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 trRNA (tRNA\textsuperscript{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\textsuperscript{Met}, attributable to increased turnover rather than decreased synthesis of pre-tRNA\textsuperscript{Met}. Remarkably, the lethality of a \textit{GCD10} deletion was suppressed by high-copy-number IMT4, indicating that its role in expression of mature tRNA\textsuperscript{Met} is the essential function of Gcd10p. A gcd14-2 mutant also showed reduced amounts of mature tRNA\textsuperscript{Met}, but in addition, displayed a defect in pre-tRNA\textsuperscript{Met} processing. Gcd10p and Gcd14p were found to be subunits of a protein complex with prominent nuclear localization, suggesting a direct role in tRNA\textsuperscript{Met} maturation. The chromatographic behavior of elongator and initiator tRNA\textsuperscript{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\textsuperscript{1}A) is undetectable in gcd10Δ tRNA. Interestingly, gcd10 and gcd14 mutations had no effect on processing or accumulation of elongator tRNA\textsuperscript{Met}, which also contains m\textsuperscript{1}A at position 58, suggesting a unique requirement for this base modification in 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\textsuperscript{Met} (Met-tRNA\textsuperscript{Met}) to the 40S ribosomal subunit to form a 43S preinitiation complex. Initiation factor 2 (elf2) delivers Met-tRNA\textsuperscript{Met} to the ribosome in a ternary complex with GDP. Exchange of the GDP bound to elf2 for GTP is catalyzed by the guanine nucleotide exchange factor elf2B. Phosphorylation of the α subunit of elf2 (elf2α) prevents the recycling of elf2 by elf2B, inhibiting the formation of elf2·GTP·Met-tRNA\textsuperscript{Met} ternary complexes (Hershey 1991; Hinnebusch 1996). In Saccharomyces cerevisiae, elf2α 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 elf2 and elf2B derepress GCN4 translation in cells lacking Gcn2p (for review, see Hinnebusch 1996). Mutations in \textit{GCD10} also derepress GCN4 translation in the absence of elf2 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 elf3 (Garcia-Barrio et al. 1995). Given their effect on GCN4 translation, it was proposed that gcd10 mutations reduce the ability of elf3 to stimulate ternary complex binding to 40S ribosomes,
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 communoprecipitated 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 mutants. Analysis of these suppressors revealed that mutation 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 non-essential 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. gcn10 mutations lead to derepression of GCN4 translation in the absence of eIF2 phosphorylation by Gcn2p (Gcd^− phenotype), conferring resistance to 3-AT (AT') in a gcn2 strain background (Harashima and Hinnebusch 1986). All of the dosage suppressors overcame the 3-AT^− 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 (hclMT3) 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_{Met} or Lhp1p is required for suppression.

We asked whether overexpression of tRNA_{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 hclMT4 or hclLHP1 (Fig. 1A). Furthermore, neither hclMT4 nor hclLHP1 suppressed the growth defects of sul2-1 or gcd1-501 alleles, encoding defective subunits of eIF2a and eIF2B, 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^{Met} using sequences complementary to the tRNA^{Met} coding region as a probe. In addition to mature tRNA^{Met}, this probe hybridized to two larger species that appear to be precursors of tRNA^{Met} bearing different 5' and 3' terminal extensions encoded by the various IMT genes (see below). The amount of mature tRNA^{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^{Met}). In contrast, levels of the putative tRNA^{Met} precursors were virtually indistinguishable between the mutant and wild-type strains (Fig. 1B; pre-tRNA^{Met}), leading to ratios of precursors to mature tRNA^{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^{Met} abundance occurs at a post-transcriptional step.

Interestingly, the levels of mature tRNA^{His} and tRNA^{Ser}_{Car} were indistinguishable between the wild-type and gcd10 strains at the permissive and nonpermissive temperatures (data not shown), whereas we consis-
tently observed a modest increase in mature elongator tRNA\textsubscript{Met} levels in the gcd10 mutant at 36°C (Fig. 1B,C; tRNA\textsubscript{Met}). The level of pre-tRNA\textsubscript{UAU}\textsuperscript{Ile} decreased at 36°C slightly more in the gcd10 mutant than it did in the wild type (Fig. 1B; pre-tRNA\textsubscript{UAU}\textsuperscript{Ile}). However, the mature tRNA\textsubscript{UAU}\textsuperscript{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\textsubscript{Met}.

To investigate whether the lowered expression of mature tRNA\textsubscript{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\textsubscript{Ile} level was ~34% lower in the sui2 mutant versus the SUI2 strain; however, the mature tRNA\textsubscript{Ile} level decreased by only 15% in the sui2 cells, leading to a small

Figure 1. High-copy suppressors of gcd10-504 overcome a defect in accumulation of tRNA\textsubscript{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\textsubscript{Met}, stripped and reprobed with radiolabeled oligonucleotides specific for tRNA\textsubscript{UAU}\textsuperscript{Ile} or tRNA\textsubscript{UAU}\textsuperscript{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\textsubscript{Met} species, mature tRNA\textsubscript{Met}, pre-tRNA\textsubscript{UAU}\textsuperscript{Ile}, and mature tRNA\textsubscript{UAU}\textsuperscript{Ile} are indicated at left. The relative intensities of the hybridization signals were quantified for mature tRNA\textsubscript{Met} and pre-tRNA\textsubscript{Met}, and the ratios of pre-tRNA\textsubscript{Met} to mature tRNA\textsubscript{Met} are listed under the appropriate lanes. The species that migrated just above mature tRNA\textsubscript{UAU}\textsuperscript{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.
reduction in the precursor-to-mature ratio (0.08 (su12) versus 0.16 (SU12)). Thus there was a much greater reduction in mature tRNA$_{\text{M}et}^\text{M105}$ levels in the gcd10 mutant (a factor of 6, Fig. 1B) versus the su12 mutant (15%), although pre-tRNA$_{\text{M}et}^\text{M105}$ expression declined in the su12 cells but not in the gcd10 cells. These data are consistent with the idea that gcd10 mutants are defective in maturation of pre-tRNA$_{\text{M}et}^\text{M105}$ at 36°C.

The dosage suppressors increase mature tRNA$_{\text{M}et}^\text{M105}$ levels in gcd10 mutants

It seemed likely that suppression of gcd10 mutations by hclMT genes occurred by increasing the abundance of mature tRNA$_{\text{M}et}^\text{M105}$. In accordance with this expectation, we found that hclMT4 in the gcd10 mutant increased mature tRNA$_{\text{M}et}^\text{M105}$ 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 hclMT4 transformant also showed increased amounts of the smaller of two putative tRNA$_{\text{M}et}^\text{M105}$ 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 hclMT1, hclMT2, hclMT3, or hclMT4 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 hclMT3 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$_{\text{M}et}^\text{M105}$ 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 hclMT3 transformants contained relatively less mature tRNA$_{\text{M}et}^\text{M105}$ compared to the other hclMT transformants (Fig. 2B), which can explain why hclMT3 suppressed the phenotypes of gcd10-505 less completely than did the other three hclMT genes (data not shown).

HclLHP1 also increased the level of precursor and mature tRNA$_{\text{M}et}^\text{M105}$ in the gcd10 mutant (Fig. 2A, lanes 1–4 vs. lanes 13–16); however, mature tRNA$_{\text{M}et}^\text{M105}$ did not reach the wild-type level at 36°C (Fig. 2A, lane 16 vs. lane 8), explaining why hclLHP1 only partially suppressed partially the ts phenotype of gcd10-504 (see Fig. 1A). HclLHP1 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$_{\text{M}et}^\text{M105}$ (species d and e). The presence of species d and e suggests that Lhp1p overexpression interferes with exonucleolytic trimming of pre-tRNA$_{\text{M}et}^\text{M105}$. 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$_{\text{M}et}^\text{M105}$ when Lhp1p is overexpressed, it could be responsible for the higher levels of full-length pre-tRNA$_{\text{M}et}^\text{M105}$ and mature tRNA$_{\text{M}et}^\text{M105}$ seen under these conditions, provided that endonucleolytic cleavage is more precise than exonucleolytic trimming in 3’ end maturation.
GCD10 is nonessential in yeast strains overexpressing initiator tRNA^{Met}

To test the possibility that overexpression of tRNA^{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 for cell viability in the presence of hcMet. We verified by immunoblot analysis that one such strain (YJA146) containing hcMT4 and lacking GCD10 had no detectable Gcd10p (data not shown).

Although the gcd10Δ hcMT4 strain is viable, it grows poorly at temperatures >26°C (Fig. 3A). We observed little difference in mature tRNA^{Met} levels between the isogenic gcd10Δ hcMT4 and GCD10 hcMT4 strains at 26°C (Fig. 3B, lanes 4.7). Thus, at low temperature, the requirement for GCD10 in mature tRNA^{Met} expression appears to be largely bypassed by overproduction of tRNA^{Met}. In contrast, mature tRNA^{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^{Met} at 36°C, even when tRNA^{Met} is being overexpressed. The gcd10Δ hcMT4 transformant accumulated higher levels of pre-tRNA^{Met} than did the GCD10 hcMT4 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^{Met}-to-mature tRNA^{Met} ratio was much greater in the gcd10Δ hcMT4 transformant compared to the GCD10 hcMT4 strain, particularly at 36°C (Fig. 3B). It is also noteworthy that the gcd10Δ hcMT4 transformant accumulated species with mobilities greater than that of mature tRNA^{Met}, which were not observed in the GCD10 hcMT4 transformant (Fig. 3B, cf. lanes 4–6 and lanes 7–9, species g). These observations suggest that Gcd10p is not required for efficient processing of pre-tRNA^{Met}, particularly at elevated growth temperatures, and that in its absence, much of the unprocessed pre-tRNA^{Met} is degraded. The gcd10Δ hcMT4 transformant showed no detectable reduction in the accumulation of other mature tRNA^{Met}.

**Figure 3.** GCD10 is dispensable for cell viability in the presence of hcMT4. (A) Growth of strain YJA146 (gcd10Δ +hcMT4) and a transformant of its parental GCD10 strain BJ5464 bearing p1775 (GCD10 +hcMT4) 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 +hcMT4 and GCD10 +hcMT4 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^{Met} and stripped and reprobed for tRNA^{Met} as described in Fig. 1. The different RNA species detected are indicated at left. The various forms of tRNA^{Met} species are labeled 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^{Met} and stripped and reprobed for tRNA^{Met} as described in Fig. 1. Indicated with arrowheads inside the blot and labeled to the right are the various tRNA^{Met} species described in Fig. 2 and above. The indicated ratios of pre-tRNA^{Met} to mature tRNA^{Met} were calculated from the relative intensities of hybridization signals quantitated by PhosphorImager analysis.
tRNAs when compared to the GCD10 + hclMT4 transformant, including tRNA_Met (Fig. 3B; tRNA_e_Met), tRNA_AUA_Met, and tRNA_CGA_Met (data not shown).

Despite the reduction in mature tRNA_Met expression at 36°C in the gcd10Δ hclMT4 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 hclMT4 transformant at 36°C suggests that mature tRNA_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_Met decreased by a factor of 1.7, whereas pre-tRNA_Met increased ~1.5-fold in a gcd14-2 mutant after 8 hr at 36°C, increasing the ratio of precursor to mature tRNA_Met from 0.28 to 0.79 (Fig. 3C). These results suggest strongly that Gcd14p is required for processing of nascent tRNA_Met transcripts. Interestingly, a tRNA_Met species migrating faster than the wild-type mature form, exhibiting a similar mobility to the aberrant tRNA_Met seen in the gcd10Δ hclMT4 transformants, is present in the gcd14-2 mutant under all conditions (Figs. 3B,C, species g). Thus, 5′- and 3′-trimmed tRNA_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_Met in vivo

We used pulse-chase analysis to investigate whether the reduced amount of mature tRNA_Met in the gcd10-504 mutant results from rapid degradation of tRNA_Met transcripts. After incubating isogenic Gcd10 and gcd10-504 strains for ~2 hr at 36°C, cells were pulse-labeled with [3H]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 radioactive activity were hybridized to immobilized oligonucleotides complementary to initiator tRNA_Met or elongator tRNA_Met. The proportion of [3H]labeled tRNA_e_Met in both mutant and wild-type cells, and of tRNA_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 [3H]labeled tRNA_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_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_Met transcripts detected by pulse-chase analysis in the gcd10-504 mutant represented nascent tRNA_Met molecules that were degraded rapidly in the nucleus. To eliminate the alternative possibility that mature tRNA_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_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_Met after the temperature shift (lanes 1–4 and 8–11). In contrast, thiolutin treatment largely eliminated the reduction in mature tRNA_Met levels at 36°C (Fig. 4B, lanes 5–7 and 12–14). The disappearance of pre-tRNA_Met species after thiolutin treatment is the expected result of inhibiting IMT transcription without preventing processing (or degradation) of the pre-existing pre-tRNA_Met transcripts. The fact that little or no change in mature tRNA_Met abundance occurred at 36°C in the presence of thiolutin suggests that the mature tRNA_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_Met seen in the absence of thiolutin most likely occurs by dilution of stable preexisting mature tRNA_Met, coupled with a failure to produce new mature tRNA_Met during cell divisions at the nonpermissive temperature. We suggest that the unstable tRNA_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\textsubscript{Met}\textsuperscript{N}\textsubscript{1}\textsuperscript{N} 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 2.2 hr before the addition of 5.0 mCi [5,6-3H]uracil (37 Ci/m mole, 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 cpns was hybridized to membrane-bound oligonucleotides complementary to full-length tRNA\textsubscript{Met}\textsuperscript{N}\textsubscript{1}\textsuperscript{N} (top) and tRNA\textsubscript{Met}\textsuperscript{N}\textsubscript{1}\textsuperscript{N} (bottom) in hybridization solution (500 mM NaCl, 24 mM NaH\textsubscript{2}PO\textsubscript{4}, 2.4 mM EDTA (pH 7.4), 30% formamide, 5x 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 (gcd10\textsuperscript{A} + hclMT4) 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 thiolulin 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 (−) thiolulin treatment were probed with a labeled oligonucleotide that specifically hybridized to both pre-tRNA\textsubscript{Met}\textsuperscript{N}\textsubscript{1}\textsuperscript{N} and mature tRNA\textsubscript{Met}\textsuperscript{N}\textsubscript{1}\textsuperscript{N} as described in Fig. 1. Labeled at right are various tRNA\textsubscript{Met}\textsuperscript{N}\textsubscript{1}\textsuperscript{N} species described in Figs. 2 and 3.

Ni\textsuperscript{2+}–NTA agarose with the GCD10–His extract but not with the isogenic GCD10 extract. In contrast, the PRT1-encoded subunit of elf3 (Naranda et al. 1994) in both extracts did not bind to the resin. These findings indicate that Gcd10p and Gcd14p are components of a hetero-meric complex that is not stably associated with elf3.

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 elf3; Naranda et al. 1997) tagged with the coding sequences of the integral subunit of elf3, 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\textsubscript{Met}\textsuperscript{N} at position 58.
Each clarified extract was batch-bound to 50 µl of Ni²⁺–agarose (Qiagen) in H₂O (50% vol/vol) for 1 hr at 4°C. Proteins bound to Ni²⁺–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-HA epitope (at 20 µg/ml; Boehringer Mannheim) was used to probe strains expressing HA-tagged proteins and the isogenic control strain YJA142 (data not shown). Together, these results provide strong evidence that Gcd10p and Gcd14p are required for 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) and the same molecular mass as protonated m1A (282 Da) as determined by mass spectrometry using ionization electrospray (data not shown). Second, the nucleoside present in the peak eluting at 13.5 min had the UV spectrum of m1A (Gehrke and Kuo 1990) and the same molecular mass as protonated m1A (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 tRNA⁹ met and in 16 other tRNA s, but is not found at any other positions in yeast tRNAs (Sprinzl et al. 1998).
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 tRNAi 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 tRNAi 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 hclIMT or hclHP1 (R. Cuesta, O. Calvo, J. Anderson, M. Garcia-Barrio, A. Hinnebusch, and M. Tamame, in prep.). The reductions in mature tRNAi 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 tRNAi Met suppressed the lethality of a gcd10Δ deletion (Fig. 3A), indicating that the essential function of GCD10 is to promote expression of mature tRNAi 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-tRNAi Met or the appearance of novel tRNAi Met species, but only to reduced amounts of mature tRNAi Met (Fig. 1B). On the basis of the results of pulse-chase experiments indicating that a large fraction of newly synthesized tRNAi Met is unstable in gcd10Δ cells (Fig. 4), we concluded that the decrease in mature tRNAi Met arose from increased degradation rather than diminished synthesis of pre-tRNAi 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-tRNAi Met species containing both 5' and 3' extensions, plus an aberrant species shorter than mature tRNAi Met were observed in addition to a specific reduction in the amount of mature tRNAi Met (Fig. 3C). These findings strongly suggest that processing of pre-tRNAi Met is impaired by gcd14-2. Similar phenotypes were observed in the gcd10Δ hclMT4 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\textsubscript{Met}\textsuperscript{Met} in gcd14-2 and gcd10a hclMT4 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\textsubscript{Met}\textsuperscript{Met} that migrates faster than mature tRNA\textsubscript{i}\textsuperscript{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 gcd10a hclMT4 strains. We observed no significant differences in the rate of IMT3 transcription or the efficiency of pre-tRNA\textsubscript{Met}\textsuperscript{Met} processing between gcd14-2 and Gcd14 extracts (J. Anderson and A.G. Hinnenbusch, unpubl.), providing evidence that Gcd14p is not required for 5′- or 3′-end trimming of pre-tRNA\textsubscript{i}\textsuperscript{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\textsubscript{Met}\textsuperscript{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\textsubscript{Met}\textsuperscript{Met}. Experimental support for this hypothesis came from our finding that both initiator and elongator tRNA\textsubscript{Met}\textsuperscript{Met} isolated from gcd10a 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\textsuperscript{1}G\textsubscript{10}, m\textsuperscript{G}\textsubscript{46}, m\textsuperscript{C}\textsubscript{48}, and m\textsuperscript{A}\textsubscript{58} (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 pre-tRNA\textsubscript{i}\textsuperscript{Met}. This finding is consistent with the idea that Gcd10p stabilizes the conformation of hypomethylated pre-tRNA\textsubscript{i}\textsuperscript{Met}, as the absence of Gcd10p would be expected to have more severe consequences at elevated temperatures where isomerization of pre-tRNA\textsubscript{i}\textsuperscript{Met} to aberrant conformations should be favored.

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′ → 5′ exonucleases (Yoo and Wolin 1997). Interestingly, a mutation in yeast tRNA\textsubscript{CGA}\textsuperscript{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 mechanism resembles the situation in gcd10a mutants where the absence of m\textsuperscript{1}A\textsubscript{58}, combined with loss of the putative Gcd10p chaperone function, would result in degradation of pre-tRNA\textsubscript{Met}\textsuperscript{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\textsubscript{i}\textsuperscript{Met} from degradation.

It is conceivable that Gcd10p accompanies mature tRNA\textsubscript{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 gcd10a mutations had little or no effect on the levels of mature forms of several tRNAs, including elongator tRNA\textsubscript{Met}\textsuperscript{Met}, tRNA\textsubscript{His}\textsuperscript{His}, tRNA\textsubscript{CGA}\textsuperscript{Ser} and tRNA\textsubscript{CGA}\textsuperscript{His}. Elongator tRNA\textsubscript{Met}\textsuperscript{Met} contains m\textsuperscript{A}\textsubscript{58}, whereas tRNA\textsubscript{His}\textsuperscript{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\textsuperscript{A}\textsubscript{58} is impaired by gcd10 or gcd14 mutations. Given that the gcd10a mutant was rescued by overexpression of initiator tRNA\textsubscript{Met}\textsuperscript{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\textsuperscript{M*} 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\textsuperscript{+} with a 6.6-kb XbaI-XhoI fragment containing the \textit{gcd10α}:hisG::URA3::hisG allele (Aliani et al. 1987) from pLPY1. A Ura\textsuperscript{−} gcd10α::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 \textit{gcd10α}:hisG::URA3::hisG fragment from pLPY1, and the \textit{gcd10α}:hisG::URA3::hisG allele was converted to \textit{gcd10α}:hisG as described above. One such Ura\textsuperscript{−} strain, LPY25, was transformed to Ura\textsuperscript{+} with plasmid pLPY5, bearing GCD10 and URA3, sporulated and a Ura\textsuperscript{+} 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\textsuperscript{+} transformants as described previously (Hoffman and Winston 1987) and shown to confer a ts\textsuperscript{+} phenotype with reintroduction into H2457. The ends of the genomic inserts were sequenced using primers complementary to the sequences flanking the BamHI 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 BssHII DNA fragment isolated from \lambda phage DNA into a unique BssHII site present in each gene, generating

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

| Strain     | Genotype                                      | Plasmid | Source or reference                  |
|------------|-----------------------------------------------|---------|-------------------------------------|
| H2457      | MAT\textit{a}, gcd10-504, gcn2-101, his1-29, ura3-52, inol (HIS4-lacZ, ura3-52) | pLPY251 | M. Garcia-Barrio (NIH) and M. Tamame (Harashima and Hinnebusch 1986) |
| H62        | MAT\textit{a}, gcd10-503, his1-29, gcn2-101, gcn3-101, ura3-52, (HIS4-lacZ, ura3-52) | pLPY252 | Garcia-Barrio et al. (1995) |
| Hm296      | MAT\textit{a}, gcd10-505, gcn2-101, his1-29, ura3-52, inol (HIS4-lacZ, ura3-52) | p2632   | E. Jones |
| BJ5464     | MAT\textit{a}, ura3-52, trp1, leu2Δ11, his3Δ200, pep::HIS4, prb1Δ1.6, can1 Gal\textsuperscript{+} | p2704   | this work |
| YJA142     | MAT\textit{a}, gcd10α::hisG, ura3-52, trp1, leu2Δ11, his3Δ200, pep::HIS4, prb1Δ1.6, can1 Gal\textsuperscript{+} | p2705   | this work |
| YJA143     | MAT\textit{a}, gcd10α::hisG, ura3-52, trp1, leu2Δ11, his3Δ200, pep::HIS4, prb1Δ1.6, can1 Gal\textsuperscript{+} | p2704   | this work |
| YJA146     | MAT\textit{a}, gcd10α::hisG, ura3-52, trp1, leu2Δ11, his3Δ200, pep::HIS4, prb1Δ1.6, can1 Gal\textsuperscript{+} | p1775  | this work |
| Hm296      | MAT\textit{a}, gcd14-2, his1-29, gcn2-101, gcn3-101, ura3-52, inol (HIS4-lacZ, ura3-52) | p2633   | Cuesta et al. (1998) |
| H1676      | MAT\textit{a}, prt1-1, leu2-3, leu2-112, ura3-52 | p2632   | M. Ramirez (NIH) and A.G. Hinnebusch |
| TD-304-10  | MAT\textit{a}, leu2-3-112, ura3-52, his4-303(ATT), sul2-1 | p1775  | T. Donahue (Indiana University, Bloomington) |
| H56        | MAT\textit{a}, gcd1-501, his 1-29, ura3-52, gcn2-101, gcn3-101, (HIS4-lacZ, ura3-52) | p2705   | Harashima and Hinnebusch (1986) |
| KAY8       | MAT\textit{a}, tif34Δ1, his1-29, gcn2-508, ura3-52, leu2-3-112, (HIS4-lacZ, ura3-52) | p2633   | Asano et al. (1998) |
| CH1305     | MAT\textit{a}, ade3, leu2, ura3, lys2, can1, Gal\textsuperscript{+} | p1775  | C. Holm (UCSD) |
| K2348      | MAT\textit{a}, ade2-1, ade3, trp1-1, can1-100, leu2-3, his3-11,15, ura3, Gal\textsuperscript{+} psi\textsuperscript{*} | p2633   | 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 Tn10 insertion that destroyed the suppressor function of p2636 interrupted the LH1P1 open reading frame. Plasmid p2626 (LH1P1) was created by inserting a 1.7-kb Sall–Pvull fragment containing LH1P1 into YEp24 digested with SalI and Smal. p2626 showed suppressor activity similar to that of p2636, proving that LH1P1 is the suppressor gene in p2636. The high-copy plasmids pC44 or p2626, bearing tested for the ability to suppress the ts− phenotypes of strains that showed suppressor activity similar to that of p2636, proving containing IMT4 SalI fragment was analyzed by HPLC according to the method of Gehrke et al. (1982), 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 of a component required for the initiation of translation, science 242: 93–97.

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 [3H]methionine (70 Ci/mmole, Amersham) or [35S]methionine (100 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 Heathery 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 hydrolysate was analyzed by HPLC according to the method of Gehrke and Kuo (1990).

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