mrp1-ND90* deletes sequences internal to *Nco*I and *Dra*I sites and mutation *mrp1-RD5* removes sequences contained within a small *Rsa*I–*Dra*I fragment. Two-step PCR site-directed mutagenesis was used to generate mutation *mrp1-B7*. The oligonucleotides used for PCR site-directed mutagenesis were: degenerate oligonucleotides 50B, 5′-TGTTGATGT(G/A)TCA(A/C)AATGGGGCTT-3′, and 3C, 5′-CCCCATTGTGA(C/T)ACATCAACAAC-3′, and flanking oligonucleotides KOC-1, 5′-TGCTATGTCGGTCAATTGCTCGTCGA-3′, and KOC-2, 5′-ACGCTATCGCGTTTCAGTGCTTGAC-3′. Nucleotides in parenthesis are present in a 50% mixture. Mutation *mrp1-TOs* was also generated in two steps. Oligonucleotides TO-P1, 5′-CAATCTCCATGGAGATCAAGACGCAAGAGTCCGGGGACTTTCCCCCTCAACGAGCTG-3′, and TO-P2, 5′-TACCTTTGAGCTCGAACGATCGTGGTTCCTCAAATTCAAACGC-3′, were used to generate a PCR fragment containing RNase P RNA (nucleotides 33–74, K1 RNA) flanked by RNase MRP RNA sequence (see Figure 1 and Table II). The PCR fragment was digested with *Nco*I and *Sac*I and cloned into the same unique sites in *mrp1*, replacing the original sequence. Mutation E4 was obtained as a spurious mutation. Oligonucleotides used for sequencing of *mrp1* have been described (Paluh and Clayton, 1995). All *mrp1* mutations are contained on a 1.2 kb fragment in the *S.pombe* plasmid, pURN18 (Barbet *et al.*, 1992).

Extraction of total RNA, primer extension and Northern hybridization

Denaturing polyacrylamide gel electrophoresis, primer extension and Northern hybridization were performed using 8–20 μg of total cellular RNA isolated from cultures of 50–100 ml of cells grown to mid-log phase in either YEA or supplemented MSA minimal media. The cell concentration was determined using a hemocytometer. For the analysis of temperature-sensitive mutations, cells were first grown at 30°C to early log phase then diluted 1/50 and shifted to 36°C. RNA was extracted and analyzed from cells harvested at several time points (1, 3, 9, 18 and 24 h) following temperature shift. Cells were disrupted using glass beads as described (Moreno *et al.*, 1991). Lower molecular weight RNA species were resolved by electrophoresis through denaturing polyacrylamide gels containing either 10 or 20% polyacrylamide–7 M urea in 67 mM Tris–Cl, pH 8.3, 67 mM borate, 1 mM EDTA (TBE) buffer. Loading buffer for RNA samples contained 90% formamide and 7 M urea. Samples were denatured by heating for 10 min at 85°C in loading buffer and snap cooling on ice before loading. Gels were stained in TBE buffer containing 1 μg/ml ethidium bromide and destained for 10 min in TBE before photography. Oligonucleotides used for Northern analysis were sp5.8S, 5′-CGGAATTCTGCAATTCACATTACGTATCGC-3′; sp5S, 5′-CTTAACTGCAGTGATCGGACGGGAACTG-3′; and sup9+, 5′-TAGATGACTAGAATACAGGATTCAAG-3′ (Potashkin and Frendewey, 1990). Primer extension reactions contained 50 μg of total RNA and 200 U of MMLV reverse transcriptase (Gibco BRL) and were performed essentially as described (Sambrook *et al.*, 1989). Two oligonucleotides were used for primer extension of 5.8S rRNA and gave similar results (sp5.8S, and C006-07, 5′-TTTCGCTGCGTTCTTCATC-3′). Primer extension products were resolved on 6% polyacrylamide–7 M urea sequencing gels.

Cell fixation, DNA staining and immunofluorescence

Cells were fixed in either 30 or 70% ethanol, or by aldehyde as previously described (Hagan and Hyams, 1988). For aldehyde fixation, essentially 10^7 cells/ml were fixed with 3% formaldehyde and 0.2% glutaraldehyde at room temperature for 60 min. Cell walls were digested with Zymolyase (100T, ICN Biochemicals). Unreacted glutaraldehyde was quenched with sodium borohydride. Cells were resuspended in either PEMBAL (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9, 1% bovine serum albumin, 0.1% NaN_3, 100 mM lysine HCl) or phosphate-buffered saline (PBS, Moreno *et al.* 1991) for antibody incubations and staining with DAPI (20 μg/ml, 4,6, diamidino-2-phenylindole, Sigma), bisbenzamide (20 μg/ml) and calcofluor (1 mg/ml; Sigma), essentially as described (Mitchison and Nurse, 1985). For tubulin staining, *Drosophila* mAb (clone 4A2, Piperno and Fuller, 1985) directed against α-tubulin was used at 1/10 dilution, or undiluted, along with Texas red-conjugated secondary antibody (Amersham) at 1/200 dilution. The *Drosophila* antibody was a generous gift from Dr Margaret Fuller (Stanford University, Stanford). Differential interference contrast (DIC) images were obtained using a Bio-Rad MRC 1000 laser scanning confocal microscope equipped with CoMos Imaging software. Cells were immobilized in low melting temperature agarose for DIC imaging. All other images were obtained from a Nikon epifluorescence microscope equipped with Lookscan software.

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