The La protein functions redundantly with tRNA modification enzymes to ensure tRNA structural stability

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ABSTRACT

Although the La protein stabilizes nascent pre-tRNAs from nucleases, influences the pathway of pre-tRNA maturation, and assists correct folding of certain pre-tRNAs, it is dispensable for growth in both budding and fission yeast. Here we show that the *Saccharomyces cerevisiae* La shares functional redundancy with both tRNA modification enzymes and other proteins that contact tRNAs during their biogenesis. La is important for growth in the presence of mutations in either the arginyl tRNA synthetase or the tRNA modification enzyme Trm1p. In addition, two pseudouridine synthases, *PUS3* and *PUS4*, are important for growth in strains carrying a mutation in tRNA^{Arg}_{CCG} and are essential when La is deleted in these strains. Depletion of Pus3p results in accumulation of the aminoacylated mutant tRNA^{Arg}_{CCG} in nuclei, while depletion of Pus4p results in decreased stability of the mutant tRNA. Interestingly, the degradation of mutant unstable forms of tRNA^{Arg}_{CCG} does not require the Trf4p poly(A) polymerase, suggesting that yeast cells possess multiple pathways for tRNA decay. These data demonstrate that La functions redundantly with both tRNA modifications and proteins that associate with tRNAs to achieve tRNA structural stability and efficient biogenesis.

Keywords: La protein; tRNA; tRNA modification; aminoacyl-tRNA synthetase; subcellular localization; RNA stability

INTRODUCTION

A protein that functions in the earliest stages of the biogenesis of many noncoding RNAs is an abundant nuclear phosphoprotein known as the La protein. La binds the UUU_{OH} found at the 3' ends of all nascent RNA polymerase III transcripts. RNAs bound by La include pre-tRNAs, pre-5S rRNAs, nascent U6 snRNA, SRP RNA, and Y RNAs (Wolin and Cedervall 2002). In the yeast *Saccharomyces cerevisiae*, the La ortholog Lhp1p also binds precursors to the RNA polymerase II-transcribed U1, U2, U4, and U5 snRNAs and certain small nucleolar RNAs that have been processed to end in UUU_{OH} (Kufel et al. 2000; Xue et al. 2000; Inada and Guthrie 2004). Binding by La protects these RNAs from 3' to 5' exonucleases and assists correct

folding of certain pre-tRNAs (Yoo and Wolin 1997; Fan et al. 1998; Pannone et al. 1998; Chakshusmathi et al. 2003).

As La proteins are abundant components of virtually all eukaryotic nuclei, and associate with many essential RNAs, it might be expected that these proteins would be required for viability. However, while La is essential in *Drosophila melanogaster* and *Trypanosoma brucei* (Bai and Tolias 2000; Arhin et al. 2005; Foldynova-Trantirkova et al. 2005), La is dispensable in both budding and fission yeast (Yoo and Wolin 1994; Van Horn et al. 1997). Moreover, while intron-containing pre-tRNAs accumulate during RNA interference-induced knock-down of La in *T. brucei* (Foldynova-Trantirkova et al. 2005) and mature tRNA^{Met} levels decline by ~50% (Arhin et al. 2005), steady state levels of other RNAs examined were unchanged, suggesting that other proteins function redundantly with La to assist noncoding RNA biogenesis.

To identify components that function redundantly with La, we carried out genetic screens to identify mutations in other genes that cause the *S. cerevisiae* La protein Lhp1p to become essential for growth. These screens revealed that certain mutations in the core proteins of spliceosomal small

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nuclear ribonucleoproteins cause yeast to require Lhp1p. Lhp1p is required to stabilize newly synthesized U6 snRNA in the presence of mutations in components of the Lsm2–Lsm8 ring (Pannone et al. 1998, 2001). In the presence of a mutation in the snRNP core protein Smd1p, Lhp1p is required for assembly of U4 snRNA into its functional form, the U4/U6 snRNP (Xue et al. 2000). Thus, Lhp1p functions redundantly with other proteins that contact nascent small nuclear RNAs to stabilize these RNAs and/or assist their assembly into RNPs.

In the case of pre-tRNAs, the mutations that caused yeast to require Lhp1p resided within the RNAs, rather than in interacting proteins. Specifically, Lhp1p is required to stabilize nascent pre-tRNAs when the pre-tRNA structure is compromised by mutations that disrupt base-pairing in conserved stems or interfere with conserved tertiary interactions (Yoo and Wolin 1997; Long et al. 2001; Johansson and Bystrom 2002; Chakshumathi et al. 2003). Also, Lhp1p is required for efficient folding of pre-tRNA^{Arg}_{CCG} when cells are grown at low temperature or the structure of the pre-tRNA is perturbed by mutation (Chakshumathi et al. 2003). These data suggest that some of the functional redundancy that allows yeast to live without Lhp1p resides within the normally stable pre-tRNA structures, in that mutations that weaken these structures cause a requirement for Lhp1p.

Like La, the many modified nucleotides found in tRNA, as well as other proteins that contact tRNAs during their biogenesis and function, may contribute to tRNA structural stability. In vitro, modified tRNAs exhibit greater thermodynamic stability than the corresponding unmodified tRNAs (Hall et al. 1989; Maglott et al. 1998; Serebrov et al. 1998; Vermeulen et al. 2005). Consistent with a role for modified nucleotides in stabilizing tRNAs, pre-tRNA^{Met}_i is unstable in cells containing a mutation in the m¹A methyltransferase (Kadaba et al. 2004). Similarly, strains carrying a mutant tRNA^{Ser}_{CGA} require the methyltransferases *TRM1* and *TRM2* and the pseudouridine synthase *PUS4* for accumulation of the tRNA during growth at elevated temperatures (Johansson and Bystrom 2002). Moreover, as a catalytically inactive form of Trm2p can substitute for the wild-type Trm2p in allowing accumulation of the mutant tRNA^{Ser}_{CGA}, tRNA modification enzymes may have a chaperone function that is distinct from their catalytic activity (Johansson and Bystrom 2002). However, while many tRNA modifications are conserved in all organisms, the majority of tRNA modification enzymes are not required for efficient growth in yeast (Hopper and Phizicky 2003). Thus, many of these enzymes share with Lhp1p the curious property of being conserved but dispensable.

Here we report that Lhp1p shares functional redundancy with certain tRNA modification enzymes and other proteins that contact tRNAs during their biogenesis. We find that a mutation in the arginyl-tRNA synthetase causes yeast to require Lhp1p for efficient growth. Aminoacylation of tRNA^{Arg}_{CCG}, a tRNA that requires Lhp1p for efficient folding

(Chakshumathi et al. 2003), is severely affected by the synthetase mutation, suggesting that binding by Lhp1p to pre-tRNA^{Arg}_{CCG} is required for residual aminoacylation of the mature tRNA by the mutant synthetase. By creating strains lacking *LHP1* and also lacking specific modification enzymes, we show that Lhp1p is essential for growth at elevated temperature in strains lacking the tRNA methyltransferase *TRM1*. Moreover, in strains carrying a mutation in tRNA^{Arg}_{CCG} and lacking *LHP1*, two pseudouridine synthases, *PUS3* and *PUS4*, are required for viability. Depletion of Pus3p results in accumulation of the mutant tRNA^{Arg}_{CCG} in nuclei, consistent with a block in export. In contrast, Pus4p depletion results in decreased stability of the mutant tRNA. As a catalytically inactive form of *PUS4* does not rescue the requirement for this gene, this likely reflects a requirement for the modification, rather than a separate role of Pus4p. Our data are consistent with a model in which Lhp1p functions together with both tRNA modifications and proteins that contact tRNAs to achieve tRNA structural stability and efficient biogenesis.

RESULTS

Yeast cells containing a mutation in the anticodon-binding domain of arginyl-tRNA synthetase require Lhp1p for efficient growth

During screens for mutations that cause yeast cells to require Lhp1p (Xue et al. 2000), we identified a mutation in *RRS1*, which encodes the yeast arginyl-tRNA synthetase. The mutation consisted of a G to A that changed the valine at position 570 to an isoleucine. In the crystal structure of the complex formed by yeast arginyl-tRNA synthetase, tRNA^{Arg}_{ICG}, and L-arginine, the amide nitrogen of V570 likely forms a hydrogen bond with cytosine 35 in the anticodon loop (Delagoutte et al. 2000). Other residues in this region also interact with the anticodon loop, both through their side chains and through backbone interactions. Mutating V570 to the larger isoleucine likely results in perturbation of the protein backbone and consequently of interactions between the protein and the anticodon region. We named the mutant allele *rrs1-100*. Cells carrying this mutation grew slowly, relative to wild-type yeast, at all temperatures (Fig. 1A).

Interestingly, the requirement for Lhp1p in the *rrs1-100* strains depended on the temperature at which the cells were grown. Although at 30°C the growth of *rrs1-100 LHP1* cells was similar to that of *rrs1-100* cells lacking Lhp1p, growth of *rrs1-100* cells lacking Lhp1p was retarded at 25°C. Strikingly, *rrs1-100* cells lacking Lhp1p were severely impaired in growth at both 16°C and 37°C (Fig. 1A).

During cloning of the *rrs1-100* gene, we isolated a genomic DNA fragment that partly suppressed the growth defect and the requirement for *LHP1* (Fig. 1B). Subcloning in a low copy vector revealed that the suppressor was *TRR4* (tRNA^{Arg}₄), which encodes tRNA^{Arg}_{CCG}. This tRNA^{Arg} iso-

FIGURE 1. A mutation in *RRS1* causes yeast to require *LHP1* for efficient aminoacylation of tRNA^{Arg}_{CCG}. (A) Fivefold serial dilutions of wild-type cells (*RRS1 LHP1*), cells containing a mutation in the arginyl-tRNA synthetase (*rrs1-100 LHP1*), cells lacking *LHP1* (*RRS1 lhp1Δ*), and *rrs1-100* cells lacking *LHP1* (*rrs1-100 lhp1Δ*) were spotted onto YPD agar and grown at the indicated temperatures. (B) Fivefold serial dilutions of wild-type cells (*RRS1 LHP1*), *rrs1-100 LHP1* cells carrying either *TRR4* on the centromeric plasmid pRS316 (pTRR4) or the pRS316 plasmid alone (vector), and *rrs1-100 lhp1Δ* cells carrying either pTRR4 or pRS316 were spotted on YPD agar and grown at 16°C (left) or 25°C (right). (C) RNA from wild-type (*RRS1 LHP1*), *rrs1-100* (*rrs1-100 LHP1*), *lhp1Δ* (*RRS1 lhp1Δ*), and *rrs1-100 lhp1Δ* cells grown at 25°C was extracted under acidic conditions and fractionated in acidic acrylamide gels. The blot was probed to detect tRNA^{Arg}_{CCG} (Arg4), tRNA^{Arg}_{CCU} (Arg1), tRNA^{Arg}_{ACG} (Arg2), tRNA^{Arg}_{UCU} (Arg3), and tRNA^{Thr}_{CGU}.

acceptor, which is encoded by an essential single-copy gene, has a fragile anticodon stem that has a propensity to misfold (Chakshumathi et al. 2003). At low temperature, Lhp1p binding to the pre-tRNA is required for efficient aminoacylation of the mature tRNA in vivo, most likely because Lhp1p binding stabilizes the anticodon stem and facilitates correct folding (Chakshumathi et al. 2003).

As *TRR4* overexpression suppressed the growth defect and the requirement for *LHP1*, we suspected that the levels or aminoacylation of tRNA^{Arg}_{CCG} was reduced in the *rrs1-100* strain. Northern analyses of cells grown at 25°C, 30°C, and 37°C revealed that the levels of all four tRNA^{Arg} isoacceptors were unchanged (data not shown). To examine aminoacylation, total RNA was extracted under acidic conditions to stabilize aminoacyl-tRNA linkages and fractionated in acidic acrylamide gels (Varshney et al. 1991). Northern blotting revealed dramatic differences in aminoacylation in the presence of the *rrs1-100* mutation. At all temperatures, tRNA^{Arg}_{CCG} aminoacylation was most affected. For example, during growth at 25°C, PhosphorImager quantitation revealed that tRNA^{Arg}_{CCG} aminoacylation declined from 38% in wild-type cells to 14% in *rrs1-100 LHP1* and 7% in *rrs1-100 lhp1Δ* cells (Fig. 1C). In contrast, aminoacylation of tRNA^{Arg}_{CCU} declined from 64% in wild-type cells to 56% in *rrs1-100*

LHP1 cells; tRNA^{Arg}_{ACG}, from 77% in wild-type cells to 67% in *rrs1-100 LHP1* cells; and tRNA^{Arg}_{UCU}, from 62% in wild-type cells to 55% in *rrs1-100 LHP1* cells (Fig. 1C). Moreover, for tRNA^{Arg}_{CCU}, tRNA^{Arg}_{ACG}, and tRNA^{Arg}_{UCU}, aminoacylation levels were similar between *rrs1-100 LHP1* cells and *rrs1-100 lhp1Δ* cells.

The finding that the *rrs1-100* mutation primarily affects tRNA^{Arg}_{CCG} aminoacylation could have several explanations. As tRNA^{Arg}_{CCG} diverges in sequence from the three other arginine isoacceptors (Fender et al. 2004), the *rrs1-100* mutation may decrease recognition of this tRNA without significantly affecting recognition of other isoacceptors. An alternative but not exclusive possibility is that the synthetase functions redundantly with Lhp1p to stabilize the fragile anticodon stem of tRNA^{Arg}_{CCG} in the correct conformation, and this function is impaired by the *rrs1-100* mutation. In support of this scenario, in vitro structure probing revealed that the weakly base-paired anticodon stem of pre-tRNA^{Arg}_{CCG} becomes single-stranded at 37°C and that Lhp1p binding prevents the stem from opening (Chakshumathi et al. 2003).

Although the *rrs1-100* cells require *LHP1* for efficient growth on agar at 16°C, 25°C, and 37°C (Fig. 1A), and this requirement is alleviated by extra copies of tRNA^{Arg}_{CCG} (Fig.

1B), we detected only small differences in the aminoacylation of this tRNA between *rrs1-100 LHP1* cells and *rrs1-100 lhp1Δ* cells at 25°C, and no differences at 30°C or 37°C (Fig. 1C; data not shown). Thus, these cells may have different requirements for growth in liquid than they do on agar plates. Alternatively, the fact that tRNA^{Arg}_{CCG} aminoacylation in *rrs1-100 LHP1* cells is already very low may make it difficult to detect further reductions in aminoacylation. Nonetheless, our finding that Lhp1p enhances the growth of *rrs1-100* cells at low and high temperatures (Fig. 1A), together with experiments showing that binding by Lhp1p to pre-tRNA^{Arg}_{CCG} contributes to efficient aminoacylation of the mature tRNA (Chakshusmathi et al. 2003), is consistent with the idea that Lhp1p binding to pre-tRNA^{Arg}_{CCG} enhances aminoacylation of the mature tRNA in the *rrs1-100* strain.

Strains lacking the tRNA methyltransferase *TRM1* require Lhp1p for growth at elevated temperature

To test the possibility that tRNA modification enzymes might function redundantly with Lhp1p, we generated strains lacking each of the modifying enzymes *TRM1*, *TRM2*, *TRM3*, *PUS3*, and *PUS4*. *TRM1*, *TRM2*, and *TRM3* encode methyltransferases while *PUS3* and *PUS4* encode pseudouridine synthases. Trm1p catalyzes formation of dimethylguanosine at position 26 in many tRNAs, Trm2p is required for formation of 5-methyluridine at position 54 (Hopper et al. 1982), and Trm3p catalyzes 2'-O-methylguanosine formation at G18 in the D loop of several tRNAs (Cavaille et al. 1999). Pus3p is required for formation of pseudouridine at positions 38 and 39 in the anticodon loop of most tRNAs (Lecointe et al. 1998), while Pus4p catalyzes pseudouridylation of position 55 in all known tRNAs (Becker et al. 1997) (Fig. 2A).

Interestingly, while strains lacking *TRM1* and *LHP1* exhibited wild-type growth at 16°C, 25°C and, 30°C, they were inviable at 37°C (Fig. 2B). Thus, at least one essential tRNA in *S. cerevisiae* likely requires the presence of either Trm1p or Lhp1p to function at elevated temperature. In contrast, strains lacking *TRM2*, *TRM3*, or *PUS4* and also lacking *LHP1* (*trm2Δ lhp1Δ* and *trm3Δ lhp1Δ* and *pus4Δ lhp1Δ*) grew normally at all temperatures (data not shown). Similarly, while strains lacking *PUS3* exhibit reduced growth and are inviable at 37°C (Lecointe et al. 1998; Fig. 3B, below) and 16°C (data not shown), the growth of *pus3Δ* strains was not affected by deletion of *LHP1*.

Strains carrying a mutant tRNA^{Arg}_{CCG} and lacking *LHP1* require *PUS3* and *PUS4*

Previously, we identified a mutation in tRNA^{Arg}_{CCG}, *trr4-1*, which is a C-to-U change that further weakens the already fragile anticodon stem (Fig. 3A). The mutation increases the propensity of the pre-tRNA to misfold and also results in decreased levels of the mature tRNA. In *trr4-1* strains, Lhp1p

FIGURE 2. Cells lacking Lhp1p require *TRM1* for growth at 37°C. (A) Sites of *TRM1*, *TRM2*, *TRM3*, *PUS3*, and *PUS4* modification are shown on a diagram of a generic tRNA (based on Hopper and Phizicky 2003). (B) Serial dilutions of wild-type cells (*TRM1 LHP1*), cells lacking Lhp1p (*TRM1 lhp1Δ*), cells lacking Trm1p (*trm1Δ LHP1*), and cells lacking both Lhp1p and Trm1p (*lhp1Δ trm1Δ*) were spotted on YPD agar and grown at the indicated temperatures.

binding to the pre-tRNA is required for efficient growth and for aminoacylation of the mature tRNA (Chakshusmathi et al. 2003). Thus, the *trr4-1* allele provides a sensitive assay for determining if modifying enzymes contribute to tRNA biogenesis. Although tRNA^{Arg}_{CCG} modifications have not been mapped, it is likely modified by *TRM2* and *PUS4*, as modifications catalyzed by these enzymes are conserved in eukaryotic and bacterial tRNAs. Also, as tRNA^{Arg}_{CCG} contains G18 and U38, it could be a substrate for *TRM3* and *PUS3*. However, tRNA^{Arg}_{CCG} lacks identity elements for *TRM1* dimethylation (G-C base pairs at positions G10-C25 and C11-G24 and a variable loop of at least 5 nt) (Edqvist et al. 1994). To test whether any of these enzymes were important for stability or function of the mutant tRNA^{Arg}_{CCG}, we combined the *trr4-1* allele with a null allele of each gene.

Although no growth defects were detected when null alleles of *TRM1*, *TRM2*, and *TRM3* were combined with the *trr4-1* mutation, *trr4-1* cells required *PUS3* and *PUS4* for efficient growth. Although very small *trr4-1 pus3Δ* and *trr4-1 pus4Δ* colonies could be isolated, they were drastically impaired in growth (Fig. 3B,C). Similar to *trr4-1* cells lacking *LHP1* (Chakshusmathi et al. 2003), the *trr4-1 pus3Δ* and *trr4-1 pus4Δ* cells had a high rate of spontaneous reversion, making biochemical analyses difficult. Consistent with *PUS3* and *PUS4* sharing redundant functions with *LHP1*, the triple mutants *trr4-1 pus3Δ lhp1Δ* and *trr4-1 pus4Δ lhp1Δ* were inviable (data not shown).

FIGURE 3. Yeast cells containing the *trr4-1* mutation require *PUS3* and *PUS4* for efficient growth. (A) Secondary structures of pre-tRNA^{Arg}_{CCG} (left) and mature tRNA^{Arg}_{CCG} (right) are shown. The position of the *trr4-1* mutation is indicated, as are the potential sites of modification by TRM2, TRM3, PUS3, and PUS4. (B) Serial dilutions of wild-type cells (*PUS3 LHP1 TRR4*), cells lacking Pus3p (*pus3Δ LHP1 TRR4*), *trr4-1* cells (*PUS3 LHP1 trr4-1*), *trr4-1* cells lacking Pus3p (*pus3Δ LHP1 trr4-1*), cells lacking Lhp1p (*PUS3 lhp1Δ TRR4*), cells lacking Pus3p and Lhp1p (*pus3Δ lhp1Δ TRR4*), and *trr4-1* cells lacking Lhp1p (*PUS3 lhp1Δ trr4-1*) were spotted on YPD agar and grown at 25°C. (C) Serial dilutions of wild-type cells (*PUS4 LHP1 TRR4*), *trr4-1* cells (*PUS4 LHP1 trr4-1*), cells lacking Pus4p (*pus4Δ LHP1 TRR4*), *trr4-1* cells lacking Pus4p (*pus4Δ LHP1 trr4-1*), cells lacking Lhp1p (*PUS4 lhp1Δ TRR4*), *trr4-1* cells lacking Lhp1p (*PUS4 lhp1Δ trr4-1*), and cells lacking Pus4p and Lhp1p (*pus4Δ lhp1Δ TRR4*) were spotted on YPD agar and grown at 25°C. (D) Plasmids containing *PUS4* or *pus4*[D75N] in the low copy vector pRS314 were introduced into *pus4Δ lhp1Δ trr4-1* cells carrying *PUS4* in the URA3-containing pRS316 vector. As a control, the strain was also transformed with the pRS314 plasmid. Transformants were streaked on 5-FOA-containing medium and grown at 25°C.

To determine whether the requirements for *PUS3* and *PUS4* were due to requirements for the tRNA modifications, we examined whether catalytically inactive mutants could alleviate the requirements for these genes. For *PUS3*, we used a mutant in which the aspartate at position 151 was converted to alanine [D151A] (Lecointe et al. 2002). This aspartate is conserved in all known pseudouridine synthases and is likely involved in catalysis (Huang et al. 1998; Hoang and Ferre-D'Amare 2001). For *PUS4*, we used a mutant in

which this aspartate, which occurs at position 75 in *PUS4*, was mutated to asparagine [D75N]. As structural information is not yet available for *TRM1* or its bacterial orthologs, we were unable to test catalytically inactive forms of this enzyme.

To assay for function, plasmids containing the mutant forms of *PUS3* and *PUS4* were introduced into *lhp1Δ trr4-1* strains in which the only wild-type copy of these genes was present on an URA3-containing plasmid. We examined whether the cells were able to lose the wild-type *PUS3* or *PUS4* and survive on media containing 5-fluoroorotic acid (5-FOA), which selects against the URA3 gene. For *PUS3*, the results were ambiguous, as even introduction of an empty vector resulted in some growth on 5-FOA, due to the high rate of reversion of these cells (data not shown). However, for *PUS4*, the catalytically inactive mutant was unable to substitute for the wild-type gene in allowing growth in *trr4-1* cells (Fig. 3D). Thus, while we cannot rule out trivial explanations for the lack of rescue, such as a defect in protein folding of the *pus4*[D75N] mutant, it is likely the Ψ55 modification which stabilizes the mutant tRNA, rather than the *PUS4* protein itself.

***PUS3* is not required for accumulation or aminoacylation of the mutant tRNA^{Arg}_{CCG}**

To examine the role of *PUS3*, we created strains in which the glucose-repressible *GAL1* promoter and a triple hemagglutinin tag (3HA) were fused to *PUS3*. Following growth in galactose to allow expression, cells were switched to glucose-containing media to repress the *GAL1* promoter and deplete Pus3p. Western blotting with anti-HA antibodies revealed that upon glucose

addition, the levels of Pus3p declined, becoming undetectable by 12 h (data not shown). At approximately the same time, the *GAL-PUS3 trr4-1* cells slowed in growth, compared with *PUS3 trr4-1* cells (data not shown).

Within 6 h of the switch to glucose-containing media, *trr4-1* tRNA^{Arg}_{CCG} levels declined in both *PUS3 trr4-1* and *GAL-PUS3 trr4-1* cells (Fig. 4A, lanes 5–6,13–14), possibly because the more rapid growth of yeast in glucose results in higher rates of tRNA turnover. However, as the levels of the

FIGURE 4. *PUS3* is not required for accumulation or aminoacylation of the mutant tRNA^{Arg}_{CCG}. (A) Wild-type cells (*PUS3 TRR4*), *trr4-1* cells (*PUS3 trr4-1*), cells containing *PUS3* under control of the *GAL1* promoter (*GAL-PUS3 TRR4*), and *trr4-1* cells containing *PUS3* under control of the *GAL1* promoter (*GAL-PUS3 trr4-1*) were grown at 25°C in galactose-containing media and switched to media containing glucose at time 0. At the indicated intervals, RNA was extracted and subjected to Northern analysis to detect tRNA^{Arg}_{CCG} (top) and tRNA^{Arg}_{UCU} (bottom). (B) At intervals after the switch to glucose media, RNA was extracted from the above cells under acidic conditions and fractionated in acidic acrylamide gels. The Northern blot was probed to detect tRNA^{Arg}_{CCG} (top) and tRNA^{Arg}_{UCU} (bottom). (Lane 1) Deacylated wild-type tRNA. As described in Chakshumathi et al. (2003), charged and uncharged forms of wild-type tRNA^{Arg}_{CCG} migrate slightly faster than these forms of *trr4-1* tRNA^{Arg}_{CCG}.

mutant tRNA in *PUS3 trr4-1* and *GAL-PUS3 trr4-1* cells were similar through 24 h of growth in glucose (Fig. 4A, cf. lanes 5–8 and 13–16), *Pus3p* is not required for accumulation of the *trr4-1* tRNA^{Arg}_{CCG}.

To examine whether *Pus3p* was required for aminoacylation, RNA was extracted under acidic conditions and fractionated in acidic acrylamide gels. As noted above, the levels of the mutant tRNA^{Arg}_{CCG} declined in both *PUS3 trr4-1* and *GAL-PUS3 trr4-1* cells within 6 h in glucose (Fig. 4B, lanes 6–9, 14–17). In *PUS3 trr4-1* cells, this was largely due to loss of the aminoacylated tRNA^{Arg}_{CCG}, although the fraction of aminoacylation recovered to ~50% by 24 h (Fig. 4B, lanes 6–9). This may be due to increased utilization or turnover of the aminoacylated tRNA in glucose, as the aminoacylated form of tRNA^{Arg}_{UCU} also decreased slightly within 6 h (Fig. 4B). In *GAL-PUS3* cells, tRNA^{Arg}_{CCG} aminoacylation was ~58% before the switch to glucose (Fig. 4B, lane 14), suggesting that *Pus3p* overexpression (due to the strong *GAL1* promoter) resulted in increased aminoacylation. Surprisingly, the decrease in aminoacylated tRNA^{Arg}_{CCG} in glucose

media was less severe in *trr4-1* cells depleted of *Pus3p* (Fig. 4B, cf. lanes 6–9 and 14–17). Thus, while overexpression of *Pus3p* may result in increased aminoacylation, *Pus3p* is not required for charging of the mutant tRNA.

The mutant tRNA^{Arg}_{CCG} accumulates in nuclei when *PUS3* is depleted

Since aminoacylation occurs in both the nucleus and cytoplasm (Lund and Dahlberg 1998; Sarkar et al. 1999; Groschans et al. 2000), we used in situ hybridization to examine the subcellular location of tRNA^{Arg}_{CCG} in wild-type and *GAL-PUS3* strains at 24 h after the switch to glucose media. In wild-type cells, *GAL-PUS3* cells and *PUS3* strains containing the *trr4-1* mutation, tRNA^{Arg}_{CCG} was detected in both the nucleus and cytoplasm in ~40% of the cells (Fig. 5A; Table 1). As a control tRNA, tRNA^{Gly}_{GCC}, was mostly cytoplasmic (Fig. 5B; data not shown), export of tRNA^{Arg}_{CCG} may be inefficient even in wild-type cells. However, after depletion of *Pus3p* in *GAL-PUS3 trr4-1* cells, the mutant tRNA^{Arg}_{CCG} was found in both the nucleus and cytoplasm in 62% of the cells, with 31% of the cells showing an entirely nuclear localization (Fig. 5A; Table 1). We conclude that *Pus3p* is important for cytoplasmic accumulation of the mutant tRNA.

PUS4 is important for stable accumulation of the mutant tRNA^{Arg}_{CCG}

We used strains in which *PUS4* was epitope-tagged and placed under control of the *GAL1* promoter to examine the requirement for *Pus4p* in *trr4-1* cells. As above, cells were grown in galactose and then switched to glucose-containing media to repress transcription of *Pus4p*. Within 12 h, *Pus4p* was undetectable by Western blotting and the *GAL-PUS4 trr4-1* cells slowed in growth (data not shown).

Northern analysis revealed that the levels of the mature tRNA^{Arg}_{CCG} declined approximately fourfold as *Pus4p* was depleted from *GAL-PUS4 trr4-1* cells (Fig. 6A, cf. lanes 13 and 16; 6B, right panel). In contrast, while the levels of tRNA^{Arg}_{CCG} in *PUS4 trr4-1* cells declined slightly within 6 h of the switch to glucose media, the levels of this tRNA did not decline further, even after 24 h in glucose (Fig. 6A, lanes 5–8; 6B). Consistent with a stability defect, examination of aminoacylation revealed that while the levels of both charged and uncharged forms of the mutant tRNA^{Arg}_{CCG} decreased in *GAL-PUS4 trr4-1* cells during growth in glucose, the fraction of the mutant tRNA^{Arg}_{CCG} that was aminoacylated at each time point was similar to that of *PUS4 trr4-1* cells (data not shown). Thus, *Pus4p* is required for stable accumulation of the mutant tRNA.

Degradation of mutant forms of tRNA^{Arg}_{CCG} does not require the *TRF4* poly(A) polymerase

Recently, a hypomodified pre-tRNA^{Met}_i, lacking m¹adenosine at position 58, was shown to be degraded through a

FIGURE 5. The *trr4-1* pre-tRNA^{Arg}_{CCG} accumulates in nuclei when *PUS3* is depleted. (A) Wild-type cells (*PUS3 TRR4*), *trr4-1* cells (*PUS3 trr4-1*), cells containing *PUS3* under control of the *GAL1* promoter (*GAL-PUS3 TRR4*), and *trr4-1* cells containing *PUS3* under control of the *GAL1* promoter (*GAL-PUS3 trr4-1*) were grown at 25°C in galactose-containing media and switched to glucose media at time 0. After 24 h, cells were subjected to in situ hybridization to detect tRNA^{Arg}_{CCG} (panels a, c, e, g). Filled arrowheads denote cells in which tRNA^{Arg}_{CCG} was detected in both the nucleus and the cytoplasm, while open arrowheads denote cells where tRNA^{Arg}_{CCG} was primarily detected in nuclei. Nuclei were visualized by staining DNA with DAPI (shown in blue in panels b, d, f, h). (B) As a control, *GAL-PUS3 trr4-1* cells were subjected to in situ hybridization to detect tRNA^{Gly}_{GCC} (right). DNA was visualized with DAPI (left). In this strain, as well as in all strains shown in A, tRNA^{Gly}_{GCC} was primarily cytoplasmic.

pathway involving polyadenylation by the Trf4p poly(A) polymerase and degradation by the nuclear exosome (Kadaba et al. 2004). We determined if the reduced levels of tRNA^{Arg}_{CCG} in *trr4-1* cells are due to Trf4p-dependent degradation. We also examined a second mutant allele of *TRR4*, *trr4-2*, which disrupts the acceptor stem, reduces mature tRNA, and results in temperature-sensitivity (Chak-

shusmathi et al. 2003). We constructed strains in which each mutant allele was combined with a null allele of *TRF4*. Loss of Trf4p did not ameliorate the growth defects of the *trr4-1* and *trr4-2* strains (data not shown). Northern analyses and PhosphorImager quantitation revealed that the level of mutant tRNA^{Arg}_{CCG} was approximately twofold elevated in *trr4-1* strains and was not significantly altered in *trr4-2* cells lacking *TRF4* (Fig. 7). Thus, mutant forms of tRNA^{Arg}_{CCG} are largely degraded through a pathway that does not require Trf4p.

DISCUSSION

The La protein Lhp1p is required for pre-tRNA end-maturation and correct folding when yeast cells contain mutations that disrupt pre-tRNA structural elements (Yoo and Wolin 1997; Chakshusmathi et al. 2003). Here we found that Lhp1p is important for growth when cells contain a mutation in the arginyl tRNA synthetase or lack the tRNA modification enzyme Trm1p. Moreover, the pseudouridine synthases Pus3p and Pus4p function together with Lhp1p to facilitate biogenesis of a mutant pre-tRNA^{Arg}. Our results support the hypothesis that Lhp1p, tRNA modifications, and possibly also tRNA synthetases all contribute to tRNA structural stabilization and/or efficient tRNA biogenesis in vivo.

For many conserved modifications, it has been puzzling that yeast cells lacking the modifications have no growth defects. However, several modification enzymes, like Lhp1p, become important or essential for growth in the presence of mutations that destabilize tRNAs (Grosshans et al. 2001; Johansson and Bystrom 2002). Consistent with the idea that tRNA modifications function redundantly to stabilize tRNAs, strains lacking both Pus1p (which catalyzes pseudouridine formation at multiple positions [Motorin et al. 1998]) and Pus4p have severe growth defects (Grosshans et al. 2001) and strains lacking Trm1p and the methyltransferase Trm11p (which catalyzes formation of m²G10) show a slight growth defect (Purushothaman et al. 2005). Most recently, yeast lacking the m⁷G46 methyltransferase displayed growth defects when other modification enzymes were deleted (Alexandrov et al. 2006). Our results that cells lacking *TRM1* and *LHP1* are temperature-sensitive, and that Lhp1p

TABLE 1. Subcellular distribution of tRNA^{Arg}_{CCG} following Pus3p depletion

Genotype	Nuclear /			No. counted
	Nuclear	Cytoplasmic	Cytoplasmic	
<i>PUS3 TRR4</i>	0%	42%	58%	107
<i>PUS3 trr4-1</i>	0%	45%	55%	100
<i>GAL-PUS3 TRR4</i>	0%	34%	66%	112
<i>GAL-PUS3 trr4-1</i>	31%	62%	7%	98

FIGURE 6. *PUS4* is required for stable accumulation of the mutant tRNA^{Arg}_{CCG}. (A) Wild-type cells (*PUS4 TRR4*), *trr4-1* cells (*PUS4 trr4-1*), cells containing *PUS4* under control of the *GAL1* promoter (*GAL-PUS4 TRR4*), and *trr4-1* cells containing *PUS4* under control of the *GAL1* promoter (*GAL-PUS4 trr4-1*) were grown at 25°C in galactose-containing media and switched to glucose media at time 0. At intervals, RNA was extracted and subjected to Northern analysis to detect tRNA^{Arg}_{CCG} (top) and tRNA^{Arg}_{UCU} (bottom). (B) Graphic representation of the data in A. The intensity of each band was determined using a PhosphorImager, and the values for tRNA^{Arg}_{CCG} at each time point were normalized relative to the amount of the control tRNA^{Arg}_{UCU}. Due to the large differences in intensities, the wild-type (left) and *trr4-1* (right) samples were graphed separately.

is required for viability in *pus3Δ* and *pus4Δ* cells carrying a mutation in tRNA^{Arg}_{CCG}, support the idea that tRNAs use multiple mechanisms, including Lhp1p binding and acquisition of modified nucleotides, to ensure structural stability. Consistent with a redundant role for Lhp1p in stabilizing pre-tRNAs, strains containing a mutation in the *TRM61* subunit of the m¹A methyltransferase and lacking Lhp1p exhibit a synthetic growth defect and decreased tRNAⁱ_{Met} (Calvo et al. 1999).

Since La proteins bind pre-tRNAs, not mature tRNAs, does Lhp1p exhibit functional redundancy exclusively with modification enzymes that act on pre-tRNAs containing 3' trailers? Consistent with this idea, those enzymes that exhibit genetic interactions with Lhp1p (*TRM1*, *TRM61*, *PUS3*, and *PUS4*) are nuclear, while *TRM3* is cytoplasmic and the location of *TRM2* is unknown (Rose et al. 1995; Anderson et al. 1998; Lecointe et al. 1998; Huh et al. 2003). However, a study in which tRNA modifications were analyzed on yeast pre-tRNA^{Tyr} following injection into *Xenopus* oocytes revealed that, while Ψ55, m¹A58, and some m²G26 (catalyzed by *PUS4*, *TRM61*, and *TRM1*, respectively) are present on pre-tRNAs that have not undergone end-maturation, Ψ38/39 and most m²G26 (catalyzed by *PUS3* and *TRM1*, respectively) were found largely on end-

matured pre-tRNA^{Tyr} (Nishikura and De Robertis 1981). If these results can be generalized to yeast, some enzymes that exhibit genetic interactions with Lhp1p may function after removal of Lhp1p from the pre-tRNA.

The finding that cells lacking *LHP1* and *TRM1* are temperature-sensitive is consistent with proposals that both Lhp1p and the *TRM1*-catalyzed m²G26 modification stabilize correctly folded anticodon stems (Steinberg and Cedergren 1995; Chakshumathi et al. 2003). Because certain tRNAs that have the potential to form alternate anticodon stems contain m²G26, it was proposed that m²G26, which disrupts cytidine base-pairing, prevents misfolding (Steinberg and Cedergren 1995). For Lhp1p, chemical modification revealed that Lhp1p stabilizes the anticodon stem of tRNA^{Arg}_{CCG} from opening at elevated temperatures (Chakshumathi et al. 2003). Thus, Lhp1p and m²G26 may function redundantly to prevent the anticodon stems of certain pre-tRNAs from adopting alternate structures.

As tRNAs shuttle between the nucleus and cytoplasm (Shaheen and Hopper 2005; Takano et al. 2005), the nuclear accumulation of *trr4-1* tRNA^{Arg}_{CCG} that occurs on Pus3p depletion could reflect either a failure of nuclear export, increased nuclear import, or a combination of both. One possibility is that tRNA export receptors, such as Los1p and Msn5p (Hopper and Phizicky 2003; Takano et al. 2005), do not recognize the mutant tRNA^{Arg}_{CCG} in the absence of the Pus3p-catalyzed modification. Although it is possible that nuclear export requires the pseudouridine

FIGURE 7. Degradation of mutant forms of tRNA^{Arg}_{CCG} do not require Trf4p. (A) RNA extracted from wild-type cells (lane 1), cells carrying the *trr4-1* and *trr4-2* mutations in tRNA^{Arg}_{CCG} (lanes 2,5), *trr4-1* cells lacking *TRF4* (*trr4-1 trf4Δ*; lane 3), *trf4Δ* (lane 4), and *trr4-2 trf4Δ* (lane 6) cells was probed to detect tRNA^{Arg}_{CCG}. The blot was reprobed to detect 5S rRNA. (B) Graphic representation of the data in A. The intensity of each band was determined using a PhosphorImager.

modification per se, we favor a model in which lack of the modification alters *trr4-1* tRNA conformation. This scenario would be consistent with the finding that wild-type tRNA^{Arg}_{CCG} is exported normally in *pus3Δ* cells. Alternatively, if nuclear reimport is part of a pathway for removing non-functional tRNAs from the translation machinery, aberrant tRNAs may accumulate in nuclei.

The functional redundancy we observed between Lhp1p and several modification enzymes could explain why La is dispensable for growth in budding and fission yeast. In this model, tRNA modifications, as well as proteins that contact nascent tRNAs, such as Lhp1p, modification enzymes, aminoacyl tRNA synthetases, and possibly also export factors, may all contribute to structural stabilization. One possibility is that organisms that require La for viability, such as *Drosophila melanogaster* and *T. brucei*, possess at least one essential, structurally fragile tRNA that depends upon La for stabilization of the pre-tRNA.

Lastly, our finding that unstable mutant forms of tRNA^{Arg}_{CCG} are degraded independently of Trf4p reveals that multiple pathways exist for noncoding RNA decay in yeast. Recently, a hypomodified tRNA^{Val} was also found to be degraded independently of this pathway (Alexandrov et al. 2006). Moreover, while Trf4p has been implicated in the decay of small nuclear and nucleolar RNAs (LaCava et al. 2005; Wyers et al. 2005), certain snRNAs that are unstable in cells containing a mutation in an Sm core protein (Xue et al. 2000) are degraded independently of Trf4p (R.L. Sherrer and S.L. Wolin, unpubl.). The identification and characterization of additional pathways for noncoding RNA decay may reveal how Lhp1p and other proteins that interface with newly synthesized RNAs contribute to quality control.

MATERIALS AND METHODS

Yeast media and strains

Media was prepared according to Sherman (1991). Wild-type and *lhp1::LEU2* strains were CY1 (*MATα ura3 lys2 ade2 trp1 his3 leu2 LHP1*) and CY2 (*MATα ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*). The *rrs1-100* and control strains were GC1 (*MATa RRS1 LHP1 ura3 lys2 ade2 trp1 his3 leu2*), GC2 (*MATα rrs-100 LHP1 ura3 lys2 ade2 trp1 his3 leu2*), GC3 (*MATa RRS1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2*), and GC4 (*MATα rrs1-100 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2*). Strains containing null alleles of modification enzymes were SW1 (*MATa trm1::HIS LHP1 ura3 lys2 ade2 trp1 his3 leu2*) and SW2 (*MATa trm1::HIS lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2*), LC1 (*MATa pus3::HIS LHP1 TRR4 ura3 lys2 ade2 trp1 his3 leu2*), LC2 (*MATα pus3::HIS lhp1::LEU2 TRR4 ura3 lys2 ade2 trp1 his3 leu2*), LC3 (*MATa pus3::HIS LHP1 trr4-1 ura3 lys2 ade2 trp1 his3 leu2*), LC4 (*MATα pus4::HIS LHP1 TRR4 ura3 lys2 ade2 trp1 his3 leu2*), LC5 (*MATa pus4::HIS lhp1::LEU2 TRR4 ura3 lys2 ade2 trp1 his3 leu2*), and LC6 (*MATa pus4::HIS LHP1 trr4-1 ura3 lys2 ade2 trp1 his3 leu2*). Other strains were SK151 (*MATa/α trr4-1/TRR4 lhp1::LEU2/LHP1 ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2*), SK100 (*MATα trr4-1 LHP1 ura3 lys2 ade2 trp1 his3 leu2*),

SK201 (*MATa trr4-2 LHP1 ura3 lys2 ade2 trp1 his3 leu2*), LC8 (*MATα pus3::KANMX6-GAL-3HA-PUS3 LHP1 TRR4 ura3 lys2 ade2 trp1 his3 leu2*), LC9 (*MATa pus3::KANMX6-GAL-3HA-PUS3 LHP1 trr4-1 ura3 lys2 ade2 trp1 his3 leu2*), LC10 (*MATα pus4::KANMX6-GAL-3HA-PUS3 LHP1 TRR4 ura3 lys2 ade2 trp1 his3 leu2*), LC11 (*MATa pus4::KANMX6-GAL-3HA-PUS3 LHP1 trr4-1 ura3 lys2 ade2 trp1 his3 leu2*), LS32.1 (*MATα trf4::HIS3 LHP1 ura3 lys2 ade2 trp1 his3 leu2*), LS28 (*MATα trf4::HIS3 LHP1 trr4-1 ura3 lys2 ade2 trp1 his3 leu2*), and LS16 (*MATα trf4::HIS3 LHP1 trr4-2 ura3 lys2 ade2 trp1 his3 leu2*).

Null alleles of *TRM2*, *TRM3*, *PUS4*, and *TRF4* were created using PCR to replace the coding sequence with *HIS3*. For each gene, the forward primer contained 50–70 nt upstream of the ORF followed by CTCTTGGCCTCCTCTAGTAC, which is complementary to sequences upstream of *HIS3* in pRS313 (Sikorski and Hieter 1989). The reverse primer contained 60 nt downstream from the ORF followed by AGCGCGCCTCGTTCAGAATG, which is downstream from *HIS3*. Following amplification using pRS313 as template, fragments were transformed into the diploid strain SK151. For *TRM1*, nucleotides 37–1679 of the ORF were replaced with *HIS3*. For *PUS3*, nt 170–709 of the ORF were replaced. Integration of the *GAL1* promoter and 3HA upstream of *PUS3* and *PUS4* was as described (Longtine et al. 1998) using pFA6a-kanMx6-PGAL1–3HA.

The *rrs1-100* allele was isolated in a screen described previously (Xue et al. 2000). To clone *RRS1*, a genomic library in YCp50 was introduced into the mutant and transformants screened at 16°C for loss of cold sensitivity. Only one genomic clone fully rescued the cold sensitivity. Subcloning revealed that a fragment of genomic DNA containing *RRS1* and 360 nt of 5′ flanking sequence and 33 nt of 3′ flanking sequence alleviated the requirement for *LHP1* and the inability to grow at 16°C and 37°C. The mutation was identified by sequencing DNA from the *rrs1-100* strain. The *trr4-1* and *trr4-2* alleles were described (Chakshusmathi et al. 2003).

Northern analyses

Total RNA was extracted from yeast using hot phenol as described (Ausubel et al. 1998). For Northern analyses, 5 μg of RNA was fractionated in 5% polyacrylamide / 8 M urea gels and transferred to ZetaProbe GT (Bio-Rad) in 0.5 TBE at 150 mA for 16 h. For aminoacylation analyses, RNA was isolated by lysing cells in pH 4.5 phenol (Varshney et al. 1991). Deacylation was performed at pH 9.0. To separate aminoacylated and deacylated tRNAs, RNAs were fractionated in 6.5% polyacrylamide, 8 M urea, 0.1 M NaOAc (pH 5.0) gels at 4°C (Varshney et al. 1991). Following transfer to membranes as described above, hybridization was performed with oligonucleotide probes: tRNA^{Arg}_{CCU}, 5′-CCGCAGTCTTCTCCTTA GGAGGGAGACGCGTTA-3′; tRNA^{Arg}_{ACG}, 5′-CCTGGAATCTTCTG GTTCGTAGCCAGACGCCGTGA-3′; tRNA^{Arg}_{UCU}, 5′-CCCATAAT CTTCTGATTAGAAGTCAGACGCGTTG-3′; tRNA^{Arg}_{CCG}, 5′-ACT CGAACCCGGATCACAGCCACCCGGAAGAATGCATGCTAACC ATT-3′; tRNA^{Thr}_{CGU}, 5′-AATTGAACCCACGATCCCCGCATTACG AGTGCGATGCCTTACCACT-3′.

Plasmids and mutagenesis

The plasmids pRS315–PUS3 and pRS315–pus3[D151A] (Lecoine et al. 2002) were gifts of Henri Grosjean (CNRS, Gif-sur-Yvette,

France). To generate pRS316–PUS3, the SalI/XbaI fragment from pRS315–PUS3 was cloned into pRS316. Plasmid pRS315–PUS4 (Grosshans et al. 2001), carrying a 3.3 kb PstI fragment containing *PUS4*, was a gift of George Simos (University of Thessaly, Greece). The PstI fragment was excised and cloned into the PstI site of pRS314. The *pus4*[D75N] mutation was made using the Quik-Change site-directed mutagenesis kit (Stratagene). To generate pRS316–PUS4, *PUS4* was excised from pRS315–PUS4 with SalI and SpeI and cloned into pRS316.

In situ hybridization

To detect tRNA^{Arg}_{CCG}, we used a clone in which the pre-tRNA was placed behind the T7 promoter in pSP64 (Chakshusmathi et al. 2003). For transcription of anti-sense RNA, the plasmid was digested with EcoRI, transcribed and labeled with digoxigenin-UTP using the DIG RNA kit and SP6 RNA polymerase (Roche). To detect tRNA^{Gly}_{CCC}, oligonucleotides 5′-GCATTTAGGTGACACTATAGAA TGCGCAAGCCCGGAATCGAACCGGGGCCCAAC-3′ and 5′-GCGCAAGTGGTTTAGTGGTAAAATCCAACGTTGCCATCGTTG GGCCCCCGGTTTCGATTCC-3′ were annealed and amplified using Pfu Turbo DNA polymerase (Stratagene). The resulting DNA was transcribed and labeled with digoxigenin-UTP as above. In situ hybridization was performed as described (Fernandez et al. 2004).

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