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A La protein requirement for efficient pre-tRNA folding

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The La protein protects the 3′ ends of many nascent small RNAs from exonucleases. Here we report that La is required for efficient folding of certain pre-tRNAs. A mutation in pre-tRNA$^{Arg}_{CCG}$ causes yeast cells to be cold-sensitive and to require the La protein Lhp1p for efficient growth. When the mutant cells are grown at low temperature, or when Lhp1p is depleted, mature tRNA$^{Arg}_{CCG}$ is not efficiently aminoacylated. The mutation causes the anticodon stem of pre-tRNA$^{Arg}_{CCG}$ to misfold into an alternative helix *in vitro*. Intragenic suppressor mutations that disrupt the misfolded helix or strengthen the correct helix alleviate the requirement for Lhp1p, providing evidence that the anticodon stem misfolds *in vivo*. Chemical and enzymatic footprinting experiments suggest a model in which Lhp1p stabilizes the correctly folded stem. Lhp1p is also required for efficient aminoacylation of two wild-type tRNAs when yeast are grown at low temperature. These experiments reveal that pre-tRNAs can require protein assistance for efficient folding *in vivo*.

Keywords: aminoacylation/La autoantigen/pre-tRNA folding

Introduction

Although it is well accepted that newly synthesized proteins can require molecular chaperones to assist folding, the role of RNA-binding proteins in assisting RNA folding is less clear. As studies of RNA folding *in vitro* have revealed that RNA often becomes kinetically trapped in alternative helices, it has been proposed that RNA-binding proteins assist RNA folding *in vivo* (reviewed by Herschlag, 1995). Several RNA-binding proteins, such as *Escherichia coli* StpA, S12 and Hfq, as well as fragments of hnRNP A1 and HIV nucleocapsid proteins, promote formation of correctly folded RNA and/or enhance RNA:RNA pairing *in vitro* (Herschlag, 1995; Schroeder *et al*., 2002). These proteins, when overexpressed in *E.coli*, resolve an alternative helix that functions as a folding trap, indicating these proteins can also assist folding *in vivo* (Clodi *et al*., 1999; Schroeder *et al*., 2002; Moll *et al*., 2003). However, of these proteins, only Hfq (Zhang *et al*., 2002) has been demonstrated to have a normal, physiologic role in facilitating RNA folding in cells.

To date, the only well understood examples of protein-assisted RNA folding *in vivo* involve group I and group II introns. For these introns, specific RNA-binding proteins assist folding by stabilizing correct tertiary structures (reviewed by Schroeder *et al*., 2002). In addition, folding of one group I intron requires an ATP-dependent RNA helicase to disrupt misfolded structures that act as kinetic traps in the folding pathway (Mohr *et al*., 2002). However, for all other classes of RNA, nothing is known of the protein requirements for folding *in vivo*.

A protein that has often been proposed to facilitate RNA folding is the La protein. This nuclear protein binds many newly synthesized small RNAs, including pre-tRNAs, pre-5S rRNA, U6 snRNA and 7SL RNA (reviewed by Wolin and Cedervall, 2002). As part of the binding site for La is the sequence UUU_{OH}, the majority of bound RNAs are polymerase III transcripts. In *Saccharomyces cerevisiae*, La also binds nascent polymerase II-transcribed small RNAs that terminate in UUU_{OH} (Kufel *et al*., 2000; Xue *et al*., 2000). For pre-tRNAs and most La-bound RNAs, the UUU_{OH} is removed during the maturation process. Thus, La does not bind the mature RNAs. In mammals, La has been implicated in internal initiation of mRNA translation (Wolin and Cedervall, 2002). As La binds many RNAs and has been linked to several processes, it was proposed that La binding facilitates formation of higher order structures (Svitkin *et al*., 1994).

Genetic and biochemical studies have revealed that a major role of La is to protect nascent RNAs from nucleases. In the yeasts *S.cerevisiae* and *Schizosaccharomyces pombe*, binding by La to pre-tRNAs prevents exonucleolytic nibbling, allowing removal of the 3′ trailers by an endonuclease (Van Horn *et al*., 1997; Yoo and Wolin, 1997; Intine *et al*., 2000). In *S.cerevisiae*, binding by the La protein Lhp1p stabilizes precursors to spliceosomal U snRNAs and the small nucleolar U3 RNA (Pannone *et al*., 1998; Kufel *et al*., 2000; Xue *et al*., 2000). In the case of pre-U4 snRNA, Lhp1p stabilizes a precursor that is preferentially bound by the snRNP protein Smd1p, suggesting that Lhp1p binding contributes to efficient snRNP assembly (Xue *et al*., 2000).

While there is good evidence that La protects nascent RNAs from nucleases, whether La contributes to RNA folding has remained unclear. Yeast containing a mutation that disrupts the anticodon stem of tRNA$^{Ser}_{CGA}$ require Lhp1p for maturation of the pre-tRNA. As restoring basepairing in the stem eliminated the Lhp1p requirement, Lhp1p binding was proposed to stabilize the correctly folded pre-tRNA (Yoo and Wolin, 1997). However, as the mature tRNA did not accumulate without Lhp1p, an alternative possibility was that Lhp1p protected the mutant

pre-tRNA from nucleases (Yoo and Wolin, 1997). Consistent with a role in nuclease protection, a mutant pre-tRNA$^{Ser}_{CGA}$ with an altered variable loop requires Lhp1p for accumulation (Johansson and Bystrom, 2002). Thus, a role for La in assisting RNA folding has not been demonstrated.

Here we show that Lhp1p is required for efficient folding of certain pre-tRNAs in yeast cells. A mutation in the essential gene encoding tRNA$^{Arg}_{CCG}$ causes yeast to be cold-sensitive and to require *LHP1* for efficient growth at 25°C. Deacylated tRNA$^{Arg}_{CCG}$ accumulates when Lhp1p is depleted or the cells are grown at low temperature. *In vitro*, the mutation causes the anticodon stem of pre-tRNA$^{Arg}_{CCG}$ to misfold. Chemical and enzymatic probing experiments are consistent with a model in which Lhp1p stabilizes the correctly folded stem. As intragenic suppressor mutations that weaken the incorrect helix or strengthen the correct helix alleviate the cold-sensitivity and *LHP1* requirement, the pre-tRNA likely also misfolds *in vivo*. Moreover, Lhp1p is required for efficient aminoacylation of two wild-type tRNAs. Our experiments reveal that certain pre-tRNAs require protein assistance for efficient folding *in vivo*.

Results

A mutation in yeast tRNA$^{Arg}_{CCG}$ causes cold-sensitivity and a requirement for LHP1

During screens for mutations that cause yeast to require *LHP1* (Pannone *et al*., 2001; also see Materials and methods), we identified three new mutations in tRNA genes. Two mutations, *trr4-1* and *trr4-2*, were members of one complementation group, while the third, *trt2-1*, defined a second group. Cloning of the genes revealed that the gene encoding tRNA$^{Arg}_{CCG}$ complemented the *LHP1* requirement in *trr4* strains while the gene encoding tRNA$^{Thr}_{CGU}$ complemented the *trt2* strain. We named the genes *TRR4* (tRNA arg4) and *TRT2* (tRNAthr2). DNA sequencing revealed that *trr4-1* is a C-to-U change that weakens the anticodon stem, while *trr4-2* is a G-to-A change that disrupts the acceptor stem of tRNA$^{Arg}_{CCG}$ (Figure 1A). The *trt2-1* mutation is a G-to-A at position 41 of tRNA$^{Thr}_{CGU}$ that disrupts the anticodon stem (data not shown). In addition to requiring *LHP1* for efficient growth at 25°C, the *trr4-1* strain grew poorly at 16 and 37°C, while the *trr4-2* strain was inviable at 37°C. Gene disruption experiments revealed that both *TRR4* and *TRT2* are essential (data not shown).

Although we had previously identified temperature-sensitive mutations in tRNA$^{Ser}_{CGA}$ that caused cells to require *LHP1* for maturation of the pre-tRNA (Yoo and Wolin, 1997; Long *et al*., 2001), the *trr4-1* allele was unusual in that it also resulted in cold-sensitivity. At 25°C, small *trr4-1* colonies could be isolated lacking *LHP1*, revealing that Lhp1p was not absolutely required for viability. These strains grew very slowly (Figure 1B) and had a high rate of reversion, making them unsuitable for biochemical analyses. The *trr4-1* strains containing chromosomal *LHP1* grew well at 25°C, but not at the same rate as wild-type cells. However, at 16°C, these cells were retarded in growth (Figure 1B). When the sole copy of *LHP1* was present on a centromeric plasmid, which raises Lhp1p levels ~2-fold (data not shown), *trr4-1* cells

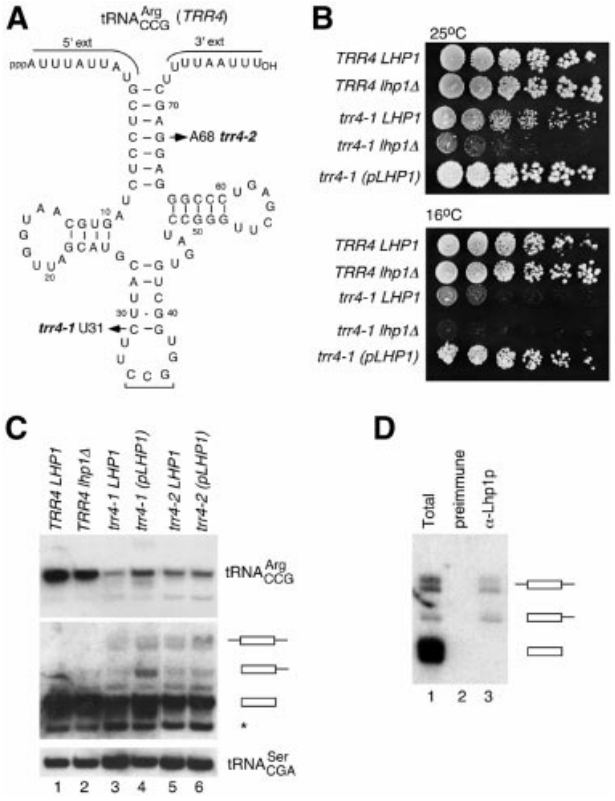

Fig. 1. The *trr4-1* mutation results in cold-sensitivity and a requirement for *LHP1*. (**A**) Positions of *trr4-1* and *trr4-2* mutations are shown on pre-tRNA$^{Arg}_{CCG}$. Leader and trailer lengths were estimated from pre-tRNA sizes on denaturing gels. (**B**) Five-fold serial dilutions of wild-type cells (*TRR4 LHP1*), cells lacking *LHP1* (*TRR4 lhp1*Δ), *trr4-1* cells lacking *LHP1* (*trr4-1 lhp1*Δ) and *trr4-1* cells carrying chromosomal (*trr4-1 LHP1*) or plasmid *LHP1* (*trr4-1 pLHP1*) were spotted onto YPD agar and grown for four days at 25°C (top) or six days at 16°C (bottom). (**C**) RNA from wild-type cells (lane 1), cells lacking *LHP1* (lane 2), *trr4-1* cells with chromosomal or plasmid *LHP1* (lanes 3 and 4) and *trr4-2* cells with chromosomal or plasmid *LHP1* (lanes 5 and 6) was subjected to northern analysis to detect tRNA$^{Arg}_{CCG}$ (top). To detect pre-tRNAs, the blot was overexposed (middle). Asterisk, cross-hybridization with another isoacceptor. The blot was reprobed to detect tRNA$^{Ser}_{CGA}$ (bottom). (**D**) A *trr4-1* cell extract was incubated with pre-immune (lane 2) or anti-Lhp1p antibodies (lane 3). RNAs in the immunoprecipitate and an equivalent amount of extract (lane 1) were subjected to northern analysis to detect tRNA$^{Arg}_{CCG}$.

grew nearly as well as wild-type cells at 25 and 16°C (Figure 1B). Thus, in addition to being required for efficient growth at 25°C, *LHP1* is a low-copy suppressor of the *trr4-1* mutation.

To determine the effects of the mutations on tRNA levels, we grew the strains at 25°C and extracted RNA. Northern blotting to detect tRNA$^{Arg}_{CCG}$ and tRNA$^{Thr}_{CGU}$ revealed that for both *trr4* strains (Figure 1C) and the *trt2-1* strain (data not shown), the mutations resulted in decreased mature tRNAs (Figure 1C, lanes 2–6, top). As overexposure of the blot (middle panel) revealed pre-tRNA accumulation, processing of the mutant pre-tRNAs may be slow relative to wild-type pre-tRNAs. With plasmid *LHP1*, pre-tRNA$^{Arg}_{CCG}$ and mature tRNA levels increased ~2-fold in the *trr4-1* strain (Figure 1C, lane 4; see also Figure 3A). Thus, as described for pre-tRNA$^{Met}_{i}$ (Anderson *et al*., 1998), overexpressed Lhp1p may stabilize *trr4-1* pre-tRNA$^{Arg}_{CCG}$ allowing more efficient maturation. Immunoprecipitations confirmed that Lhp1p

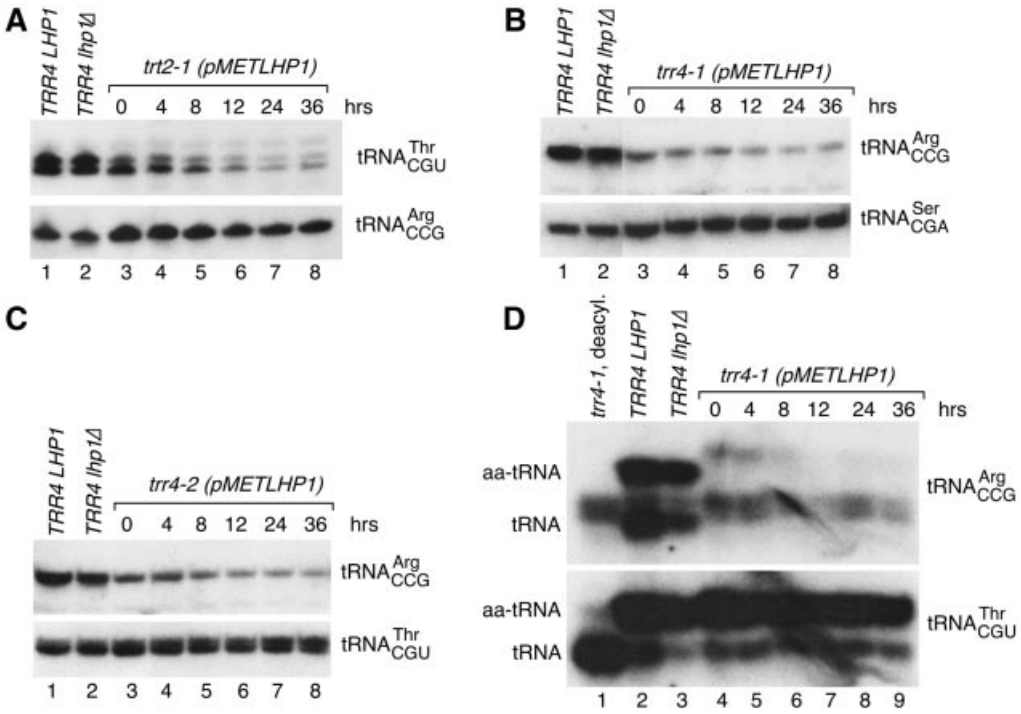

Fig. 2. Depletion of *LHP1* from *trr4* and *trt2* cells. *trt2-1* (**A**), *trr4-1* (**B**) and *trr4-2* cells (**C**) containing pMETLHP1 were grown at 25°C in media lacking methionine and switched to 2 mM methionine media at time 0. At intervals (lanes 3–8), RNA was subjected to northern analysis to detect $tRNA^{Thr}_{CGU}$ (A, top; C, bottom), $tRNA^{Arg}_{CCG}$ (A, bottom; B and C, top) or $tRNA^{Ser}_{CGA}$ (B, bottom). RNA was also analyzed from wild-type (lanes 1) and *lhp1*Δ cells (lanes 2). For unknown reasons, $tRNA^{Thr}_{CGU}$ runs as a doublet. (**D**) At intervals after the switch to 2 mM methionine, RNA was extracted from *trr4-1* cells under acidic conditions and fractionated in acidic acrylamide gels. The northern blot was probed to detect $tRNA^{Arg}_{CCG}$ and $tRNA^{Thr}_{CGU}$. Lane 1, deacylated *trr4-1* RNA. RNA was also analyzed from wild-type cells (lane 2) and cells lacking *LHP1* (lane 3). Consistent with an altered structure, charged and uncharged forms of wild-type $tRNA^{Arg}_{CCG}$ migrate differently than these forms of *trr4-1* $tRNA^{Arg}_{CCG}$. Lane 3 is underloaded.

bound wild-type and mutant pre-$tRNA^{Arg}_{CCG}$ containing 3′ trailers, but not mature tRNA (Figure 1D and data not shown).

LHP1 is required for efficient aminoacylation of $tRNA^{Arg}$ in trr4-1 strains

To determine why *LHP1* was required, we placed *LHP1* under control of the methionine-repressible *MET3* promoter. When strains were grown in media lacking methionine, Lhp1p levels were similar to that of wild-type cells. Upon addition of 2 mM methionine, Lhp1p declined, becoming undetectable by 12 h. Also at 12 h, the *trt2-1*, *trr4-1* and *trr4-2* strains slowed in growth (data not shown).

RNA was extracted from the cells and subjected to northern blotting. Levels of mature $tRNA^{Thr}_{CGU}$ declined 3.5-fold as Lhp1p was depleted from *trt2-1* cells (Figure 2A, lanes 3–8). Overexposure of the blot revealed that pre-$tRNA^{Thr}_{CGU}$ levels were unchanged (data not shown), consistent with a requirement for Lhp1p in tRNA maturation rather than transcription. Both findings were similar to that observed when Lhp1p was depleted from cells carrying a mutation in $tRNA^{Ser}_{CGA}$ (Yoo and Wolin, 1997).

In contrast, Lhp1p depletion from the two *trr4* strains resulted in less severe decline in $tRNA^{Arg}_{CCG}$ levels (Figure 2B and C). This was most apparent for *trr4-1* (Figure 2B, lanes 3–8), where PhosphorImager quantitation revealed that mature $tRNA^{Arg}_{CCG}$ declined <2-fold. To determine whether the tRNA was functional, RNA was extracted under acidic conditions to stabilize aminoacyl-tRNA linkages and fractionated in acidic acrylamide gels (Varshney *et al*., 1991). Northern analyses revealed that while approximately half the wild-type $tRNA^{Arg}_{CCG}$ was aminoacylated (Figure 2D, lane 2), only 20% of *trr4-1* $tRNA^{Arg}_{CCG}$ was charged in the presence of Lhp1p (lane 4). Upon Lhp1p depletion, aminoacylated $tRNA^{Arg}_{CCG}$ declined, becoming undetectable after 12 h (lanes 4–9). Reprobing for $tRNA^{Thr}_{CGU}$ revealed that aminoacylation of this tRNA was unaffected (Figure 2D). Thus, Lhp1p is required in *trr4-1* cells for efficient aminoacylation of $tRNA^{Arg}_{CCG}$.

As Lhp1p does not bind mature $tRNA^{Arg}_{CCG}$ (Figure 1D), it was unlikely that Lhp1p functioned directly in aminoacylation. However, aminoacylation is sensitive to changes in tRNA structure (Giege *et al*., 1993), as is the export of mature tRNA from the nucleus, which uses both nuclear aminoacylation and binding of tRNA-specific export receptors as proofreading steps (Hopper and Phizicky, 2003). Thus, one possibility was that *trr4-1* pre-tRNA misfolded in the absence of Lhp1p, such that the resulting mature tRNA was either incompetent for export and/or unable to undergo charging. Consistent with a structural alteration, both charged and uncharged forms of *trr4-1* $tRNA^{Arg}_{CCG}$ migrated slower than these forms of the wild-type tRNA (Figure 2D, lanes 2–4).

Uncharged $tRNA^{Arg}_{CCG}$ accumulates in trr4-1 cells grown at low temperature

Since cold-sensitive mutations are associated with trapping of RNA in competing helices at low temperature

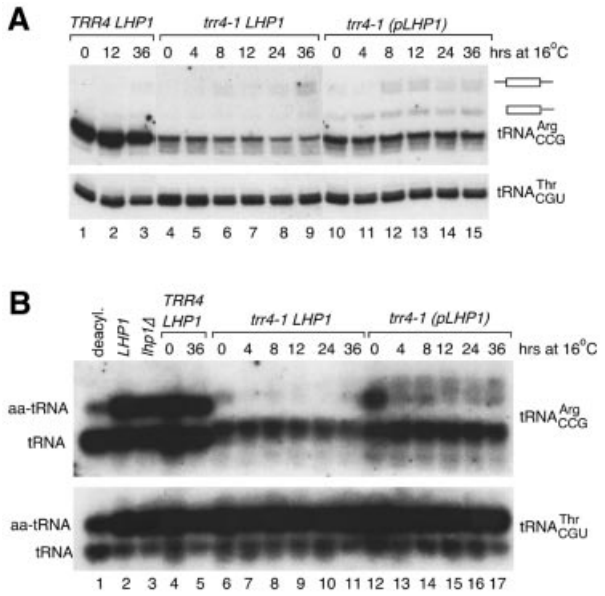

Fig. 3. Aminoacylated $tRNA^{Arg}_{CCG}$ declines when *trr4-1* cells are grown at 16°C. (**A**) Wild-type (lanes 1–3) and *trr4-1* cells carrying chromosomal (lanes 4–9) or plasmid *LHP1* (lanes 10–16) were grown at 25°C and switched to 16°C at time 0. At indicated times, RNA was extracted and subjected to northern analysis to detect $tRNA^{Arg}_{CCG}$ and $tRNA^{Thr}_{CGU}$. (**B**) At intervals, RNA was extracted under acidic conditions, fractionated in acidic acrylamide gels, and subjected to northern analysis to detect $tRNA^{Arg}_{CCG}$ and $tRNA^{Thr}_{CGU}$.

(Dammel and Noller, 1993; Zavanelli *et al*., 1994), we examined whether deacylated $tRNA^{Arg}_{CCG}$ accumulated at 16°C. Wild-type and *trr4-1* cells were grown at 25°C and then shifted to 16°C. RNA was analyzed by northern blotting. While $tRNA^{Arg}_{CCG}$ levels were initially lower in *trr4-1* cells (Figure 3A, lanes 4 and 10), mature tRNA declined <2-fold during growth at 16°C (lanes 4–9 and 10–15).

To examine aminoacylation, RNA was extracted and subjected to electrophoresis under acidic conditions. At 25°C, the fraction of charged $tRNA^{Arg}_{CCG}$ was higher in cells carrying plasmid *LHP1* than cells with chromosomal *LHP1* (Figure 3B, lanes 6 and 12). Within 4 h at 16°C, charged $tRNA^{Arg}_{CCG}$ was undetectable in *trr4-1* cells with chromosomal *LHP1* (Figure 3B, lane 7). In cells with plasmid *LHP1*, charged $tRNA^{Arg}_{CCG}$ also declined (lanes 12–17), but a smear of hybridization (which may correspond to pre-tRNAs) made it hard to determine whether charged tRNA declined to undetectable levels. Nonetheless, accumulation of deacylated $tRNA^{Arg}_{CCG}$ at 16°C is consistent with the hypothesis that the *trr4-1* mutation results in misfolding.

The trr4-1 mutation causes the anticodon stem of pre-$tRNA^{Arg}_{CCG}$ to misfold in vitro

To examine the structures formed by the pre-tRNAs *in vitro*, we synthesized the RNAs using T7 RNA polymerase. Preliminary experiments using ribonucleases revealed that both wild-type and *trr4-1* pre-tRNAs were grossly misfolded when isolated under denaturing conditions (electrophoresis in urea-containing gels, phenol-extraction and ethanol precipitation). This is consistent with reports that yeast $tRNA^{Arg}$ is one of several tRNAs that is isolated from cells in a form that is inactive for aminoacylation (Lindahl *et al*., 1966). Heating of the wild-type pre-tRNA in 10 mM magnesium largely alleviated the misfolding, but did not correct the *trr4-1* structure (data not shown). A similar misfolding problem has been described for unmodified *E.coli* $tRNA^{Phe}$, in that once denatured, the tRNA cannot be efficiently refolded (Uhlenbeck, 1995). To minimize the problem, *in vitro*-transcribed pre-tRNAs were isolated on DEAE–Sepharose columns without denaturants (see Materials and methods). As end-labeling reactions require denaturing gel electrophoresis to isolate a single labeled species, reactions were carried out on unlabeled RNAs and visualized by primer extension or northern analysis.

Experiments using chemical probes that modify RNA in single-stranded regions are shown in Figure 4. Sites of modification were mapped by primer extension, as reverse transcriptase stops at the nucleotide 3′ to the modification. Since pre-tRNAs are highly structured, and because some degradation is inevitable during native RNA isolation, there were many stops that were independent of chemical probes (Figure 4A and B). However, examination of the stops that depended on chemical addition revealed several differences between wild-type and *trr4-1* pre-tRNAs. Most changes were in bases of the anticodon stem. Experiments using kethoxal (which reacts with unpaired Gs) revealed that G39 and G40, which form part of the weakly basepaired anticodon stem in the canonical tRNA structure, were more accessible to the chemical in *trr4-1* pre-tRNA (Figure 4A, lanes 6, 7, 12 and 13). Also, G36 and G37, which are single-stranded in the wild-type structure, are less accessible in *trr4-1* RNA (lanes 12 and 13; also Figure 6A). Experiments with CMCT [1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate], which modifies unpaired Us, revealed that U31 in *trr4-1* pre-tRNA (which is a basepaired C in wild-type RNA) was accessible (Figure 4B, lanes 9 and 10). Lastly, G10 and G22 (at the ends of the D-stem) were more accessible in *trr4-1* RNA (Figure 4A, lanes 6 and 7, 12 and 13).

The CMCT and kethoxal data, along with experiments with dimethylsulfate (DMS) (which modifies A and C; data not shown), indicated that *trr4-1* pre-$tRNA^{Arg}_{CCG}$ adopts an altered conformation *in vitro*. The most plausible model, based on structure mapping, is shown in Figure 4D (right panel). In the model, the anticodon stem is misfolded into an alternative helix, such that the anticodon is partly basepaired. As G36 and G37 are less accessible to kethoxal in *trr4-1* pre-tRNA, suggesting they are at least partly basepaired, while their proposed pairing partners U29 and U30 are accessible to CMCT, the misfolded stem may exist as two conformers, one in which the residues are basepaired, and a second in which they are unpaired (Figure 4D, right). While our model does not change positions of G10 and G22, which are more accessible in *trr4-1* RNA, formation of tertiary interactions between D and T-loops in the misfolded tRNA may cause residues at the ends of the D-stem to become partly unpaired. Consistent with a largely intact D-stem, U11 (Figure 4B, lanes 9 and 10), C23 and A24 (not shown) are inaccessible to chemical probes. Although other models for the *trr4-1* pre-tRNA structure are possible, the proposed misfolded stem is consistent with the finding that uncharged *trr4-1*

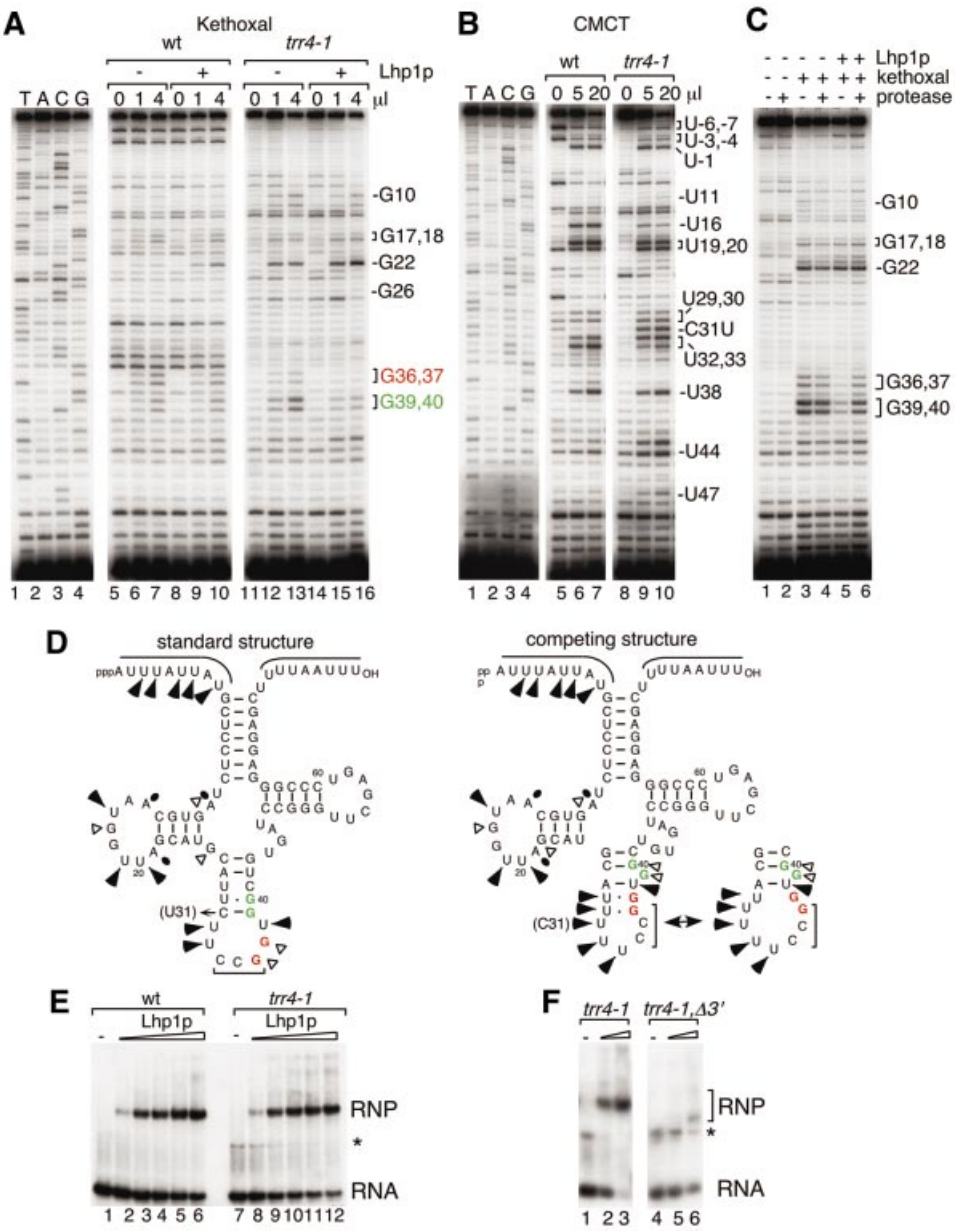

Fig. 4. *trr4-1* pre-tRNA adopts an altered conformation *in vitro*. (**A**) Wild-type (lanes 5–10) and *trr4-1* pre-tRNA$^{Arg}_{CCG}$ (lanes 11–16) (0.68 pmol each) were incubated with the indicated amounts of kethoxal in the absence or presence of 6.8 pmol Lhp1p. Modifications were detected by primer extension. Lanes 1–4, dideoxy sequencing of pre-tRNA$^{Arg}_{CCG}$. Lanes are labeled according to the RNA sequence. Each extension stop is one base below the modified nucleotide. In some experiments, modification of G26 is detected in wild-type pre-tRNA. (**B**) Wild-type (lanes 5–7) and *trr4-1* pre-tRNA$^{Arg}_{CCG}$ (lanes 8–10) were incubated with the indicated amounts of CMCT and subjected to primer extension. Lanes 1–4, dideoxy sequencing. (**C**) *trr4-1* pre-tRNA$^{Arg}_{CCG}$ was incubated in the absence (lanes 1–4) or presence (lanes 5 and 6) of Lhp1p. Proteinase K was added to digest Lhp1p (lanes 2, 4 and 6). Following a second incubation, kethoxal was added (lanes 3–6), and modifications detected as in (A). (**D**) The classic structure for pre-tRNA$^{Arg}_{CCG}$ (left) and the proposed alternative structure (right). Sites of modification are designated by arrows (CMCT), triangles (kethoxal) and dots (DMS). Two G residues that are more accessible to kethoxal in wild-type RNA are red and two Gs that are less accessible are green. The alternative structure is predicted by MFOLD to be more stable than the classic structure by 2.3 kcal/mol. (**E**) 0.34 pmol of wild-type (lanes 1–6) and *trr4-1* pre-tRNA$^{Arg}_{CCG}$ (lanes 7–12) were incubated without protein (lanes 1–7) or with 2- (lanes 2 and 8), 4- (lanes 3 and 9), 6- (lanes 4 and 10), 8- (lanes 5 and 11) or 10-fold (lanes 6 and 12) molar excess of Lhp1p. RNA and RNPs were separated in native gels and detected by northern analysis. Asterisk, a second *trr4-1* pre-tRNA conformer. (**F**) 0.68 pmol of *trr4-1* (lanes 1–3) and *trr4-1* pre-tRNA lacking the 3′ 9 nt (*trr4-1*,Δ3′) were incubated without protein (lanes 1 and 4), or with 5- (lanes 2 and 5) or 10-fold (lanes 3 and 6) molar excess of Lhp1p. Naked RNAs and RNPs were separated in native gels and detected by northern analysis. Asterisk, a conformer of *trr4-1* pre-tRNA.

tRNA accumulates *in vivo*. While tRNA end maturation and CCA addition require only that the acceptor stem and TΨC stem–loop be intact (Maizels and Weiner, 1999), tRNA^{Arg} charging requires unpaired C35 and G36 in the anticodon loop (Sissler *et al*., 1996).

Lhp1p alters the accessibility of the mutant pre-tRNA to chemical probes

To examine effects of Lhp1p, we determined the Lhp1p concentration that shifted pre-tRNA$^{Arg}_{CCG}$ into an RNA–protein complex, while minimizing formation of higher

order, less-specific complexes (Long *et al*., 2001) (Figure 4E). By comparing Lhp1p binding to full-length and 3′ truncated pre-tRNAs, we confirmed that binding was largely dependent on the 3′ trailer (Figure 4F and data not shown).

For wild-type pre-tRNA, kethoxal modifications were similar in the presence or absence of Lhp1p (Figure 4A, lanes 8–10). (As modifications are viewed by primer extension, no information is obtained about the RNA 3′ end.) Experiments with CMCT revealed that all protections were lower with Lhp1p (not shown), most likely due to side reactions that occur between CMCT and protein (Krol and Carbon, 1989).

Interestingly, Lhp1p addition to *trr4-1* pre-tRNA resulted in protection of G36, G37, G39 and G40 from kethoxal modification (Figure 4A, lanes 15 and 16). These residues are all part of the misfolded stem (Figure 4D). Changes in modification were dependent on bound Lhp1p, as digestion of the RNA–protein complex with protease prior to probing restored accessibility of the mutant pre-tRNA to modification (Figure 4C, lanes 5 and 6). The changes in modification could have several explanations. Lhp1p binding could facilitate correct folding of the anticodon stem, since G39 and G40 are basepaired in the wild-type RNA (Figure 4D). However, G22 accessibility was unchanged upon Lhp1p addition, indicating that the *trr4-1* structure did not fully convert to that of wild-type pre-tRNA. An alternative possibility is that Lhp1p protects G36, G37, G39 and G40 from modification. Thus, Lhp1p makes specific contacts with the misfolded stem and/or causes alterations of the *trr4-1* structure *in vitro*.

Intragenic suppressors that stabilize the correct helix or weaken the competing helix relieve the LHP1 requirement

To obtain evidence that the alternative conformation forms *in vivo*, we selected intragenic suppressors of the *trr4-1* cold-sensitivity. We expected to identify mutations that stabilized the correct helix or weakened the competing helix. A library of *trr4-1* genes containing random second mutations in *trr4-1* was constructed. The DNA was integrated into strains in which the only copy of *TRR4* was on a *URA3*-containing plasmid. Transformants were tested for growth on media containing 5-fluoro-orotic acid (FOA) at 25°C. As FOA selects for cells that have lost the *URA3* plasmid, cells that grew received a mutated *trr4-1* allele that functions at 25°C. Strains were tested for growth at 16°C. One strain, which changed G40 to U, was identified. This change destabilizes the central C–G basepair of the competing helix, but should have only a mild effect on the correct stem, as it disrupts a non-Watson–Crick basepair adjacent to unpaired bases (Figure 5A). Cells carrying the mutation (*trr4-1*,*U40*) grew better than *trr4-1* cells at 16°C (Figure 5B, bottom panel). Mating to a strain lacking *LHP1*, followed by tetrad dissection, revealed that cells carrying *trr4-1*,*U40* did not require *LHP1* for efficient growth at 25°C (Figure 5B). As mutations stabilizing the correct stem were not obtained (most likely because our screen did not reach saturation), we constructed such a strain by mutating G39-to-A (Figure 5A). This strain (*trr4-1*,*A39*) grew well at 16°C and did not require *LHP1* for efficient growth (Figure 5B).

To determine the mechanism of suppression, we examined $tRNA^{Arg}_{CCG}$ levels and charging. At 25 and 16°C, $tRNA^{Arg}_{CCG}$ levels in strains carrying suppressor mutations (*trr4-1*,*U40* and *trr4-1*,*A39*) were similar to the *trr4-1* strain (Figure 5C, lanes 2–4 and 6–8). However, the fraction of charged $tRNA^{Arg}_{CCG}$ was higher in the *trr4-1*,*A39* strain (51% for *trr4-1*,*A39* versus 20% for *trr4-1*) (Figure 5D, lanes 3 and 5). For the *trr4-1*,*U40* strain, the presence of shorter species (Figure 5C, lanes 3 and 7; Figure 5D, lanes 4 and 8), which may be due to inefficient CCA addition, made it difficult to quantitate the charged tRNA. However, after 4 h at 16°C, charged $tRNA^{Arg}_{CCG}$ remained detectable in the suppressors, but was undetectable in the *trr4-1* strain (lanes 7–9). Thus, both suppressors function by preventing $tRNA^{Arg}_{CCG}$ misfolding.

As mutations predicted to stabilize the correct helix (*trr4-1*, *A39*) or weaken the competing helix (*trr4-1*, *U40*) alleviate the cold-sensitivity and requirement for *LHP1*, we conclude that the *trr4-1* anticodon stem likely misfolds *in vivo*.

LHP1 is required for efficient aminoacylation of two wild-type tRNAs

Since the anticodon stem of wild-type pre-$tRNA^{Arg}_{CCG}$ is weakly basepaired, the wild-type RNA may also misfold. We used kethoxal modification to examine pre-tRNA structure as a function of temperature *in vitro* (Figure 6A). For both wild-type and *trr4-1* pre-$tRNA^{Arg}_{CCG}$, raising the temperature to 30 or 37°C resulted in enhanced accessibility of G39 and G40 to kethoxal (lanes 7, 8, 15 and 16). As decreased accessibility at G36 and G37 was not detected, enhanced modification of G39 and G40 probably reflects increased opening of the weakly basepaired wild-type and *trr4-1* anticodon stems at 37°C, rather than misfolding. Lhp1p addition largely eliminated the changes in modification at G39 and G40 (lanes 10–12 and 18–20). Thus, Lhp1p prevents opening of the anticodon stem and/or protects the unpaired residues from modification.

We examined whether efficient aminoacylation of the wild-type tRNA depended on *LHP1*. As low temperature should trap misfolded forms (Zavanelli and Ares, 1991; Dammel and Noller, 1993), we examined aminoacylation at 16°C. Cells were grown first at 25°C, and then shifted to 16°C. In wild-type cells, there was a small (~2-fold) decrease in charged $tRNA^{Arg}_{CCG}$ during growth at 16°C (Figure 6B, lanes 2–7, top). This decrease was exacerbated in cells lacking Lhp1p, as charged $tRNA^{Arg}_{CCG}$ declined >5-fold by 24 h at 16°C (lanes 8–12). Reprobing for $tRNA^{Thr}_{CGU}$ and $tRNA^{Gln}_{CUG}$ revealed that $tRNA^{Thr}_{CGu}$ aminoacylation also declined slightly in cells lacking *LHP1* during growth at 16°C, while $tRNA^{Gln}_{CUG}$ charging was unaffected. Thus, *LHP1* is required for efficient aminoacylation of at least two wild-type tRNAs at low temperature.

We examined $tRNA^{Arg}_{CCG}$ aminoacylation during growth at higher temperatures. While the fraction of $tRNA^{Arg}_{CCG}$ that was charged increased at 30 and 37°C, no differences were detected between wild-type and *lhp1*Δ strains (data not shown). Thus, if the anticodon stem opening that we detect at elevated temperatures *in vitro* (Figure 6A) occurs *in vivo*, it is not detrimental to aminoacylation. Nonetheless, the finding that Lhp1p increases aminoacylation of two wild-type tRNAs at 16°C suggests that binding by Lhp1p to these pre-tRNAs facilitates correct folding.

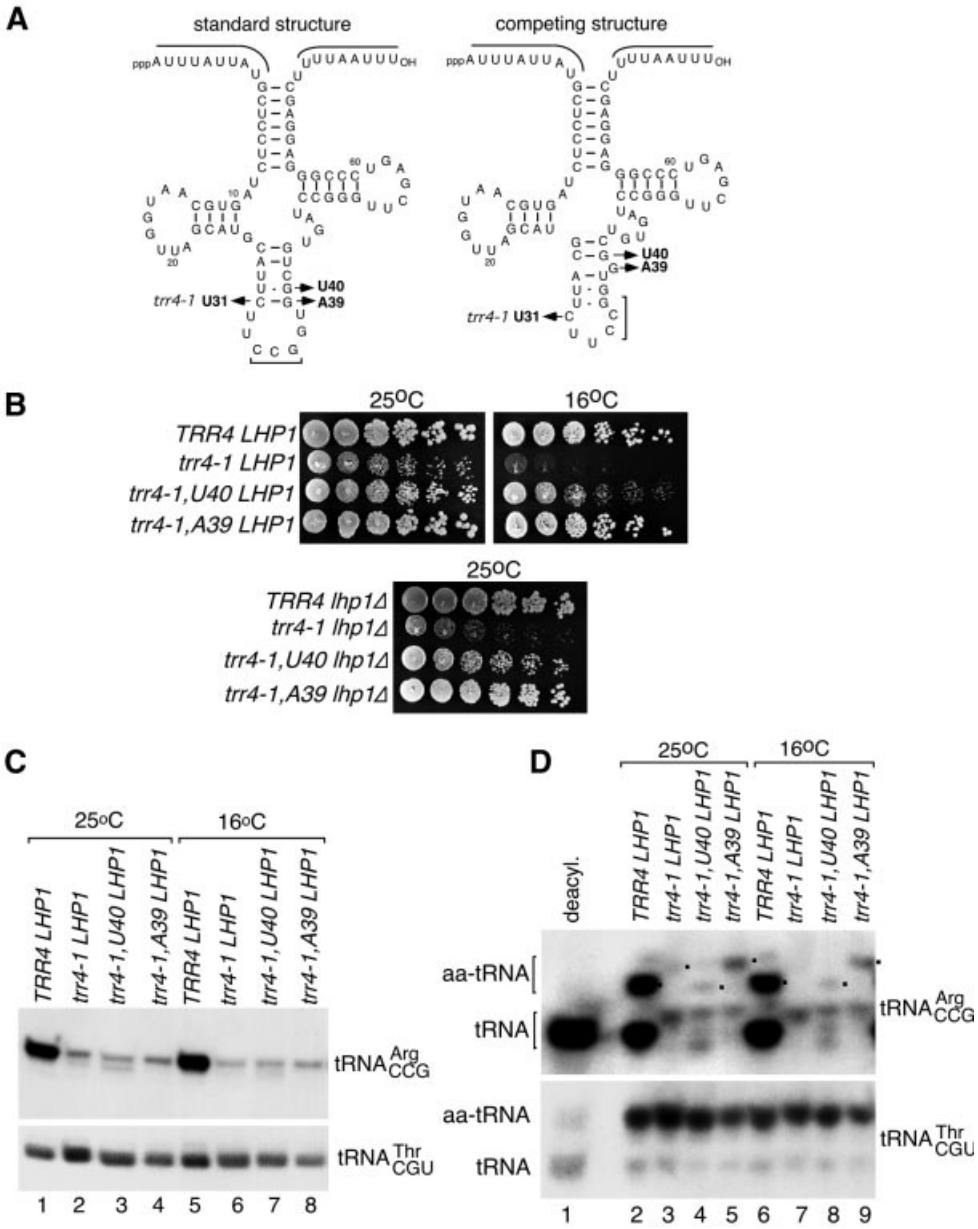

Fig. 5. Intragenic suppressor mutations favor formation of the correct helix. (**A**) Standard and proposed competing structures of pre-tRNA$^{Arg}_{CCG}$ are shown along with positions of the *trr4-1* (U31) mutation and A39 and U40 suppressors. (**B**) Top: 5-fold serial dilutions of wild-type, *trr4-1 LHP1* and *trr4-1 LHP1* strains carrying suppressors A39 (*trr4-1,A39 LHP1*) and U40 (*trr4-1,U40 LHP1*) were spotted on YPD agar and grown at 25°C for four days (left) or 16°C for six days (right). Bottom: growth of wild-type and *trr4-1* strains lacking *LHP1* at 25°C was compared with *trr4-1* strains carrying mutations A39 (*trr4-1,A39 lhp1*Δ) and U40 (*trr4-1,U40 lhp1*Δ). (**C**) RNA from wild-type (lanes 1 and 5), *trr4-1* (lanes 2 and 6), *trr4-1,U40* (lanes 3 and 7) and *trr4-1,A39* strains (lanes 4 and 8) containing *LHP1* was subjected to northern analysis to detect tRNA$^{Arg}_{CCG}$ (top) and tRNA$^{Thr}_{CGU}$ (bottom). The band below mature tRNA$^{Arg}_{CCG}$ in *trr4-1,U40* strains may represent tRNA lacking CCA. (**D**) RNA was extracted under acidic conditions and fractionated in acidic acrylamide gels. The blot was probed to detect tRNA$^{Arg}_{CCG}$ and tRNA$^{Thr}_{CGU}$. Charged species are indicated by dots. For *trr4-1,U40*, two species represent uncharged tRNA (lanes 4 and 8), one of which may lack CCA. Lane 1, deacylated *TRR4 LHP1* RNA.

Lhp1p may contact the acceptor stem and anticodon loop of pre-tRNA$^{Arg}_{CCG}$

A question raised by our studies was how a protein that binds the 3′ trailer could influence folding of the anticodon stem. However, while 3′ uridylates are a major determinant for La binding, La must also recognize other structural features (Wolin and Cedervall, 2002). To examine sites of contact between Lhp1p and pre-tRNA$^{Arg}_{CCG}$, we performed enzymatic footprinting using 5′ end-labeled RNA. As only wild-type pre-tRNA$^{Arg}_{CCG}$ could be largely refolded following denaturing gel purification, the analysis was confined to the wild-type RNA.

We performed footprinting using ribonucleases T1, T2 and V1. T1 cuts after guanosines, preferentially in single-stranded regions, T2 cuts in single-stranded regions and V1 prefers helical regions (Krol and Carbon, 1989). Cleavage of the naked RNA revealed strong V1 cuts in the acceptor and anticodon stems, but weak to undetectable cuts in the T and D stems, consistent with previous tRNA probing (Lowman and Draper, 1986). T1 and T2 yielded

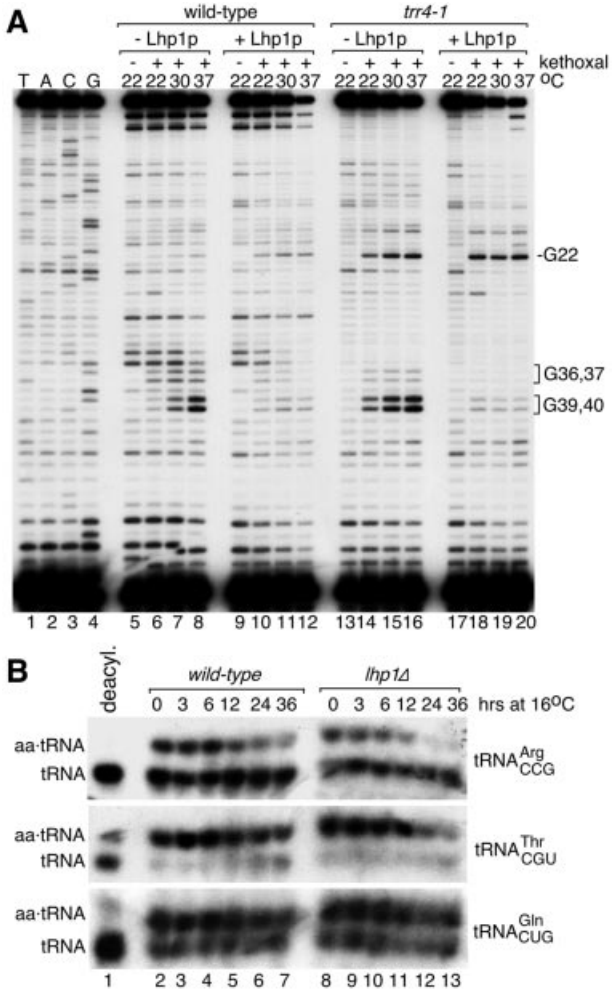

Fig. 6. *LHP1* is required for efficient aminoacylation of wild-type $tRNA^{Arg}_{CCG}$. (**A**) Wild-type (lanes 5–12) and *trr4-1* pre-$tRNA^{Arg}_{CCG}$ (lanes 13–20) were incubated at the indicated temperature in the absence or presence of Lhp1p. Following incubation, 5 µl of kethoxal was added. Modifications were detected by primer extension. Lanes 1–4, dideoxy sequencing. The primer extension stops in the wild-type tRNA (lanes 5–12) at positions 30, 34 and 35 and near the top of the gel are not kethoxal-dependent. (**B**) Wild-type (lanes 1–7) and cells lacking *LHP1* (lanes 8–12) were grown at 25°C and switched to 16°C at time 0. At intervals, RNA was extracted under acidic conditions and subjected to northern analysis to detect $tRNA^{Arg}_{CCG}$ (top), $tRNA^{Thr}_{CGU}$ (middle) and $tRNA^{Gln}_{CUG}$ (bottom panel). Lane 1, deacylated wild-type RNA.

strong cuts in the anticodon loop and weaker cleavage of the D and T loops (Figure 7A, lanes 5, 7 and 9). We also detected weak T1 and T2 cleavage in the T-stem, suggesting that a fraction of the RNA is single-stranded in this region. As these cleavages were more prominent in pre-tRNA that was not refolded, they may represent a fraction of the wild-type RNA that remains incorrectly folded.

Lhp1p protected the 3′ strand of the acceptor stem from V1 RNase digestion (Figure 7A, lanes 9 and 10; Figure 7B, top). The V1 cleavages and Lhp1p protection extended into the trailer, consistent with the proposal that the trailer and leader basepair (Lee *et al*., 1997). In contrast, V1 cleavages in the anticodon stem were unchanged with Lhp1p (Figure 7B, bottom). However, in multiple experiments, Lhp1p addition resulted in slight protection of parts of the anticodon loop (nucleotides U34–G36) from T2 ribonuclease (Figure 7A, lanes 7 and 8, asterisks). PhosphorImager quantitation confirmed that these nucleotides were less accessible in the presence of Lhp1p (Figure 7C, top). T2 cleavages in the D-loop were unchanged with Lhp1p (Figure 7C, bottom), revealing that the very weak protection of the anticodon loop was not due to underloading in the lane. While changes in nuclease accessibiity can be due to either protein binding or structural alterations, protection of the 3′ acceptor stem and trailer is consistent with the known binding of Lhp1p to 3′ ends (Wolin and Cedervall, 2002). Moreover, the slight protection of the anticodon loop from nuclease suggests that bound Lhp1p may also contact the anticodon loop. Regions of pre-$tRNA^{Arg}_{CCG}$ protected from nuclease digestion by Lhp1p are shown in red in Figure 7D.

Discussion

Our experiments have revealed that binding by Lhp1p to both wild-type and a mutant pre-$tRNA^{Arg}_{CCG}$ is important for formation of the correctly folded mature tRNAs. In the absence of Lhp1p, the pre-tRNAs undergo end maturation, but the mature tRNAs are inefficiently aminoacylated. Biochemical and genetic experiments support a role for Lhp1p in stabilizing pre-tRNA structure. To our knowledge, this is the first evidence that pre-tRNAs can require proteins to assist folding into their correct structures *in vivo*.

Previous studies have documented that certain tRNAs have a propensity to misfold. Several tRNAs are isolated from cells in two conformations, only one of which is a substrate for aminoacylation (Gartland and Sueoka, 1966; Lindahl *et al*., 1966; Herschlag, 1995). Studies of point mutations in human mitochondrial $tRNA^{Ile}$ have revealed that wild-type $tRNA^{Ile}$ has a fragile T stem that is susceptible to structural rearrangements (Kelley *et al*., 2001). The $tRNA^{Arg}_{CCG}$ may be particularly susceptible to misfolding, as it is the only *S.cerevisiae* tRNA with a mismatch in the anticodon stem. The *trr4-1* mutation further weakens the already fragile stem, increasing the fraction of RNA that misfolds. As $tRNA^{Thr}_{CGU}$ also requires Lhp1p for efficient aminoacylation at 16°C, a fraction of this tRNA may similarly misfold.

Our data are most consistent with a model in which Lhp1p stabilizes the correctly folded anticodon stem. First, as treatment of Lhp1p–pre-tRNA complexes with protease prior to kethoxal addition restores the modification pattern of the misfolded anticodon stem to that of naked RNA (Figure 4C), Lhp1p probably acts by stoichiometric binding rather than through a catalytic mechanism. Second, the weak protection of the wild-type anticodon loop from ribonuclease, coupled with the finding that Lhp1p eliminates the increased kethoxal accessibility of the *trr4-1* and wild-type anticodon stems at 30 and 37°C (Figure 6A) suggests that bound Lhp1p stabilizes the anticodon stem–loop. In our model, the mutant pre-$tRNA^{Arg}_{CCG}$ is kinetically trapped in the alternative helix at 16°C. Raising the temperature lowers the kinetic barrier, allowing more pre-tRNA to fold into the correct helix. Binding by Lhp1p stabilizes the correct anticodon stem, preventing misfolding.

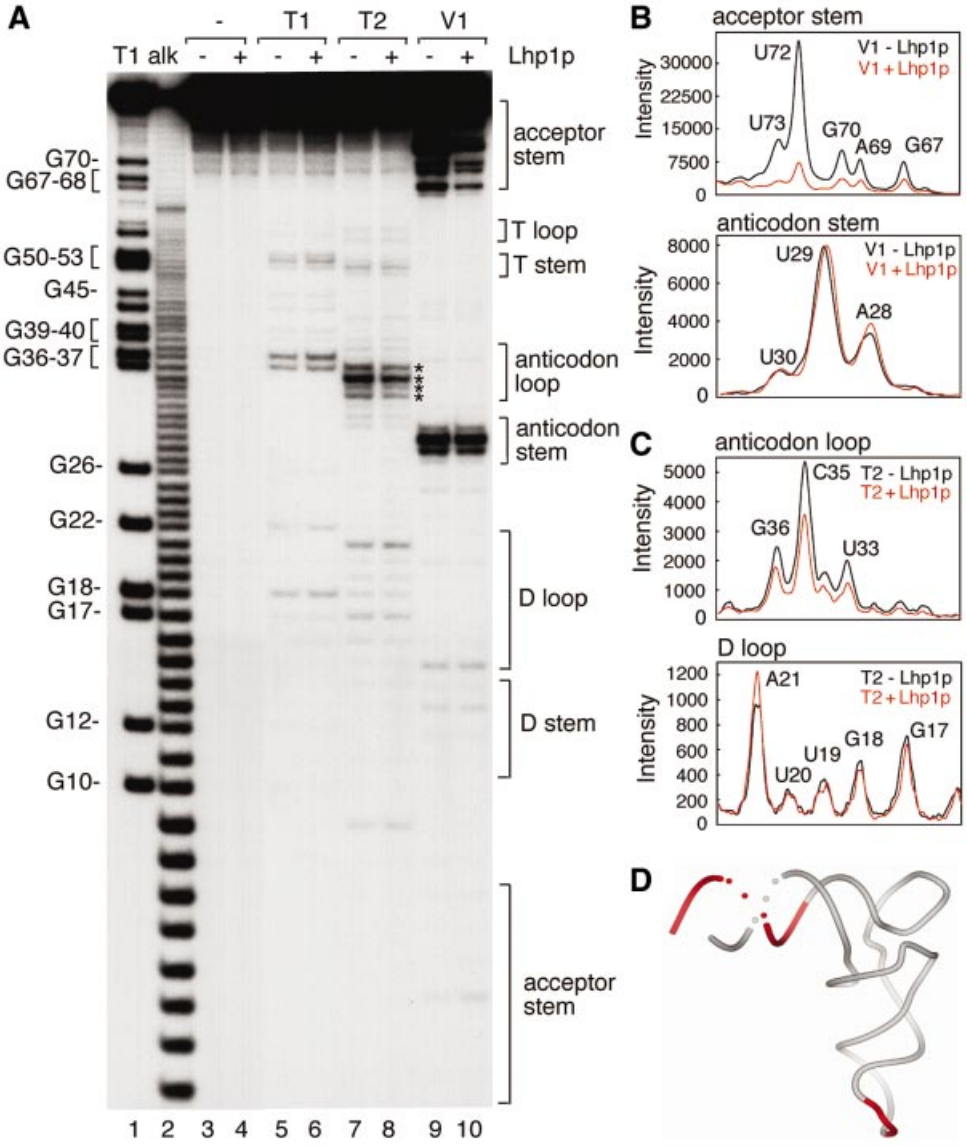

Fig. 7. Lhp1p may contact the acceptor stem and anticodon loop of pre-tRNA$^{Arg}_{CCG}$. (**A**) 5′ labeled pre-tRNA$^{Arg}_{CCG}$ was incubated without (lanes 3, 5, 7 and 9) or with (lanes 4, 6, 8 and 10) Lhp1p, followed by cleavage with T1 (lanes 5 and 6), T2 (lanes 7 and 8) or V1 (lanes 9 and 10) ribonucleases. In the experiment, ~85% of the labeled RNA was bound by Lhp1p. Lanes 1 and 2, T1 ribonuclease and alkaline hydrolysis ladders. Asterisks, sites of weak protection by Lhp1p. (**B**) Phosphorimager quantitation of V1 cleavage of the acceptor stem (top) and the anticodon stem (bottom). (**C**) Quantitation of T2 cleavage of the anticodon loop (top) and D loop (bottom). (**D**) Pre-tRNA$^{Arg}_{CCG}$ was modeled on the structure of tRNA$^{Arg}_{ICG}$ (Delagoutte *et al*., 2000) using SPOCK. Bases protected from cleavage by Lhp1p are shown in red.

As Lhp1p is removed upon cleavage of the trailer (Yoo and Wolin, 1997), the correct mature tRNA$^{Arg}_{CCG}$ structure must be maintained by other mechanisms. One possibility is that once the structure is formed, tertiary and/or base stacking interactions stabilize the correctly folded tRNA. As pre-tRNAs bound by La lack certain modifications (Rinke and Steitz, 1982), acquisition of these modified nucleotides may contribute to stability. Also, subsequent proteins, such as modifying enzymes, the aminoacyl-tRNA synthetase, tRNA export factors and elongation factor 1A may stabilize the functional conformation (Johansson and Bystrom, 2002). We note that pre-tRNA$^{Arg}_{CCG}$ regions likely contacted by Lhp1p (the acceptor stem and anticodon stem–loop), are contacted in mature tRNA by arginyl-tRNA synthetase (Delagoutte *et al*., 2000). Interestingly, we identified a mutation in yeast arginyl-tRNA synthetase that causes cells to require Lhp1p (unpublished data). Characterization of the mutation may reveal if the synthetase redundantly stabilizes folding of tRNA$^{Arg}_{CCG}$.

Although our data suggest that Lhp1p facilitates folding by stabilizing the anticodon stem–loop, other proteins could also be involved. To date, we have been unable to fully convert the misfolded *trr4-1* pre-tRNA structure to that of the wild-type RNA by adding Lhp1p. One possibility is that the misfolded structure, once formed, is thermodynamically stable *in vitro*. Alternatively, other proteins may assist in resolving the misfolded pre-tRNA *in vivo*. One candidate would be a member of the DEAD box family of RNA helicases. Human La associates with DDX-15, the orthologue of yeast Prp43, an RNA-dependent ATPase that functions in pre-mRNA splicing

(Fouraux *et al*., 2002). Such a mechanism would resemble group I intron folding in *Neurospora crassa*, where CYT-18, which stabilizes correct tertiary structure, functions together with the CYT-19 RNA helicase (Mohr *et al*., 2002). Thus far, our attempts to use cell extracts to examine roles of other proteins have been complicated by the fact that cleavage of the 3′ trailer occurs in extracts, removing Lhp1p from the pre-tRNA.

While base modifications enhance tRNA structural stability, it is unlikely that Lhp1p acts by facilitating a modification that affects basepairing. HPLC analyses of tRNA from strains lacking Lhp1p failed to detect differences in modified nucleosides (Johansson and Bystrom, 2002), making it unlikely that Lhp1p is required for an abundant modification. Also, most modified nucleosides in cytosolic tRNAs do not influence basepairing. An exception is the dimethylguanosine at position 26 in many tRNAs, which prevents G26 from pairing with C (Steinberg and Cedergren, 1995). This modification, which is carried out by *TRM1*, would weaken the proposed alternative helix. However, $tRNA^{Arg}_{CCG}$ lacks identity elements for *TRM1* dimethylation (G–C basepairs at positions G10–C25 and C11–G24 and a variable loop of at least five nucleotides) (Edqvist *et al*., 1994). Also, deletion of *TRM1* in the *trr4-1* strain did not affect the cold-sensitivity or *LHP1* requirement (unpublished data), revealing that a failure to modify G26 does not contribute to either phenotype.

Our finding that binding by Lhp1p to certain pre-tRNAs facilitates folding suggests that La may assist folding of other nascent small RNAs. In many RNA polymerase III transcripts, such as pre-tRNAs, 5S rRNA and signal recognition particle (SRP) RNA, 3′ sequences basepair with internal or 5′ sequences to form a long stem. As Lhp1p contacts the pre-$tRNA^{Arg}_{CCG}$ acceptor stem, La may contact the analogous stems in other nascent RNAs, stabilizing the helix. Moreover, our identification of an essential pre-tRNA that misfolds *in vivo* may be useful for genetic identification of other proteins that resolve misfolded RNAs or stabilize correctly folded RNA structures.

Materials and methods

Yeast media and strains

Yeast media were according to Sherman (1991). Wild-type and *lhp1::LEU2* strains were CY1 (*MAT*α *ura3 lys2 ade2 trp1 his3 leu2 LHP1*) and CY2 (*MAT*α *ura3 lys2 ade2 trp1 his3 leu2 lhp1::LEU2*). Other strains were SK100 (*MAT*α *trr4-1 LHP1 ura3 lys2 ade2 trp1 his3 leu2*), SK110 (*MAT*α *trr4-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* + pATL), SK130 (*MAT*α *trr4-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* + pMETLHP1), SK201 (*MAT***a** *trr4-2 LHP1 ura3 lys2 ade2 trp1 his3 leu2*), SK210 (*MAT***a** *trr4-2 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* + pATL), SK230 (*MAT*α *trr4-2 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* + pMETLHP1), SK310 (*MAT***a** *trt2-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* + pATL) and SK330 (*MAT***a** *trt2-1 lhp1::LEU2 ura3 lys2 ade2 trp1 his3 leu2* + pMETLHP1). Suppressors were isolated using SK160 (*ura3 lys2 ade2 his3 leu2 trr4::HIS3* + *TRR4* in pRS316).

Synthetic lethal screen

trr4-1 and *trt2-1* alleles were identified previously (Pannone *et al*., 2001). The *trr4-2* allele was isolated largely as described (Pannone *et al*., 1998). CY2 carrying pATL (*LHP1*, *TRP1*, *ADE2*; Pannone *et al*., 1998) was mutagenized with ultraviolet light to 15% survival. After plating on media containing limiting adenine, colonies were screened for the inability to lose pATL and form red sectors. Colonies that did not sector were transformed with pSLL28 (Yoo and Wolin, 1997) and tested for the ability to lose pATL. Strains that lost pATL were tested on media containing 1 μg/ml FOA to confirm they required pSLL28.

To clone *TRR4* and *TRT2*, a genomic library in YCp50 was introduced into SK110 and SK310 and transformants screened for loss of pATL. Subcloning revealed that *TRR4* with 81 bp of 5′ and 543 bp of 3′ flanking sequence eliminated the *LHP1* requirement in SK110. For SK310, *TRT2* with 286 bp of 5′ and 46 bp of 3′ sequence eliminated the requirement. Mutations were identified by sequencing genomic DNA.

Lhp1p depletion and coldshift experiments

To deplete Lhp1p, strains SK130, SK230 and SK330 carrying pMETLHP1 (Pannone *et al*., 1998) were grown in synthetic complete (SC) minus methionine at 25°C to OD_{600} = 0.5, diluted into SC + 2 mM methionine and grown for 36 h. Cells were kept below OD_{600} = 0.5. For the coldshift, CY1, SK100 and SK110 were grown at 25°C in YPD to OD_{600} = 0.4 and shifted to 16°C. Cells were kept below OD_{600} = 0.5.

Immunoprecipitations and northerns

Immunoprecipitations were as described (Xue *et al*., 2000). Total RNA was extracted using hot phenol/SDS and subjected to northern blotting (Pannone *et al*., 1998). For aminoacylation analyses, cells were lysed in pH 4.5 phenol as described (Varshney *et al*., 1991). Deacylation was at pH 9.0. RNAs were fractionated in 6.5% polyacrylamide, 8 M urea, 0.1 M NaOAc pH 5.0 gels at 4°C. Probes: $tRNA^{Arg}_{CCG}$: 5′-ACTCG-AACCCGGATCACAGCCACCGGAAGAATGCATGCTAACCATT-3′, $tRNA^{Thr}_{CGU}$: 5′-AATTGAACCCACGATCCCCGCATTACGAGTGCGA-TGCCTTACCACT-3′, $tRNA^{Gln}_{CUG}$: 5′-ATTCGAACTGGGGTTGTT-CGGATCAGAACCGAAAGTGATAACCACT-3′, $tRNA^{Ser}_{CGA}$: 5′-AGCCCAAGAGATTTCGAGTCTCTCG-3′.

RNA purification, gel shifts and structure mapping

To put pre-$tRNA^{Arg}_{CCG}$ behind the T7 promoter, 5′-CGGCGAATTCTAA-TACGACTCACTATAGTTTATTATGCTCCTCTAGTGC-3′ and 5′-GGCCGGATCCTTTAAATTAAAAGCTCCTCCCGGG-3′ were used to amplify *TRR4* or *trr4-1* DNA. The DNA was inserted into *Eco*RI/*Bam*HI sites of pSP64 (Promega). After *Dra*I cleavage and T7 transcription, reactions were diluted 10-fold with 10 mM MOPS pH 7.0, 100 mM NaCl, 2 mM $MgCl_2$ and bound to DEAE–Sepharose (100 μl) equilibrated in this buffer. Unincorporated nucleotides were removed with 250 mM NaCl in this buffer. RNAs were eluted with 500 mM NaCl. Samples were dialyzed against 10 mM HEPES pH 7.9, 2 mM $MgCl_2$, frozen on dry ice and stored at –80°C.

For gel shifts, 0.34 pmol of wild-type and *trr4-1* pre-$tRNA^{Arg}_{CCG}$ in HMK buffer (Black and Pinto, 1989) was incubated with Lhp1p (0.68–6.8 pmol) and 0.04 mg/ml *E.coli* tRNA in 10 μl for 10 min at 22°C. After 10 min on ice, samples were loaded on 5% acrylamide/5% glycerol gels (Long *et al*., 2001) and run at 15 V/cm for 3 h.

Chemical modifications using DMS, kethoxal and CMCT were as described (Black and Pinto, 1989). Pre-$tRNA^{Arg}_{CCG}$ (0.68 pmol) and 0.04 mg/ml *E.coli* tRNA were incubated for 15 min with or without 6.8 pmol of Lhp1p in modification buffer. Kethoxal stock (37 μl kethoxal/ml), CMCT (140 mg/ml) or DMS (5% in dioxane) was added and incubated 15 min. RNAs were phenol-extracted, precipitated, and modifications visualized by extending a primer complementary to nucleotides 55 to 89 of the pre-tRNA. For proteinase K digestion, 1 μl of 10 mg/ml proteinase K was added and incubated for 15 min at 22°C prior to kethoxal addition.

For enzymatic footprinting, labeled RNA was heated to 50°C in 50 mM Tris pH 8.0, 10 mM $MgCl_2$ and cooled to 22°C. 0.34 pmol pre-tRNA was incubated with 3.4 pmol Lhp1p and 0.02 mg/ml *E.coli* tRNA in 20 mM Tris pH 8, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM DTT, 100 mM NaCl, 10% glycerol, 0.01% NP-40 in 10 μl at 22°C for 20 min, then placed on ice for 30 min. Next, 5 U of RNase T2, 0.02 U RNase V1 or 0.05 U RNase T1 was added and incubated on ice for 10 min. Reactions were stopped with 300 μl 0.2 mg/ml proteinase K, 0.03 mg/ml *E.coli* tRNA, 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM EDTA, 0.5% SDS and incubated 30 min at 55°C. Following phenol extraction and ethanol precipitation, samples were fractionated in 15% polyacrylamide, 8 M urea gels.

Identification of intragenic suppressors

To construct a mutant *trr4-1* library, two oligonucleotides, degenerate in the coding sequence (99.1% correct, 0.9% incorrect nucleotides), were synthesized. After annealing and extending, DNA was ligated into *Bam*HI/*Eco*RI sites of pRS304 (Sikorski and Hieter, 1989). After transformation, DNA was pooled from 5×10^5 colonies, cut with *Bsp*MI and integrated into *TRP1* of SK160. Integrants were tested on

FOA for the ability to lose the *URA3* plasmid. After testing strains for growth at 16°C, *trr4-1* DNA was sequenced. To confirm that *trr4-1*,*U40* and *trr4-1*,*A39* rescued the cold-sensitivity, these alleles, along with *TRR4* and *trr4-1* alleles, were synthesized with PCR and integrated into the *trr4::HIS3* locus of SDK160. To examine if *trr4-1*,*U40* and *trr4-1*,*A39* strains required *LHP1*, strains were mated to CY2.

Acknowledgements

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