The essential Gcd10p–Gcd14p nuclear complex is required for 1-methyladenosine modification and maturation of initiator methionyl-tRNA

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Gcd10p and Gcd14p are essential proteins required for the initiation of protein synthesis and translational repression of *GCN4* mRNA. The phenotypes of *gcd10* mutants were suppressed by high-copy-number *IMT* genes, encoding initiator methionyl tRNA ($tRNA_i^{Met}$), or *LHP1*, encoding the yeast homolog of the human La autoantigen. The *gcd10-504* mutation led to a reduction in steady-state levels of mature $tRNA_i^{Met}$, attributable to increased turnover rather than decreased synthesis of pre-$tRNA_i^{Met}$. Remarkably, the lethality of a *GCD10* deletion was suppressed by high-copy-number *IMT4*, indicating that its role in expression of mature $tRNA_i^{Met}$ is the essential function of Gcd10p. A *gcd14-2* mutant also showed reduced amounts of mature $tRNA_i^{Met}$, but in addition, displayed a defect in pre-$tRNA_i^{Met}$ processing. Gcd10p and Gcd14p were found to be subunits of a protein complex with prominent nuclear localization, suggesting a direct role in $tRNA_i^{Met}$ maturation. The chromatographic behavior of elongator and initiator $tRNA^{Met}$ on a RPC-5 column indicated that both species are altered structurally in *gcd10Δ* cells, and analysis of base modifications revealed that 1-methyladenosine (m^1A) is undetectable in *gcd10Δ* tRNA. Interestingly, *gcd10* and *gcd14* mutations had no effect on processing or accumulation of elongator $tRNA^{Met}$, which also contains m^1A at position 58, suggesting a unique requirement for this base modification in initiator maturation.

[*Key Words*: Gcd10p–Gcp14p nuclear complex; 1-methyladenosine; tRNA; initiator maturation]

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A key step in the initiation of protein synthesis in eukaryotic cells involves the binding of the methionyl initiator $tRNA^{Met}$ (Met–$tRNA_i^{Met}$) to the 40S ribosomal subunit to form a 43S preinitiation complex. Initiation factor 2 (eIF2) delivers Met–$tRNA_i^{Met}$ to the ribosome in a ternary complex with GTP, and the eIF2 is released in an inactive binary complex with GDP. Exchange of the GDP bound to eIF2 for GTP is catalyzed by the guanine nucleotide exchange factor eIF2B. Phosphorylation of the α subunit of eIF2 (eIF2α) prevents the recycling of eIF2 by eIF2B, inhibiting the formation of eIF2 · GTP · Met–$tRNA_i^{Met}$ ternary complexes (Hershey 1991; Hinnebusch 1996). In *Saccharomyces cerevisiae*, eIF2α is phosphorylated by the protein kinase Gcn2p when cells are deprived of an amino acid and this elicits increased translation of a specific mRNA encoding Gcn4p, a transcriptional activator of amino acid biosynthetic enzymes (Hinnebusch 1996).

Because translation of *GCN4* is coupled inversely to the concentration of ternary complexes (Dever et al. 1995) mutations in subunits of eIF2 and eIF2B derepress *GCN4* translation in cells lacking Gcn2p (for review, see Hinnebusch 1996). Mutations in *GCD10* also derepress *GCN4* translation in the absence of eIF2 phosphorylation by Gcn2p (Harashima and Hinnebusch 1986). *GCD10* is essential and temperature-sensitive mutations in this gene inhibit general translation initiation at the restrictive temperature. It was found that Gcd10p copurified and coimmunoprecipitated with subunits of translation initiation factor eIF3 (Garcia-Barrio et al. 1995). Given their effect on *GCN4* translation, it was proposed that *gcd10* mutations reduce the ability of eIF3 to stimulate ternary complex binding to 40S ribosomes,

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mimicking the inhibition of ternary complex formation elicited by eIF2 phosphorylation. Recently, we purified a yeast eIF3 complex from a strain expressing a polyhistidine-tagged form of the *PRT1*-encoded subunit (Phan et al. 1998) and found that it contains all five *S. cerevisiae* proteins homologous to subunits of mammalian eIF3, but lacks Gcd10p. In addition, Gcd10p was not coimmunoprecipitated with an epitope-tagged version of the *TIF34* subunit of yeast eIF3 (Asano et al. 1998; Phan et al. 1998). Together, these findings suggested that Gcd10p is not an integral subunit of yeast eIF3, and may have a distinct function involved in the formation of ternary complexes or their recruitment to the ribosome.

To identify the in vivo function of Gcd10p, we carried out a genetic analysis by isolating dosage suppressors of the temperature-sensitive phenotype of *gcd10* mutations. Analysis of these suppressors revealed that maturation of initiator $tRNA^{Met}$ is defective in *gcd10* mutants. Our biochemical results indicate that Gcd10p resides in a nuclear complex with Gcd14p, another factor involved in *GCN4* translational control (Cuesta et al. 1998), and is required for 1-methyladenosine formation in yeast tRNA. Moreover, *GCD10* was found to be nonessential in cells overexpressing initiator $tRNA^{Met}$. It appears that the Gcd10p–Gcd14p complex is required specifically at the initiation step of translation because of a strong requirement for 1-methyladenosine at position 58 (m^1A58) in the processing and accumulation of initiator $tRNA^{Met}$.

Results

Genes encoding initiator $tRNA^{Met}$ or a La homolog are dosage suppressors of the growth defects of gcd10 *mutants*

To identify functional interactions between Gcd10p and components of the translation initiation apparatus, we identified wild-type genes that in high-copy-number enable *gcd10* mutants to grow at the nonpermissive temperature. Strains H2457(*gcd10-504 gcn2-101*) and Hm298 (*gcd10-505 gcn2-101*) were transformed with a high-copy yeast genomic library, and transformants were selected for growth at 36°C. Analysis of plasmids recovered from the ts^+ transformants (described in Materials and Methods) led to the identification of five dosage suppressors, of which four are the genes encoding initiator $tRNA^{Met}$, *IMT1*, *IMT2*, *IMT3*, and *IMT4* (Cigan and Donahue 1986; Byström and Fink 1989). The remaining suppressor gene was identified as *LHP1*, encoding a homolog of the human La protein (Yoo and Wolin 1994) previously implicated in processing of tRNAs in yeast (Yoo and Wolin 1997) (Fig. 1A). All five genes were found to suppress the effects of *gcd10* mutations on *GCN4* translation in the following way. Because increased Gcn4p synthesis is required to derepress the histidine biosynthetic enzyme inhibited by 3-AT (His3p), *gcn2* mutants are sensitive to 3-AT. *gcd10* mutations lead to derepression of *GCN4* translation in the absence of eIF2 phosphorylation by Gcn2p (Gcd^- phenotype), conferring resistance to 3-AT (AT^r) in a *gcn2* strain background (Harashima and Hinnebusch 1986). All of the dosage suppressors overcame the 3-AT^r phenotype of *gcn2-101 gcd10-504* strain H2457 (data not shown), and thus appear to restore repression of *GCN4* translation.

The presence of *IMT1*, *IMT2*, and *IMT4* in high copy suppressed the ts^- phenotypes of the *gcd10-504*, *gcd10-505*, and *gcd10-503* alleles as completely as did low-copy *GCD10*; however, high-copy-number *IMT3* (hc*IMT3*) did not suppress fully the ts^- phenotype of *gcd10-505*. High copy *LHP1* only suppressed partially all three *gcd10* alleles (data not shown). Only one additional copy of *IMT4* or *LHP1* on single-copy plasmids p2627 and 2628, respectively, did not suppress the phenotypes of *gcd10-504* in H2457 (data not shown), suggesting that overexpression of $tRNA_i^{Met}$ or Lhp1p is required for suppression.

We asked whether overexpression of $tRNA_i^{Met}$ or Lhp1p would suppress general defects in translation initiation conferred by mutations in known initiation factors. *PRT1* encodes the 90-kD subunit of eIF3 (Naranda et al. 1994) and *prt1-1* mutants are impaired for translation initiation at the restrictive temperature both in vivo and in vitro (Feinberg et al. 1982). Unlike the *gcd10* mutants, the ts^- phenotype of a *prt1-1* mutant was not suppressed by hc*IMT4* or hc*LHP1* (Fig. 1A). Furthermore, neither hc*IMT4* nor hc*LHP1* suppressed the growth defects of *sui2-1* or *gcd1-501* alleles, encoding defective subunits of eIF2α and eIF2Bγ, respectively (data not shown). These findings suggest that Gcd10p promotes translation initiation in a manner specifically involving initiator $tRNA^{Met}$ metabolism or function.

The abundance of mature initiator $tRNA^{Met}$ is reduced specifically in a gcd10-504 *mutant*

To examine whether initiator $tRNA^{Met}$ expression is reduced in *gcd10* mutants, we used Northern blot analysis to measure the steady-state levels of $tRNA_i^{Met}$ using sequences complementary to the $tRNA_i^{Met}$ coding region as a probe. In addition to mature $tRNA_i^{Met}$, this probe hybridized to two larger species that appear to be precursors of $tRNA_i^{Met}$ bearing different 5′ and 3′ terminal extensions encoded by the various *IMT* genes (see below). The amount of mature $tRNA_i^{Met}$ in the *gcd10* mutant was about twofold lower than in the wild type at 26°C, and only about one-sixth of the wild-type level after 10 hr at 36°C (Fig.1B,C; $tRNA_i^{Met}$). In contrast, levels of the putative $tRNA_i^{Met}$ precursors were virtually indistinguishable between the mutant and wild-type strains (Fig. 1B; pre-$tRNA_i^{Met}$), leading to ratios of precursors to mature $tRNA_i^{Met}$ three- to eightfold higher in the mutant over the course of the experiment (Fig. 1B). These results suggest that transcription of the *IMT* genes is not impaired in the *gcd10* mutant but that a reduction in mature $tRNA_i^{Met}$ abundance occurs at a post-transcriptional step.

Interestingly, the levels of mature $tRNA^{His}$ and $tRNA_{CGA}^{Ser}$ were indistinguishable between the wild-type and *gcd10* strains at the permissive and nonpermissive temperatures (data not shown), whereas we consis-

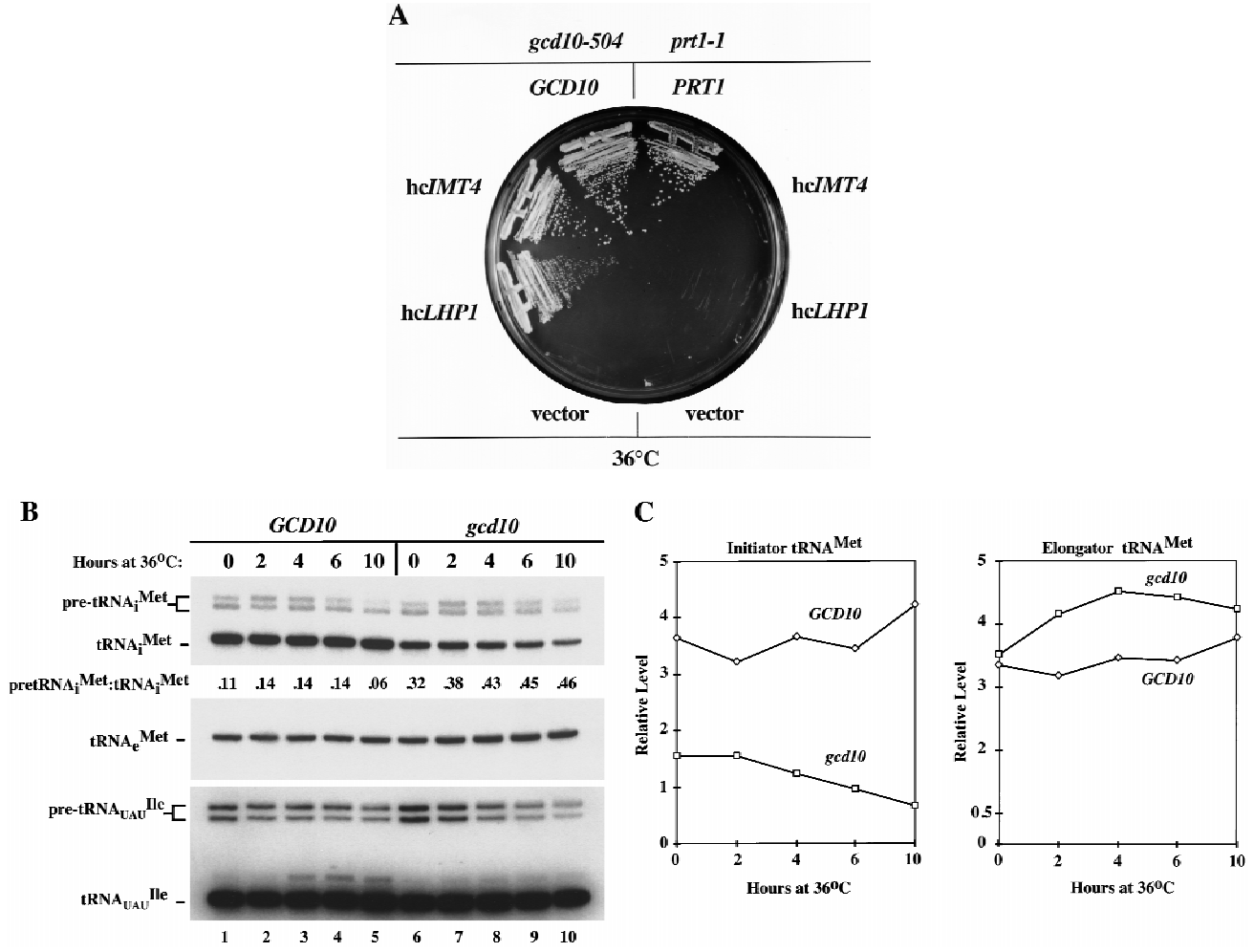

Figure 1. High-copy suppressors of *gcd10-504* overcome a defect in accumulation of $tRNA_i^{Met}$ (*A*) Transformants of strains H2457 (*gcd10-504*) and H1676 (*prt1-1*) containing low-copy plasmids bearing *GCD10* (pMG107) (Garcia-Barrio et al. 1995) or *PRT1* (p2625), respectively, or high-copy plasmids bearing *IMT4* (pC44) (Cigan et al. 1988), *LHP1* (p2626), or empty vector YEp24 were streaked for single colonies on minimally supplemented SD plates and incubated at 36°C for 2 days. (*B*) Northern blot analysis of total RNA (5 μg) isolated as described (Kohrer and Domdey 1991) from strain H2457 (*gcd10-504*) bearing *GCD10* on low-copy plasmid pMG107 (*GCD10* lanes) or empty vector YEp24 (*gcd10* lanes) grown in supplemented SD at 26°C to mid-exponential phase (0 hr at 36°C) and shifted to 36°C for 2, 4, 6, or 10 hr. The RNAs were separated on an 8% polyacrylamide-*bis*-acrylamide (19:1), 8.3 M urea gel by electrophoresis and transferred to Hybond-N+ membranes (Amersham). The blot was probed using a radiolabeled oligonucleotide that hybridized specifically to $tRNA_i^{Met}$, stripped and reprobed with radiolabeled oligonucleotides specific for $tRNA_e^{Met}$ or $tRNA_{UAU}^{Ile}$. Direct quantitation of all hybridized probes was conducted by PhosphorImager analysis using a Storm 860 apparatus (Molecular Dynamics) and ImageQuant software. The positions of pre-$tRNA_i^{Met}$ species, mature $tRNA_i^{Met}$, $tRNA_e^{Met}$, pre-$tRNA_{UAU}^{Ile}$, and mature $tRNA_{UAU}^{Ile}$ are indicated at *left*. The relative intensities of the hybridization signals were quantified for mature $tRNA_i^{Met}$ and pre-$tRNA_i^{Met}$, and the ratios of pre-$tRNA_i^{Met}$ to mature $tRNA_i^{Met}$ are listed under the appropriate lanes. The species that migrated just above mature $tRNA_{UAU}^{Ile}$ and accumulated at high temperature most likely represent spliced precursors still bearing the 3′ extension (O'Connor and Peebles 1991). (*C*) The relative intensities of the hybridization signals in *B* were quantified by PhosphorImager analysis of the autoradiograph and plotted against the time of incubation at 36°C.

tently observed a modest increase in mature elongator $tRNA^{Met}$ levels in the *gcd10* mutant at 36°C (Fig. 1B,C; $tRNA_e^{Met}$). The level of pre-$tRNA_{UAU}^{Ile}$ decreased at 36°C slightly more in the *gcd10* mutant than it did in the wild type (Fig. 1B; pre-$tRNA_{UAU}^{Ile}$); however, the mature $tRNA_{UAU}^{Ile}$ levels were identical in the two strains at all temperatures. These findings suggest that the *gcd10* mutation primarily reduces the accumulation of mature $tRNA_i^{Met}$.

To investigate whether the lowered expression of mature $tRNA_i^{Met}$ in *gcd10* cells could be a general response to reduced growth rates, we conducted Northern blot analysis on the temperature sensitive *sui2-1* mutant, and compared the results to those shown in Figure 1B for the *gcd10-504* strain (data not shown). The pre-$tRNA_i^{Met}$ level was ~34% lower in the *sui2* mutant versus the *SUI2* strain; however, the mature $tRNA_i^{Met}$ level decreased by only 15% in the *sui2* cells, leading to a small

reduction in the precursor-to-mature ratio [0.08 (*sui2*) versus 0.16 (*SUI2*)]. Thus there was a much greater reduction in mature $tRNA_i^{Met}$ levels in the *gcd10* mutant (a factor of 6, Fig. 1B) versus the *sui2* mutant (15%), although pre-$tRNA_i^{Met}$ expression declined in the *sui2* cells but not in the *gcd10* cells. These data are consistent with the idea that *gcd10* mutants are defective in maturation of pre-$tRNA_i^{Met}$ at 36°C.

The dosage suppressors increase mature $tRNA_i^{Met}$ levels in gcd10 *mutants*

It seemed likely that suppression of *gcd10* mutations by hc*IMT* genes occurred by increasing the abundance of mature $tRNA_i^{Met}$. In accordance with this expectation, we found that hc*IMT4* in the *gcd10* mutant increased mature $tRNA_i^{Met}$ to levels exceeding that observed in the isogenic *gcd10* transformant containing a low-copy plasmid bearing *GCD10* (Fig. 2A, cf. lanes 9–12 and lanes 5–8). The hc*IMT4* transformant also showed increased amounts of the smaller of two putative $tRNA_i^{Met}$ precursors detected in the wild-type strain plus an even slower migrating species not detected in wild type (Fig. 2A, cf. lanes 9–12 and lanes 5–8, species c and a, respectively).

Northern analysis of strains bearing hc*IMT1*, hc*IMT2*, hc*IMT3*, or hc*IMT4* suggests that the larger putative precursors seen in wild-type strains derive primarily from *IMT2* and *IMT3*, whereas the smaller species are produced from *IMT1* and *IMT4* (Fig. 2B, species b and c, respectively). In addition, we confirmed that the putative precursor overexpressed in the hc*IMT3* transformant hybridized with probes containing only the 5′ and 3′ terminal extensions encoded at *IMT3*, and that the putative precursor *c* hybridized with sequences derived from upstream of the *IMT4*-coding sequence (data not shown). The observed differences in pre-$tRNA_i^{Met}$ sizes between the *IMT* genes can be explained by different locations of the transcription terminators either closer to (*IMT1*, *IMT4*) or farther away (*IMT2*, *IMT3*) from the 3′ end of the mature tRNA. The hc*IMT3* transformants contained relatively less mature $tRNA_i^{Met}$ compared to the other hc*IMT* transformants (Fig. 2B), which can explain why hc*IMT3* suppressed the phenotypes of *gcd10-505* less completely than did the other three hc*IMT* genes (data not shown).

Hc*LHP1* also increased the level of precursor and mature $tRNA_i^{Met}$ in the *gcd10* mutant (Fig. 2A, lanes 1–4 vs. lanes 13–16); however, mature $tRNA_i^{Met}$ did not reach the wild-type level at 36°C (Fig. 2A, lane 16 vs. lane 8), explaining why hc*LHP1* only suppressed partially the ts^- phenotype of *gcd10-504* (see Fig. 1A). Hc*LHP1* increased differentially the two precursor species detectable in wild type (species b and c) and also led to the appearance of two novel species migrating slower than mature $tRNA_i^{Met}$ (species d and e). The presence of species d and e suggests that Lhp1p overexpression interferes with exonucleolytic trimming of pre-$tRNA_i^{Met}$. There is evidence that Lhp1p blocks exonucleolytic trimming and promotes endonucleolytic cleavage of the 3′ trailer for certain other yeast tRNA families (Yoo and Wolin 1997). If this occurs for pre-$tRNA_i^{Met}$ when Lhp1p is overexpressed, it could be responsible for the higher levels of full-length pre-$tRNA_i^{Met}$ and mature $tRNA_i^{Met}$ seen under these conditions, provided that endonucleolytic cleavage is more precise than exonucleolytic trimming in 3′ end maturation.

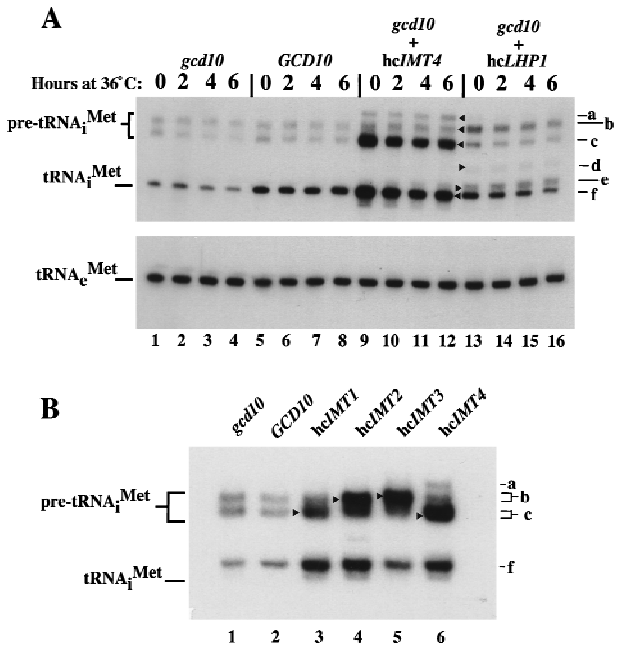

Figure 2. High-copy suppressors of *gcd10* mutations lead to increased amounts of initiator $tRNA^{Met}$ and identification of pre-$tRNA_i^{Met}$ species. (*A*) Northern blot analysis of total RNA (5 µg) isolated from transformants of strains H2457 (*gcd10-504*) carrying empty vector YEp24 (*gcd10* lanes), low-copy plasmid pMG107 bearing *GCD10* (*GCD10* lanes), high-copy plasmid pC44 bearing *IMT4* (*gcd10* + *hcIMT4* lanes), or high-copy plasmid p2626 bearing *LHP1* (*gcd10* + hc*LHP1* lanes), grown at 26°C or 36°C for the indicated times, as described in Fig. 1. The blot was probed for $tRNA_i^{Met}$ and stripped and reprobed for $tRNA_e^{Met}$ as described in Fig. 1. Indicated with arrowheads inside the blot and labeled to the *right* are the presumed positions of pre-$tRNA_i^{Met}$ containing 5′ and 3′ extensions encoded by *IMT4* and terminating downstream of the usual termination site (*a*), pre-$tRNA_i^{Met}$ containing 5′ and 3′ extensions encoded by *IMT2* and *IMT3* (*b*), pre-$tRNA_i^{Met}$ containing 5′ and 3′ extensions encoded by *IMT1* and *IMT4* (*c*), processing intermediate of *IMT3* containing the 3′ extension (*d*), processing intermediate containing partial 3′ extension (*e*), and mature $tRNA_i^{Met}$ (*f*). (*B*) Northern blot analysis of total RNA (10 µg) isolated from H2457 (*gcd10-504*) transformants containing empty vector YEp24 (*gcd10*), low-copy plasmid pMG107 bearing *GCD10*, or a high-copy plasmid bearing one of the four *IMT* genes, as indicated above the blot, grown at 26°C as described in Fig. 1. Initiator $tRNA^{Met}$ was detected by hybridization as described above and in Fig. 1. Indicated with arrowheads inside the blot and labeled to the *right* are the positions of pre-$tRNA_i^{Met}$ containing 5′ and 3′ extensions (*a–c*) and mature $tRNA_i^{Met}$ (*f*), as in *A*.

GCD10 *is nonessential in yeast strains overexpressing initiator tRNA*^{Met}

To test the possibility that overexpression of $tRNA_i^{Met}$ would allow yeast cells to survive in the absence of Gcd10p, the *gcd10Δ* strain YJA143 containing wild-type *GCD10* on a *URA3* plasmid (p2702) was transformed with a high-copy *LEU2* plasmid bearing *IMT4* (p1775) (Dever et al. 1995). The *URA3 GCD10* plasmid p2702 was readily eliminated from the resulting transformant by plasmid shuffling on medium containing 5-fluoroorotic acid (5-FOA) (Boeke et al. 1987), whereas p2702 could not be lost from isogenic transformants containing an empty *LEU2* vector. These results indicate that *GCD10* is dispensable in cells overexpressing $tRNA_i^{Met}$. We verified by immunoblot analysis that one such strain (YJA146) containing hc*IMT4* and lacking *GCD10* had no detectable Gcd10p (data not shown).

Although the *gcd10Δ* hc*IMT4* strain is viable, it grows poorly at temperatures >26°C (Fig. 3A). We observed little difference in mature $tRNA_i^{Met}$ levels between the isogenic *gcd10Δ* hc*IMT4* and *GCD10* hc*IMT4* strains at 26°C (Fig. 3B, lanes 4,7). Thus, at low temperature, the requirement for *GCD10* in mature $tRNA_i^{Met}$ expression appears to be largely bypassed by overproduction of $tRNA_i^{Met}$. In contrast, mature $tRNA_i^{Met}$ was reduced substantially after 2 or 6 hr at 36°C in the *gcd10Δ* strain (Fig. 3B, lanes 5,6 vs. 8,9), showing that *GCD10* is required for accumulation of mature $tRNA_i^{Met}$ at 36°C, even when $tRNA_i^{Met}$ is being overexpressed. The *gcd10Δ* hc*IMT4* transformant accumulated higher levels of pre-$tRNA_i^{Met}$ than did the *GCD10* hc*IMT4* transformant (Fig. 3B, cf. lanes 4–6 and lanes 7–9), supporting the conclusion that Gcd10p is not required for efficient transcription of *IMT4*. In addition, the pre-$tRNA_i^{Met}$-to-mature $tRNA_i^{Met}$ ratio was much greater in the *gcd10Δ* hc*IMT4* transformant compared to the *GCD10* hc*IMT4* strain, particularly at 36°C (Fig. 3B). It is also noteworthy that the *gcd10Δ* hc*IMT4* transformant accumulated species with mobilities greater than that of mature $tRNA_i^{Met}$, which were not observed in the *GCD10* hc*IMT4* transformant (Fig. 3B, cf. lanes 4–6 and lanes 7–9, species g). These observations suggest that Gcd10p is required for efficient processing of pre-$tRNA_i^{Met}$, particularly at elevated growth temperatures, and that in its absence, much of the unprocessed pre-$tRNA_i^{Met}$ is degraded. The *gcd10Δ* hc*IMT4* transformant showed no detectable reduction in the accumulation of other mature

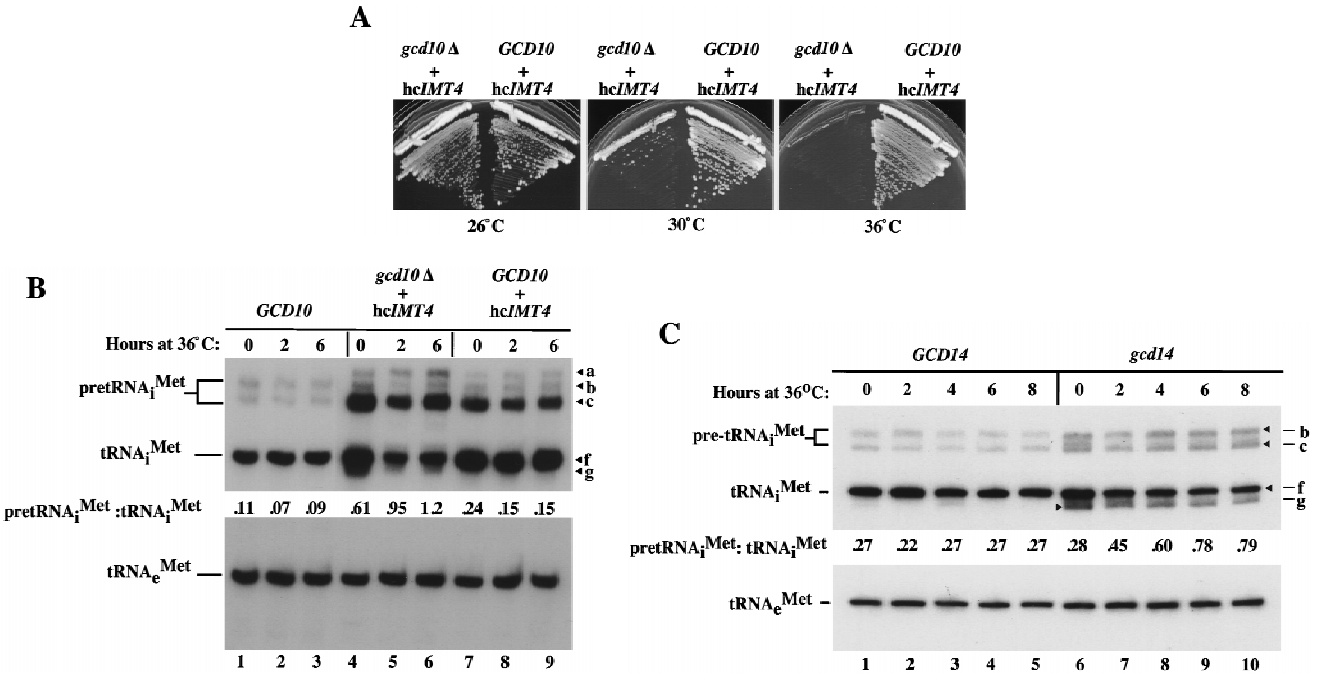

Figure 3. *GCD10* is dispensable for cell viability in the presence of hc*IMT4*. (*A*) Growth of strain YJA146 (*gcd10Δ* + hc*IMT4*) and a transformant of its parental *GCD10* strain BJ5464 bearing p1775 (*GCD10* + hc*IMT4*) on YPD medium at 26°C for 3 days, and at 30°C or 36°C for 2 days. (*B*) Northern blot analysis of total RNA (7 μg) isolated from the same two strains described in *A* (*gcd10Δ* + hc*IMT4* and *GCD10* + hc*IMT4* lanes) plus isogenic strain YJA143 containing the *gcd10Δ* chromosomal allele and single-copy *GCD10* plasmid p2704 (*GCD10*). Strains were grown at 26°C or 36°C for 2 and 6 hr as described in Fig. 1. The membrane was probed for $tRNA_i^{Met}$ and stripped and reprobed for $tRNA_e^{Met}$ as described in Fig. 1. The different RNA species detected are indicated at *left*. The various forms of $tRNA_i^{Met}$ species are labeled at *right* as in Fig. 2, with the addition of species g, which may be end-trimmed molecules lacking the CCA extension (see text). (*C*) Northern blot analysis of total RNA (5 μg) isolated from strain Hm296 (*gcd14-2*) containing wild-type *GCD14* on single-copy plasmid pRC62 (*GCD14* lanes) or empty vector YEp24 (*gcd14* lanes), grown as described in Fig. 1. The membrane was probed for $tRNA_i^{Met}$ and stripped and reprobed for $tRNA_e^{Met}$ as described in Fig. 1. Indicated with arrowheads inside the blot and labeled to the right are the various $tRNA_i^{Met}$ species described in Fig. 2 and above. The indicated ratios of pre-$tRNA_i^{Met}$ to mature $tRNA_i^{Met}$ were calculated from the relative intensities of hybridization signals quantitated by PhosphorImager analysis.

tRNAs when compared to the *GCD10* + hc*IMT4* transformant, including $tRNA_e^{Met}$ (Fig. 3B; $tRNA_e^{Met}$), $tRNA_{UAU}^{Ile}$, and $tRNA_{CGA}^{Ser}$ (data not shown).

Despite the reduction in mature $tRNA_i^{Met}$ expression at 36°C in the *gcd10Δ* hc*IMT4* transformant, it still maintained levels comparable to that seen in isogenic wild-type cells grown under the same conditions (Fig. 3B, cf. lane 3 and lane 6, species f). The presence of species g in the *gcd10Δ* hc*IMT4* transformant at 36°C suggests that mature $tRNA_i^{Met}$ is either unstable, or is processed or modified incompletely, and may not be fully functional in translation. This could explain the inviability of this strain at high temperatures without the need to propose a second function for Gcd10p.

gcd14 *mutants are defective for processing of initiator* $tRNA^{Met}$ *in vivo*

gcd14 mutants exhibit constitutively derepressed *GCN4* translation (Gcd^-) and are temperature sensitive for general translation, the same phenotypes described for *gcd10* mutations (Cuesta et al. 1998). In addition, any of the four *IMT* genes or *LHP1* on a high-copy plasmid suppressed the Gcd^- and ts^- phenotypes of *gcd14* mutants (R. Cuesta, O. Calvo, J. Anderson, M. Garcia-Barrio, A. Hinnebusch, and M. Tamame, in prep.). In accordance with these findings, we found that mature $tRNA_i^{Met}$ decreased by a factor of 1.7, whereas pre-$tRNA_i^{Met}$ increased ~1.5-fold in a *gcd14-2* mutant after 8 hr at 36°C, increasing the ratio of precursor to mature $tRNA_i^{Met}$ from 0.28 to 0.79 (Fig. 3C). These results suggest strongly that Gcd14p is required for processing of nascent $tRNA_i^{Met}$ transcripts. Interestingly, a $tRNA_i^{Met}$ species migrating faster than the wild-type mature form, exhibiting a similar mobility to the aberrant $tRNA_i^{Met}$ seen in the *gcd10Δ* hc*IMT4* transformants, is present in the *gcd14-2* mutant under all conditions (Figs. 3B,C, species g). Thus, 5′- and 3′-trimmed $tRNA_i^{Met}$ may be unstable, or incorrectly processed or modified, in *gcd14-2* cells. As in the case of *gcd10-504*, the *gcd14-2* mutation had no effect on the level or apparent length of elongator $tRNA^{Met}$ (Fig. 3C).

Gcd10p is required for the stability of total $tRNA_i^{Met}$ *in vivo*

We used pulse–chase analysis to investigate whether the reduced amount of mature $tRNA_i^{Met}$ in the *gcd10-504* mutant results from rapid degradation of $tRNA_i^{Met}$ transcripts. After incubating isogenic *GCD10* and *gcd10-504* strains for ~2 hr at 36°C, cells were pulse-labeled with [^{3}H]uracil for 1 hr, and chased for 5 hr with excess unlabeled uracil. Total RNA was isolated at different times and aliquots containing equivalent amounts of radioactivity were hybridized to immobilized oligonucleotides complementary to initiator $tRNA^{Met}$ or elongator $tRNA^{Met}$. The proportion of ^{3}H-labeled $tRNA_e^{Met}$ in both mutant and wild-type cells, and of $tRNA_i^{Met}$ in the wild-type strain, showed a small increase over the 5-hr chase period (Fig. 4A). Presumably, these increases occurred because the pool of uracil nucleotides chased slowly, allowing the proportion of label in stable RNA to increase relative to unstable mRNA. In contrast, the proportion of ^{3}H-labeled $tRNA_i^{Met}$ in the *gcd10* mutant dropped by a factor of about two during the first hour of the chase and showed little additional change throughout the remaining 4-hr chase period (Fig. 4A). These results indicate that a large fraction of $tRNA_i^{Met}$ transcripts made during the 1-hr pulse in the *gcd10* mutant was very unstable, whereas the remainder was highly stable. As expected, the *gcd10* mutation had no effect on the turnover of $tRNA_e^{Met}$.

It seemed likely that the unstable pool of $tRNA_i^{Met}$ transcripts detected by pulse–chase analysis in the *gcd10-504* mutant represented nascent $tRNA_i^{Met}$ molecules that were degraded rapidly in the nucleus. To eliminate the alternative possibility that mature $tRNA_i^{Met}$ is unstable, we treated *gcd10Δ* and *gcd10-504* cells with an inhibitor of Pol III transcription, thiolutin (Jimenez et al. 1973), coincident with the shift to 36°C to prevent cell division and new synthesis of pre-$tRNA_i^{Met}$ transcripts at the nonpermissive temperature. As shown in Figure 4B, the untreated *gcd10Δ* and *gcd10-504* cells showed the usual reduction in mature $tRNA_i^{Met}$ after the temperature shift (lanes 1–4 and 8–11). In contrast, thiolutin treatment largely eliminated the reduction in mature $tRNA_i^{Met}$ levels at 36°C (Fig. 4B, lanes 5–7 and 12–14). The disappearance of pre-$tRNA_i^{Met}$ species after thiolutin treatment is the expected result of inhibiting *IMT* transcription without preventing processing (or degradation) of the preexisting pre-$tRNA_i^{Met}$ transcripts. The fact that little or no change in mature $tRNA_i^{Met}$ abundance occurred at 36°C in the presence of thiolutin suggests that the mature $tRNA_i^{Met}$ present at the temperature shift is stable in *gcd10-504* cells. The *gcd10-504* mutant continues to grow at 36°C, albeit more slowly than the wild type, doubling in mass ~2.5 times after the temperature shift. Thus, the reduction in mature $tRNA_i^{Met}$ seen in the absence of thiolutin most likely occurs by dilution of stable preexisting mature $tRNA_i^{Met}$, coupled with a failure to produce new mature $tRNA_i^{Met}$ during cell divisions at the nonpermissive temperature. We suggest that the unstable $tRNA_i^{Met}$ molecules detected by pulse–chase analysis in the *gcd10-504* mutant (Fig. 4A) are primarily nascent transcripts that are rapidly degraded in the nucleus.

Evidence that Gcd10p and Gcd14p are components of a heteromeric nuclear complex

To investigate whether Gcd10p and Gcd14p are physically associated in vivo, we constructed a yeast strain expressing a polyhistidine-tagged form of Gcd10p (His–Gcd10p) to allow affinity purification of the protein. The *GCD10–His* allele encoding His–Gcd10p was indistinguishable from wild-type *GCD10* in complementing *gcd10* mutations in vivo (see Materials and Methods). As shown in Figure 5A, substantial fractions of both Gcd14p and Gcd10p in whole cell extracts were eluted from

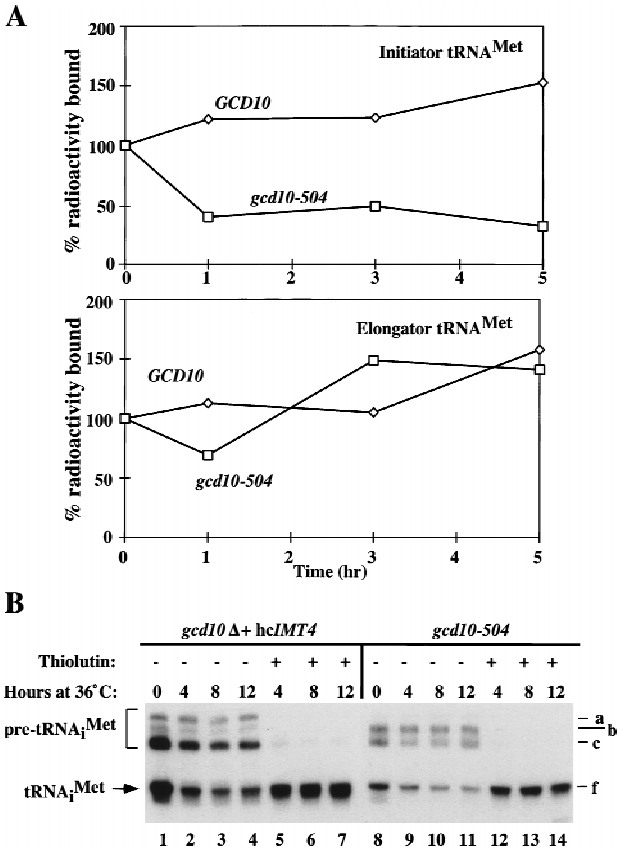

Figure 4. Evidence that newly synthesized initiator $tRNA^{Met}$ is unstable in *gcd10* mutants. (*A*) Transformants of strain H2457 (*gcd10-504*) bearing the *GCD10* plasmid pMG107 (*GCD10*) or vector YEp24 (*gcd10-504*) were grown in supplemented SD medium at 36°C for 2.25 hr before the addition of 5.0 mCi [5,6-3H]-uracil (37 Ci/mmole, 1 mCi/ml NEN). Cells were continuously labeled at 36°C for 60 min (pulse) after which 200-fold excess unlabeled uracil was added and incubation at 36°C was continued for 5 hr (chase). Total RNA was isolated from 2.0-ml aliquots at 0, 1, 3, and 5 hr after addition of unlabeled uracil and an amount of RNA representing equal cpms was hybridized to membrane-bound oligonucleotides complementary to full-length $tRNA_i^{Met}$ (*top*) and $tRNA_e^{Met}$ (*bottom*) in hybridization solution [500 mM NaCl, 24 mM NaH_2PO_4, 2.4 mM EDTA (pH 7.4), 30 % formamide, 5× Denhardt's solution, 0.1% SDS] at 40°C for 2.5 days with constant mixing. After hybridization, filters were washed once in hybridization solution at 40°C for 30 min, once in 2× SSC, 0.1% SDS for 30 min at room temperature, once in 2× SSC for 30 min, and twice with 95% ethanol. Filters were dried and counted by liquid scintillation in Econo-fluor (Packard Chemical). The cpm bound to the membranes at each time point were corrected by subtracting the cpm bound to a third membrane containing a nonspecific oligonucleotide. The corrected counts per minute are expressed as the percentage of cpm bound to the membrane at the beginning of the chase (time = 0). (*B*) Strains YJA146 (*gcd10Δ* + hc*IMT4*) and a transformant of H2457 containing vector YEp24 (*gcd10-504* + YEp24) were grown to mid-exponential phase at 26°C in SC or minimally supplemented SD medium and resuspended in the same medium prewarmed to 36°C containing 5 μg/ml thiolutin in DMSO or DMSO only. Northern blots of total RNA (10 μg) isolated from the strains at 26°C (0 hr at 36°C) or 36°C (4, 8, 12 hr at 36°C) with (+) or without (−) thiolutin treatment were probed with a labeled oligonucleotide that specifically hybridized to both pre-$tRNA_i^{Met}$ and mature $tRNA_i^{Met}$, as described in Fig. 1. Labeled at *right* are various $tRNA_i^{Met}$ species described in Figs. 2 and 3.

Ni^{+2}–NTA agarose with the *GCD10–His* extract but not with the isogenic *GCD10* extract. In contrast, the *PRT1*-encoded subunit of eIF3 (Naranda et al. 1994) in both extracts did not bind to the resin. These findings indicate that Gcd10p and Gcd14p are components of a heteromeric complex that is not stably associated with eIF3.

As most steps in tRNA processing are believed to occur in the nucleus (Hopper and Martin 1992), we used indirect immunofluorescence to determine whether Gcd10p and Gcd14p are nuclear proteins. Toward this end, we constructed alleles of *GCD10*, *GCD14*, and *TIF34* (encoding the 39-kD subunit of yeast eIF3; Naranda et al. 1997) tagged with the coding sequences for three tandem copies of the HA epitope. The tagged alleles were introduced on low-copy plasmids into yeast strains either lacking the cognate chromosomal allele, in the case of *GCD10–HA* (YJA142) and *TIF34–HA* (KAY8), or containing a temperature-sensitive allele in the case of *GCD14–HA* (see Materials and Methods). All three tagged alleles complemented the lethal effects of mutations in the corresponding genes as effectively as did the wild-type alleles (data not shown). In addition, the tagged proteins were expressed at levels similar to that of the wild-type proteins (data not shown). As expected for an integral subunit of eIF3, HA–Tif34p was found exclusively in the cytoplasm (Fig. 5B, k), whereas Nab1p showed diffuse nuclear staining characteristic of a nucleoplasmic protein (panel e) (Wilson et al. 1994). Both HA–Gcd10p and HA–Gcd14p showed prominent nuclear localization with staining indicative of nucleoplasmic factors (Fig. 5B, a,g). Because of the background staining with anti-HA antibodies of the control *GCD10* and *GCD14* strains (Fig. 5B, c,i), it was not possible to determine whether Gcd10p and Gcd14p are located in the cytoplasm in addition to the nucleus.

Gcd10p is required for the 1-methyladenosine modification of initiator $tRNA^{Met}$ at position 58

Gcd14p exhibits significant homology with *S*-adenosylmethionine-dependent methyltransferases (R. Cuesta, O. Calvo, J. Anderson, M. Garcia-Barrio, A. Hinnebusch, and M. Tamame, in prep.), suggesting the possibility that Gcd10p and Gcd14p are required for $tRNA_i^{Met}$ methylation. To test this idea, total tRNA isolated from *gcd10Δ* and wild-type strains was aminoacylated with [^{35}S]methionine or [^{3}H]methionine, respectively, and the labeled

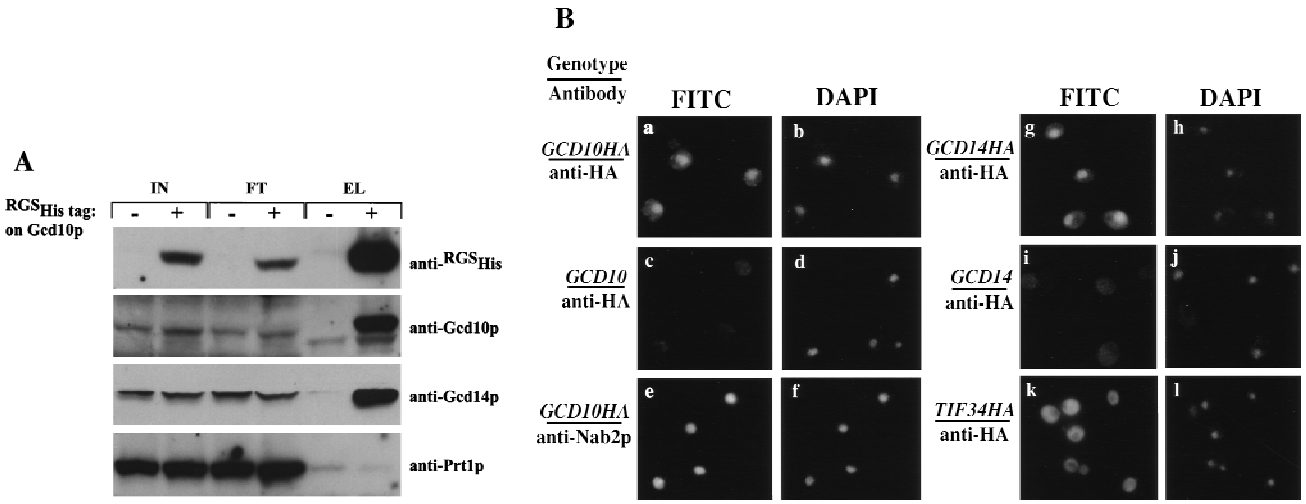

Figure 5. Gcd10p and Gcd14p form a stable nuclear complex in vivo. (*A*) Whole cell extracts were prepared from isogenic strains LPY251 (*GCD10*) and LPY252 (*GCD10–His*) containing wild-type and His-tagged Gcd10p, respectively, as described (Phan et al. 1998). Each clarified extract was batch-bound to 50 µl of Ni^{2+}–agarose (Qiagen) in H_2O (50% vol/vol) for 1 hr at 4°C. Proteins bound to Ni^{2+}–agarose were collected by centrifugation at 3000 rpm for 2 min, washed four times with 300 µl of breaking buffer, and batch-eluted with 50 µl of breaking buffer containing 250 mM imidazole. Aliquots containing 10% of the input cell extracts (IN), 10% of the flowthrough wash (FT), and 100% of the eluate (EL) were resolved by SDS-PAGE and subjected to immunoblot analysis using monoclonal anti-^{RGS}His antibodies (1:500; Qiagen) directed against the tag on His–Gcd10p, and with polyclonal antibodies directed against Gcd10p (1:500), Gcd14p (1:500), or Prt1p(1:1000). (*B*) Indirect immunofluorescence was used to study the subcellular distribution of HA epitope-tagged forms of Gcd10p, Gcd14p, and Tif34p in strains YJA142 (*GCD10–HA*; *a*,*b*), YJA143 (*GCD10*; *c*,*d*), Hm296 bearing pRC64 (*GCD14–HA*; *g*,*h*), Hm296 bearing pRC62 (*GCD14*; *i*,*j*), and KAY8 (*TIF34–HA*; *k*,*l*), as described previously (Anderson et al. 1993). All antibodies were diluted in PBS, 5% non-fat dried milk. The affinity-purified 12CA5 monoclonal antibody against the HA epitope (at 20 µg/ml; Boehringer Mannheim) was used to probe strains expressing HA-tagged proteins and the isogenic control strains lacking tagged proteins (*a*,*c*,*g*,*i*,*k*). Monoclonal antibody 1E4 (at 1:750 dilution; Wilson et al. 1994) was used to detect Nab1p in strain YJA142 (*e*). Detection of the primary antibodies was accomplished using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (*a*,*c*,*e*,*g*,*i*,*k*) and the DNA distribution was visualized by DAPI (*b*,*d*,*f*,*h*,*j*,*l*).

tRNAs were chromatographed on a RPC-5 column (Kelmers and Heatherly 1971). It was shown previously that this technique can resolve tRNAs that differ by only a single methyl group (Diamond et al. 1993). As shown in Figure 6A, wild-type tRNA contains two peaks of methionine-accepting tRNAs corresponding to initiator (fractions 20–40) and elongator $tRNA^{Met}$ (fractions 95–120) (D. Hatfield, unpubl.). In the *gcd10Δ* tRNA, the majority of both initiator and elongator methionyl-$tRNA^{Met}$ were resolved into single peaks that eluted in later fractions than did the same isoacceptors from the wild type. The differences in methionyl-$tRNA^{Met}$ elution profiles might arise from differences in tRNA length, although this seems unlikely for the 3′ end because of the obligatory requirement of CCA termini for aminoacylation. In addition, we observed no differences in length of initiator or elongator $tRNA^{Met}$ between *GCD10* and *gcd10Δ* tRNAs after separation on high resolution polyacrylamide gels and Northern blot analysis (data not shown). Together, these results provide strong evidence that Gcd10p and Gcd14p are required for modifying one or more bases in both forms of $tRNA^{Met}$. Small amounts of the initiator and elongator methionyl-tRNAs from *gcd10Δ* cells eluted earlier (initiator) or later (elongator) than the major peaks (Fig. 6A). These minor peaks may represent methionine-accepting tRNAs lacking additional modifications, or exhibiting a collapsed tertiary structure, due to the absence of a Gcd10p-dependent modification.

To examine directly whether the methionyl-tRNAs from *gcd10Δ* cells lack a modified base, total tRNA from the *GCD10* and *gcd10Δ* strains was digested completely to nucleosides and separated by high-performance liquid chromatography (HPLC) (Gehrke and Kuo 1990). The resulting chromatograms were identical except for the absence of a single peak in the *gcd10Δ* sample with a retention time of 13.5 min, identified previously as 1-methyladenosine (Gehrke and Kuo 1990) (Fig. 6B). This assignment was confirmed in two ways. First, addition of synthetic m^1A to wild-type or *gcd10Δ* tRNA hydrolyzates followed by HPLC analysis restored the missing peak to the *gcd10Δ* sample and led to a quantitative increase in the corresponding peak in the wild-type sample (data not shown). Second, the nucleoside present in the peak eluting at 13.5 min had the UV spectrum of m^1A (Gehrke and Kuo 1990) and the same molecular mass as protonated m^1A (282 Da) as determined by mass spectrometry using ionization electrospray (data not shown). These results strongly suggest that Gcd10p is required for the formation of 1-methyladenosine in tRNA. This base modification occurs at position 58 in initiator and elongator $tRNA^{Met}$ and in 16 other tRNAs, but is not found at any other positions in yeast tRNAs (Sprinzl et al. 1998).

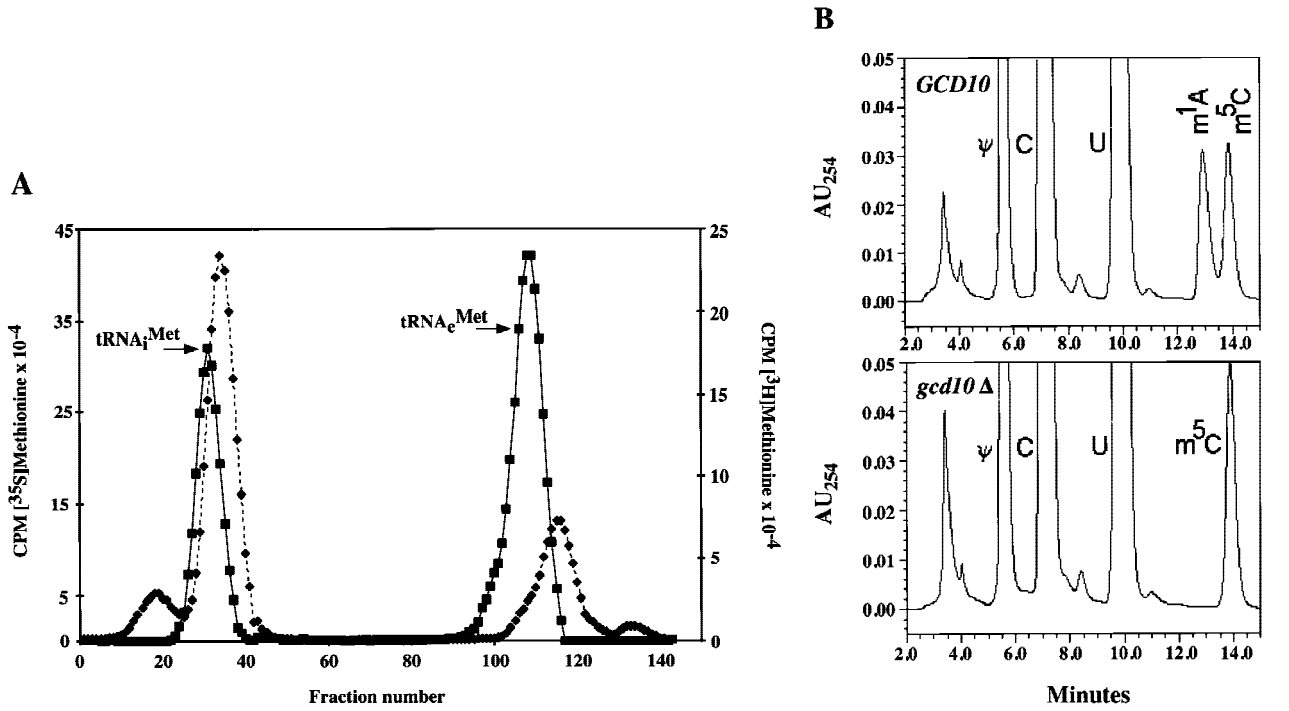

Figure 6. Evidence that methionine-accepting and other tRNAs from a *gcd10Δ* strain are hypomethylated. (*A*) Total tRNA isolated from YJA146 (*gcd10Δ*) and the p1775 transformant of Bj5464 (*GCD10*) was aminoacylated with [^{35}S]methionine and [^{3}H]methionine, respectively, and 500,000 cpm of each were chromatographed on a RPC-5 column. The radioactivity in each fraction (0.6 ml) was measured by liquid scintillation in 7 ml of Ecoscint A (National Diagnostics) and is plotted on different *y*-axes, as shown, against the fraction number. (■) Results obtained with wild-type tRNA (*GCD10*); (♦) results with *gcd10Δ* tRNA. The elution positions of the methionine-accepting tRNAs are indicated at the appropriate positions. (*B*) Transfer RNAs were digested to nucleosides and chromatographed by HPLC on a Supelcosil LC-18S column. Only the portion of the chromatogram (corresponding to retention times of 2–14.5 min) showing a difference between the *GCD10* and *gcd10Δ* samples is shown here. The peak that is absent in the *gcd10Δ* sample was identified as m^{1}A by several means (see text). The identities of other peaks in this portion of the chromatogram are indicated: (Ψ) Pseudouridine; (C) cytidine; (U) uridine; (m^{1}A) 1-methyladenosine; (m^{5}C) 5-methylcytidine. (AU$_{254}$) Absorbance units at 254 nm.

Discussion

Previously, we identified Gcd10p genetically as a factor required for translational repression of *GCN4* mRNA, indicating a role in the formation or utilization of ternary complexes. Using suppressor analysis, we have uncovered a function for Gcd10p in tRNA$_i^{Met}$ maturation. After discovering that *gcd10* mutations can be suppressed by extra copies of the *IMT* or *LHP1* genes, we found that the level of mature tRNA$_i^{Met}$ was reduced in a *gcd10-504* mutant (Fig. 1). This was also true of a *gcd14-2* mutant (Fig. 3C), which exhibits the same defect in *GCN4* translation seen in *gcd10* mutants (Cuesta et al. 1998) and can also be suppressed by hc*IMT* or hc*LHP1* (R. Cuesta, O. Calvo, J. Anderson, M. Garcia-Barrio, A. Hinnebusch, and M. Tamame, in prep.). The reductions in mature tRNA$_i^{Met}$ levels in these mutants can be expected to diminish ternary complex formation, explaining their constitutive derepression of *GCN4* translation. It was striking that overexpression of tRNA$_i^{Met}$ suppressed the lethality of a *gcd10Δ* deletion (Fig. 3A), indicating that the essential function of *GCD10* is to promote expression of mature tRNA$_i^{Met}$. In addition to the genetic links between Gcd10p and Gcd14p, both proteins show prominent nuclear localization (Fig. 5B) and are components of the same heteromeric protein complex (Fig. 5A; R. Cuesta, O. Calvo, J. Anderson, M. Garcia-Barrio, A. Hinnebusch, and M. Tamame, in prep.).

The *gcd10-504* mutation did not lead to accumulation of pre-tRNA$_i^{Met}$ or the appearance of novel tRNA$_i^{Met}$ species, but only to reduced amounts of mature tRNA$_i^{Met}$ (Fig. 1B). On the basis of the results of pulse–chase experiments indicating that a large fraction of newly synthesized tRNA$_i^{Met}$ is unstable in *gcd10-504* cells (Fig. 4), we concluded that the decrease in mature tRNA$_i^{Met}$ arose from increased degradation rather than diminished synthesis of pre-tRNA$_i^{Met}$. This conclusion is consistent with our finding that in vitro transcription of *IMT4* occurred at the same rates in extracts from *gcd10Δ* and *GCD10* strains (J. Anderson and A.G. Hinnebusch, unpubl.). In the *gcd14-2* mutant, accumulation of pre-tRNA$_i^{Met}$ species containing both 5′ and 3′ extensions, plus an aberrant species shorter than mature tRNA$_i^{Met}$ were observed in addition to a specific reduction in the amount of mature tRNA$_i^{Met}$ (Fig. 3C). These findings strongly suggest that processing of pre-tRNA$_i^{Met}$ is impaired by *gcd14-2*. Similar phenotypes were observed in the *gcd10Δ* hc*IMT4* strain (Fig. 3B).

Considering that Gcd10p and Gcd14p reside in the same complex, deletion of *GCD10* may indirectly impair Gcd14p function. Because cleavage of the 5′ extension by RNase P generally precedes trimming of the 3′ extension (O'Connor and Peebles 1991), accumulation of pre-$tRNA_i^{Met}$ in *gcd14-2* and *gcd10Δ* hc*IMT4* cells containing both 5′ and 3′ extensions (species b and c) suggests that trimming by RNase P occurs more slowly in these mutants. The predicted length of the novel $tRNA_i^{Met}$ that migrates faster than mature $tRNA_i^{Met}$ (species g) is consistent with tRNAs lacking the 3′ CCA. Thus, it is possible that CCA addition also occurs less efficiently in *gcd14-2* and *gcd10Δ* hc*IMT4* strains. We observed no significant differences in the rate of *IMT3* transcription or the efficiency of pre-$tRNA_i^{Met}$ processing between *gcd14-2* and *GCD14* extracts (J. Anderson and A.G. Hinnebusch, unpubl.), providing evidence that Gcd14p is not required for 5′- or 3′-end trimming of pre-$tRNA_i^{Met}$ in vitro.

The fact that large fractions of Gcd10p and Gcd14p are found in the nucleus, where enzymes involved in modification (Rose et al. 1995; Simos et al. 1996) and processing (Clark and Abelson 1987) of tRNAs generally reside, suggests that they function directly in one or more aspects of $tRNA_i^{Met}$ maturation. Gcd14p contains motifs (Kagan and Clarke 1994) common to *S*-adenosylmethionine-dependent methyltransferases (Cuesta et al. in prep.), raising the possibility that it methylates one of the bases in $tRNA_i^{Met}$. Experimental support for this hypothesis came from our finding that both initiator and elongator $tRNA^{Met}$ isolated from *gcd10Δ* cells eluted from a RPC-5 column in different positions than did the corresponding tRNAs from wild-type cells (Fig. 6A). As there are only four base methylations common to initiator and elongator methionine tRNAs, m^2G10, m^7G46, m^5C48, and m^1A58 (Sprinzl et al. 1998), these results led to the prediction that the Gcd10p–Gcd14p complex is required for one of these modifications. Analysis of all modified nucleosides in total tRNA (Fig. 6B) indicated that 1-methyladenosine, found at position 58 in 18 yeast tRNAs, was undetectable in *gcd10Δ* cells. Recently, we repeated the HPLC analysis of modified nucleosides in purified intitiator $tRNA^{Met}$ and again failed to detect m^1A (J. Anderson, G.R. Björk, and A.G. Hinnebusch, unpubl.). Thus, we conclude that initiator $tRNA^{Met}$ produced in *gcd10Δ* cells lacks m^1A58. Importantly, this residue plays a central role in determining a unique tertiary substructure not observed in elongator tRNAs, involving three residues unique to eukaryotic initiators, A60, A54, and A20 (Basavappa and Sigler 1991). Therefore, the absence of this modification might alter the tertiary structure of the initiator, and impair its processing and stability, without similarly affecting elongator $tRNA^{Met}$ and other tRNAs bearing m^1A58. Several of the genes that function in modifying tRNAs have been cloned and none is essential for cell viability (Hopper and Martin 1992). If the Gcd10p–Gcd14p complex functions only in the production of m^1A58, this would be the first instance of an essential tRNA modification in yeast.

In addition to showing a defect in processing, it appears that unprocessed pre-$tRNA_i^{Met}$ molecules are degraded more rapidly in *gcd10Δ* mutants. These results suggest that Gcd10p is required to protect hypomethylated pre-$tRNA_i^{Met}$ from degradation in addition to promoting the function of Gcd14p in m^1A58 formation. The maturation of $tRNA_i^{Met}$ occurs very inefficiently in *gcd10Δ* hc*IMT4* cells grown at 36°C, such that mature $tRNA_i^{Met}$ is not overproduced despite the increased copy number of *IMT4* (Fig. 3B, cf. lanes 2 and 3 with lanes 5 and 6). This finding is consistent with the idea that Gcd10p stabilizes the conformation of hypomethylated pre-$tRNA_i^{Met}$, as the absence of Gcd10p would be expected to have more severe consequences at elevated temperatures where isomerization of pre-$tRNA_i^{Met}$ to aberrant conformations should be favored. Furthermore, Gcd10p has strong RNA-binding activity (Garcia-Barrio et al. 1995), which might enable it to perform this proposed RNA chaperone function.

Recently, it was proposed that Lhp1p functions as an RNA chaperone in facilitating endonucleolytic trimming of the 3′ trailers of many yeast pre-tRNAs. Through binding to the poly(U) stretch at the 3′ end of pre-tRNA, Lhp1p would stabilize the conformation needed for endonucleolytic 3′-trimming and block access by $3' \rightarrow 5'$ exonucleases (Yoo and Wolin 1997). Interestingly, a mutation in yeast $tRNA_{CGA}^{Ser}$ that renders its processing dependent on Lhp1p leads to degradation of processing intermediates when Lhp1p is depleted from cells (Yoo and Wolin 1997). This might resemble the situation in *gcd10Δ* mutants where the absence of m^1A58, combined with loss of the putative Gcd10p chaperone function, would result in degradation of pre-$tRNA_i^{Met}$. Perhaps overexpression of Lhp1p in *gcd10* mutants allows it to substitute partially for the chaperone function of Gcd10p, increasing the probability of accurate processing and protecting the hypomethylated pre-$tRNA_i^{Met}$ from degradation.

It is conceivable that Gcd10p accompanies mature $tRNA_i^{Met}$ from the nucleus to the cytoplasm, where it could promote formation of ternary complexes with eIF2 and GTP. This could explain why it copurified with eIF3 activity through several chromatographic separations (Garcia-Barrio et al. 1995), as stabilizing the ternary complex is one function ascribed to eIF3 (Peterson et al. 1979). Although we did not observe a stable interaction between Gcd10p and the *PRT1* subunit of eIF3 under conditions where Gcd14p was tightly associated with Gcd10p (Fig. 5A), interaction between eIF3 and Gcd10p may be highly sensitive to differences in strain background, extract preparation, or purification scheme.

The *gcd10-504* and *gcd10Δ* mutations had little or no effect on the levels of mature forms of several tRNAs, including elongator $tRNA^{Met}$, $tRNA^{His}$, $tRNA_{UAU}^{Ile}$, and $tRNA_{CGA}^{Ser}$. Elongator $tRNA^{Met}$ contains m^1A58, whereas $tRNA^{His}$ does not, and the sequences of the other two tRNAs are unknown (Sprinzl et al. 1998). It will be interesting to determine whether expression of any other tRNAs containing m^1A58 is impaired by *gcd10* or *gcd14* mutations. Given that the *gcd10Δ* mutant was rescued by overexpression of initiator $tRNA^{Met}$,

it is likely that Gcd10p and Gcd14p play an essential role in maturation and accumulation of only this tRNA. Accordingly, it is conceivable that these proteins provide a novel means of regulating translation initiation, whereby modulating the maturation and stability of pre-$tRNA_i^{Met}$ in the nucleus would affect the formation of ternary complexes in the cytoplasm.

Materials and methods

Plasmid and yeast strain constructions

Table 1 contains the genotypes of all yeast strains used in this work. Details of plasmid constructions will be provided on request. Yeast strains YJA142, YJA143, and YJA146 were constructed by introducing plasmid p2705 (*GCD10-HA*) into strain BJ5464 (gift of E. Jones, Carnegie Mellon University, Pittsburgh, PA) and then deleting chromosomal *GCD10* by transformation to Ura^+ with a 6.6-kb *Xba*I–*Xho*I fragment containing the *gcd10Δ*::*hisG*::*URA3*::*hisG* allele (Alani et al. 1987) from pLPY1. A Ura^- *gcd10Δ*::*hisG* derivative (YJA142) was selected by growth on SC medium containing 1 μg/ml 5-FOA (SC + FOA). YJA143 was constructed by replacing plasmid p2705 in YJA142 with plasmid p2704 by plasmid shuffling (Boeke et al. 1987). Strain YJA146 was constructed from YJA143 as described in the Results section. To construct strains LPY251 and LPY252, a diploid from a cross between CH1305 and K2348 (a gift from K. Nasmyth, Research Institute of Molecular Pathology, Vienna, Austria) was transformed with the 6.6-kb *gcd10Δ*::*hisG*::*URA3*::*hisG* fragment from pLPY1, and the *gcd10Δ*::*hisG*::*URA3*::*hisG* allele was converted to *gcd10Δ*::*hisG* as described above. One such Ura^- strain, LPY25, was transformed to Ura^+ with plasmid pLPY5, bearing *GCD10* and *URA3*, sporulated and a Ura^+ ascospore (LPY251B) was isolated by tetrad analysis. After introduction of the *LEU2* plasmids pLPY2 or pLPY3 bearing *GCD10* or *GCD10–His*, LPY251B was cured of plasmid pLPY5 on 5-FOA medium to produce LPY251 and LPY252.

High copy suppressor analysis

Standard genetic techniques and culture media (SD, SC, and YPD) (Sherman et al. 1974) and the growth assays for sensitivity to 3-AT (Hinnebusch and Fink 1983) were described previously. Yeast strains H2457 and HM298 were transformed with a high-copy genomic library constructed in YEp24 (Carlson and Botstein 1982) and transformants were plated on minimally supplemented SD plates at 36°C. Plasmids were isolated from ts^+ transformants as described previously (Hoffman and Winston 1987) and shown to confer a ts^+ phenotype with reintroduction into H2457. The ends of the genomic inserts were sequenced using primers complementary to the sequences flanking the *Bam*HI site in YEp24, and compared to the complete *S. cerevisiae* sequence (http://genome-www.Stanford.edu/Saccharomyces/) to identify the end points of the genomic inserts. Tn10 insertion libraries were constructed for each plasmid as described previously (Huisman et al. 1987). A Tn10 insertion that destroyed the suppressor function of plasmid p2634 was found to interrupt the *IMT3* gene. On the basis that the other three *IMT* genes were present on the genomic inserts in suppressor plasmids p2632 (*IMT1*), p2633 (*IMT2*), and p2635 (*IMT4*), the *IMT* coding regions in all four plasmids were disrupted by inserting a 604-bp *Bss*HII DNA fragment isolated from λ phage DNA into a unique *Bss*HII site present in each gene, generating

Table 1. *Genotypes of yeast strains used in this study*

Strain	Genotype	Plasmid [name: relevant gene]	Source or reference
H2457	*MAT*α, *gcd10-504, gcn2-101, his1-29, ura3-52, inol (HIS4–lacZ, ura3-52)*		M. Garcia-Barrio (NIH) and M. Tamame
H62	*MAT*α, *gcd10-503, his1-29, gcn2-101, gcn3-101, ura3-52, (HIS4–lacZ, ura3-52)*		Harashima and Hinnebusch (1986)
Hm298	*MAT***a**, *gcd10-505, gcn2-101, his1-29, ura3-52, inol (HIS4–lacZ, ura3-52)*		Garcia-Barrio et al. (1995)
BJ5464	*MAT*α, *ura3-52, trp1, leu2Δ1, his3Δ200, pep*::*HIS4, prb1Δ1.6, can1* Gal^+		E. Jones
YJA142	*MAT*α, *gcd10Δ*::*hisG, ura3-52, trp1, leu2Δ1, his3Δ200, pep*::*HIS4, prb1Δ1.6, can1* Gal^+	[p2705:*GCD10HA*]	this work
YJA143	*MAT*α, *gcd10Δ*::*hisG, ura3-52, trp1, leu2Δ1, his3Δ200, pep*::*HIS4, prb1Δ1.6, can1* Gal^+	[p2704:*GCD10*]	this work
YJA146	*MAT*α, *gcd10Δ*::*hisG, ura3-52, trp1, leu2Δ1, his3Δ200, pep*::*HIS4, prb1Δ1.6, can1* Gal^+	[p1775:*IMT4*]	this work
Hm296	*MAT*α, *gcd14-2, his1-29, gcn2-101, gcn3-101, ura3-52, inol (HIS4–lacZ, ura3-52)*		Cuesta et al. (1998)
H1676	*MAT***a**, *prt1-1, leu2-3; leu2-112, ura3-52*		M. Ramirez (NIH) and A.G. Hinnebusch
TD-304-10	*MAT***a**, *leu2-3,-112, ura3-52, his4–303*(ATT), *sui2-1*		T. Donahue (Indiana University, Bloomington)
H56	*MAT*α, *gcd1-501, his 1-29, ura3-52, gcn2-101, gcn3-101, (HIS4–lacZ, ura3-52)*		Harashima and Hinnebusch (1986)
KAY8	*MAT*α, *tif34Δ1, his1-29, gcn2-508, ura3-52, leu2-3,-112, (HIS4–lacZ, ura3-52)*	[YCpL*TIF34HA*]	Asano et al. (1998)
CH1305	*MAT***a**, *ade3, leu2, ura3, lys2, can1,* Gal^+		C. Holm (UCSD)
K2348	*MAT*α, *ade2-1, ade3, trp1-1, can1-100, leu2-3, his3-11,15, ura3,* Gal^+ psi^+		K. Nasmyth

plasmids pJA104 (*IMT2*), pJA105 (*IMT3*), pJA106 (*IMT4*), and pJA107 (*IMT1*). None of the plasmids containing the disrupted *IMT* genes had suppressor activity in H2457, identifying the *IMT* genes as the dosage suppressors in plasmids p2623–p2625. A Tn*10* insertion that destroyed the suppressor function of p2636 interrupted the *LHP1* open reading frame. Plasmid p2626 (*LHP1*) was created by inserting a 1.7-kb *Sal*I–*Pvu*II fragment containing *LHP1* into YEp24 digested with *Sal*I and *Sma*I. p2626 showed suppressor activity similar to that of p2636, proving that *LHP1* is the suppressor gene in p2636. The high-copy plasmids pC44 or p2626, bearing *IMT4* or *LHP1*, respectively, were tested for the ability to suppress the ts^- phenotypes of strains H1676 (*prt1-1*), H56 (*gcd1-501*), and TD304-10B (*sui2-1*) in comparison to the low-copy plasmids p2625, p256 (Hill and Struhl 1988), or p1234 (Vazquez de Aldana et al. 1993) bearing wild-type copies of *PRT1*, *GCD1*, or *SUI2*, respectively.

Analysis of tRNA modification

Total RNA was extracted from 300 grams (wet weight) of yeast cells grown in YPD medium (Rubin 1975) and tRNA was purified by DEAE–cellulose chromatography and deacylated (Hatfield et al. 1979), all as previously described. Transfer RNAs were aminoacylated with [^{3}H]methionine (70 Ci/mmole, Amersham) or [^{35}S]methionine (1000 Ci/mmole; Amersham) under conditions of limiting tRNA and the labeled tRNAs were fractionated by reversed-phase chromatography on a RPC-5 column (Kelmers and Heatherly 1971) essentially as described (Hatfield et al. 1979), except that elution of aminoacyl-tRNAs was carried out using a linear gradient of 0.45–0.65 M NaCl in the presence of 10 mM magnesium acetate. For HPLC analysis of base modifications, the tRNA was digested to nucleosides by nuclease P1 and alkaline phosphatase (Gehrke et al. 1982) and the hydrolyzate was analyzed by HPLC according to the method of Gehrke and Kuo (1990).

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