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The A1 · U72 Base Pair Conserved in Eukaryotic Initiator tRNAs Is Important Specifically for Binding to the Eukaryotic Translation Initiation Factor eIF2

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The formation of a specific ternary complex between eukaryotic initiation factor 2 (eIF2), the initiator methionyl-tRNA (Met-tRNA), and GTP is a critical step in translation initiation in the cytoplasmic protein-synthesizing system of eukaryotes. We show that the A1 · U72 base pair conserved at the end of the acceptor stem in eukaryotic and archaebacterial initiator methionine tRNAs plays an important role in this interaction. We changed the A1 · U72 base pair of the human initiator tRNA to G1 · C72 and expressed the wild-type and mutant tRNA genes in the yeast *Saccharomyces cerevisiae* by using constructs previously developed in our laboratory for expression of the human initiator tRNA gene in yeasts. We show that both the wild-type and mutant human initiator tRNAs are aminoacylated well in vivo. We have isolated the wild-type and mutant human initiator tRNAs in substantially pure form, free of the yeast initiator tRNA, and have analyzed their properties in vitro. The G1 · C72 mutation affects specifically the binding affinity of eIF2 for the initiator tRNA. It has no effect on the subsequent formation of 40S or 80S ribosome initiator Met-tRNA-AUG initiation complexes in vitro or on the puromycin reactivity of the Met-tRNA in the 80S initiation complex.

A special methionine tRNA is used to initiate protein synthesis in all organisms studied. Of the two classes of methionyl-tRNA (Met-tRNA), the initiator is used for the initiation of protein synthesis whereas the elongator is used for insertion of methionine into internal peptide bonds (27, 36). Eubacteria, mitochondria, and chloroplasts initiate protein synthesis by using formyl-Met-tRNA, whereas the cytoplasmic system of eukaryotes uses Met-tRNA (22, 45).

Because of their unique function, initiator tRNAs possess several properties which are distinct from those of elongator tRNAs. For eukaryotic initiator tRNAs, these include (i) the highly specific formation of a ternary complex between the initiator Met-tRNA, eukaryotic initiation factor 2 (eIF2), and GTP; (ii) the binding of the initiator Met-tRNA to the P site on the 80S ribosome; and (iii) exclusion of the initiator Met-tRNA from the ribosomal A site. In contrast, the elongator tRNAs form a ternary complex with the elongation factor EF-1 and GTP and then bind to the ribosomal A site.

The initiator tRNA also plays an important role in the formation of the 40S and 80S ribosomal initiation complexes (21, 33, 46). Once the eIF2 · GTP · Met-tRNA ternary complex is formed, it binds to the 40S ribosomal subunit. This new complex, consisting of the 40S ribosome, eIF2, GTP, and Met-tRNA, then binds to the capped 5′ end of mRNA in a reaction requiring several initiation factors. The 40S ribosome containing the bound eIF2 · GTP · Met-tRNA ternary complex then scans the mRNA until it reaches the AUG codon (28). Donahue and coworkers (8, 12) have shown that in *Saccharomyces cerevisiae*, the initiator Met-tRNA is directly involved in helping the scanning ribosome select the appropriate initiation codon on the mRNA through codon-anticodon base pairing. eIF2 plays an important role in start site selection by monitoring this base pairing. With the 40S ribosomal subunit correctly positioned on the AUG codon, eIF5 then interacts with the 40S initiation complex, GTP is hydrolyzed, and the resulting eIF2 · GDP is released from the 40S ribosome · mRNA · Met-tRNA complex (3, 7, 33). The 60S ribosomal subunit now joins this complex to form the 80S initiation complex in which the initiator tRNA is located on the ribosomal P site.

Along with their distinctive properties, initiator tRNAs also have several unique sequence and structural features that are not found in most elongator tRNAs (36). These include an A1 · U72 base pair at the end of the acceptor stem, (ii) three consecutive G · C base pairs (G29 · C41, G30 · C40, and G31 · C39) in the anticodon stem, and (iii) A54 and A60 in the TψC loop instead of the T54 and pyrimidine 60 found in virtually all elongator tRNAs (Fig. 1).

We are interested in understanding the role of these conserved features in specifying the distinctive properties of initiator tRNAs. In previous work, we have mutagenized each of the conserved features in human initiator tRNA to those found in human elongator methionine tRNA, expressed the mutant tRNAs in CV-1 monkey kidney cells, and analyzed the overall activities of the mutant tRNAs in initiation of protein synthesis in vitro in rabbit reticulocyte and wheat germ cell-free systems (13). These studies showed that the A1 · U72 base pair at the end of the acceptor stem and the G · C base pairs in the anticodon stem were important for the activity of the tRNA in initiation in vitro. In parallel studies with yeast cells in which all four of the initiator tRNA genes have been inactivated by disruption, von Pawel-Rammingen et al. evaluated the ability of plasmid-borne mutant initiator tRNA genes to rescue cell growth as a measure of the function of the tRNA in vivo (51). Their results showed that mutation of A1 · U72 to G1 · C72 abolished the function of the initiator tRNA in vivo.

The above studies indicated the importance of some of the

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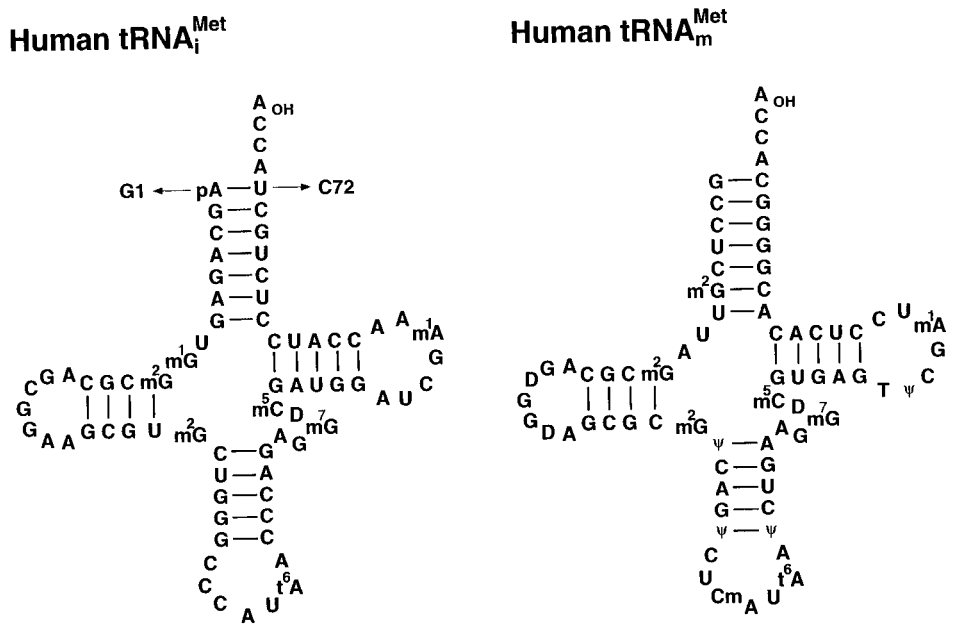

FIG. 1. Cloverleaf structures of human initiator and human elongator methionine tRNAs. The conserved first base pair, A1 · U72, of human initiator tRNA was changed to the G1 · C72 base pair found at the corresponding position of the human elongator tRNA. Arrows indicate the sequence changes made in the human initiator tRNA gene.

conserved features in the initiator tRNA for its function. However, understanding of their precise role requires a detailed analysis of the function of the mutant tRNAs at each of the steps, including studies of their binding to eIF2 and their role in the formation of the 40S and 80S initiation complexes. A limiting factor for such studies has been the amount of mutant initiator tRNAs that can be generated and separated from wild-type initiator tRNA. Previously, we described constructs for expression of human initiator tRNA genes in *S. cerevisiae* (17). By transforming yeast cells with a 2μm-based multicopy vector carrying these tRNA gene constructs and the *leu-2d* gene as a selectable marker (15), we have isolated large amounts of substantially pure wild-type (A1 · U72) and G1 · C72 mutant human initiator tRNAs free of any endogenous yeast initiator or elongator methionine tRNAs. This system has allowed us to study in detail the binding of wild-type and mutant initiator tRNA to eIF2 and its ability to form the 40S and 80S initiation complexes. We show that the G1 · C72 mutant initiator tRNA is impaired specifically in binding to eIF2 · GTP. The mutation weakens the affinity of eIF2 · GTP for the initiator tRNA by a factor of 10 to 17. However, once the ternary complex is formed, the G1 · C72 mutant tRNA works as well as the wild type in the formation of 40S and 80S initiation complexes and in the formation of methionyl-puromycin.

MATERIALS AND METHODS

General. Radiochemicals were obtained from NEN-DuPont or Amersham. Restriction enzymes were from Boehringer Mannheim or New England Biolabs. *Escherichia coli* S100 was used as a source of Met-tRNA synthetase (MetRS) for routine aminoacylation assays of column fractions (37). Purified *E. coli* MetRS provided by M. Dyson was used for large-scale aminoacylation of tRNAs used in functional studies. Purified *E. coli* tRNA nucleotidyltransferase added to the large-scale aminoacylation of baker's yeast elongator methionine tRNA was provided by M. Dyson. Samples of eIF2 purified from rabbit reticulocytes (11, 39) were kindly provided by N. K. Gupta, University of Nebraska, and A. J. Wahba, University of Mississippi Medical Center. The factor was also prepared by an adaptation of the procedure of Dholakia and Wahba (11) as described elsewhere (5). Homogeneous recombinant rat eIF5 was prepared as described previously (6). Previously purified yeast and rabbit liver initiator tRNAs were used as markers (43, 44). Yeast elongator methionine tRNA is a partially purified preparation obtained by BD-cellulose chromatography of total yeast tRNA.

Strains. *Escherichia coli* TG1 [K-12 Δ (*lac-proAB*) *supE thi* Δ(*hsdM-mcrB*)*5* (r_K^- m_K^- McrB$^-$)/F′ *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*] was used for mutagenesis. *E. coli* DH5α [*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* (ϕ80 d*lac*Δ (*lacZ*)*M15*] was used for routine plasmid construction and purification. *S. cerevisiae* SHY4 (α *ste-VC9 ura3-52 trp1-289 leu2-3 leu2-112 his3-Δ1*) was obtained from D. Botstein (2).

Oligonucleotides. The following oligonucleotides were used for mutagenesis of the A1 · U72 base pair in the human initiator tRNA gene to G1 · C72, 5′-CAA ATAGTAGGCAGAGTG-3′ for A1 to G1 and 5′-CCTCTGCCAGGTCCT-3′ for U72 to C72. Oligonucleotides 5′-AGCACGCTTCCGCTGCGCCACTCT-3′ and 5′-TGCGCGCTTCCACTGCGCCACGGC-3′, each 24 nucleotides long, were used as Northern (RNA) blot probes against nucleotides 4 to 28 of human and yeast initiator tRNA, respectively. The standard nucleotide numbering system for tRNAs based on the sequence of yeast phenylalanine tRNA is described elsewhere (48). Because the human and yeast initiator tRNAs both contain only 7 nucleotides in the dihydrouridine loop (18, 43) instead of the more common 8 nucleotides found in most tRNAs, the probe directed against nucleotides 4 to 28 is 24 nucleotides long instead of 25.

Plasmids and mutagenesis. Plasmid pScM carrying a 238-bp yeast arginine tRNA gene 5′-flanking sequence joined to the human initiator tRNA coding sequence in a 2μm vector has been described previously (17). An *Eco*RI-*Bam*HI fragment containing the human initiator tRNA gene was cloned into M13mp18 for mutagenesis. The mutagenized tRNA gene was excised from M13 replicative-form DNA as an *Eco*RI-*Bam*HI fragment and cloned into the *Bam*HI site of pMP78-1 by blunt-end ligation.

Yeast methods. Transformation was done by the spheroplast method (1, 42). Small-scale preparation of total tRNA and analytical RPC5 column chromatography of aminoacyl-tRNAs were done as described previously (17).

Isolation and electrophoresis of total RNA from *S. cerevisiae* under acidic conditions. The procedure is based on an earlier protocol for work with *E. coli* but is scaled up for use with *S. cerevisiae* (49). Cultures of *S. cerevisiae* (50 ml) were grown to late log phase. Cells were pelleted and resuspended in 4 ml of 0.3 M sodium acetate (pH 4.5)–10 mM EDTA. An equal volume of phenol was added, and tRNA was isolated by phenol extraction followed by ethanol precipitation from the aqueous layer. Details can be found in reference 16. The total yield of tRNA from 50 ml of yeast culture was ~0.5 to 0.8 A_{260} unit. A 1-μl aliquot of each tRNA sample was analyzed on a 1.2% agarose gel containing 5 μg of ethidium bromide per ml in 1× Tris-borate-EDTA (TBE) buffer. The remainder of the samples were quick-frozen on dry ice and stored at −70°C.

For Northern blot hybridization, 0.05 A_{260} unit of each tRNA sample was applied to the acid-urea gel. Controls showed that from 0.025 to 0.1 A_{260} unit could be loaded on these gels without any problem. The human initiator tRNA migrated more slowly than the *E. coli* initiator tRNA; therefore, the region of the

gel to a few centimeters above the xylene cyanol dye was cut out for transfer to Nytran membrane.

Northern blot hybridization. Northern blotting was carried out as described previously (17), except that prehybridization and hybridization were performed at 25°C. Oligonucleotide probes were 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Purification of human initiator tRNAs from *S. cerevisiae*. (i) Growth of cells. Medium (10 liters) for fed-batch fermentation of *S. cerevisiae* was the one used by O'Connor et al. (34). The fermentor was inoculated with a 1-liter culture of late-log-phase yeast cells grown in synthetic medium (SD + Ura, Trp, and His). Growth was carried out at 30°C at a pH of 5 to 6 (maintained by automatic NH_4OH feed-in). The sugar, amino acids, and vitamins were added at a rate corresponding to the growth rate of the cells as determined by withdrawal of samples at various times and measurements of A_{600} (38).

(ii) Isolation and purification of tRNA. tRNA was isolated and purified as described in reference 16.

Large scale aminoacylation of human initiator tRNA. Aminoacylations were carried out in 20 mM imidazole HCl (pH 7.5)–0.1 M EDTA–2 mM ATP–150 mM NH_4Cl–10 μg of bovine serum albumin (BSA) per ml–4 mM $MgCl_2$–25 μM L-methionine–1 μM [^{35}S]methionine. The reaction mixture (1 ml) contained 2 A_{260} units of wild-type or G1 · C72 human initiator tRNA or 10 A_{260} units of yeast elongator methionine tRNA fraction, 100 μl (1 mCi) of [^{35}S]methionine, and 0.03 U of MetRS (a gift from M. Dyson [1 U = 1 μmol of aminoacyl-tRNA formed in 5 min at 37°C]). For the yeast elongator methionine tRNA, 1 μl of purified tRNA-nucleotidyltransferase (a gift from M. Dyson) was added along with 0.2 mM CTP and $MgCl_2$ to 15 mM. The yeast elongator $tRNA^{Met}$ requires a higher Mg^{2+} concentration than does the initiator tRNA for charging by *E. coli* MetRS (37). After incubation at 37°C for 10 min, the reaction mixtures were extracted with 0.5 ml of H_2O-saturated phenol and then with H_2O-saturated ether. To the aqueous layer was added 0.1 volume of 3 M lithium acetate (pH 5.2) and 2.5 volumes of ethanol, and the mixture was left in dry ice for 15 min. The precipitated [^{35}S]Met-tRNA was collected by centrifugation at 10,000 × *g* for 20 min, resuspended in 100 μl of 10 mM sodium acetate (pH 4.5)–1 mM EDTA, and then separated from unreacted [^{35}S]methionine, ATP, and CTP by passage through a G25 Quick-Spin column (Boehringer). The purified [^{35}S]Met-tRNA was then stored at −80°C.

Assay for the formation of eIF2 · GTP · [^{35}S]Met-tRNA ternary complex. The buffer for assay of ternary complex formation by nitrocellulose filtration contained 24 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.4), 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 20 μg of acetylated BSA (New England Biolabs) per ml, 25 mM creatine phosphate, 5% glycerol, and 0.02% Nonidet P-40. For the binding experiments, the above buffer with 1 mM GTP, 0.03 U of creatine kinase per μl, and eIF2 were preincubated at 37°C for 5 min. Unless otherwise noted, *E. coli* total tRNA was used at ~10- to 20-fold excess over eIF2. [^{35}S]Met-tRNA was added, and the incubation was continued at 37°C for 4 min. After 1 ml of ice-cold 20 mM HEPES-KOH (pH 7.4)–100 mM KCl was added, the whole sample was applied to a prewet nitrocellulose filter (Millipore HA; pore size, 0.45 μm) on a Millipore vacuum manifold. The tubes were rinsed with 1 ml of buffer which was added to the filter, and the filters were washed twice with 1 ml of buffer. The filters were dried at 65°C for 30 min and then counted in Ecoscint fluid.

For the gel mobility shift assays, reaction mixtures were prepared and incubated as for the nitrocellulose filter-binding experiments. The samples were quick-chilled on ice, 1 μl of load dye was added, and the mixtures were applied to a 10% (30:0.8 acrylamide/bisacrylamide ratio) native acrylamide gel in 0.5× TBE (prechilled at 4°C and prerun at 10 V/cm). The samples were electrophoresed at 20 V/cm for 15 min and then at 15 V/cm until the bromophenol blue dye had migrated about halfway through the gel. The gel was fixed, dried on Whatman 3MM filter paper, and exposed to film.

Assay for transfer of [^{35}S]Met-tRNA from the eIF2 · GTP · [^{35}S]Met-tRNA ternary complex to 40S and 80S initiation complexes. The assay of 40S ribosomal initiation complex formation was carried out in two stages and under two different conditions (see below). The first stage consisted of forming the eIF2 · GTP · Met-tRNA ternary complex with various amounts of either the wild-type or the mutant [^{35}S]Met-tRNA. Reaction mixtures for ternary complex formation (50 μl) contained 20 mM Tris HCl (pH 7.5), 100 mM KCl, 5 mM β-mercaptoethanol, 0.3 mM GTP, 0.5 A_{260} unit of *E. coli* tRNA, 6 pmol of purified rabbit reticulocyte eIF-2, and various amounts of [^{35}S]Met-tRNA. Following incubation at 37°C for 4 min, the reaction mixtures were filtered through nitrocellulose (pore size 0.45 μm) membrane filters. The amount of eIF2 · GTP · [^{35}S]Met-tRNA ternary complex formed was measured by counting radioactivity retained on the filter as above.

For assaying the transfer of [^{35}S]Met-tRNA to 40S ribosomal subunits, after incubation of reaction mixtures at 37°C for 4 min as above to form the eIF2 · GTP · Met-tRNA ternary complex, $MgCl_2$ was added to the reaction mixtures to 5 mM, and then 40S ribosomal subunits (0.4 A_{260} unit) and AUG codon (0.05 A_{260} unit) were added. After a second incubation at 37°C for 4 min, the reaction mixtures were chilled on ice and then layered onto 5 ml of 7.5 to 30% (wt/vol) sucrose gradients containing 100 mM KCl, 20 mM Tris HCl (pH 7.5), 5 mM β-mercaptoethanol, and 5 mM $MgCl_2$. Following centrifugation for 105 min at 48,000 rpm in Beckman SW 50.1 rotors, fractions of 0.3 ml were collected from the bottom of each tube and counted for radioactivity. Radioactivity sedimenting near the bottom of the gradient at a position corresponding to 40S ribosomal subunits is indicative of the 40S initiation complex, while that at the top represents unbound [^{35}S]Met-tRNA.

To determine the amounts of 80S initiation complex formed, the same reactions were carried out as described for the assay for 40S initiation complex formation, except that after incubation to form the 40S initiation complex, 60S ribosomal subunits (0.5 A_{260} unit) and purified recombinant eIF5 (200 ng) were added to the reaction mixtures and this was followed by a third incubation at 37°C for 4 min. After sedimentation through sucrose gradients, ^{35}S radioactivity in the fractions was determined to quantitate the formation of 80S initiation complex (3).

Binding of [^{35}S]Met-tRNA to 40S ribosomal subunits was also carried out at 1 mM Mg^{2+} in the presence of eIF3 and eIF1A (formerly eIF4C). Conditions of incubation with the 40S ribosome and AUG were as above, except that the Mg^{2+} concentration was 1 mM and the incubation mixture contained 500 ng of eIF1A and 2 μg of eIF3. Sedimentation of the 40S initiation complex was carried out in sucrose gradients containing 1 mM $MgCl_2$.

Assay for puromycin reactivity of [^{35}S]Met-tRNAs in the 80S initiation complex. The puromycin activity assay was carried out as described previously (3) by the method of Merrick (32).

RESULTS

Expression of the wild-type and the G1 · C72 mutant human initiator tRNA genes in yeast cells. We showed previously that although the human initiator tRNA gene, in the context of its own 5′- and 3′-flanking sequences, is not transcribed in *S. cerevisiae* (14), switching the human 5′-flanking sequence with that of the yeast arginine tRNA gene resulted in expression of the human initiator tRNA (17). The plasmid used for this purpose, pScM, contained the human initiator tRNA gene in a 2μm vector and produced the human initiator tRNA at approximately half the level of the yeast initiator tRNA in *S. cerevisiae.*

To obtain a higher level of expression of the human initiator tRNA, we have, in the current work, cloned the human initiator tRNA gene into a very high copy number vector, pMP78-1 (15). This plasmid, which has a mutant version of the *LEU2* gene designated *leu-2d* as a selectable marker, must be maintained at a very high copy number (~4- to 5-fold higher than normal 2μm vectors) in order to confer Leu^+ phenotype to a *leu2* yeast strain. The wild-type or mutant human initiator tRNA gene fragment, together with the yeast arginine 5′ flank, was excised from pScM and inserted into pMP78-1. The resulting pMP78-1 vectors were transformed into the yeast SHY4 strain, which is *leu2.*

Figure 2 shows the level of expression of the wild-type and the G1 · C72 mutant human initiator tRNAs in *S. cerevisiae*. A small amount of tRNA isolated from SHY4 transformants was aminoacylated in vitro with [^{35}S]methionine and the [^{35}S]Met-tRNAs were chromatographed on an analytical RPC5 column. Under the conditions used, the human initiator tRNA elutes first and is quite well separated from the yeast initiator and the elongator methionine tRNAs. Quantitation of ^{35}S radioactivity in the peaks indicates that the wild-type human initiator tRNA is overexpressed fourfold relative to the endogenous yeast initiator tRNA (Fig. 2A). The G1 · C72 mutant tRNA yields a similar ^{35}S radioactivity profile (Fig. 2B) and is overproduced to approximately twofold over the yeast initiator tRNA.

Aminoacylation of wild-type and mutant human initiator tRNAs in *S. cerevisiae*. tRNA was isolated under acidic conditions from SHY4 transformants expressing the human initiator tRNAs and then separated by electrophoresis on an acid urea–6.5% polyacrylamide gel (49). The two forms of the human initiator tRNA, uncharged tRNA and aminoacyl-tRNA, were detected by Northern blot hybridization with a probe directed against nucleotides 4 to 28 of the human initiator tRNA. Under the conditions of gel electrophoresis, the aminoacyl-tRNA migrates more slowly than uncharged tRNA. Results in Fig. 3 show that the wild-type and the G1 · C72 mutant human ini-

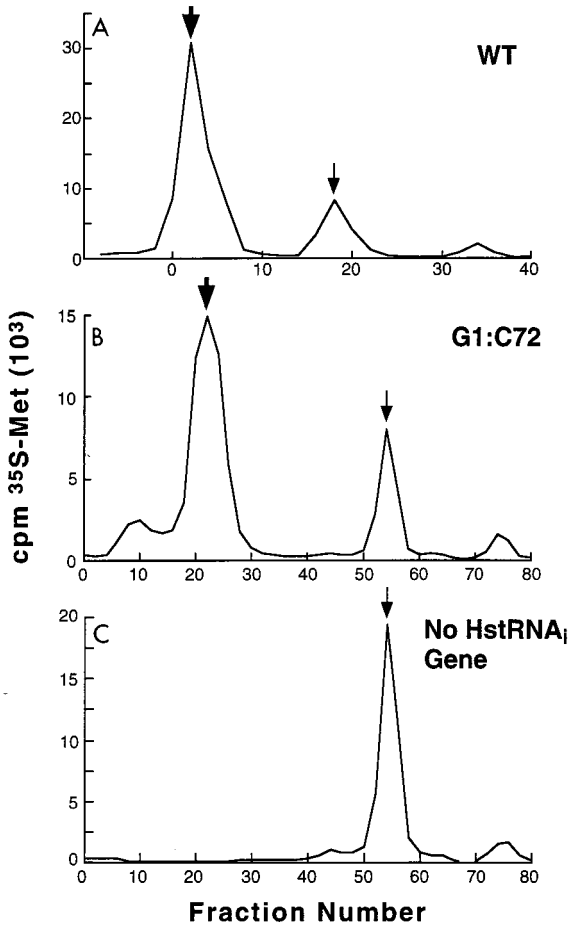

FIG. 2. Analysis by RPC5 column chromatography of human initiator tRNAs made in the yeast SHY4 transformants. Total tRNA was isolated from SHY4 yeast strains carrying the human initiator tRNA genes on pMP78-1. The tRNA was aminoacylated with [^{35}S]Met and chromatographed on an RPC5 column (wild type [wt] [A], G1 · C72 mutant [B], no human tRNA gene [C]). The gradient used for elution of the wild-type tRNA was 0.4 M to 0.6 M NaCl, whereas for the other samples a 0.375 M to 0.525 M NaCl gradient was used. The large arrow indicates the peak corresponding to human initiator Met-tRNA, while the small arrow indicates the position of yeast initiator Met-tRNA eluted from the column. The smaller third peak corresponds to yeast elongator Met-tRNA. The wild-type and G1 · C72 mutant human initiator genes both produce tRNA at levels much higher than does the endogenous yeast initiator.

tiator tRNAs are mostly aminoacylated in vivo (lanes −). Only a small amount of uncharged tRNA, the faster-migrating species, is visible in both cases. Both aminoacyl-tRNAs can be deacylated to tRNA by treatment with base (Fig. 3, lanes +).

Large-scale isolation and purification of wild-type and mutant human initiator tRNAs. Cultures (10 liters) of the SHY4 transformed with pMP78-1 plasmid carrying the wild-type or G1 · C72 mutant human initiator tRNA genes were grown in a fermentor containing a synthetic medium. The yields of cells were 720 and 1,420 g (wet weight) for cells expressing the wild-type and mutant human initiator tRNAs, respectively. The yields of total tRNA were 16,000 and 33,000 A_{260} units, respectively.

The steps for purification of the human initiator tRNAs produced in *S. cerevisiae* were similar to those used previously for purification of yeast initiator tRNA (37). These include chromatography of total tRNA on DEAE-Sephadex A-50 followed by chromatography on an RPC5 column. Electrophoresis on a 15% polyacrylamide gel was used as the last step in the purification. On the basis of methionine acceptor activity, the tRNAs were judged to be 65 to 85% pure (1,000 to 1,400 pmol of methionine accepted per A_{260} unit). Most importantly for the work described here, the wild-type and G1 · C72 mutant human initiator tRNAs were totally free of any contamination by yeast initiator tRNA (see below).

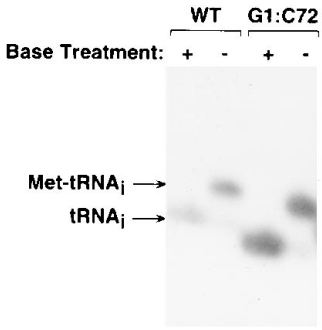

FIG. 3. Acid-urea gel analysis of the human initiator tRNA expressed in *S. cerevisiae*. Total tRNA was isolated under acidic conditions from L2718 carrying the human initiator tRNA genes on pScM (17). The tRNAs were separated by electrophoresis on a 6% polyacrylamide gel at pH 4.5, transferred to Nytran membrane, and subjected to Northern blot analysis. The positions of charged (Met-tRNA$_i$) and uncharged (tRNA$_i$) human initiator tRNA are shown. The wild-type (WT) and G1 · C72 mutant initiator tRNAs appear to be present in *S. cerevisiae* mostly as aminoacyl-tRNA. Base treatment generates uncharged tRNAs.

Figure 4 shows a Northern blot analysis of the G1 · C72 mutant human initiator tRNA at various stages of purification, using a probe complementary to nucleotides 4 to 28 of yeast initiator tRNA. This probe, which has 18 of 20 contiguous nucleotides complementary to nucleotides 7 to 27 of the human initiator tRNA, cross-hybridizes to the human initiator tRNA (lanes 1 to 4). The locations of the yeast and human initiator tRNAs were determined by the use of marker tRNAs. On the 40-cm-long gels used for this purpose, the yeast initiator tRNA is separated clearly from the human initiator tRNA. It can be seen that while the total tRNA isolated from cells expressing the G1 · C72 mutant human initiator tRNA contains, as expected, the endogenous yeast initiator tRNA

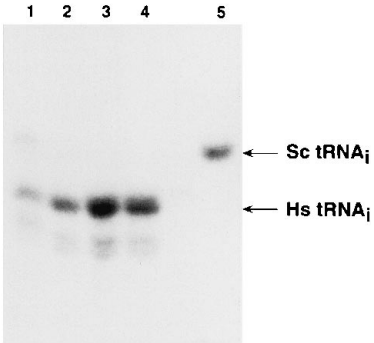

FIG. 4. Northern blot analysis of G1 · C72 mutant human initiator tRNA at different stages of purification. tRNAs were separated on a 40-cm-long 15% native polyacrylamide gel and analyzed by Northern blotting with a probe directed against nucleotides 4 to 28 of yeast initiator tRNA. The identity of the yeast and human tRNA bands was confirmed by UV shadowing to visualize purified human and yeast initiator tRNA samples. Lanes: 1, total tRNA; 2 and 3, peak 3 from the DEAE-Sephadex column; 4, RPC5-purified fraction; 5, total tRNA from strain SHY4 not carrying any human initiator tRNA gene. Sc, *S. cerevisiae*; Hs, human.

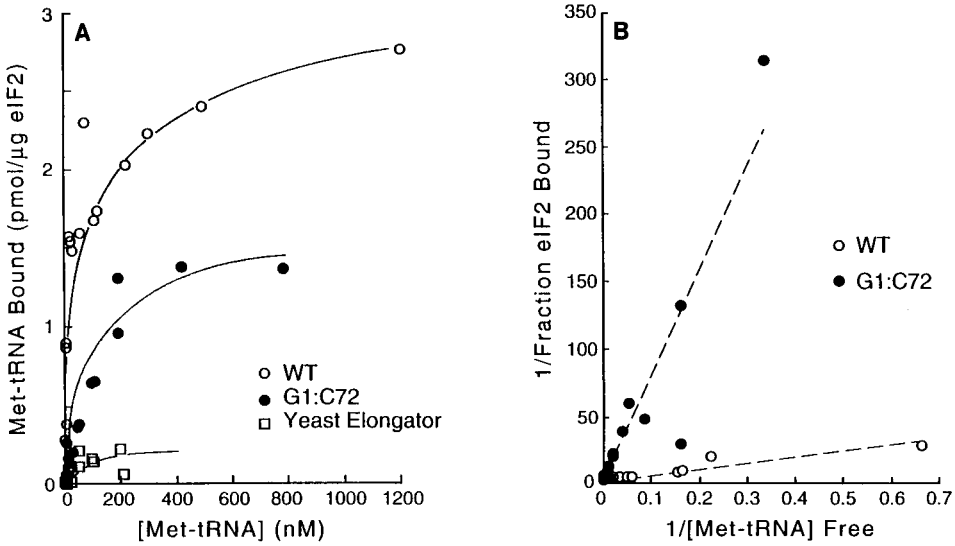

FIG. 5. Binding of human initiator Met-tRNA to eIF2. The amount of [^{35}S]Met-tRNA in ternary complexes was measured by nitrocellulose filter binding. eIF2 was present at 1.4 μg/ml (11 nM), and *E. coli* tRNA was present at 220 nM. All points are the mean of duplicate experiments with the blank (no eIF2 added) value subtracted. Lines shown on the double-reciprocal plot (B) are calculated for the best-fit values of the dissociation constant. Dissociation constants obtained were 4.7×10^{-8} M and 7.9×10^{-7} M for wild-type (WT) and G1 · C72 human initiator Met-tRNA, respectively.

(lane 1), the DEAE-Sephadex-purified fractions (lanes 2 and 3) and the RPC5-purified fractions (lane 4) are free of any yeast initiator tRNA.

The same fractions as above were also subjected to Northern blot hybridization with a probe complementary to nucleotides 4 to 28 of the human initiator tRNA (data not shown). The hybridization pattern obtained is identical to the one in Fig. 4, except that the band corresponding to the yeast initiator tRNA in lanes 1 and 5 was absent, presumably because the human initiator tRNA probe does not cross-hybridize to the yeast initiator tRNA under the conditions used.

The identities of the weakly hybridizing bands in lanes 1 to 4, which migrate faster than the human initiator tRNA and which hybridize to either of the probes used, are not known. They could be fragments of the human initiator tRNA or a small fraction of the tRNA which is lacking one or more of the modified bases.

Comparison of binding affinities of eIF2 for the wild-type and G1 · C72 mutant initiator tRNAs. Formation of the eIF2 · GTP · initiator Met-tRNA ternary complex was measured by retention of [^{35}S]Met-tRNAs on nitrocellulose filters. In initial experiments, a considerable amount of GTP-independent binding of the initiator Met-tRNA to eIF2 was found for all preparations of eIF2. Purified eIF2 is known to bind tRNAs, rRNAs, and mRNAs nonspecifically in the absence of GTP and other components of initiation (6, 23, 47). The GTP-independent nonspecific binding of initiator Met-tRNA to eIF2 was abolished by addition of either total *E. coli* tRNA or purified yeast phenylalanine tRNA as competitors. At an eIF2 concentration of 11 nM and a competitor tRNA concentration of 1.1 μM, the ratio of GTP-dependent binding to GTP-independent binding was greater than 200-fold (data not shown).

Figure 5A shows the effect of increasing the concentration of Met-tRNA on the formation of the eIF2 · GTP · Met-tRNA ternary complex. The tRNAs used were the wild-type and the G1 · C72 mutant human initiator tRNAs and yeast elongator methionine tRNA as a negative control. The G1 · C72 mutant initiator tRNA binds poorly to eIF2 compared with the binding of the wild-type initiator tRNA. The yeast elongator methionine tRNA binds very poorly to eIF2, if at all. From the double-reciprocal plot (52) shown in Fig. 5B, the apparent dissociation constants (K_D) for eIF2 binding to the wild-type and G1 · C72 mutant initiator tRNAs are 47 ± 4.6 and 790 ± 74 nM, respectively, a 17-fold difference in binding affinity. Similar experiments carried out with another preparation of eIF2 yielded K_D values of 28 ± 2.0 and 270 ± 15 nM, respectively, for the wild-type and mutant initiator tRNAs (data not shown), yielding a ~10-fold difference in binding affinity. The 10- to 17-fold difference in K_D corresponds to a difference of 1.4 to 1.7 kcal/mol (5.9 to 7.1 kJ/mol) in the free energy of binding. The K_D values of 28 and 47 nM for eIF2 binding to wild-type human initiator tRNA determined in this work are in good agreement with previous estimates of 50 nM for yeast initiator tRNA (52) and 20 nM for rabbit liver initiator tRNA binding to rabbit reticulocyte eIF2 (26, 31). It should be noted that the sequences of vertebrate initiator tRNAs, including those of rabbit liver and human initiator tRNAs, are identical (18, 19, 44).

Gel retardation assays for ternary complex formation. The binding of a nucleic acid to a protein or to another nucleic acid can be monitored by retardation of the nucleic acid on native polyacrylamide gels run at low ionic strength (reviewed in reference 29). Figure 6 shows that this assay can also be used to monitor the formation of the eIF2 · GTP · Met-tRNA ternary complex. As in the case of nitrocellulose filter binding, GTP dependence of eIF2 binding to Met-tRNA required the addition of total *E. coli* tRNA as a competitor (data not shown). The gel retardation assay shows the same trend for binding of eIF2 to the three Met-tRNAs as was quantitated by nitrocellulose filter binding assays. At a fixed concentration of Met-tRNA, the wild-type human initiator tRNA shows strong binding to eIF2, whereas the G1 · C72 mutant and the yeast elongator Met-tRNA display progressively weaker binding. The gel retardation assays show more than one shifted band; the reason for this is not known.

Activity of wild-type and G1 · C72 mutant human initiator tRNAs in formation of 40S and 80S ribosome · AUG · Met-tRNA initiation complexes. In further functional studies, we

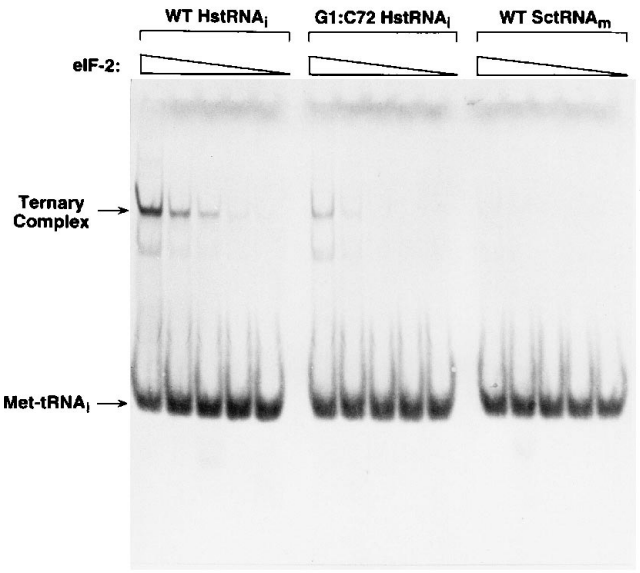

FIG. 6. Gel mobility shift of human initiator [^{35}S]Met-tRNA by eIF2. [^{35}S]Met-tRNAs were present at 50 nM, and *E. coli* total tRNA was present at 440 nM. The eIF2 concentration was varied in this experiment instead of the [^{35}S]Met-tRNA concentration. Within each set of lanes, eIF2 concentrations are 120, 40, 13, 4.4, and 1.5 nM.

have analyzed the effect of the G1 · C72 mutation on the transfer of Met-tRNA from the eIF2 · GTP · Met-tRNA ternary complex to 40S · AUG · eIF2 · GTP · Met-tRNA and 80S · AUG · Met-tRNA initiation complexes. The ternary complex was incubated with 40S ribosomes and AUG for the formation of the 40S initiation complex and subsequently with 60S ribosome and eIF5 for the formation of the 80S initiation complex. Reactions were monitored by sucrose gradient centrifugation. Figure 7 shows the profile obtained with the wild-type and G1 · C72 mutant initiator tRNAs at a Met-tRNA concentration of 64 nM. The amount of G1 · C72 mutant initiator tRNA in the 40S and 80S initiation complexes is much smaller than that of the wild-type initiator tRNA. However, this is solely due to decreased efficiency of the mutant tRNA to form the eIF2 · GTP · Met-tRNA ternary complex. Both the wild-type and mutant Met-tRNAs present in the ternary complex can essentially quantitatively form the 40S and the 80S ribosome · AUG · Met-tRNA initiation complexes.

Table 1 shows the effect of changing the Met-tRNA concentration on formation of the ternary complex and the 40S and 80S initiation complexes. The percentage of Met-tRNA transferred from the ternary complex to both 40S and 80S initiation complexes is about the same (~80 to 90%) for both the wild-type and G1 · C72 mutant human initiator tRNAs. Thus, at all the concentrations of Met-tRNA tested, while the amount of ternary complex formed with the G1 · C72 mutant initiator tRNA is always smaller than that formed with the wild-type initiator tRNA, the mutation in the first base pair does not affect the subsequent transfer of the bound [^{35}S]Met-tRNA to 40S or 80S initiation complexes. Also, for both the wild-type and mutant initiator tRNAs, the [^{35}S]Met-tRNA bound to the 80S initiation complex is puromycin reactive (Table 2). These results show that the effect of the G1 · C72 mutation is specifically on the binding of the initiator Met-tRNA to eIF2 · GTP.

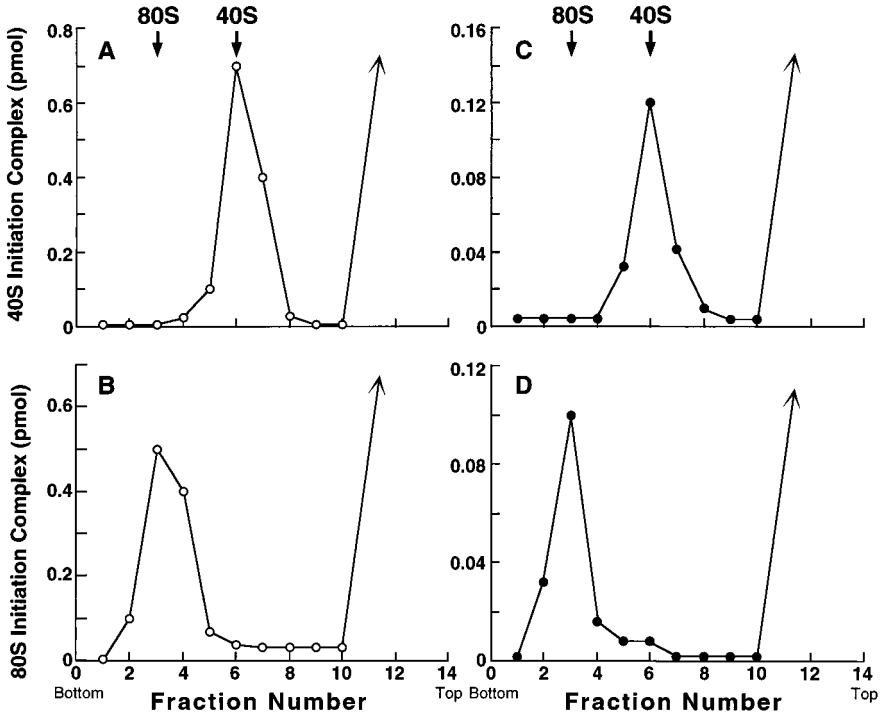

FIG. 7. Transfer of [^{35}S]Met-tRNA to 40S and 80S initiation complexes as analyzed by sucrose density gradient centrifugation. Reaction mixtures were prepared and incubated as described in Materials and Methods. (A and B) Preformed wild-type eIF2 · GTP · Met-tRNA ternary complex (1.6 pmol) was used to form the ribosomal initiation complexes; (C and D) 0.3 pmol of mutant eIF2 · GTP · Met-tRNA ternary complex formed with the G1 · C72 mutant tRNA was used. Following centrifugation through sucrose gradients for 1 h 45 min at 48,000 rpm in SW 50.1 rotors to separate 80S, 40S, and ternary complexes, fractions (0.3 ml) were collected from the bottom of each tube and counted for radioactivity. (A and C) Incubation for 40S initiation complex formation; (B and D) incubation for 80S initiation complex formation. ○, wild-type [^{35}S]Met-tRNA; ●, G1 · C72 mutant [^{35}S]Met-tRNA.

TABLE 1. Effect of varying Met-tRNA concentrations on the formation of ternary complex and the 40S and 80S initiation complexes[a]

tRNA	Amt of Met-tRNA used (pmol)	Amt of ternary complex formed (pmol)	Amt of 40S initiation complex (pmol)[b]	Amt of 80S initiation complex (pmol)
Wild-type initiator	0.8	0.3	ND	ND
	1.6	0.6	0.4	ND
	3.2	1.2	1.0	0.9
	6.4	1.5	1.3	1.2
	8.0	1.6	1.4 (1.5)	1.3
	64	2.1	ND	ND
G1 · C72 mutant	0.8	0.06	ND	ND
	1.6	0.07	0.05	0.04
	3.2	0.17	0.15	0.10
	8.0	0.30	0.25 (0.28)	0.20
	25.0	0.48	ND	ND
	50.0	1.03	0.90	0.80
	100.0	0.90	ND	ND
Yeast elongator methionine	1.6	0.026	ND	ND
	3.2	0.048	0.03	0.02
	6.4	0.062	0.04	0.03
	8.0	0.075	0.04 (0.03)	0.03
	64	0.180	ND	ND

[a] The incubation mixture (50 μl) contained ~6 pmol of eIF2.
[b] Numbers in parentheses are the results of 40S initiation complex formed at 1 mM $MgCl_2$ and in the presence of eIF1A (formerly eIF4C) and eIF3.
[c] ND, not determined.

We have also measured 40S initiation complex formation in the presence of eIF3 and eIF1A (formerly eIF4C) at 1 mM Mg^{2+}. Under these conditions also once the eIF2 · GTP· Met-tRNA ternary complex is formed, subsequent binding of this complex to the 40S subunit containing bound AUG is the same for the wild-type and the G1 · C72 mutant initiator tRNAs (Table 1).

DISCUSSION

Role of the A1 · U72 base pair in function of human initiator tRNA. A clear result of this work is that the A1 · U72 base pair conserved at the end of the acceptor stem in virtually all eukaryotic cytoplasmic initiator tRNAs plays an important role in the formation of the eIF2 · GTP · Met-tRNA ternary complex, the first step in the initiation of protein synthesis in the cytoplasm of eukaryotes. Mutation of the A1 · U72 base pair to G1 · C72 reduces the binding affinity of eIF2 for the mutant initiator tRNA by a factor of 10 to 17 compared with that for the wild-type initiator tRNA. This mutation is therefore likely to reduce substantially the steady-state levels of the eIF2 · GTP · Met-tRNA ternary complex in vivo. Interestingly, this mutation has no effect on the subsequent formation of 40S and 80S initiation complexes in reaction mixtures containing the initiation factors eIF1A, eIF3 and eIF5 and on puromycin reactivity of the Met-tRNA in the 80S initiation complex. Therefore, the difference in binding affinity alone could account for the substantially reduced activity of the G1 · C72 mutant tRNA in initiation of protein synthesis in rabbit reticulocyte and wheat germ cell extracts (13) and for the very poor function of the corresponding mutant yeast initiator tRNA in *S. cerevisiae* (51). Dever et al. have recently shown that in *S. cerevisiae*, the in vivo concentration of eIF2 · GTP · Met-tRNA ternary complex is the critical factor which determines the site of ribosome reinitiation within the multiple short open reading frames of *GCN4* mRNA and thereby translational regulation of *GCN4* mRNA (10). This finding further highlights the important role of the A1 · U72 base pair not only in the overall activity of the tRNA in initiation but also in the translational regulation of mRNAs such as the GCN4 mRNA.

TABLE 2. Transfer of methionine from Met-tRNA in 80S initiation complexes to puromycin

tRNA	Amt of Met-tRNA used (pmol)	Amt of ternary complex formed (pmol)	Amt of Met-puromycin formed (pmol)
Wild-type initiator	1.6	0.48	0.45
	3.2	0.98	0.92
G1 · C72 mutant	3.2	0.14	0.13
	8.0	0.30	0.25
Yeast elongator methionine	3.2	0.04	0.02
	8.0	0.08	0.04

The important role of the A1 · U72 base pair in eIF2 binding explains the near universal conservation of this base pair in eukaryotic cytoplasmic initiator tRNAs and its absence in any eukaryotic elongator tRNA (36, 48). The only known exception to the universal conservation of the A1 · U72 base pair is the initiator tRNA from *Schizosaccharomyces pombe*, which has a ψ1 · A72 base pair (25). Assuming that the *S. pombe* eIF2 is similar to other eukaryotic eIF2s, some structural feature common to A1 · U72 and ψ1 · A72 base pairs may be part of an important determinant on the initiator tRNA for eIF2 · GTP. A mutant of *S. cerevisiae* initiator tRNA in which the A1 · U72 base pair is changed to U1 · A72 is also reported to function in vivo, at least in allowing the growth of strains dependent exclusively on the initiator tRNA expressed from a high-copy-number vector (4). Whether the U1 in this tRNA is modified to ψ1 as in the *S. pombe* initiator tRNA is, however, not known.

The A1 · U72 base pair is also conserved in all the archaebacterial initiator tRNAs sequenced so far (20, 48). Combined with the fact that archaebacteria, like the eukaryotic cytoplasmic systems, also initiate protein synthesis by using Met-tRNA, this conservation raises the possibility that the A1 · U72 base

pair plays a role in binding to initiation factor in archaebacteria. Whether archaebacteria contain an initiation factor homologous to the three-subunit eIF2 of the eukaryotic cytoplasm is, however, not known (24). Also, while the A1 · U72 base pair is not generally found among archaebacterial elongator tRNAs, the glutamine tRNA of halobacteria is an exception in having an A1 · U72 base pair (20). Thus, if the A1 · U72 base pair in archaebacterial initiator tRNAs plays a similar role in binding to the archaebacterial initiation factor, there must be other important determinants in the tRNA for the initiation factor. One of these could be the amino acid methionine that is attached to the tRNA (see below).

Extensive work on the structure and function of *E. coli* initiator tRNA has established the critical role of nucleotides at positions 1 and 72 in this tRNA also (reviewed in reference 35). Nucleotides 1 and 72 in eubacteria are unique in that they cannot form a Watson-Crick base pair. The results described in this work with the human initiator tRNA and their possible extension to archaebacterial initiator tRNAs (see above) suggest that nucleotides 1 and 72 of initiator tRNA could play an important role in the function of initiator tRNAs from all organisms.

The G1 · C72 mutation does not reduce the eIF2-binding affinity of the tRNA down to the level seen with yeast elongator Met-tRNA. This is probably because eIF2 also interacts with some other features in the initiator tRNA besides the A1 · U72 base pair.

The amino acid methionine attached to the initiator tRNA is likely to be another element involved in recognition of initiator Met-tRNA by eIF2 · GTP. Wagner et al. have shown that yeast initiator tRNA aminoacylated with isoleucine binds very poorly to eIF2 (a difference in K_D of over 400-fold) compared with the same tRNA aminoacylated with methionine (52). Similarly, Drabkin and RajBhandary (14a) have shown that an anticodon sequence mutant of human initiator tRNA, which is aminoacylated with glutamine, is unable to initiate protein synthesis in vitro or in vivo by using the complementary sequence UAG as an initiation codon. The corresponding mutant of *E. coli* initiator tRNA initiates protein synthesis in *E. coli* with UAG (50).

***S. cerevisiae* as a system for overproduction and subsequent purification of wild-type and mutant human initiator tRNA.** We have shown that plasmid pMP78-1 can be used to achieve high-level expression of human initiator tRNA in *S. cerevisiae*. With the use of fermentors for the growth of yeast cells, this system can now be used, even in selective medium, for the large-scale isolation of many mutant human initiator tRNAs. This, combined with the methods used here for purification and separation of the mutant human initiator tRNAs from the endogenous yeast initiator and elongator methionine tRNAs, should greatly facilitate the detailed biochemical analysis of mutant human initiator tRNAs.

Both wild-type and G1 · C72 mutant human initiator tRNAs expressed in *S. cerevisiae* were aminoacylated by yeast MetRS in vivo. The aminoacylation of the wild-type initiator tRNA was expected from our previous work showing that the human initiator tRNA could partially rescue the slow-growth phenotype of *S. cerevisiae* L2718 caused by limiting amounts of the endogenous initiator tRNA (17). The aminoacylation of the G1 · C72 mutant human initiator tRNA is also expected because it has the CAU anticodon, a major identity element for MetRS recognition of tRNAs (9, 30, 40, 41). Nevertheless, the use of acid-urea gels for analysis of aminoacylation in vivo allows a quantitative measure of charging of wild-type or mutant tRNAs which are overproduced; our results (Fig. 3) show that the tRNAs that we have generated are essentially fully charged.

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