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Mutational Analysis of Conserved Positions Potentially Important for Initiator tRNA Function in *Saccharomyces cerevisiae*

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The conserved positions of the eukaryotic cytoplasmic initiator tRNA have been suggested to be important for the initiation of protein synthesis. However, the role of these positions is not known. We describe in this report a functional analysis of the yeast initiator methionine tRNA ($tRNA_i^{Met}$), using a novel in vivo assay system which is not dependent on suppressor tRNAs. Strains of *Saccharomyces cerevisiae* with null alleles of the four initiator methionine tRNA (*IMT*) genes were constructed. Consequently, growth of these strains was dependent on $tRNA_i^{Met}$ encoded from a plasmid-derived gene. We used these strains to investigate the significance of the conserved nucleosides of yeast $tRNA_i^{Met}$ in vivo. Nucleotide substitutions corresponding to the nucleosides of the yeast elongator methionine tRNA ($tRNA_M^{Met}$) have been made at all conserved positions to identify the positions that are important for $tRNA_i^{Met}$ to function in the initiation process. Surprisingly, nucleoside changes in base pairs 3-70, 12-23, 31-39, and 29-41, as well as expanding loop I by inserting an A at position 17 (A_{17}) had no effect on the tester strain. Nucleotide substitutions in positions 54 and 60 to cytidines and guanosines (C_{54}, G_{54}, C_{60}, and G_{60}) did not prevent cell growth. In contrast, the double mutation U/r$T_{54}C_{60}$ blocked cell growth, and changing the A-U base pair 1-72 to a G-C base pair was deleterious to the cell, although these tRNAs were synthesized and accepted methionine in vitro. From our data, we suggest that an A-U base pair in position 1-72 is important for $tRNA_i^{Met}$ function, that the hypothetical requirement for adenosines at positions 54 and 60 is invalid, and that a U/rT at position 54 is an antideterminant distinguishing an elongator from an initiator tRNA in the initiation of translation.

In the translational decoding of mRNA, codons are read by a unique set of tRNA species. Each isoacceptor is usually encoded by more than one copy of the gene. To study the function of a tRNA in protein synthesis, a common strategy is to generate tRNA mutations. To date, phenotypic changes in vivo have been scored either by different types of suppressor tRNAs (nonsense, missense, and frameshift, i.e., a tRNA with an already existing mutation) or by an in vivo system in which the rate of aminoacyl-tRNA selection is measured in competition with a natural $tRNA_2^{Leu}$ frameshifter (14, 19, 39). The former strategy has some limitations. First, the anticodon is a major identity element for some tRNA synthetases (41, 49), including the methionyl-tRNA synthetase from *Escherichia coli* (50). Therefore, the translational efficiency of the suppressor tRNA cannot be distinguished from its capacity to be aminoacylated. Second, in the translational process, a stop codon is a signal for the release factors to stop translation. Thus, the translational efficiency of the suppressor tRNA will be masked, as the tRNA must compete with release factors for the stop codon. In this report, an in vivo system which overcomes these obstacles by being dependent on one single *IMT* gene (mutated or wild type), located on either low- or high-copy-number plasmids, is described.

The $tRNA_i^{Met}$ plays a pivotal role by participating only in the initiation of protein synthesis, not in the elongation process (28, 55). Several unique features distinguish initiator tRNAs from elongator tRNAs (33, 56). Four conserved G-C pairs are found at identical positions in the initiator methionine tRNAs (9). Eukaryotic initiator tRNAs have in position 54 an A instead of a T; at position 60 an A is found instead of a pyrimidine, and an A-U base pair is present in position 1-72 in the acceptor stem (9, 33). Higher-order eukaryotes have a C in position 33 instead of U, which was thought to be invariable (33). Furthermore, loop I of eukaryotic initiator tRNAs consists of seven nucleotides, in contrast to the eight- or nine-membered loop of elongator tRNAs (9), and, finally, initiators have a different conformation of the anticodon region compared with elongators (48, 61). The importance of some of these features has been partly investigated; loop IV and the presence of a 2′-ribosylated purine in position 64 of yeast $tRNA_i^{Met}$ have been suggested by in vitro experiments to exclude the initiator tRNA from participating in translational elongation (31, 59). To understand the significance of the conserved structures of the $tRNA_i^{Met}$, we have undertaken a mutational analysis of the conserved positions of the *Saccharomyces cerevisiae* initiator methionine tRNA gene. Nucleotide substitutions at these positions which corresponded to the same positions of the elongator methionine tRNA were made, and their importance was investigated in vivo.

MATERIALS AND METHODS

Designations and abbreviations. *IMT* and *imt* designate the genotypes of the wild-type and mutant alleles of the initiator methionine tRNA gene. Materials are abbreviated as follows: 5-FOA, the orotidine 5′-phosphate analog 5-fluoroorotic acid, used in the selections for *ura3* (6); SDS, sodium dodecyl sulfate; SSC, standard saline citrate (0.15 M NaCl plus 0.015 M sodium citrate); BSA, bovine serum albumin. Ribosylthymine and pseudouridine are abbreviated rT and ψ, respectively. Other abbreviations are as follows: $tRNA_i^{Met}$, initiator methionine tRNA; $tRNA_M^{Met}$, elongator methionine tRNA; f-met-$tRNA_f^{Met}$, formylated methionine initiator tRNA; IF-2, initiation factor 2; eIF-2, eukaryotic initiation

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TABLE 1. Strains used

Strain	Genotype	Source or reference
S. cerevisiae		
ASB65-1A	*MATα trp1Δ1 ura3-52 leu2-3 leu2-112 IMT1 imt2::TRP1 IMT3 IMT4*	This work
ASB66-6D	*MATα trp1Δ1 ura3-52 leu2-3 leu2-112 IMT1 IMT2 IMT3 imt4::TRP1*	This work
ASB67-6B	*MATa trp1Δ1 ura3-52 leu2-3 leu2-112 imt1::TRP1 IMT2 IMT3 IMT4*	This work
ASB68-4A	*MATa trp1Δ1 ura3-52 leu2-3 leu2-112 IMT1 IMT2 imt3::TRP1 IMT4*	This work
ASB155-1A	*MATα trp1Δ1 ura3-52 leu2-3 leu2-112 IMT1 imt2::TRP1 imt3::TRP1 imt4::TRP1*	This work
ASB215-33B	*MATa trp1Δ1 ura3-52 leu2-3 leu2-112 IMT1 IMT2 imt3::TRP1 imt4::TRP1*	This work
ASB216-2A	*MATα trp1Δ1 ura3-52 leu2-3 leu2-112 imt1::TRP1 imt2::TRP1 IMT3 IMT4*	This work
ASB217-32C	*MATα trp1Δ1 ura3-52 leu2-3 leu2-112 imt1::TRP1 imt2::TRP1 imt3::TRP1 imt4::TRP1*/pBY161	This work
ASB217-54B	*MATa trp1Δ1 ura3-52 leu2-3 leu2-112 imt1::TRP1 imt2::TRP1 imt3::TRP1 imt4::TRP1*/pBY161	This work
ASB219	*MATa/α trp1Δ1/trp1Δ1 ura3-52/ura3-52 leu2-3/leu2-3 leu2-112/leu2-112 imt1::TRP1/imt1::TRP1 imt2::TRP1/imt2::TRP1 imt3::TRP1/imt3::TRP1 imt4::TRP1/imt4::TRP1*/pBY161	This work
ASB938	*MATa trp1Δ1 ura3-52 leu2-3 leu2-112 imt1::TRP1 imt2::TRP1 imt3::TRP1 imt4::TRP1*/YCp50-*IMT*	This work
ASB939	*MATα trp1Δ1 ura3-52 leu2-3 leu2-112 imt1::TRP1 imt2::TRP1 imt3::TRP1 imt4::TRP1*/YCp50-*IMT*	This work
ASB940	*MATa/α trp1Δ1/trp1Δ1 ura3-52/ura3-52 leu2-3/leu2-3 leu2-112/leu2-112 imt1::TRP1/imt1::TRP1 imt2::TRP1/imt2::TRP1 imt3::TRP1/imt3::TRP1 imt4::TRP1/imt4::TRP1*/YCp50-*IMT*	This work
E. coli		
TG1	Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZΔM15*	22
DH5α	F$^-$ *endA1 hsdR17* (r_K^-, m_K^+) *supE44 thi-1* d$^-$ *recA gyrA96 relA1* Δ(argF-lacZYA)*U169* ϕ80d*lacZΔM15*	Bethesda Research Laboratories
CJ236	*dut1 ung1 thi relA*/pCJ105 (Cm^r)	35

factor 2; EF-Tu, bacterial elongation factor Tu; EF-1α, eukaryotic elongation factor 1α; EOP, efficiency of plating.

Materials. Hybridizations were carried out on Zeta-Probe blotting membranes from Bio-Rad. The NACS-20 material was purchased from Bethesda Research Laboratories. Restriction enzymes were purchased from New England Biolabs, Pharmacia, or Boehringer Mannheim. T4 DNA ligase, DNA polymerase I, T7 DNA polymerase, deoxytriphosphates, and dideoxytriphosphates were all purchased from Pharmacia. T4 polynucleotide kinase was purchased from Boehringer Mannheim. Crude yeast tRNA synthetase was purchased from Sigma. 5-FOA was purchased from SCM Specialty Chemicals, Gainesville, Fla. Sequencing gels and Northern (RNA) blots were autoradiographed on Amersham MP hyperfilm or Kodak X-Omat AR5 film. Isotopes ^{35}S-ATP (600 Ci/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol), and L-[^{35}S]methionine (1,000 Ci/mmol) were purchased from Amersham. Oligonucleotides used in site-directed mutagenesis were purchased from Symbicom AB, Umeå, Sweden.

Strains and plasmids. The sources and genotypes of yeast and bacterial strains used are listed in Table 1. Plasmids used are listed in Tables 2 and 3.

Genetic procedures. Yeast genetic manipulations were performed as described by Rose et al. (46). Yeast transformations were performed by the lithium acetate procedure according to the method of Ito et al. (29) or by electroporation. In the electroporation procedure, fresh logarithmically cultivated yeast cells were concentrated to an optical density at 600 nm of 15 in YPD containing 1 M sorbitol and transformed with 0.1 to 1 μg of DNA at 500 V, 600 ohms, and 25 μF. Bacterial transformations followed the Hanahan procedure (23).

TABLE 2. Plasmids used[a]

Plasmid	Genotype	Source or reference
pUC18	*bla* ColE1 *ori*; pUC18 polylinker	62
M13mp18	M13 *ori*; pUC18 polylinker	62
pCGS42	*URA3* 2μm *tet bla* ColE1 *ori*	Collaborative Genetics, through G. R. Fink
pGEM3zf(+)	*bla* ColE1 *ori* f1(+) *ori*; Bluescript polylinker	Stratagene
YCp50	*URA3 CEN4 ARS1 tet bla* ColE1 *ori*	45
YCp50-*IMT*	As YCp50 but with *IMT* gene	This work
pRS315	*LEU2 CEN6 ARSH4 bla* ColE1 *ori* f1(+) *ori*; Bluescript polylinker	54
pRS423	*HIS3* 2μm *bla* ColE1 *ori* f1(+) *ori*; Bluescript polylinker	P. Hieter
pFM5	YIp5 with a *Sal*I-*Bam*HI fragment of *MES1* gene	38
pRS423-*MES1*	As pRS423 but with *MES1* gene	This work
pSÅ03	pRS315 with polylinker of pGEM3zf(+)	This work
YEp351	*LEU2* 2μm *bla* ColE1 *ori*; pUC18 polylinker	25

[a] Plasmids used for the construction of hybrid *IMT* genes on YCp50 (5′-*IMT4/2*-3′) or pSÅ03 and YEp351 (5′-*IMT2/4*-3′) are described in Materials and Methods.

TABLE 3. *IMT* and *imt* plasmid constructs used in the in vivo assay

Plasmid	Genotype	Plasmid	Genotype
pIMT10	pSÅ03-*IMT* (wild type)	pIMT100	YEp351-*IMT* (wild type)
pIMT11	pSÅ03-*imt* (G_1-C_{72})	pIMT111	YEp351-*imt* (G_1-C_{72})
pIMT12	pSÅ03-*imt* (T_3-A_{70})	pIMT112	YEp351-*imt* (T_3-A_{70})
pIMT13	pSÅ03-*imt* (T_{12}-A_{23})		
pIMT14	pSÅ03-*imt* (T_{31}-T_{39})	pIMT114	YEp351-*imt* (T_{31}-T_{39})
pIMT15	pSÅ03-*imt* (A_{29}-T_{41}) (T_{31}-T_{39})	pIMT115	YEp351-*imt* (A_{29}-T_{41}) (T_{31}-T_{39})
pIMT16	pSÅ03-*imt* (A_{17} insertion)	pIMT116	YEp351-*imt* (A_{17} insertion)
pIMT17	pSÅ03-*imt* (C_{33})		
pIMT18	pSÅ03-*imt* (C_{54})	pIMT118	YEp351-*imt* (C_{54})
pIMT18a	pSÅ03-*imt* (G_{54})	pIMT118a	YEp351-*imt* (G_{54})
pIMT18b	pSÅ03-*imt* (T_{54})	pIMT118b	YEp351-*imt* (T_{54})
pIMT19	pSÅ03-*imt* (C_{60})	pIMT119	YEp351-*imt* (C_{60})
pIMT19a	pSÅ03-*imt* (G_{60})	pIMT119a	YEp351-*imt* (G_{60})
pIMT19b	pSÅ03-*imt* (T_{60})	pIMT119b	YEp351-*imt* (T_{60})
pIMT20	pSÅ03-*imt* (C_{60}-T_{54})	pIMT120	YEp351-*imt* (C_{60}-T_{54})
pIMT21	pSÅ03-*imt* (T_{60}-C_{54})	pIMT121	YEp351-*imt* (T_{60}-C_{54})

The efficiency by which a mutated *imt* gene could support growth in the test system was investigated as follows. Strain ASB940 was transformed with the mutated *imt* gene located on *LEU2* vector pSÅ03 (low copy) or YEp351 (high copy), and prototrophs were selected on solid synthetic complete medium lacking uracil and leucine. Prototrophs were purified by streaking on the same type of plate. Five milliliters of synthetic complete medium lacking uracil and leucine was inoculated with the mutant to be tested. Serial dilutions of the saturated culture were plated on synthetic complete medium lacking leucine but containing 5-FOA (1 mg/ml) and on YEPD plates. The titration ratio of the culture on synthetic complete medium lacking leucine but containing 5-FOA to YEPD plates determined the EOP of the mutant strain. In this assay, the wild-type *IMT* gene located on the pSÅ03 or YEp351 vector gave an EOP of 2.0×10^{-2} or 1.4×10^{-2}, respectively. In Table 4, *imt* mutant EOP values are given relative to values of a strain having the wild-type *IMT* gene on the *LEU2* vector. From all mutants surviving the plasmid shuffling, plasmids were reisolated and the *imt* genes were sequenced to confirm the mutation. From mutants not surviving or having a low EOP, plasmids were reisolated before plasmid shuffling and sequenced.

Plasmid constructs and in vitro oligonucleotide-directed mutagenesis. Cloning of the *IMT* genes used in these constructions has been described previously (8). A 166-bp *Hin*cII-*Hin*dIII fragment containing 5′-26 bp-*IMT4*-65 bp-3′ was subcloned into the corresponding site of pUC18, generating construct pBY140. A 180-bp *Bam*HI-*Hin*dIII fragment from this plasmid was subcloned into the 2μm *URA3* vector pCGS42, giving vector pBY161. This plasmid was initially used as a rescue vector for strains having their chromosomal *IMT1-4* genes disrupted. A 162-bp *Rsa*I-*Hpa*II fragment containing 5′-26 bp-*IMT2*-62 bp-3′ was cloned into the *Sma*I-*Acc*I fragment of phage M13mp18, generating construct pBY174. With the two constructs pBY140 and pBY174, hybrid *IMT* genes were constructed by using a unique *Bss*HII restriction site within the *IMT* gene. The replicative form of construct pBY174 (*IMT2*) was digested with *Eco*RI-*Bss*HII or *Bss*HII-*Hin*dIII, and corresponding fragments were exchanged with an *Eco*RI-*Bss*HII or *Bss*HII-*Hin*dIII fragment from plasmid pBY140 (*IMT4*). Thus, hybrid constructs 5′-*IMT4/2*-3′ (pBY191) and 5′-*IMT2/4*-3′ (pBY192) were made. The *Eco*RI-*Hin*dIII fragment of *IMT* hybrid pBY191 was cloned into the *URA3* low-copy-number vector YCp50, generating plasmid YCp50-*IMT*, which was used as the rescue plasmid in the test system described below. Plasmid pSÅ03 was constructed from pRS315 by exchanging the multiple cloning cassette from pGEM3zf(+) by a *Pvu*II cloning. The *Eco*RI-*Hin*dIII fragment of *IMT* hybrid pBY192 was subcloned into low- and high-copy-number vectors pSÅ03 and YEp351, respectively, which were used as positive controls in the test system.

The single-stranded M13 DNA containing the hybrid *IMT* gene of construct pBY192 was the starting DNA for oligonucleotide site-directed mutagenesis. Oligonucleotide site-directed in vitro mutagenesis was performed according to the method of Kunkel (35) or Nakamaye and Eckstein (40). The mutations were confirmed by DNA sequencing. Hybrid genes were used to reduce potential recombinational events between the mutant and wild-type genes and their corresponding chromosomally disrupted *IMT* genes.

To test wild-type and mutated constructs in yeast cells, the *Eco*RI-*Hin*dIII fragments were isolated from the M13 replicative form and cloned into the corresponding sites of plasmids pSÅ03 and YEp351. Correct constructs were transformed into UMY940 (*MATα/**a***) and tested for activity as shown in Fig. 1. Plasmid pRS423-*MES1* was constructed by subcloning a 3,000-bp *Sal*I-*Bam*HI fragment containing a functional methionyl-tRNA synthetase (*MES1*) gene of *S. cerevisiae* from plasmid pFM5 into the corresponding sites of the 2μm *HIS3* vector pRS423.

Construction of strains. Strains ASB217-54B and ASB217-32C, in which the chromosomal *IMT* genes are disrupted by functional *TRP1* genes and the viability of the cell is dependent on a plasmid-borne *IMT* gene, were constructed by the following crosses. Double-disruptant ASB215-33B (*imt3*::*TRP1 imt4*::*TRP1*) was made by mating ASB68-4A with ASB66-6D. As the strains are isogenic except for the disrupted *imt* gene and *MAT* locus, zygotes were isolated by micromanipulation. Strains disrupted for two *imt* genes were identified in tetrads in which the Trp phenotype segregated 2 Trp^+:2 Trp^-. These results were confirmed by Southern blot analysis as previously described (8). Strain ASB216-2A (*imt1*::*TRP1 imt2*::*TRP1*) was constructed in an identical manner by crossing ASB67-6B with ASB65-1A. The quadruply disrupted strain was obtained by a similar strategy. However, the following modifications had to be made. The

diploid in the cross between ASB216-2A and ASB215-33B was transformed with plasmid pBY161 (*IMT4*). In the concomitant tetrad analysis, tetrads were identified in which the Trp phenotype segregated 2 Trp^+:2 Trp^-. The two Trp^+ spores had no intact *IMT* genes on the chromosome and were rescued by the *URA3*-based plasmid pBY161 (*IMT4*). Spores with the above-mentioned properties were printed onto synthetic complete medium plates containing 5-FOA. A strain with all of the *IMT* genes of the chromosomes disrupted and in which the viability is dependent on the plasmid-based *IMT4* on a *URA3* 2μm vector (pBY161) should not grow on a plate containing 5-FOA. Spores with these properties were finally confirmed by Southern blot analysis to have all of the chromosomal *IMT* genes disrupted. Strains fulfilling these criteria were ASB217-54B (*MAT***a**) and ASB217-32C (*MAT*α). A diploid with the same properties was obtained by mating these strains with each other, and the zygote was isolated by micromanipulation. This strain was designated ASB219. The high-copy-number *URA3* 2μm plasmid pBY161 of the above-mentioned strains was exchanged for the low-copy-number *URA3 CEN4 ARS1 IMT4/2* plasmid (YCp50-*IMT*), to generate strains UMY938 (*Mat***a**), UMY939 (*Mat*α), and UMY940 (*Mat***a**/α). During the exchange, the viability of the strains was maintained by the *LEU2* vector YEp351, which carried a wild-type *IMT* gene. The precaution of generating a strain with the wild-type *IMT* gene on the low-copy-number YCp50-*IMT* was taken to reduce recombinational events between wild-type and mutated *IMT* genes due to copy number effects.

DNA methods. Isolation of plasmid DNA, restriction enzyme analysis, and agarose gel electrophoresis were performed according to the method of Maniatis et al. (36). Isolation of chromosomal DNA from yeast cells for Southern blot analysis was performed as described by Boeke et al. (5). Minipreparations of plasmid DNA from *E. coli* were made according to the method of Holmes and Quigley (26). Sequencing was performed by the chain termination method of Sanger et al. (47). Cloning into M13 phage was done as described by Messing et al. (37). The use of the 18-mer radiolabeled oligonucleotide (5′-CGATCCGAGGACATCA GG-3′) in Southern blot analysis of genomic DNA, which recognized both the intact and disrupted *IMT* gene, has been described before (8).

For oligonucleotide site-directed mutagenesis, oligonucleotides that were complementary to the $tRNA_i^{Met}$ RNA sequence except at the site of mutation were designed. The corresponding position numbers in $tRNA_i^{Met}$ are given. When similar oligonucleotides were used, the mutation sites are indicated by (M). Brackets indicate nucleotide extensions which were not present in all oligonucleotides. Some double mutations were introduced in two steps; in these cases, both oligonucleotides are described. Sites of mutations are indicated by subscript numbers. Mutations G_1 and T_3, 5′-dCACGGC(M/3)C(M/1)GCTAAATCATG-3′; mutations A_{70} and C_{72}, 5′-dGAAATGAAAAATT(M/72)G(M/70) GCCGCTC[GG]-3′; mutation T_{12}-A_{23} and insertion A_{17}, 5′-d[CCC]TGCGC(M/23)CTTCC(M/17)CTG(M/12)GCCA CG[GC]-3′; mutations C_{33}, T_{31}-T_{39} and A_{29}-T_{41}/T_{31}-T_{39}, 5′-d [CCGAGGACAT]CA(M/41)G(M/39)TTATG(M/33)G(M/31) C(M/29)TGCGCG[CTTCC]-3′; mutations in position 54, 5′-d [TC]GGTTTCGA(M/54)CCGAG[GACAT]-3′; and mutations in position 60, 5′-d[C]CGCTCGG(M/60)TTCGATCC [G]-3′. Radiolabeled oligonucleotides used as probes in Northern blot hybridizations and slot blot analysis were as follows: for wild-type $tRNA_i^{Met}$, 5′-dGGTTTCGATCCGAG-3′; for the elongator $tRNA_M^{Met}$, 5′-dTGCTCCAGGAGAGG TTCGAAC-3′; for the C_{60}-T_{54} mutant, 5′-dGGGTTCGAAC CGAG-3′; for the T_{60} mutant: 5′-dCGCTCGGATTCGATC C-3′; and for the G_1-C_{72} mutant: 5′-dTGGCGCCCGCTCGG -3′.

RNA methods. For Northern blot hybridizations and aminoacylations, total yeast tRNA was prepared according to the methods of Carlson and Botstein (11) and Avital et al. (1). In Northern blot analysis, 2 μg of total tRNA was separated on 8% polyacrylamide gels at 10 W in 1× TBE (89 mM Tris-borate [pH 8.0], 2 mM EDTA) and transferred to a Zeta-Probe membrane with a semidry electrophoretic transfer cell (Bio-Rad) for a period of 30 min at 15 V. The filters were UV cross-linked at 302 nm for 10 min, using a Transilluminator model TM-36. For prehybridization, the filters were soaked in a solution of 0.90 M NaCl, 90 mM Tris (pH 7.6), 1 mM EDTA, 1% SDS, and 100 μg of salmon sperm DNA per ml for 1 h at 35°C. For the hybridization, 1 pmol of ^{32}P-kinase-treated oligonucleotide was added to the prehybridization solution, and the filters were heated to 70°C for 5 min in a water bath and cooled slowly to room temperature. For probing of mutant or wild-type tRNAs, 14 pmol of unlabeled wild-type or mutant oligonucleotide was used for competition studies during the hybridization. The filters were washed twice in 6× SSC containing 0.1% SDS (5 min at 37°C) and exposed to an autoradiogram film. For slot blot analysis of NACS-20 column peak fractions, 10 μl of undiluted, 10-fold-diluted, or 100-fold-diluted peak fractions was used per slot. The samples were probed with radiolabeled oligonucleotides specific for the wild-type $tRNA_i^{Met}$, for the mutant $tRNA_i^{Met}$, and for the elongator $tRNA_M^{Met}$. For positive controls, 40 ng of plasmid DNA corresponding to wild-type $tRNA_i^{Met}$, mutant $tRNA_i^{Met}$, and the elongator $tRNA_M^{Met}$ was also blotted. Slot blot filters were treated as described above.

Aminoacylation of $tRNA^{Met}$. Approximately 1.0 A_{260} unit of total crude tRNA preparations was aminoacylated with 50 μCi of [^{35}S]methionine (specific activity, 1,000 Ci/mmol), using the homologous yeast tRNA synthetase. The following assay conditions were used: 20 mM Tris-HCl (pH 7.6), 2 mM ATP, 5 mM $MgCl_2$, 150 mM NH_4Cl, 100 μM EDTA, 0.25 μM methionine, and 10 μg of BSA per ml. The aminoacylation reaction was started by incubating the reaction mixture (200 μl) at 37°C. Samples of 5 μl were taken at 0, 5, 10, 15, 20, 25, and 30 min, trichloroacetic acid precipitated on 3MM Whatman paper, and counted in a scintillation counter to monitor the progress of the reaction. At 30 min, the reaction was stopped by adding 100 μl of phenol (pH 5.2). For further analysis by NACS-20 column chromatography, the sample was phenol extracted (pH 5.2) and precipitated with 2.5 volumes of ethanol. The aminoacylation was found to be completed within 10 to 15 min, and the plateau was reached with the same kinetics for all tRNA preparations (including the wild type). That tRNA was limiting in the reaction was shown in control experiments by adding more tRNA or synthetase after 30 min of the reaction. Synthetase did not increase [^{35}S]methionine incorporation, whereas the addition of the same amount of tRNA increased the incorporation twofold.

Overexpression of methionyl-tRNA synthetase (*MES1*). Existing techniques to detect in vivo-aminoacylated tRNA were not satisfactory and gave imprecise results (63). tRNAs defective in aminoacylation in vivo respond to an increase in the synthetase concentration and can be aminoacylated by overexpressing the wild-type tRNA synthetase (13, 63). To exclude the possibility that the mutant initiator tRNAs were deficient in the velocity of aminoacyl acceptance in vivo,

mutations which were located on the low-copy-number vector pSÅ03 and had relative EOP values below 10^{-1} were also tested by performing the shuffling assay in a strain containing the *MES1* gene on the 2μm high-copy-number vector pRS423.

NACS-20 column chromatography. Aminoacylated tRNAs were applied to a NACS-20 column (1 by 15 cm) in 1 ml of buffer I (0.2 M NaCl, 10 mM $MgCl_2$, 10 mM acetate, pH 4.5). The column was washed with 2 volumes of buffer I, and the aminoacylated tRNAs were eluted with a linear salt gradient ranging from 0.4 to 0.8 M NaCl in buffer I. The chromatography was performed at 25°C. Fractions (0.5 ml) were collected, and 250 μl was mixed with 5 ml of scintillation fluid ACS (Amersham) and counted in an LKB Rackbeta Excel liquid scintillation counter. Except for the A_{17} insertion, all of the $tRNA_i^{Met}$ mutated species separated from the elongator $tRNA_M^{Met}$ (see Results), which was used as an internal control to estimate the ratio of $tRNA_i^{Met}$ to elongator $tRNA_M^{Met}$ (see Fig. 2). Identification of the different tRNA species was made by slot blot analysis of each peak fraction against oligonucleotides specific for the wild-type $tRNA_i^{Met}$, $tRNA_M^{Met}$, and mutated $tRNA_i^{Met}$. The amount of each species was calculated as the total radioactivity in each peak from the NACS-20 chromatogram. The ratio of $tRNA_i^{Met}$ to endogenous $tRNA_M^{Met}$ in a strain having only a wild-type *IMT* gene on the low-copy-number vector YCp50 is 0.4. This ratio was arbitrarily set to 1.0, and all numbers are given as a ratio relative to this number.

RESULTS

An in vivo assay system dependent on a plasmid-borne *IMT* gene. We have constructed a set of strains in which all four *IMT* genes have been disrupted by a functional *TRP1* gene. The viability of the strains was maintained by a wild-type *IMT* gene on the *URA3*-based low-copy-number vector YCp50-*IMT* (Fig. 1). In addition to a *ura3* selection marker, a *leu2* selection marker is present in the strains. By using a *LEU2*-containing vector, a second *IMT* gene, mutated or wild type, was introduced into the strain. Thus, a strain that harbored both the *URA3* plasmid (wild-type *IMT* gene) and the *LEU2* plasmid (mutated *imt* gene) was obtained. If such a strain was plated on medium supplemented with 5-FOA, the *URA3* vector was selected against, since the active *URA3* gene product converted 5-FOA to a toxic compound. By this plasmid shuffling procedure (first described by Boeke et al. [7]), the phenotype of a plasmid-borne mutant *imt* gene was uncovered. To quantitate the plasmid shuffling efficiency, data are given as the EOP with the mutant *imt* construct on the *LEU2* vector relative to the EOP of a wild-type *IMT* gene on the *LEU2* vector (Table 4; see Materials and Methods).

No elongator tRNA can act as an initiator. To test the assay system, a *LEU2* vector containing either no insert or a functional *IMT* gene was introduced into the tester strains. A *LEU2* vector without insert gave no growth on plates containing 5-FOA, which selects against the *URA3* vector (Fig. 1). However, the strain with a wild-type *IMT* gene on the *LEU2* vector survived (Fig. 1 and Table 4). The fact that a *LEU2* vector with no insert did not support growth shows that no endogenous elongator tRNA can substitute for the $tRNA_i^{Met}$.

Mutations not affecting the tester strains. Independent of species, initiator tRNAs have been suggested to have four conserved G-C pairs (9). The conserved G-C pairs at positions 3-70, 12-23, and 31-39 were mutagenized to the corresponding nucleosides of the yeast elongator methionine tRNA. As position 30-40 is a G-C pair also in the elongator $tRNA^{Met}$, no mutants were made in this position. Also, a double mutation at base pairs in positions 29-41 and 31-39 was made. All mutants showed a growth rate similar to that of a strain having the wild-type *IMT* gene on a low-copy-number vector (Table 5). Under steady-state growth conditions, the $tRNA_i^{Met}$ of the three mutants U_{12}-A_{23}, U_{31}-U_{39}, and U_3-A_{70} and of the double mutant A_{29}-U_{41}/U_{31}-U_{39} were produced in ratios to the endogenous elongator $tRNA_M^{Met}$ of 1.2, 1.3, 2.0, and 2.4, respectively (Fig. 2A).

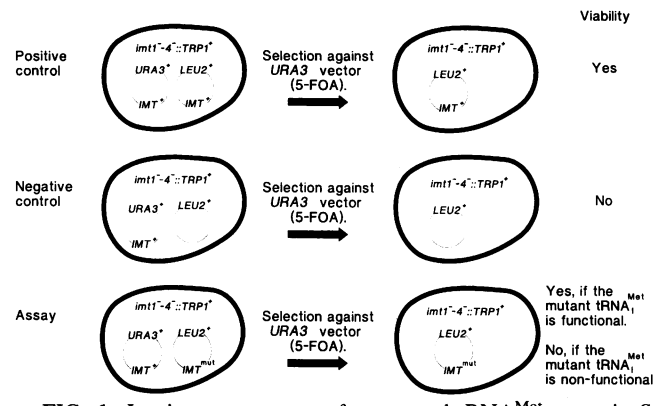

FIG. 1. In vivo assay system for mutated $tRNA_i^{Met}$ genes in *S. cerevisiae*. Yeast strain S288C has four *IMT* genes on four different chromosomes. All four have been disrupted by the insertion of a functional *TRP1* gene in the structural part of each *IMT* gene. The viability of the strains is maintained by having a wild-type hybrid *IMT* gene (5′-*IMT4*/*IMT2*-3′) on a low-copy-number *URA3 CEN4 ARS1*-based vector YCp50, generating YCp50-*IMT*. In addition to a *ura3* selection marker, a *leu2* selection marker is present in the strain. By using a low- or high-copy-number *LEU2* vector, pSÅ03 or YEp351, a second hybrid *IMT* gene (5′-*IMT2*/*IMT4*-3′), mutated or wild type, can be introduced into the strain. Thus, a strain that harbors both the *URA3* plasmid (wild-type *IMT* gene) and the *LEU2* plasmid (mutated *imt* gene) can be obtained. If such a strain is plated on medium supplemented with 5-FOA, the *URA3* vector will be selected against, since the active *URA3* gene product converts 5-FOA to a toxic compound (7). By this plasmid shuffling procedure, the phenotype of a *LEU2* plasmid-borne and mutated *imt* gene can be uncovered. All *imt* constructs described in this report were tested in strain ASB940.

In position 33, initiator $tRNA^{Met}$ of higher eukaryotes has a C instead of the U present at the same position in the lower eukaryotes. Moreover, loop I of eukaryotic initiators has a seven-membered nucleotide loop instead of the eight- or nine-nucleotide loop present in elongators. This is due to the lack of a nucleotide at position 17 of the initiators. Both of these features were investigated either by mutating the U at position 33 to a C or by inserting an A at position 17. Neither of these mutants showed any defects in the in vivo test system or in growth rates of strains being dependent on the mutated initiator tRNA (Tables 4 and 5). Strains harboring the C_{33}-mutated $tRNA_i^{Met}$ gene produced mutant $tRNA_i^{Met}$ in a 1.2 ratio to elongator $tRNA^{Met}$ (Fig. 2A). However, the insertion of an A at position 17 made the mutated $tRNA_i^{Met}$ comigrate with the elongator methionine (data not shown). A ratio to the elongator $tRNA_i^{Met}$ could therefore not be calculated.

Conserved adenosines in positions 54 and 60 are not essential. Adenosines in positions 54 and 60 are unique for eukaryotic cytoplasmic initiator tRNAs (33). The depen-

TABLE 4. EOP of *imt* mutants relative to that of the wild type

Mutation	EOP with *imt* gene on pSÅ03[a]	
	−High-copy-number vector pRS423-*MES1*	+High-copy-number vector pRS423-*MES1*
Wild type[b]	1.0	4.7×10^{-1}
A_1-T_{72} to G_1-C_{72}	8.0×10^{-4}[c]	2.5×10^{-3}
C_3-G_{70} to T_3-A_{70}	7.0×10^{-2}[d]	3.5×10^{-2}
G_{12}-C_{23} to T_{12}-A_{23}	5.5×10^{-1}	ND
A_{17} insertion	2.1×10^{-1}	ND
G_{31}-C_{39} to T_{31}-T_{39}	1.3	ND
G_{29}-C_{41} to A_{29}-T_{41} G_{31}-C_{39} to T_{31}-T_{39}	7.0×10^{-1}	ND
T_{33} to C_{33}	4.0×10^{-1}	ND
A_{54} to C_{54}	6.5×10^{-3}[d]	8.5×10^{-3}
A_{54} to G_{54}	2.6×10^{-1}	ND
A_{54} to T_{54}	$<1.0 \times 10^{-6}$[c]	$<1.6 \times 10^{-6}$
A_{60} to C_{60}	1.3×10^{-1}	ND
A_{60} to G_{60}	2.4×10^{-1}	ND
A_{60} to T_{60}	$<1.1 \times 10^{-6}$[c]	$<1.8 \times 10^{-6}$
A_{54}-A_{60} to C_{54}-T_{60}	3.8×10^{-2}[d]	4.2×10^{-3}
A_{54}-A_{60} to C_{60}-T_{54}	$<1.3 \times 10^{-6}$[c]	$<5.6 \times 10^{-7}$

[a] Determined as titration ratio of cells plated on synthetic complete medium containing 5-FOA to cells plated on YEPD media. ND, not determined.

[b] Absolute EOP values for strains containing a wild-type *IMT* gene on low- and high-copy-number vectors pSÅ03 and YEp351 are 2×10^{-2} and 1.4×10^{-2}, respectively.

[c] Corresponding values when the *imt* gene is harbored on high-copy-number vector YEp351: for G_1-C_{72}, 2.2×10^{-2}; for T_{54}, 5.5×10^{-4}; for T_{60}, $<2.7 \times 10^{-6}$; and for C_{60}-T_{54}, 2.8×10^{-3}.

[d] Corresponding values are $>10^{-1}$ when the *imt* gene is harbored on high-copy-number vector YEp351: for T_3-A_{70}, 1.3×10^{-1}; for C_{54}, 1.6; and for C_{54}-T_{60}, 2.4×10^{-1}.

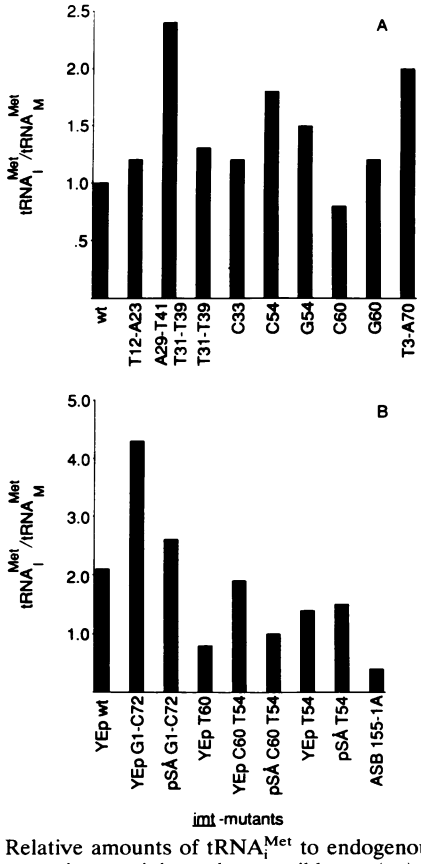

FIG. 2. Relative amounts of $tRNA_i^{Met}$ to endogenous $tRNA_M^{Met}$. The ratio in a strain containing only one wild-type (wt) *IMT* gene on a low-copy-number vector (0.4) was set to 1.0 (see Fig. 4A). (A) Ratio in shuffled strains containing an *imt* gene on low-copy-number vector pSÅ03; (B) ratio in nonshuffled strains containing an *imt* gene on low- or high-copy-number vector pSÅ03 or YEp351 in addition to a wild-type *IMT* gene on low-copy-number vector YCp50-*IMT*. Mutants U/rT_{54} and C_{60}-U/rT_{54} separated from the wild-type initiator, whereas mutants G_1-C_{72} and U_{60} did not. ASB 155-1A designates a strain in which three of the four chromosomal *IMT* genes are disrupted by a functional *TRP1* gene. The amount of $tRNA^{Met}$ species was calculated from the total radioactivity in each peak of the NACS-20 chromatogram.

dence on the highly conserved A_{54} for eukaryotic $tRNA_i^{Met}$ function was investigated by substituting other nucleosides for the A_{54} nucleoside. Mutants having G_{54} and C_{54} were viable (Table 4) but showed a reduced growth rate (Table 5). In addition, mutant C_{54}, when present on a low-copy-number vector, showed a reduced EOP independent of the absence or presence of the overproduced methionyl-tRNA synthetase (*MES1*) gene product (Table 4). This is not due to a frequently occurring suppressor, because when the shuffled C_{54} mutant was transformed with the YCp50-*IMT* rescue vector and replated, the same low EOP was obtained (data not shown). Relative to wild-type $tRNA_i^{Met}$, the mutants containing a G_{54} and C_{54} were overproduced 1.5- and 1.8-fold, respectively (Fig. 2A). Similarly, position 60 in $tRNA_i^{Met}$ was changed from A_{60} to G_{60} and C_{60}. Although C_{60} is a feature of the elongator tRNA, no effects on EOP, steady-state growth, or expression of the mutated *imt* gene were observed (Tables 4 and 5; Fig. 2A). Similarly, no effect was observed with mutation G_{60} (Tables 4 and 5; Fig. 2A). In conclusion, for $tRNA_i^{Met}$ function there is no absolute dependence on the conserved adenosines at position 54 or 60.

TABLE 5. Growth rates of shuffled[a] strain ASB940

Mutant	Growth rate[b]	
	h^{-1}	% wt
Wild type	0.57	100
C_3-G_{70} to T_3-A_{70}	0.54	95
G_{12}-C_{23} to T_{12}-A_{23}	0.55	97
A_{17} insertion	0.50	95
G_{31}-C_{39} to T_{31}-T_{39}	0.56	98
G_{29}-C_{41} to A_{29}-T_{41} G_{31}-C_{39} to T_{31}-T_{39}	0.55	97
T_{33} to C_{33}	0.58	102
A_{54} to C_{54}	0.46	81
A_{54} to G_{54}	0.46	81
A_{60} to C_{60}	0.55	97
A_{60} to G_{60}	0.58	102
A_{54}-A_{60} to C_{54}-T_{60}	0.48	84

[a] Growth is dependent on one *imt* gene located on vector pSÅ03 containing the indicated mutation.

[b] Determined in synthetic complete medium lacking leucine at 30°C.

A U/rT at position 54 of loop IV is detrimental. The single mutations U/rT_{54} and U_{60} were each lethal on both low- and high-copy-number vectors (Table 4). The levels of $tRNA_i^{Met}$ were therefore determined in a strain which contained a wild-type *IMT* gene in addition to the mutant *imt* construct. On NACS-20 chromatography, an extra peak of [^{35}S]methionine-labeled aminoacylated material was obtained from the U/rT_{54} mutant tRNA but not from the U_{60} mutant. The U/rT_{54} mutant tRNA was produced in a 1.5 ratio relative to the elongator tRNA when the mutant gene was located on a

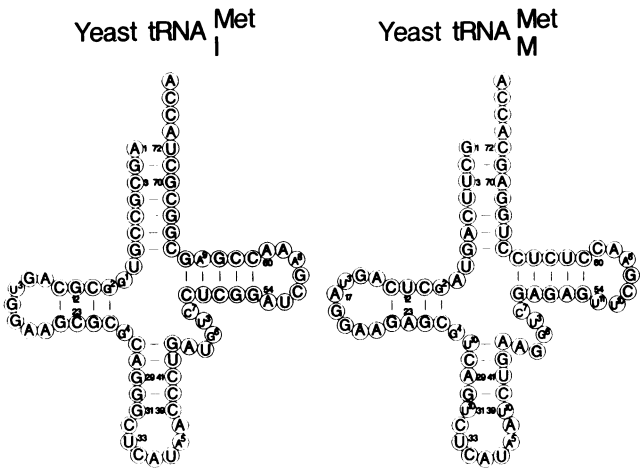

FIG. 3. Schematic two-dimensional drawing of yeast $tRNA_i^{Met}$ and $tRNA_M^{Met}$. The sequences and numbering system are from Sprinzl et al. (56). Nucleosides are circled, and numbers within the circles represent the following modifications: 1, 1-methylguanosine; 2, N^2-methylguanosine; 3, dihydrouridine; 4, N^2,N^2-dimethylguanosine; 5, *N*-(*N*-(9-β-D-ribofuranosylpurine-6-yl)carbamoyl) threonine; 6, 7-methylguanosine; 7, 5-methylcytidine; 8, 1-methyladenosine; 9, 2′-*O*-ribosylated adenosine; 10, pseudouridine; 11, 5-methyluridine. Numbers outside the circles represent positions of $tRNA_i^{Met}$ that have been mutagenized by oligonucleotide site-directed mutagenesis.

low-copy-number vector and in a 1.4 ratio when the mutant gene was located on a high-copy-number vector (Fig. 2B). The amount of $tRNA_i^{Met}$ observed in the U_{60} mutant probably originated from the rescue vector YCp50-*IMT*, and accordingly no U_{60} transcript could be detected by Northern blot hybridization (Fig. 2B and data not shown).

From the secondary structure of the $tRNA_i^{Met}$, it is evident that the introduction of U/rT_{54} or of U_{60} might induce additional base pairs in the stem of loop IV between U/rT_{54}-U/ψ_{55}–A_{59}-A_{60} and A_{54}-U_{55}–A_{59}-U_{60} (Fig. 3). To establish whether this additional base pairing per se would be lethal, the double mutants U/rT_{54}-C_{60} and C_{54}-U_{60} were constructed. These mutant combinations should hypothetically prevent the additional base pairs. Mutant C_{54}-U_{60} supported growth in the test system, although when it was present on a low-copy-number vector a reduced EOP and reduced growth rate of the shuffled strain were observed (Tables 4 and 5). Mutant U/rT_{54}-C_{60} did not support growth in the test system, although a transcript of the correct size was made in vivo (Table 4 and data not shown). tRNA from this mutant accepted methionine in vitro and was separable from the wild-type $tRNA_i^{Met}$ on a NACS-20 column (Fig. 4C and D). The aminoacylated mutant tRNA was present in about the same amount as the wild-type when the gene was located on a low-copy-number vector and in 1.9-fold excess when the gene was located on a high-copy-number vector (Fig. 2B). These amounts of aminoacylated mutant tRNA should not be limiting for growth, since the slowly growing strain ASB155-1A, which has three of the four *IMT* genes disrupted (8), has a relative ratio of $tRNA_i^{Met}$ to $tRNA_M^{Met}$ of 0.4 (Fig. 2B). To our knowledge, this is the lowest level of $tRNA_i^{Met}$ which gives a viable phenotype. The mutant phenotype could not be rescued by overproducing the *MES1* gene product (Table 4). Conclusively, the single mutant C_{60} supported growth but the double mutant U/rT_{54}-C_{60} did not, although the tRNA was produced and aminoacylated in vitro

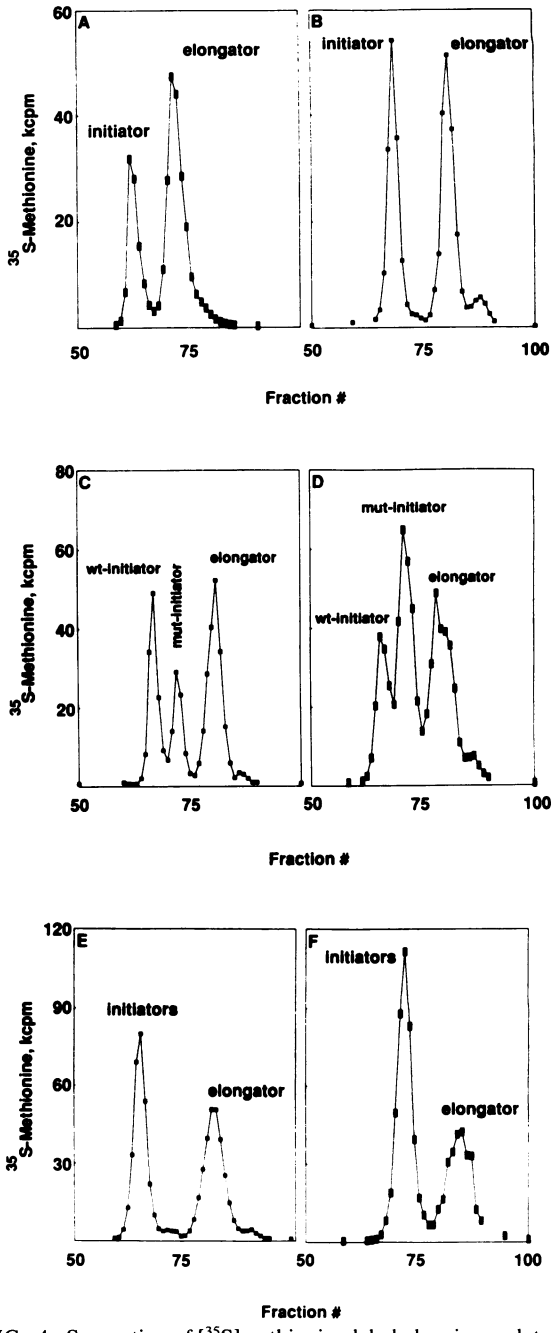

FIG. 4. Separation of [^{35}S]methionine-labeled aminoacylated tRNAs on NACS-20 column chromatography. In all cases, tRNA was prepared from an *imt1-4 TRP1* disrupted strain containing a wild-type *IMT* gene on the *URA3*-based vector YCp50-*IMT*. In addition to the YCp50-*IMT* vector, the strains contained pSÅ03 (no insert) (A), YEp351-*IMT* (wild type [wt]) (B), pSÅ03-*imt* (C_{60}-T_{54}) (C), YEp351-*imt* (C_{60}-T_{54}) (D), pSÅ03-*imt* (G_1-C_{72}) (E), and YEp351-*imt* (G_1-C_{72}) (F). All $tRNA_i^{Met}$ species (wild type and mutant) separated from the elongator $tRNA_M^{Met}$. Different peaks were identified by slot blot hybridizations (data not shown). Note the different ordinate scales.

in sufficient amounts. This finding suggests that a U/rT in position 54 restricts $tRNA_i^{Met}$ from functioning in the initiation of translation.

The A-U base pair at position 1-72 is essential. An A-U base

pair at position 1-72 is a conserved feature of eukaryotic initiator tRNAs (9). The A_1-U_{72} base pair of the yeast initiator was exchanged for G_1-C_{72} of the yeast elongator methionine tRNA and probed for function in the in vivo test system. When the mutated tRNA gene was present on a low- or high-copy-number vector, the relative EOP value was 8.0×10^{-4} or 2.2×10^{-2}, respectively (Table 4). The survivors of the shuffling procedure, which contained the mutant *imt* gene on low-copy-number vector pSÅ03, were retransformed with YCp50-*IMT* and replated on 5-FOA plates. A relative EOP of 1.0 was observed (data not shown). Therefore, it is likely that a genetic event has occurred in the survivors which suppressed the detrimental phenotype of mutation G_1-C_{72}. Overexpression of a *MES1* gene in the shuffling assay did not result in an increase in EOP values (Table 4), suggesting that the mutant tRNA is not deficient in aminoacylation in vivo. Furthermore, a correct-size transcript was detected on Northern blots (data not shown). The crude tRNA was aminoacylated with [^{35}S]methionine and assayed on NACS-20 chromatography. The mutant tRNA does not separate from the wild-type $tRNA_i^{Met}$ (Fig. 4E and F). When the corresponding mutant gene was located on a low- or high-copy-number vector, the total amount of $tRNA_i^{Met}$ (wild type and mutant) corresponded to a 2.6- or 4.3-fold overproduction compared with the wild-type $tRNA_i^{Met}$ produced from vector YCp50-*IMT* (Fig. 2B). However, since we know the amounts of $tRNA^{Met}$ produced from plasmid YCp50-*IMT*, the additional $tRNA^{Met}$ was most likely derived from the mutant $tRNA_i^{Met}$. Thus, a G-C base pair in position 1-72 is suggested to prevent initiator tRNA function in the initiation of translation.

Gene dosage effects of *imt* mutants. With the exception of mutant U_{31}-U_{39}, all mutations when located on low-copy-number vector pSÅ03 gave an EOP value slightly or in some cases dramatically lower than that of the wild type. This result implies that these mutations affect, probably by not having an optimal structure, the efficiency by which the mutant tRNA can participate in initiation in translation. Generally, this efficiency increased (higher EOP values) when the mutated *imt* gene was located on the high-copy-number vector YEp351, thereby generating a higher gene dosage of the mutated *imt* gene. Mutants with relative EOP values below 10^{-1} when located on the low-copy-number pSÅ03 vector can be divided into two classes when investigated on the high-copy-number YEp351 vector. Class I mutations T_3-A_{70}, C_{54}, and C_{54}-T_{60} all had relative EOP values over 10^{-1} (1.3×10^{-1}, 1.6, and 2.4×10^{-1}, respectively). Class II mutations G_1-C_{72}, T_{54}, T_{60}, and C_{60}-T_{54} had relative EOP values below 10^{-1} (2.2×10^{-2}, 5.5×10^{-4}, $<2.7 \times 10^{-6}$, and 2.8×10^{-3}, respectively). Thus, the mutations in the second class affected the function of the $tRNA_i^{Met}$ so dramatically that even an increase in gene dosage could not overcome the deleterious effect of the mutation. We also observed that tRNAs, especially with mutations A_{29}-U_{41}/U_{31}-U_{39}, G_1-C_{72}, U_3-A_{70}, C_{54}, and G_{54}, when located on the low-copy-number vector pSÅ03 were overproduced compared with wild-type $tRNA_i^{Met}$. This observation may be due to variations in the copy number of the vector carrying the mutated *imt* gene, increased stability of such a tRNA, or autoregulation of the yeast $tRNA_i^{Met}$.

DISCUSSION

The conserved positions of $tRNA_i^{Met}$ have been suggested to be important for initiator function and to restrict the $tRNA_i^{Met}$ from functioning in the elongation process. In this study, we describe a functional analysis of the conserved positions of the yeast $tRNA_i^{Met}$ in a novel in vivo test system. *S. cerevisiae* strains with null alleles for each of the four *IMT* genes were constructed which could be made entirely dependent on a wild-type or mutated *IMT* gene, using a two-plasmid system. In this in vivo test system, the initiator tRNA may be completely depleted, and under these conditions, no endogenous elongator tRNA could act as an initiator at AUG codons. Earlier, it was shown that no codon other than AUG was physiologically relevant to translational initiation at *HIS4* and *CYC1* of *S. cerevisiae* (16, 53), although weak initiation had been observed at UUG and AUA codons (64). For the mutational analysis, the conserved positions of yeast $tRNA_i^{Met}$ were changed to the corresponding positions of the yeast $tRNA_M^{Met}$ and tested for function in the in vivo assay. Surprisingly, most of the positions conserved among eukaryotic initiator tRNAs do not seem to be essential for its function in vivo. Changing the U believed to be invariable in position 33 to the conserved C present in the $tRNA_i^{Met}$ of higher-order eukaryotes supported growth in the test system (Tables 4 and 5). This result is in accordance with the observation that there is no preference for a U in position 33 and that the human $tRNA_i^{Met}$ carrying a C in this position is functional in yeast cells (2, 3, 21). An A was introduced in position 17 of loop I to generate the larger loop identical to the yeast methionine elongator. No effect on viability was observed (Tables 4 and 5). Four G-C pairs, located at positions 3-70, 12-23, 31-39, and 30-40, have been suggested to be universally conserved among initiators (9). However, exceptions for the two first positions have been found (56). The first three base pairs and a double base pair, involving positions 29-41 and 31-39, were changed to the corresponding positions of the yeast $tRNA_M^{Met}$ and tested in the in vivo functional assay. No effect was seen for any of the alterations (Table 4 and Table 5). Previously, the three G-C base pairs in the anticodon stem of *E. coli* $tRNA_i^{Met}$ were shown, in vitro, to be important for ribosomal P-site binding and for initiation of protein synthesis (52). Unless the 2.4-fold overexpression of the mutant initiator tRNA can diminish the need for the conserved G-C pairs, our observation suggests a difference in the P-site recognition process between the prokaryotic and eukaryotic translational initiation machinery.

Our mutational analysis of the conserved positions in loop IV revealed that nucleoside A_{60} or A_{54} is not a prerequisite for a functional *IMT* gene, since the mutations G_{60}, C_{60}, C_{54}, and G_{54} maintained a functional $tRNA_i^{Met}$ (Tables 4 and 5). However, there is a preference to have an A at position 54, as the C_{54} and G_{54} mutants have a reduced growth rate (Table 5). According to our EOP data, a G might function better than a C in position 54 (Table 4). No such preference was observed for A at position 60 (Tables 4 and 5).

When the human $tRNA_i^{Met}$ gene was mutated from dA_{54} to dT_{54}, no effect on production of tRNA was observed in vitro in HeLa cell extracts, but the mutant tRNA was produced to a lesser extent in vivo in monkey kidney CV-1 cells (17, 18). In our in vivo system, mutants with U/rT_{54} or U_{60} were detrimental to cell growth (Table 4). Whereas the U/rT_{54}-containing $tRNA_i^{Met}$ was detected by NACS-20 chromatography, the U_{60} mutant $tRNA_i^{Met}$ could not be observed, and no U_{60} transcript could be detected in Northern blot hybridizations (Fig. 2B and data not shown). However, the introduction of U/rT_{54} or U_{60} might induce additional base pairs in the stem of loop IV, between U/rT_{54}-U/ψ_{55}–A_{59}-A_{60} and A_{54}-U_{55}–A_{59}-U_{60} which may affect tRNA structure and

maturation (30, 34, 43). To circumvent this potential problem and to investigate the importance of U/rT and U in positions 54 and 60, the double mutants U/rT_{54}-C_{60} and C_{54}-U_{60} were constructed. Mutant C_{54}-U_{60} supported growth in the test system (Tables 4 and 5). The reduced EOP value and reduced growth rate of this mutant are probably due to effects of the C_{54} mutation, since the single mutant C_{54} displayed the same behavior. This was investigated by retransforming the shuffled strain with the wild-type plasmid YCp50-*IMT* and replating it on 5-FOA. Again, the low EOP was observed (data not shown). Thus, we conclude that any nucleoside is functionally allowed at position 60. Mutation T_{54}-C_{60} was lethal on a low-copy-number vector, and the mutant was drastically affected when the mutant gene was located on a high-copy-number vector, although steady-state levels of this mutant tRNA were produced in sufficient amounts (Table 4 and Fig. 2B). Since the mutant with only C_{60} in the tRNA was viable, we suggest that this nucleoside per se cannot be important for the activity of the $tRNA_i^{Met}$. Therefore, U/rT_{54} seems to interfere with the functional activity of the initiator tRNA. As other nucleosides (C_{54} and G_{54}) only partially inactivated the initiator tRNA, we suggest that U/rT_{54} is an antideterminant in the initiation of translation in eukaryotes distinguishing an elongator from an initiator tRNA. We do not know whether U_{54} is modified to rT_{54} or whether such a modification might play a role in the discrimination of elongator and initiator tRNAs. However, yeast mutants lacking this modification do exist and show no obvious physiological defect (27). Moreover, another fundamental difference in the initiation of translation between eukaryotes and prokaryotes may be implicated, since both the elongator and the initiator tRNAs in prokaryotes contain an rT in position 54. Mutants of the initiator methionine tRNA in which the conserved A-U base pair in position 1-72 was changed to a G-C pair do not support growth in the test system, although the mutant tRNA was produced in sufficient amounts and aminoacylated in vitro (Fig. 4E and F; Fig. 2B). Furthermore, by using the test system, the $tRNA_i^{Met}$ has been identified as an essential component in Ty1 retrotransposition (12). In those experiments, changing base pair A_1-U_{72} to U_1-A_{72} in addition to swapping base pairs 2-71, 3-70, and 6-67 and mutating A_{73} to a G_{73} did not affect the viability of the tester strain (12). Thus, it seems important to have an A-U base pair in position 1-72 independent of the orientation.

The aminoacyl acceptance of the mutations T_{54}-C_{60} and G_1-C_{72} was most likely not affected in vivo, as there was no large difference in EOP values observed with or without extra copies of the *MES1* gene (Table 4). Conclusively, we showed that a G-C base pair in position 1-72 and an rT/U in position 54 have detrimental effects on the initiator tRNA function. This finding suggests that these positions are involved in the discrimination of an elongator tRNA from the initiator tRNA in the translation in eukaryotes.

In the tertiary structure of yeast $tRNA^{Phe}$ and yeast $tRNA_i^{Met}$, position 54 and position 1 are located at one surface of the tRNA molecule (Fig. 5) (4, 32, 44). This surface also includes position 64, where the 2′-*O*-ribosylated adenosine of yeast $tRNA_i^{Met}$ has been shown in vitro to restrict the interaction with bacterial EF-Tu (15, 31). Lack of this modification allows the yeast initiator, in vitro, to function in initiation and in elongation (31). Thus, this surface seems to interact with a component(s) that will discriminate between initiator and elongator tRNAs. The most obvious candidates are eIF-2 and EF-1α, which correspond to IF-2 and EF-Tu in *E. coli*, which were shown to interact with this surface (20, 24, 42, 51, 57, 58, 60). We can only hypothesize what the recognition/restriction elements of these factors might be. The presence of the 2′-*O*-ribosylated modification of position 64 in $tRNA_i^{Met}$ prevents the interaction with the heterologous EF-Tu (31); the presence of U/rT at position 54 might enhance EF-1α binding, and the lack of U/rT at this position might restrict EF-1α binding but, alternatively, enhance eIF-2 binding. It is difficult to make any suggestions regarding the importance of position 1-72, as some elongator tRNAs have an A-U base pair at this position. A suppressor analysis of *imt* mutants having detrimental phenotypes is in progress in order to address these questions.

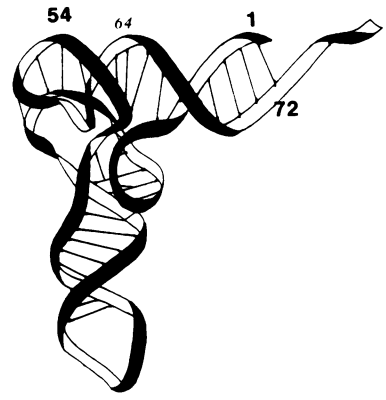

FIG. 5. Tertiary structure of yeast $tRNA^{Phe}$. Position numbers are according to Sprinzl et al. (56). The positions of mutations G_1-C_{72} and T_{54}, which were found to be detrimental in our in vivo system, are indicated by bold numbers. Position 64 (2-ribosylated A) was shown to exclude $tRNA_i^{Met}$ from participating in elongation (31) and is indicated in italics. Position 1 of base pair 1-72, as well as positions 54 and 64, are all located at one surface of the tRNA molecule. The representation is based on a drawing by Irving Geis (10).

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