METHOD

Preparation and activity of synthetic unmodified mammalian tRNA_i^{Met} in initiation of translation in vitro

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ABSTRACT

Translation of eukaryotic mRNA is initiated by a unique amino-acyl tRNA, Met-tRNA_i^{Met}, which passes through a complex series of highly specific interactions with components of the translation apparatus during the initiation process. To facilitate in vitro biochemical and molecular biological analysis of these interactions in fully reconstituted translation initiation reactions, we generated mammalian tRNA_i^{Met} by in vitro transcription that lacked all eight base modifications present in native tRNA_i^{Met}. Here we report a method for in vitro transcription and aminoacylation of synthetic unmodified initiator tRNA_i^{Met} that is active in every stage of the initiation process, including aminoacylation by methionyl-tRNA synthetase, binding of Met-tRNA_i^{Met} to eIF2-GTP to form a ternary complex, binding of the ternary complexes to 40S ribosomal subunits to form 43S complexes, binding of the 43S complex to a native capped eukaryotic mRNA, and scanning on its 5' untranslated region to the correct initiation codon to form a 48S complex, and finally joining with a 60S subunit to assemble an 80S ribosome that is competent to catalyze formation of the first peptide bond using the [³⁵S]methionine residue attached to the acceptor terminus of the tRNA_i^{Met} transcript.

Keywords: eukaryotic initiation factor; initiator tRNA; ribosome; translation

INTRODUCTION

Translation of eukaryotic mRNA is initiated by a unique aminoacyl-tRNA, Met-tRNA_i^{Met} (Housman et al., 1970; Smith & Marcker, 1970), which passes through a complex series of highly specific interactions with components of the translation apparatus before the methionine residue attached to this initiator tRNA is incorporated into a protein. Eukaryotic initiator tRNAs are 76 nt long and contain eight residues that have additional base modifications (Fig. 1B). The tRNA_i^{Met} moiety is aminoacylated by methionyl tRNA synthetase (MetRS) and Met-tRNA_i^{Met} is then specifically bound by eukaryotic initiation factor 2 (eIF2) to form a stable ternary complex with GTP (Dettman & Stanley, 1972; Levin et al., 1973) that subsequently binds to the 40S ribosomal subunit with eIF3 in an mRNA-independent reaction, resulting in formation of a 43S preinitiation complex

(Darnborough et al., 1973; Schreier & Staehelin, 1973). The 43S complex binds mRNA in either a 5'-cap-dependent manner or by direct association with an internal ribosomal entry site (IRES) within the 5'-untranslated region (5' UTR; Jackson, 2000). Initiator tRNA occupies the ribosomal P-site in the 48S complex and is directly involved in mediating selection of the start site of translation by the ribosome as it scans in a 5'-3' direction on the 5' UTR (Cigan et al., 1988; Pestova & Hellen, 1999). After base pairing between the initiation codon and the anticodon of initiator tRNA has been established, eIF5 and eIF5B mediate displacement of initiation factors from the 40S subunit and joining of a 60S ribosomal subunit to the residual [40S/Met-tRNA_i^{Met}] complex (Pestova et al., 2000a, 2000b). The aminoacyl terminus of initiator tRNA is placed in the peptidyltransferase center of the resulting 80S ribosome, a second (elongator) tRNA is placed in the ribosomal A-site by eukaryotic elongation factor 1A (eEF1A) and the first peptide bond is formed. AUG (methionine) codons at internal positions within an open reading frame are not recognized by tRNA_i^{Met} and are instead

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FIGURE 1. A: Construction of a plasmid for transcription of mammalian cytoplasmic tRNA^{Met}. The T7 RNA polymerase promoter is shown in lower case letters, and the start site of transcription at the 5' end of the tRNA sequence and the cleavage site of BstNI at the 3' end of each tRNA sequence are indicated. The tRNA sequence shown corresponds to that for wild-type tRNA_i^{Met} and is common to human, rabbit, murine, and rat moieties (Sprinzl et al., 1998). **B:** The cloverleaf structure of native tRNA_i^{Met} showing modified nucleotides and the positions of mutations present in the tRNA_i^{Met} transcripts derived from pTRM2 (an A14G substitution), pTRM3 (insertion of a C residue after residue G10), and pTRM4 (this C insertion combined with deletion of residues C31 and C32).

decoded by the elongator methionine tRNA (tRNA_m^{Met}). In contrast to tRNA_i^{Met}, this elongator tRNA forms a ternary complex with eEF1A and GTP and binds to the A-site (Negrutski & El'skaya, 1998). The fidelity of translation initiation therefore depends on the specificity of the complex interactions between tRNA_i^{Met} and eIF2 and the 40S ribosomal subunit, and on the exclusion of elongator tRNAs (including tRNA_m^{Met}) from these interactions. However, very little is known about the structural determinants of these interactions: biochemical

investigation of them has been hampered by the difficulty of purifying individual native tRNA_i^{Met} free of other tRNAs. Demonstration that synthetic tRNA_i^{Met} produced in vitro is fully active in all stages of translation initiation would greatly facilitate such analyses.

The development of methods for in vitro transcription of cloned synthetic tRNA genes by bacteriophage T7 polymerase has already permitted extensive, detailed studies of structure–function relationships in tRNA (Sampson & Uhlenbeck, 1988; Saks et al., 1994). This

approach has primarily been used to identify determinants of *Escherichia coli* tRNA identity (i.e., determinants of the precise recognition of tRNAs by their cognate aminoacyl tRNA synthetases). Synthetic unmodified tRNAs are aminoacylated by their cognate synthetases with kinetics similar to those of native tRNA (reviewed by Schulman, 1991).

We have adopted this approach to generate unmodified mammalian tRNA_i^{Met} for use in in vitro dissection of the molecular mechanism of translation initiation in eukaryotes. Here we describe methods for the transcription and specific aminoacylation of synthetic unmodified mammalian tRNA_i^{Met} and report that its activity in each stage of the initiation process, up to and including formation of the first peptide bond by 80S ribosomes assembled from fully fractionated translation components is comparable to that of native initiator tRNA. This finding will now permit the activity of various tRNA_i^{Met} mutants to be examined to elucidate the unique features of this tRNA responsible for recognition by eIF2 and binding to the ribosomal P-site.

RESULTS

Transcription of unmodified tRNA_i^{Met}

Synthetic DNA oligomers were used to construct the plasmid pTRM-1, which contains a T7 promoter upstream and directly adjacent to a mammalian cytoplasmic tRNA_i^{Met} gene and a *Bst*N1 site at the 3' end of the gene (Fig. 1A). This plasmid was designed such that runoff transcription after *Bst*N1-digestion would yield a 76-nt RNA with a sequence identical to that of unmodified human tRNA_i^{Met} (Fig. 1B). Sequence analysis identified three adventitious mutants, pTRM2, pTRM3, and pTRM4 that had mutations in the D stem-loop and in the anticodon stem (Fig. 1B). Analysis of transcription products on an 8% polyacrylamide gel revealed that a single RNA product was synthesized from each plasmid (Fig. 2A). The relatively low level of transcription is consistent with observations that although T7 RNA polymerase has a strong preference for initiation with GTP, it will initiate transcription accurately but less efficiently with ATP (Imburgio et al., 2000).

Aminoacylation of tRNA_i^{Met} transcripts

The methionine acceptor activity of tRNA_i^{Met} transcripts was determined using *E. coli* methionyl-tRNA synthetase in aminoacylation reactions. This enzyme aminoacylates native eukaryotic initiator tRNA but does not aminoacylate mammalian elongator methionine tRNA (Stanley, 1974; Drabkin & Rajbhandary, 1985). [³⁵S]Methionine-labeled tRNA_i^{Met} was resolved by polyacrylamide gel electrophoresis. The wild-type tRNA transcript and the A14G mutant transcript derived from pTRM2 were aminoacylated to similar extents (Fig. 2B,

FIGURE 2. Transcription and aminoacylation of synthetic tRNA_i^{Met} transcripts and aminoacylation by *E. coli* methionyl tRNA synthetase. **A:** DNA corresponding to plasmids pTRM1–pTRM4, as indicated, after restriction with *Bst*N1 and separation on an 8% polyacrylamide gel (lanes 1–4) and with tRNA_i^{Met} transcripts (indicated by an arrowhead) after transcription with T7 RNA polymerase (lanes 5–8). **B:** Visualization of tRNA_i^{Met} aminoacylated with [³⁵S]methionine. Aminoacylated RNA substrates (indicated by an arrowhead) were separated from [³⁵S]methionine-labeled enzyme by phenol extraction, and from free [³⁵S]methionine by electrophoresis in 10% polyacrylamide gel in TBE buffer (pH 8.0) at 4°C. The dried gel was exposed to Kodak X-Omat AR film overnight at –80°C.

lanes 1 and 2) but the pTRM3 and pTRM4 mutant transcripts were aminoacylated poorly or not all (Fig. 2B, lanes 3 and 4). These results indicate that aminoacylation was specific. They are consistent with the importance of the anticodon stem-loop and the D-stem for binding and subsequent aminoacylation of initiator tRNA (Senger et al., 1992, 1995). The efficiency of aminoacylation of the wild-type tRNA transcript was 70–80%.

Purification of native aminoacylated tRNA_i^{Met}

To obtain native aminoacylated tRNA_i^{Met}, we aminoacylated total native rabbit tRNA. However, the pres-

ence of large amounts of other tRNAs and other high and low molecular weight RNA contaminants in such preparations may influence the activity of native aminoacylated tRNA_i^{Met} in different assays. To be able to directly compare the activities of synthetic transcribed and native tRNA_i^{Met}, we therefore purified native aminoacylated tRNA_i^{Met} from other tRNAs and RNA contaminants using gel filtration. The first round of gel filtration on Superdex-75 allowed us to separate tRNA from high molecular weight RNA contaminants (Fig. 3A). After

this stage, the partially purified preparation of aminoacylated native tRNA_i^{Met} was incubated with eIF2 and GMPPNP to form eIF2/tRNA_i^{Met}/GMPPNP-ternary complexes. The resulting reaction mixture was subjected to a second round of gel filtration on Superdex-75 (Fig. 3B). The ternary complex migrated much more quickly than other tRNAs and the low molecular weight RNA contaminants. After phenol extraction of ternary complexes and ethanol precipitation we obtained pure individual fully aminoacylated native tRNA_i^{Met} (Fig. 3C,D), which

FIGURE 3. Purification of native aminoacylated initiator tRNA_i^{Met}. **A,B:** Typical chromatograms of **(A)** total aminoacylated tRNA and **(B)** eIF2/GTP/initiator tRNA ternary complexes on Superdex G-75 column. The positions of total tRNA, eIF2/GTP/initiator tRNA ternary complex, and RNA contaminants are indicated. **C,D:** Electrophoresis of 1 pmol of [³⁵S]methionine-aminoacylated synthetic initiator tRNA_i^{Met} transcripts (specific activity 100,000 cpm/pmol; lane 1) and 1 pmol of individual native initiator tRNA (specific activity 100,000 cpm/pmol; lane 2) in 14% polyacrylamide gel in TBE buffer (pH 8.0) at 4 °C. Initiator tRNA in **C** was visualized by staining with ethidium bromide; the gel was then dried and exposed to X-ray film overnight at -80 °C (**D**).

we used for direct comparison with synthetic transcribed aminoacylated tRNA^{Met} in ternary complex formation and methionylpuromycin synthesis assays.

Incorporation of unmodified tRNA^{Met} transcripts into ternary eIF2-GTP-tRNA complexes

The biological activity of unmodified aminoacylated initiator tRNA was first studied by investigating its ability to form stable ternary complexes with GMPPNP and purified rabbit eIF2 (Fig. 4). Synthetic or native aminoacylated tRNA^{Met} (0.8 pmol) was incubated with increasing amounts of eIF2. Ternary complex formation was analyzed using a filter-binding assay. We did not detect any significant difference in the activities of native and synthetic tRNAs in this assay. The activities of synthetic mutant tRNAs in ternary complex formation are summarized in Table 1. The A14G mutant tRNA was incorporated into ternary complexes only slightly less well than wild-type tRNA transcripts. Incorporation of both tRNAs increased in a linear manner as the amount of eIF2 in the reaction mixtures was increased from 8 pmol (equivalent to the amount of tRNA) to 24 pmol (a threefold molar excess over the amount of tRNA).

Incorporation of unmodified tRNA^{Met} transcripts into ribosomal 43S and 48S complexes

Unmodified [³⁵S]Met-tRNA^{Met} was incorporated efficiently and specifically into 43S ribosomal complexes (Fig. 5A). Incorporation was dependent on the presence of eIF2. The A14G mutant tRNA was incorporated into 43S complexes significantly less well than the wild-type tRNA (Fig. 5B) indicating that this mutant has a functional defect in addition to slightly reduced binding

FIGURE 4. Incorporation of aminoacylated native and synthetic transcript [³⁵S]Met-tRNA^{Met} into eIF2/GTP/initiator tRNA ternary complexes using indicated amounts of eIF2 and 0.8 pmol of [³⁵S]Met-tRNA^{Met} (100,000 cpm/pmol) and assayed by filter binding.

TABLE 1. Incorporation of aminoacylated [³⁵S]Met-tRNA^{Met} transcripts into eIF2/GTP/initiator tRNA ternary complexes.

	eIF2 (8 pmol)	eIF2 (24 pmol)
Wild type	7,335 (16.9%)	21,273 (49.1%)
A14G mutant	5,437 (12.5%)	15,559 (35.9%)

Values represent incorporation of [³⁵S]Met-tRNA^{Met} with specific activity 10,000 cpm/pmol (cpm, and in parenthesis, percentage of input [³⁵S]Met-tRNA^{Met}) into ternary complexes in the presence of the indicated amounts of eIF2.

to eIF2. This result provides evidence that initiator tRNA plays a critical role in the stability of the 43S complex. Assembly of 48S complexes at the initiation codon of native capped β -globin mRNA requires the initiation factors eIFs 1, 1A, 2, 3, 4A, 4B, and 4F (Pestova et al., 1998a), and can be assessed using a toe-printing assay. 48S complexes assembled on β -globin mRNA using native aminoacylated tRNA^{Met} yielded stops 15, 16, and 17 nt downstream of the initiation codon (Fig. 6, lane 3). Stops were also detected at these positions when unmodified tRNA transcripts with the wild-type

FIGURE 5. Incorporation of aminoacylated [³⁵S]Met-tRNA^{Met} transcripts into 43S preinitiation complexes, fractionated by centrifugation in 10–30% linear sucrose gradients. Dependence on eIF2 (**A**) and effect of an A14G substitution in tRNA^{Met} (**B**). Complexes were assembled as described in Materials and Methods in the presence or absence of eIF2, or in the presence of eIF2 and either wild-type or mutant A14G [³⁵S]Met-tRNA^{Met} (specific activity 10,000 cpm/pmol) as indicated. Sedimentation was from right to left. Upper fractions from the gradient have been omitted for clarity.

FIGURE 6. Toe-print analysis of 48S complexes assembled on β -globin mRNA. The full-length cDNA product is marked E, and the toe-prints due to 48S complexes assembled at the initiation codon of β -globin mRNA terminated 15–17 nt downstream from this codon. It is indicated to the left of the reference lanes (G, A, T, and C), which show the β -globin cDNA sequence derived using the same primer.

sequence were used in this assay (Fig. 6, lane 1) indicating that they are also fully active in 48S complex formation. We noted that the intensity of the +15 toe-print was slightly stronger relative to the toe-prints at +16 and +17 in reactions that contained the unmodified tRNA transcript than in those that contained native initiator tRNA.

Activity of unmodified tRNA^{Met} transcripts in the peptidyltransferase center of assembled 80S ribosomes

The final stage in translation initiation is the process of ribosomal subunit joining, in which initiation factors are displaced from the 48S complex and it joins with a 60S subunit to form an 80S ribosome. The activity of 80S ribosomes assembled in this way can be determined by assaying the reactivity of [³⁵S]Met-tRNA_i^{Met} in the ribosomal P site with the puromycin in the ribosomal A site to form methionylpuromycin. This reaction mimics the formation of the first peptide bond in the peptidyltransferase center of assembled 80S ribosomes (Leder & Bursztn, 1966).

We assayed the reactivity of [³⁵S]Met-tRNA_i^{Met} with puromycin in 80S ribosomes assembled on an AUG triplet in the presence of different combinations of the factors eIF1, eIF3, eIF5, and eIF5B (Fig. 7A). 48S complex formation on AUG triplets is very efficient and sim-

FIGURE 7. A: Methionylpuromycin synthesis by active 80S ribosomes assembled using 48S complexes and 60S subunits with recombinant eIF5 and full-length native eIF5B, as indicated. 48S complexes were assembled using 40S subunits, aminoacylated [³⁵S]Met-tRNA_i^{Met} transcripts (specific activity 80,000 cpm/pmol), AUG triplets, and factors as indicated. **B:** Time-course of methionylpuromycin synthesis by ribosomes assembled using 48S complexes and 60S subunits with recombinant eIF5 and full-length native eIF5B. 48S complexes were assembled using 40S subunits, AUG triplets, factors, and either aminoacylated native or synthetic transcript [³⁵S]Met-tRNA_i^{Met} (specific activity 100,000 cpm/pmol) as indicated.

ple, and does not require the full set of initiation factors necessary for 48S complex formation on a capped mRNA. It can occur in the presence of only eIF2. However, we have found previously that eIF1 and eIF3 in-

fluence the factor requirements for subunit joining to 48S complexes assembled on AUG triplets in the presence of native initiator tRNA (Pestova et al., 2000b). We therefore assayed joining of 60S subunits to 48S complexes assembled using unmodified tRNA_i^{Met} transcripts and compared these results with those obtained previously using native initiator tRNA. As described previously for reactions containing native initiator tRNA (Pestova et al., 2000b), in the absence of eIF3 and the presence of eIF1, both eIF5 and eIF5B were able to mediate subunit joining (Fig. 7A, lanes 2 and 4). Inclusion of eIF3 and omission of eIF1 enhanced the activity of eIF5B (Fig. 7A, lane 5) and reduced the activity of eIF5 (Fig. 7A, lane 3) in promoting assembly of active 80S ribosomes. Both eIF5 and eIF5B are required for subunit joining in reactions that contain eIF1 and eIF3 simultaneously (Pestova et al., 2000b) and in experiments described here, assembly reactions that contained all four of these factors yielded significant amounts of active 80S ribosomes (Fig. 7A, lane 6). We therefore did not detect any qualitative difference in the subunit joining process when native initiator tRNA or unmodified tRNA_i^{Met} transcripts were used in the reaction. To assay the relative activities of native and transcript initiator tRNA in methionylpuromycin synthesis quantitatively, we performed kinetic studies (Fig. 7B). Reaction mixtures contained AUG triplets, 40S and 60S subunits, eIF2, eIF3, eIF1, eIF1A, eIF5, and eIF5B. Methionylpuromycin synthesis occurred at a similar linear rate over the entire 30 min of reaction using both native and synthetic initiator tRNAs. We therefore did not detect any difference between native Met-tRNA_i^{Met} and unmodified transcript Met-tRNA_i^{Met} in this assay.

DISCUSSION

We report here that synthetic eukaryotic initiator tRNA_i^{Met} transcripts lacking all eight posttranscriptional base modifications are active in all stages of translation initiation reconstituted *in vitro* from full fractionated translation components. These stages include aminoacylation by methionyl-tRNA synthetase, binding of Met-tRNA_i^{Met} to eIF2-GTP to form a ternary complex, binding of the ternary complexes to 40S ribosomal subunits to form 43S complexes, binding of the 43S complex to a native capped eukaryotic mRNA and scanning on its 5' untranslated region to the correct initiation codon to form a 48S complex, and finally joining with a 60S subunit to assemble an 80S ribosome that is competent to catalyze formation of the first peptide bond using the [³⁵S]methionine residue that is covalently attached to the acceptor terminus of the tRNA_i^{Met} transcript. The observations reported here that unmodified tRNA_i^{Met} transcript are active in each of these stages are consistent with previous observations that modification of tRNA residues serves primarily to exclude initiator tRNA from the elongation stage in translation

(Kiesewetter et al., 1990; Åström et al., 1993; Förster et al., 1993; Åström & Byström, 1994). We have not yet investigated whether the unmodified tRNA_i^{Met} transcript described here can be recognized by eEF-1 α and be used in the process of translation elongation. Modified bases in other tRNAs contribute to conformational stability (Hall et al., 1989; Kintanar et al., 1994) and may also do so in initiator tRNA. The 1-methyladenosine (m¹A) modification at position 58 of initiator tRNAs is a determinant of a unique tertiary substructure not observed in elongator tRNAs (Basavappa & Sigler, 1991). The absence of this modification leads to severe defects in processing and stability of initiator tRNA in yeast (Anderson et al., 1998; Calvo et al., 1999). We have shown in this report that this and other base modifications in initiator tRNA are not required for translation initiation, and it is therefore possible that some of the other modifications may also have *in vivo* roles in initiator tRNA processing or stability.

Eukaryotic initiator tRNAs also contain several conserved sequence elements that are either uncommon or do not occur at all in elongator tRNAs: an A1-U72 base pair at the end of the aminoacyl acceptor stem, a short (seven-membered) D loop, residue A20, three consecutive G-C base pairs in the anticodon stem, the bases A54 and U55 (in place of the ribothymidine and pseudouridine residues found in elongator tRNAs), and residue A60. A role for some of these conserved sequence elements in determining the structure and identity of tRNA_i^{Met} has been established but these studies have largely been limited to dissecting the interactions of initiator tRNA with methionyl-tRNA synthetase and to a lesser extent eIF2 (Basavappa & Sigler, 1991; von Pawel-Ramingen et al., 1992; Senger et al., 1992, 1995; Drabkin et al., 1993; Farruggio et al., 1996; Drabkin & Rajbhandary, 1998). The data reported here, which show that initiator tRNA transcripts are active in all subsequent stages of the initiation process will allow us to assess the importance of unique conserved sequence elements in initiator tRNA for its function in other stages in initiation. For example, the observations that A14G mutant tRNA was incorporated into ternary complexes as well as wild-type tRNA but that it had a defect in incorporation into 43S complexes indicates that initiator tRNA plays a critical role in the stability of the 43S complex.

Of equal importance, the ability to prepare active tRNA_i^{Met} by *in vitro* transcription will also permit the specific incorporation of photoreactive or fluorescent nucleotides at defined positions in initiator tRNA. Initiator tRNA derivatized in this way could be used for high resolution crosslinking studies (Barta et al., 1984; Wower et al., 1994), directed probing experiments to establish which elements of the eukaryotic ribosome are in close physical proximity to initiator tRNA (Joseph & Noller, 1996; Joseph et al., 2000), kinetic analyses, and to probe interactions with other components of the trans-

lation apparatus or structural rearrangements that occur during scanning, displacement of eIF2-GDP from 48S complexes, and ribosomal subunit joining (e.g., Robertson et al., 1986; Rodnina et al., 1995; Lago et al., 2001).

MATERIALS AND METHODS

Enzymes and reagents

DNA restriction endonucleases and DNA modifying enzymes were from New England BioLabs (Beverly, Massachusetts). RQ1 RNase-free DNase and avian myeloblastosis virus (AMV) reverse transcriptase were purchased from Promega Corp. (Madison, Wisconsin). Native rabbit α - and β -globin mRNAs were from Life Technologies (Grand Island, New York) and native rabbit tRNA was from Novagen (Madison, Wisconsin). Unlabeled NTPs were from Amersham Pharmacia (Piscataway, New Jersey) and unlabeled methionine was from Fluka Chemical Corp. (Ronkonkoma, New York). Radiochemicals [35 S]methionine (44 TBq/mmol), [35 S]dATP (37 TBq/mmol), and [32 P]dATP (220 TBq/mmol) were purchased from Amersham Corp. (Arlington Heights, Illinois) and ICN Radiochemicals (Irvine, California). *E. coli* methionyl-tRNA synthetase was purified from *E. coli* strain MRE 600 (American Type Culture Collection, Manassas, Virginia) as described (Stanley, 1974). Ribosomal 40S and 60S subunits and native and recombinant translation initiation factors were purified as described (Pestova et al., 1996, 1998a, 1998b, 2000a).

Construction of plasmid DNA

Four synthetic, partially overlapping DNA oligomers were purified, phosphorylated, annealed, and inserted between the *Hind*III and *Bam*HI sites of plasmid pBR322 to construct the plasmid pTRM1, in a manner similar to that described (Sampson & Uhlenbeck, 1988). This plasmid contains a bacteriophage T7 promoter directly adjacent to the mammalian cytoplasmic tRNA_i^{Met} gene and a *Bst*NI site at the 3' end of the gene (Fig. 1A). The sequence of this *wt* construct was confirmed (and three adventitious mutants were identified) using Sequenase according to the manufacturer's directions (U.S. Biochemicals, Cleveland, Ohio). These mutants were termed pTRM2, pTRM3, and pTRM4, and contained an A14G substitution, an insertion of a C residue after residue G10 and a deletion of residues C31 and C32, and insertion of a C residue after residue G10, respectively.

In vitro transcription

pTRM1-pTRM4 were linearized by digestion with *Bst*NI and transcribed in vitro with *wt* recombinant T7 RNA polymerase (Roche) at 37 °C for 45 min. Because tRNA transcription was initiated at an adenine residue (rather than guanine) it was relatively inefficient, so we found that it was important to include high concentrations of plasmid DNA in reactions (10–20 μ g/100 μ L). T7 RNA polymerase was used at a concentration of 16 U/100 μ L reaction volume. Plasmid DNA templates were digested with RQ1 RNase-free DNase according to the manufacturer's instructions, and synthetic

transcripts were then purified using Nuc-trap columns from Stratagene Cloning Systems (La Jolla, California), as described (Pestova et al., 1991).

Aminoacylation of tRNA

Native rabbit tRNA (1 mg) or synthetic tRNA from a 200- μ L transcription reaction was aminoacylated in vitro using aminoacyl tRNA synthetase, essentially as described (Stanley, 1974) in 200- μ L reaction mixtures that contained 1 mCi/mL [35 S]methionine ($\sim$ 1250 Ci/mmol) and 0.1 mM or 0.01 mM unlabeled methionine yielding about 10,000 cpm/pmol or 100,000 cpm/pmol of [35 S] tRNA_i^{Met}, respectively. The reaction mixture was incubated for 30 min at 37 °C, phenol extracted, filtered using a Nuc-trap column, and ethanol precipitated.

Purification of native aminoacylated tRNA_i^{Met}

Total native tRNA (2 mg) was aminoacylated (specific activity 100,000 cpm/pmol) essentially as described above and was then purified from high molecular weight contaminants by gel filtration by FPLC on Superdex G-75 using a 25-mL column. Gel filtration buffer contained 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2.5 mM MgAc, 1 mM DTT (see Fig. 3A,B). Fractions that contained [35 S]Met-tRNA_i^{Met} were collected and ethanol precipitated. Precipitated [35 S]Met-tRNA_i^{Met} was then incubated for 10 min at 37 °C with 60 μ g purified eIF2 in a reaction mixture (200 μ L) with buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2.5 mM MgAc, 1 mM DTT, and 1 mM GMP-PNP to form ternary eIF2-GTP-[35 S]tRNA_i^{Met} complex. The reaction mixture was reappplied to the Superdex G-75 column which, permitted the separation of eIF2-GTP-[35 S]tRNA_i^{Met} complex from all other tRNAs and low molecular weight RNA contaminants. Fractions that contained [35 S]Met-labeled ternary complex were collected, phenol extracted, and ethanol precipitated.

Ternary complex formation

Aminoacylated synthetic or native [35 S]Met-tRNA_i^{Met} with specific activities of either 10,000 or 100,000 cpm/pmol (as indicated in figure legends) was bound to different amounts of purified eIF2 by incubation in 50 μ L buffer (20 mM Tris-HCl, pH 7.6, 100 mM potassium acetate, 2.5 mM magnesium acetate, and 2 mM DTT) containing 1 mM GTP, for 10 min at 37 °C. Samples were diluted with 0.5 mL of the same ice-cold buffer after incubation and were then subjected to a filter-binding assay. The radioactivity retained on nitrocellulose filters was counted directly to quantitate ternary eIF2-GTP-tRNA_i^{Met} complex formation.

Formation of 43S preinitiation complexes

43S preinitiation complexes were assembled by incubating eIF2 (2 μ g) and eIF3 (7 μ g) with [35 S]Met-tRNA_i^{Met} (3 pmol with specific activity of 10,000 cpm/pmol) and 40S ribosomal subunits (12 pmol) in 100 μ L buffer (20 mM Tris-HCl, pH 7.6; 100 mM potassium acetate; 2.5 mM magnesium acetate; 2 mM DTT; 0.1 mM spermidine) containing 1 mM GTP for 10 min at 37 °C. These complexes were isolated by centrifugation.

gation for 15 h at 4 °C and 24,000 rpm in 10–30% linear sucrose gradients in buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, and 2 mM DTT, using a Beckman SW41 rotor.

Assembly and analysis of ribosomal complexes

48S ribosomal complexes were assembled by incubation of 0.3 µg of a mixture of native α and β -globin mRNAs for 5 min at 37 °C in a reaction mixture that contained buffer (20 mM Tris, pH 7.5, 100 mM KAc, 2 mM DTT, 2.5 mM MgAc), 1 mM ATP, 0.4 mM GTP, 0.25 mM spermidine, 6 pmol native or transcript [³⁵S]Met-tRNA, 5 pmol 40S subunits, 2 µg eIF2, 6 µg eIF3, 2 µg eIF4A, 1 µg eIF4B, 1 µg eIF4F, 0.3 µg eIF1 and 0.3 µg eIF1A.

Primer extension (toe-printing) analysis of 48S complexes was done using the primer 5'-GCATTTGCAGAGGACAGG-3' (complementary to nt 177–194 of β -globin mRNA and AMV reverse transcriptase in the presence of [α -³²P]dATP (~6,000 Ci/mmol) as described (Pestova et al., 1998a). cDNA products were ethanol precipitated, resuspended, and analyzed by electrophoresis through 6% polyacrylamide sequencing gels. cDNA products were compared with an appropriate deoxynucleotide sequence ladder.

Methionyl-puromycin synthesis

For factor dependency experiments, reaction mixtures (40 µL) containing 3 pmol 40S subunits, 2 pmol transcript [³⁵S]Met-tRNA (specific activity 80,000 cpm/pmol), 0.1 mM GTP, 1 nmol AUG triplet, 2 µg eIF2, 5 µg eIF3, 0.5 µg eIF1, and 0.5 µg eIF1A were incubated at 37 °C for 5 min in buffer B (20 mM Tris, pH 7.5, 100 mM KAc, 2 mM DTT, 2.5 mM MgAc). The MgAc concentration in this reaction mixture containing preassembled 48S complexes was elevated to 5 mM after addition of 1 mM puromycin, 3 pmol 60S subunits, 0.3 µg eIF5 and 0.5 µg eIF5B, and incubation was continued at 37 °C for 30 min. Methionylpuromycin was then extracted and counted as described (Pestova et al., 1998b). For time course experiments, a reaction mixture (150 µL) containing 6 pmol transcript or native purified individual [³⁵S]Met-tRNA (specific activity 100,000 cpm/pmol), 20 pmol 40S subunits, 0.1 mM GTP, 1 nmol AUG triplet, 8 µg eIF2, 25 µg eIF3, 2 µg eIF1, and 2 µg eIF1A were incubated at 37 °C for 5 min in buffer B. The MgAc concentration was elevated to 5 mM and 1 mM puromycin, 20 pmol 60S subunits, 2 µg eIF5, and 2 µg eIF5B were added to the reaction mixture. Aliquots of 20 µL were taken after 2, 4, 6, 8, 15, and 30 min of incubation at 37 °C and methionylpuromycin was then extracted and counted as described (Pestova et al., 1998b).

ACKNOWLEDGMENTS

This work was supported by grant R01 GM59660 from the National Institute of General Medical Sciences to C.U.T.H. and T.V.P.

Received March 19, 2001; returned for revision May 18, 2001; revised manuscript received July 23, 2001

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