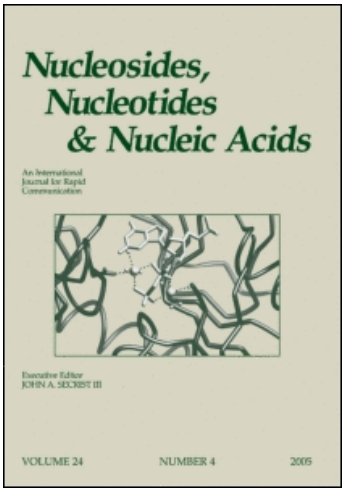

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Synthesis and Properties of an Initiation Codon Analog Consisting of 2'-O-Methyl Nucleotides

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SYNTHESIS AND PROPERTIES OF AN INITIATION CODON ANALOG CONSISTING OF 2'-O-METHYL NUCLEOTIDES

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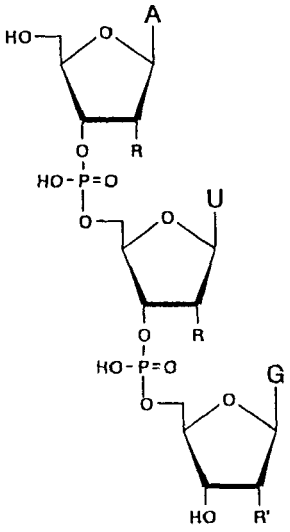
Abstract

An initiation codon analog consisting of 2'-O-methyl nucleotides (AmUmG) was synthesized and examined for its binding efficiency to *E. coli* ribosomes with fMet-tRNA^{fMet} and also its stability in binding assay systems for comparison with those of r(AUG) and d(ATG). AmUmG was found completely resistant to nucleases under the conditions used.

INTRODUCTION

2'-Modified RNAs may possibly be abundantly present in *in vivo* and *in vitro* systems due to their greater ability to resist nucleases than the corresponding RNAs and DNAs[1]. Recently, 2'-O-methyl-RNAs have come to be used as antisense oligonucleotides for inhibiting the human immunodeficiency virus[2] and for the antisense probing of U2[3] and U4/U6 snRNP[4]. Oligo(2'-O-methyl)ribonucleotides form stable duplexes with RNA and DNA fragments[5] and 2'-O-methyladenosine tetramer binds to ribosomes with their corresponding aminoacyl-tRNAs[6].

To apply 2'-O-methylated RNAs to protein synthesis systems in place of native mRNAs, an AUG fragment containing 2'-O-methyladenosine and 2'-O-methyluridine (AmUmG, Fig. 1) was first synthesized and then examined for its binding ability to *E. coli* ribosomes with fMet-tRNA, and ability to resist nuclease degradation which may possibly occur in the *in vitro* prokaryotic translation system.



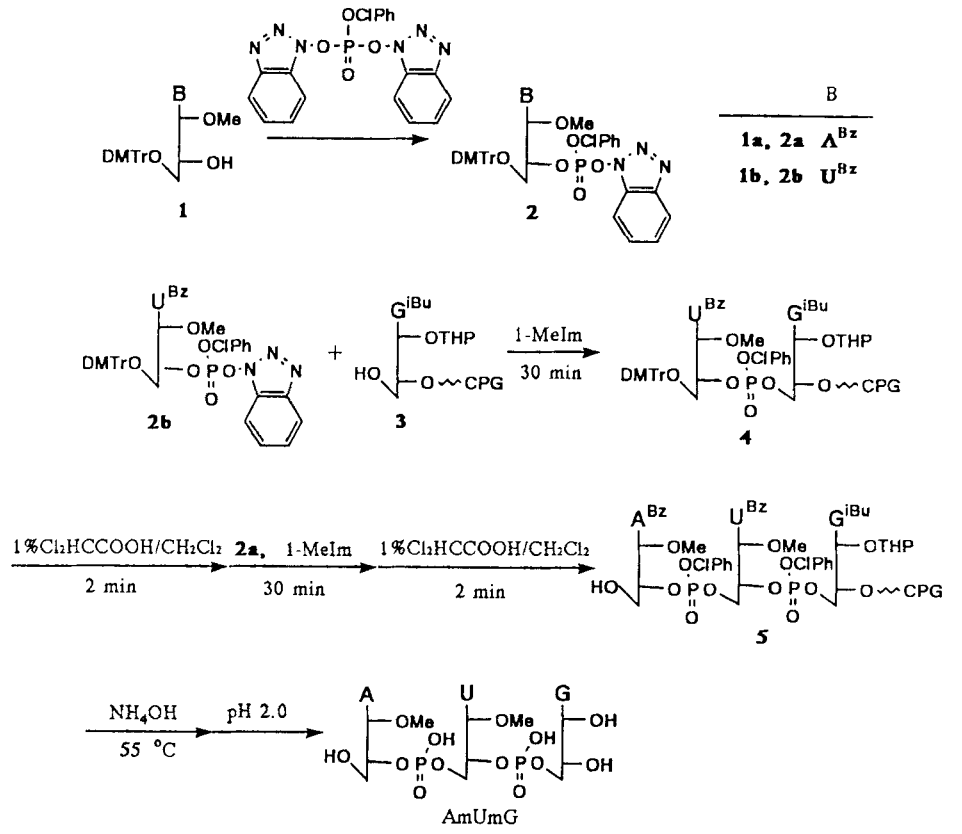
	R	R'
AmUmG	OMe	OH
r(AUG)	OH	OH
d(ATG)	H	H

FIG. 1. Chemical structures of AUG analogs, whose modifications are specified in the column. For d(ATG), U in the base moiety has been replaced by T.

Results and Discussion

Nucleoside units N^6-benzoyl-5'-O-dimethoxytrityl-2'-O-methyl-adenosine (**1a**) and N^3-benzoyl-5'-O-dimethoxytrityl- 2'-O-methyluridine (**1b**) were prepared according to the literature[5]. Compounds **1a** and **1b** were phosphorylated with 2-chlorophenyl-O,O-bis(1-benzotriazolyl) phosphate[7] in dioxane, and their phosphate derivatives (**2a** and **2b**) were directly used for coupling reactions. The synthesis was conducted as previously described (Scheme 1)[8,9] using a ZEON Genet manual DNA synthesizer (NIPPON ZEON Co., Ltd.). N^2-Isobutyryl-2'-O-tetrahydropyranylguanosine-bound-long-chain aminoalkyl-CPG[8] (63 mg, 1.63 μmol) was detritylated by 1% dichloroacetic acid in dichloromethane at 30°C. To the CPG-resin (**3**) in the reaction vessel was added **2** (0.06 mmol) in acetonitrile (0.25 ml) and 1-methylimidazole (0.025 ml) and the coupling reaction was carried out for 15 min at 30°C. The average coupling yield was 90%.

Following the synthesis, the protected AmUmG (**5**) was deprotected by treatment with conc. aqueous ammonia at 55°C for 14 h, and then with 0.01 M HCl (pH 2.0) for 21 h. The crude AmUmG was purified by reversed-phase C-18 high performance liquid chromatography (HPLC) (Fig. 2), and the fractions corresponding to the main peak were collected.



Scheme 1

The total yield of AmUmG (11 OD_{260} units) from guanosine-bound CPG resin was 21% as determined from the hypochromicity of AmUmG.

The purity of AmUmG was assessed as being almost 100%, in that the ^{32}p-labeled sample showed a single band on a 20% polyacrylamide - 7 M urea gel-electrophoresis (Fig. 3). The mobility of ^{32}p-labeled AmUmG was similar to that of labeled r(AUG) but less than that of labeled d(ATG).

AmUmG was subsequently examined for template activity in directing the binding of fMet-tRNA to ribosomes. Concentrations of the oligomers were determined from their hypochromicity which was estimated as the absorbance difference monitored at 260 nm between the oligomer and component mononucleosides from the complete degradation of each fragment with snake venom phosphodiesterase and bacterial phosphodiesterase. The hypochromicities of AmUmG, r(AUG) and d(ATG)

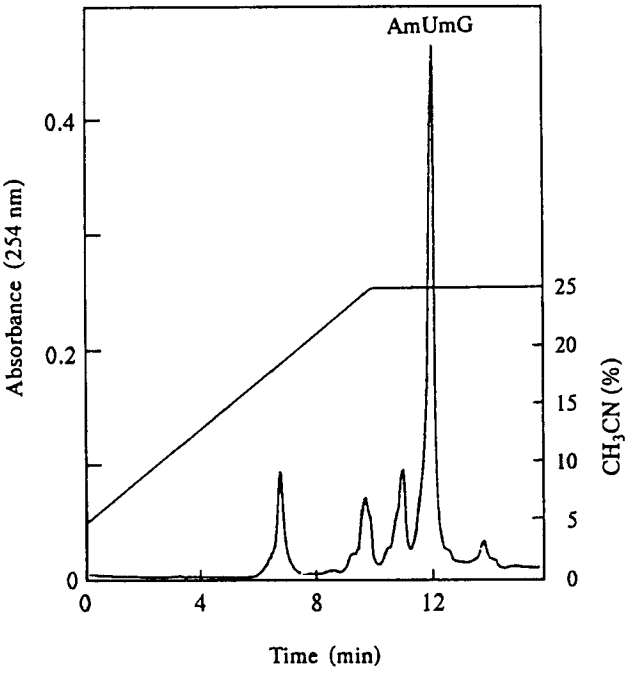


FIG. 2. Reversed-phase HPLC profile for purification and analysis of crude product mixture containig AmUmG.

were 8.96, 9.21 and 8.81%, respectively. Figure 4 shows the time dependent binding of ^{35}S-labeled fMet-tRNA to *E. coli* Q13 70S ribosomes in the presence of the initiation factors (IFs) directed by AmUmG, r(AUG) or d(ATG).

Although r(AUG) showed the most efficient binding, AmUmG also bound to an appreciable extent to the ribosomes.

With d(ATG), binding efficiency decreased with the time of incubation, possibly as a result of the enzymatic degradation of d(ATG) in the assay system. The stability of these fragments under the binding assay conditions was then examined (Fig. 5). 5'-^{32}P-Labeled fragments were analyzed by 20% polyacrylamide - 7 M urea gel-electrophoresis following incubation with 70S ribosomes and IFs of *E. coli* Q13. d(ATG) was completely digested in 1 h, and this would account for the decrease in the binding efficiency of d(ATG) with ribosome, as shown in Figure 4. In case of another strain *E. coli* S296, the degradation of r(AUG) was also observed, but no

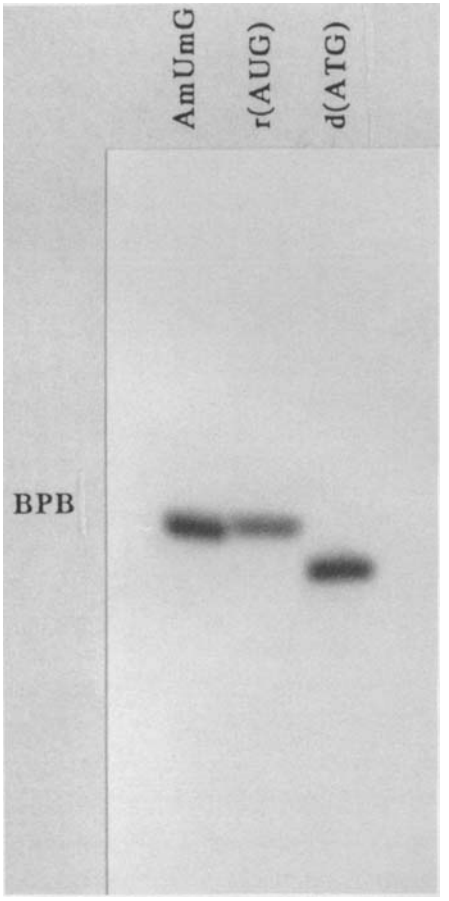


FIG. 3. 20% Polyacrylamide - 7 M urea gel-electrophoresis of ^{32}P-labeled AmUmG, r(AUG) and d(ATG).

degradation of AmUmG was observed (Data not shown). Furthermore, AmUmG was stable in the S-30 fraction (*E. coli* MRE600)(Amersham) for cell-free protein synthesis, but both r(AUG) and d(ATG) gave digested bands on 20% polyacrylamide - 7 M urea gel (Fig. 6), since the S-30 fraction contains more nucleases than isolated ribosomes and IFs.

While the binding efficiency of AmUmG with ribosome is less than that of r(AUG), AmUmG is completely resistant to nucleases in the translation system of *E. coli*. Price and Rottman[6] found the 2'-O-methyladenosine tetramer to be approximately 30-40% as active as

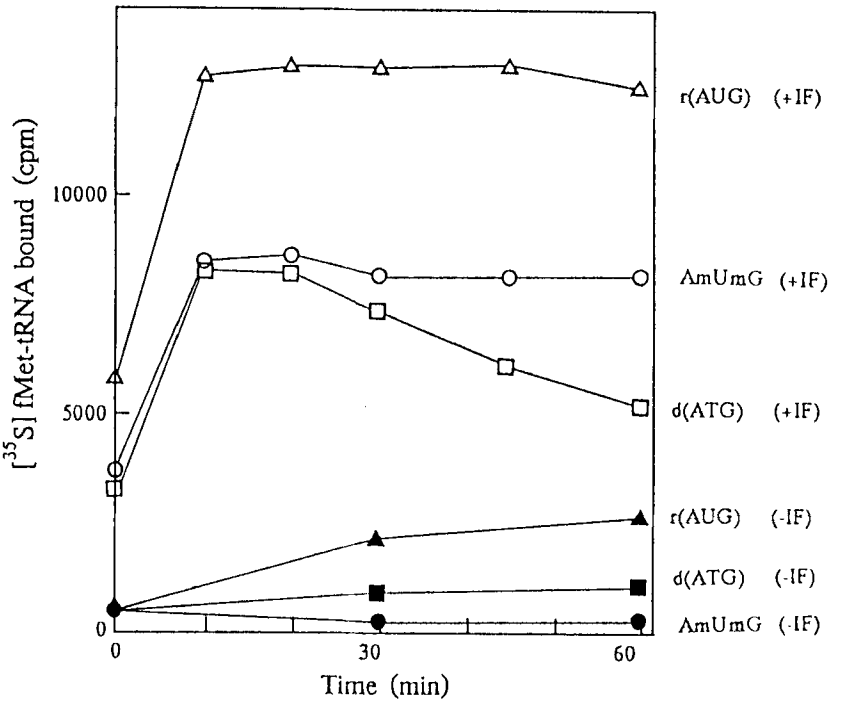


FIG. 4. Time dependence of the binding of AmUmG, r(AUG) and d(ATG) to ribosomes with fMet-tRNA in the presence or absence of IFs.

the corresponding adenosine tetramer for binding to ribosomes with lysyl-tRNA and the presence of a 2'-O-methyl residue to possibly not hinder the translational activity of the codon. An efficient system for protein synthesis can be constructed using 2'-O-methyl RNAs or modified RNAs whose 2'-O-methyl groups are partially introduced, for the purpose of enhancing both the stability and protein synthesis efficiency of mRNAs.

EXPERIMENTAL SECTION

The reagents and solvents were purified and dried by the method of van Boom *et al.*[10] and our own procedure[9].

HPLC was conducted by the Gilson gradient chromatography system with an M&S PACK C-18 column (4.6 mmID x 15 cm, M&S Co.) for reversed-phase HPLC, using an elution buffer that was a mixture of CH_3CN and 0.1 M triethylammonium acetate (pH 7.0).

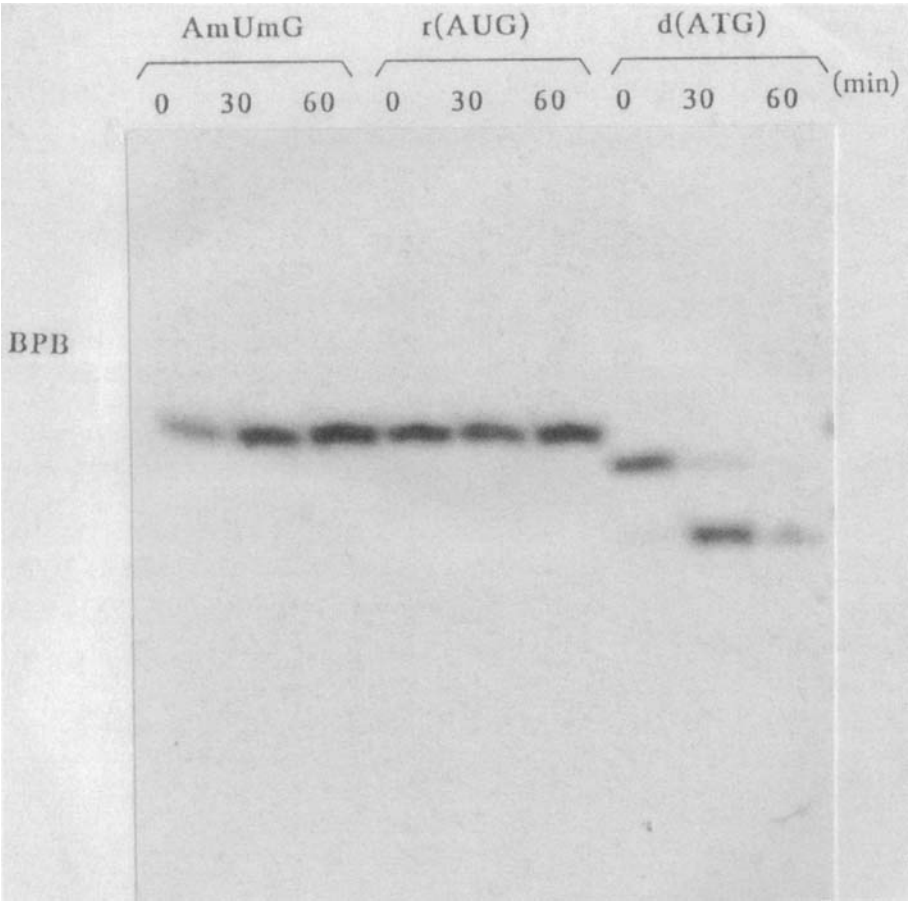


FIG. 5. Time dependent stability of AUG analogs, analyzed with 20% polyacrylamide - 7 M urea gel-electrophoresis. AmUmG, r(AUG) and d(ATG) were incubated at 37°C for 0, 30 and 60 min in the binding assay system of *E. coli* Q13.

Snake venom phosphodiesterase and calf intestine alkaline phosphatase were purchased from Boehringer mannhaim.

Synthesis of AmUmG

N^6-Benzoyl-5'-O-dimethoxytrityl-2'-O-methyladenosine (1a) (40.8 mg, 0.062 mmol) and N^3-benzoyl-5'-O-dimethoxytrityl-2'-O-methyluridine (1b) (43.0 mg, 0.065 mmol) were phosphorylated with 0.2 M 2-chlorophenyl-O,O-bis(1-benzotriazolyl) phosphate[10] (0.91 mol equiv.) in dioxane for 30 min at room temperature. A small amount of

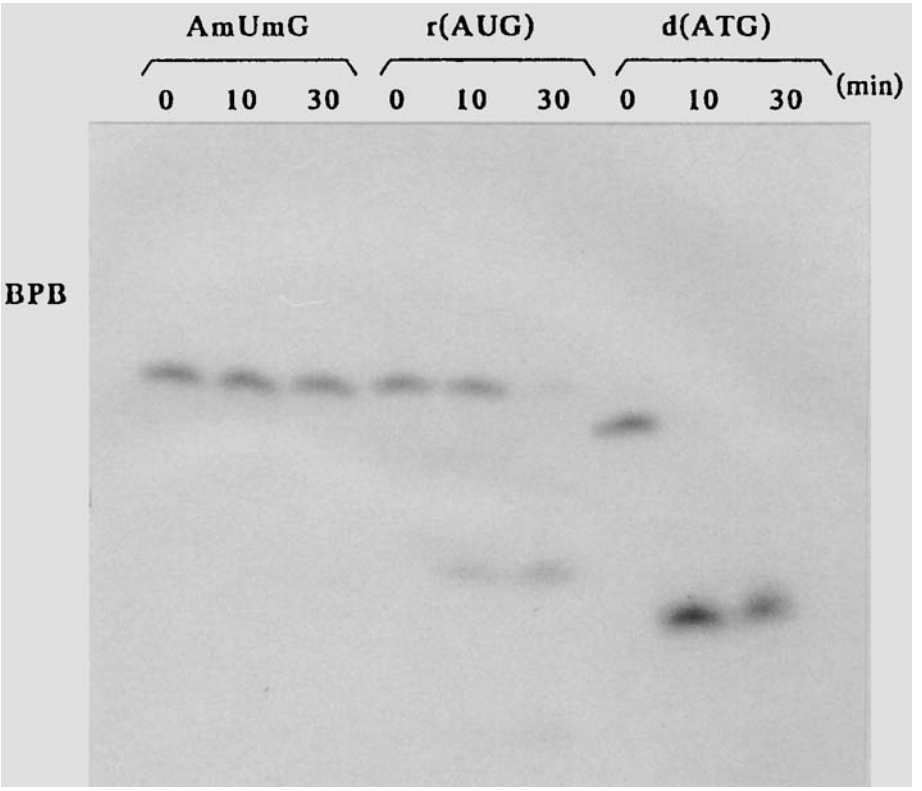


FIG. 6. Time dependent stability of AUG analogs, analyzed with 20% polyacrylamide - 7 M urea gel- electrophoresis. AmUmG, r(AUG) and d(ATG) were incubated at 37°C for 0, 10 and 30 min in the S-30 fraction of *E. coli* MRE600.

pyridine was then added to the mixture to neutralize the free 1-hydroxybenzotriazole[11], and the solvent subsequently evaporated. The residue (2) was dissolved in acetonitrile and which was directly used for the AmUmG synthesis. The trimer synthesis was conducted by our own method[11] using a ZEON Genet DNA manual synthesizer (NIPPON ZEON Co.) and LCA-CPG resin (63 mg, 1.63 μmol) derived from N^2-isobutyryl-2'-O-tetrahydropyranylguanosine. N-Acylated oligomer (5) was treated with conc. aqueous ammonia at 55°C for 14 h. The filtrate was treated with 0.01 M HCl (pH 2.0) for 21 h at room temperature. The crude AmUmG was then purified by reversed-phase C-18 HPLC. From the 1.63-micromole of the starting nucleoside, 11 OD_{260} units of purified AmUmG were obtained.

The purity of AmUmG was almost 100% as determined by 20% polyacrylamide - 7 M urea gel-electrophoresis of its 5'-labeled oligomer, as shown in Figure 2.

^{32}P-Labeling of oligomers and their polyacrylamide gel-electrophoresis

Oligomers were labeled at their 5'-terminus with [γ-^{32}P] ATP (Amersham) using T4-polynucleotide kinase (Boehringer)[11]. Each sample was directly electrophoresed on 20% polyacrylamide gel containing 7 M urea, 0.08 M Tris-phosphate and 0.002 M EDTA[12].

Binding assay

E. coli 70S ribosomes, IFs and unfractionated ^{35}S-labeled fMet-tRNA were prepared as previously described[13], except that the ribosomes were treated with bentonite[14]. The filter assay was carried out according to Nirenberg and Leder[15]. Each reaction mixture contained ribosomes (80 pmol), ^{35}S-labeled fMet-tRNA (80 pmol) and oligomer (2 nmol) in 50 mM Tris-HCl (pH 7.4), 0.1 M ammonium chloride, 15 mM magnesium acetate, 1 mM DTT, and 1 mM GTP in the presence (0.019 OD_{280} units) or absence of IFs. The mixture was incubated at 30°C. At a specified time, a portion of the reaction mixture (4 µl) was added to a buffer (0.8 ml) of 10 mM Tris-HCl (pH 7.4), 100 mM ammonium chloride and 10 mM magnesium acetate and then applied onto nitrocellulose filter (Millipore type HA, 0.45 µm, HAWPO2500). The filter was washed with the buffer (2 ml x 5), dried and then counted in toluene-based scintillation fluid with a scintillation counter (WALLAC 1216 RACKBETA, LKB Co.).

Labeled oligomer stability in the binding-assay system

^{32}P-labeled oligomer (1 nmol), ^{35}S-labeled fMet-tRNA (20 pmol), 70S ribosomes (20 pmol), and IFs (0.0049 OD_{280} units) were mixed with a buffer (16 µl) of 50 mM Tris-HCl (pH 7.4), 0.1 M ammonium chloride, 15 mM magnesium acetate, 1 mM DTT, and 1 mM GTP for the binding assay, followed by incubating the mixture at 30°C. At a specified time, a portion of the mixture (4 µl) was added to a dye containing 10 M urea (4 µl) and analyzed by 20% polyacrylamide gel electrophoresis containing 7 M urea. Stability in an *E. coli* S-30 fraction was

assessed with the S-30 fraction (2µl) of *E. coli* MRE600 (Amersham Prokaryotic DNA-directed translation kit) instead of 70S ribosomes and IFs in the buffer (16 µl).

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