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Mechanism of inhibition of eukaryotic translational initiation by the trinucleotide ApUpG

Thomas Wagner*, Paul B. Sigler+ and Martin Gross†

Departments of Biochemistry & Molecular Biology and †*Pathology, The University of Chicago, 920 E. 58th Street, Chicago, IL 60637, USA*

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ApUpG, the oligoribonucleotide homologous to the initiation codon, as well as the tetranucleotides ApUpGpA and ApUpGpG block initiation of protein synthesis in the rabbit reticulocyte lysate. These oligonucleotides are recognized as translational initiation sites by the ribosomes, leading to a very large accumulation of complete, but inactive, 80 S initiation complexes, containing methionylated initiator tRNA and ApUpG in a 1:1 stoichiometry. ApUpG appears to inhibit by competing with endogenous globin mRNA for 80 S ribosomal couples, since the inhibition of protein synthesis by ApUpG can be largely relieved by increasing the globin mRNA. The 80 S · Met-tRNA$_i^{Met}$ · ApUpG complexes are not formed in the absence of hemin, demonstrating that their formation requires the active recycling of eukaryotic initiation factor 2. In addition the trinucleotide correctly directs the Met-tRNA$_i^{Met}$ into the ribosomal donor site, since the methionyl residue is puromycin-reactive.

Reticulocyte lysate; Protein synthesis; Initiation codon; Oligoribonucleotide; 80 S complex

1. INTRODUCTION

The translation of eukaryotic mRNAs generally starts at the 5′-proximal AUG codon [1]. The start codon is selected according to secondary structure in the 5′-flanking region of the mRNA and sequence context of the respective AUG initiation codon [2]. In the selection process the methionylated initiator tRNA first forms a ternary complex with eukaryotic initiation factor 2 (eIF-2) and GTP [3], which binds to the 40 S ribosomal subunit [4,5]. This ribosomal preinitiation complex scans the mRNA until it encounters the initiating AUG [2]. Upon its interaction with the anticodon of the initiator tRNA, eIF-2 · GDP dissociates and the 60 S ribosomal subunit joins resulting in a complete 80 S initiation complex [4,5] now ready for the binding of an elongator aminoacyl-tRNA.

Previous studies have shown that the addition of the oligoribonucleotide ApUpG to a cycloheximide-inhibited rabbit reticulocyte lysate causes a shift from 40 S-associated initiation complexes to 80 S initiation complexes [6,7]. This suggests that the trinucleotide ApUpG can be recognized by the ribosomal subunits as a start site for translation and leads to the formation of complete initiation complexes. We recently reported that of a large number of oligoribonucleotides tested, only those containing the sequence C_pA inhibit protein synthesis in the rabbit reticulocyte lysate and that the inhibition is at the level of polypeptide chain elongation [8]. In this report we will demonstrate that ApUpG and two analogous tetranucleotides also inhibit protein synthesis in the rabbit

Correspondence address: M. Gross, Dept of Pathology, The University of Chicago, 5841 S. Maryland, Chicago, IL 60637, USA

* *Present address:* Enichem SpA, Laboratori di Biotecnologia, Via E. Ramarini 32, 00015 Monterotondo, Italy

+ *Present address:* Yale University and The Howard Hughes Medical Institute, Department of Molecular Biophysics and Biochemistry, 260 Whitney Avenue, JWG 402, New Haven, CT 06511, USA

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reticulocyte lysate, but at the level of polypeptide chain initiation.

2. EXPERIMENTAL

2.1. *Synthesis and purification of oligoribonucleotides*

The trinucleoside diphosphate ApUpG was synthesized and purified as described [8]. Radiolabeled ApUp[^{3}H]G was obtained using [^{3}H]GDP (Amersham), specific activity 5 Ci/mmol. The tetranucleotides ApUpGpA and ApUpGpG were synthesized in reaction mixtures (0.15 ml) containing 50 mM Hepes·KOH, pH 8.3, 20 mM $MgCl_2$, 3 mM DTT, 5 mM ATP, 3 mM ApUpG, 5 mM adenosine or guanosine 3′,5′-diphosphate (Sigma) and 240 U/ml of T4 RNA ligase (BRL) by incubating at 4°C for 14 h. After adding 1/10 volume of 1 M ammonium acetate, pH 9, and 10 U/ml of bacterial alkaline phosphatase (Sigma), the mixtures were further incubated at 37°C for 1 h. The oligoribonucleotides were purified by reverse phase HPLC and characterized by their nucleoside composition according to published procedures [8,9].

2.2. *Cell-free protein synthesis and sucrose gradient analysis*

Protein synthesis in rabbit reticulocyte lysate was determined by the incorporation of L-[^{14}C]leucine (10 cpm/pmol, 0.3 mM) into acid precipitable macromolecules and the relative amounts of ribosomal complexes formed were quantitated by separating them on sucrose density gradients as described elsewhere [10]. Specifically, all incubations were at 34°C, and, unless otherwise indicated, hemin was added at a final concentration that was optimal for protein synthesis (20 μM). When samples were analyzed on sucrose gradients or by the puromycin reaction (see below) nonradioactive L-leucine replaced L-[^{14}C]leucine. Globin mRNA was purified from rabbit reticulocyte ribosomes as described [11].

2.3. *Puromycin reaction*

Standard rabbit reticulocyte lysate protein synthesizing mixtures (55 μl), received rabbit reticulocyte [^{35}S]Met-tRNA$_i^{Met}$ (1.25×10^5 cpm/pmol, final concentration 60 nM) after 10 min of incubation and puromycin dihydrochloride (Sigma, 0.32 mM final concentration) after 10.5 min. Aliquots (15 μl) were removed at the indicated times after the puromycin addition and diluted into 0.9 ml of 0.1 M potassium phosphate, pH 8. The dilutions were extracted with 3 ml of ethyl acetate at room temperature and 2 ml of the extracts were mixed with 8 ml of Aquasol (NEN, New Brunswick, MA) to measure their content of radioactivity.

3. RESULTS

3.1. *Short oligoribonucleotides analogous to the initiation codon inhibit protein synthesis*

At a concentration of 200 μM the oligoribonucleotides ApUpG, ApUpGpG and ApUpGpA strongly inhibit translation in the rabbit reticulocyte lysate, with ApUpG being somewhat less inhibitory than the tetranucleotides and ApUpGpA having the most marked effect (fig.1). The degree of inhibition approaches that observed when the lysate is incubated in the absence of hemin, but, unlike hemin deficiency, these oligoribonucleotides inhibit immediately upon the start of incubation (fig.1). The effect is highly sequence specific, since a 200 μM concentration of a large number of other tri- and tetranucleotides (except those containing the sequence CpA) has no effect on protein synthesis in this system [8].

Dose dependence experiments with ApUpG showed that inhibition approaches a maximum of about 50% between 100 and 200 μM during the first 20 min of incubation (fig.2). With longer incubation, however, the inhibitory effect of ApUpG is quite different. At 20–90 min, up to 50 μM ApUpG causes little change, while 200 μM ApUpG inhibits protein synthesis by about 80%. The transient inhibition seen at low concentrations of ApUpG is presumably due to the action of a rabbit reticulocyte 3′-exonuclease affecting short oligoribonucleotides as demonstrated earlier [8]. With small amounts of ApUpG and given enough time, this enzyme would convert most of the trinucleotide into the noninhibitory dinucleotide ApU and GMP.

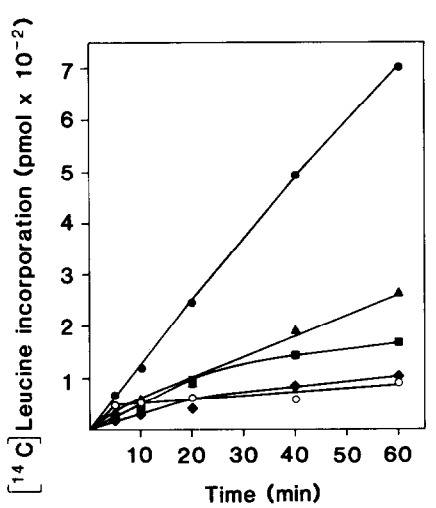

Fig.1. Inhibition of protein synthesis by oligoribonucleotides homologous to the initiation codon. Cell-free samples (55 μl) were incubated in the absence of hemin (○) or in the presence of hemin plus no further addition (●), 200 μM ApUpG (▲), 200 μM ApUpGpG (■), or 200 μM ApUpGpA (◆). Aliquots (10 μl) were removed after the indicated times, and the acid precipitable radioactivity was determined.

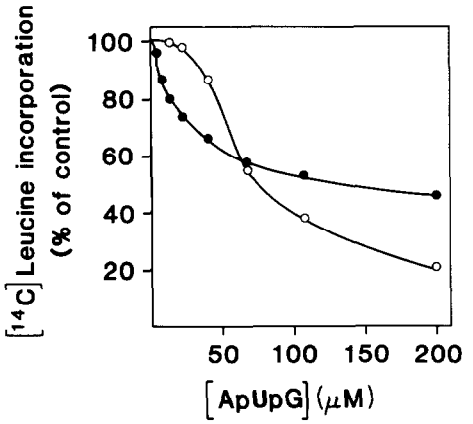

Fig.2. Dose dependence of the inhibition of protein synthesis by ApUpG. Reaction mixtures (55 μl) were incubated in the presence of increasing concentrations of ApUpG. The acid precipitable radiolabeled macromolecules (10 μl aliquots) were quantified between 0 and 20 min (closed circles) and 20 and 90 min (open circles).

We tested whether the ApUpG-mediated inhibition is influenced by the level of mRNA. We found that supplementing protein synthesizing lysate with purified globin mRNA largely overcomes the inhibitory effect of 100 μM ApUpG (table 1). The optimal reversing concentration of exogenous mRNA is about equal to that present in the lysate. This result suggests that ApUpG may inhibit by competing with the endogenous mRNA for one or more components of the translation system.

Table 1

Effect of additional globin mRNA on the translational inhibition by ApUpG

Addition	Protein synthesis	
	nmol Leu/ ml of lysate	% of control
None	134	100
ApUpG	77	57
ApUpG + mRNA (23 nM)	114	85
ApUpG + mRNA (40 nM)	122	91
mRNA (40 nM)	138	103

Duplicate cell-free samples, containing 100 μM ApUpG (where added) and the indicated final concentrations of exogenous globin mRNA, were incubated in the presence of hemin in a final volume of 44 μl. The incorporation of L-[^{14}C]leucine into protein was determined after 90 min

3.2. *Complete 80 S ribosomes containing stoichiometric amounts of ApUpG and methionylated initiator tRNA accumulate*

Sucrose density gradient analysis of reticulocyte lysate, in which protein synthesis was inhibited by the addition of ApUpG, ApUpGpA or ApUpGpG, revealed that (as expected) these oligoribonucleotides act at the level of polypeptide chain initiation, since the polyribosomes become partially disaggregated. The inhibition manifests itself as a 20-fold increase in 80 S initiation complexes, containing bound [^{35}S]Met-tRNA$_i^{Met}$, when compared to the noninhibited protein synthesizing lysate (fig.3). Analysis of alkali-treated, gradient purified 80 S-bound radiolabeled material [10] from reticulocyte lysate containing ApUpG demonstrated that it is entirely in the form of methionine as opposed to methionyl-peptide (data not shown). When the lysate was incubated with L-[^{35}S]methionine and ApUp[^{3}H]G, we found that the 80 S complexes that accumulate contain Met-tRNA$_i^{Met}$ and ApUpG in approximately stoichiometric amounts (fig.4). Furthermore, the addition of the cap analogue, 7-methyl-GTP, which inhibits the ribosomal binding of globin mRNA [12], increases the formation of 80 S·Met-tRNA$_i^{Met}$·ApUpG complexes about 1.3-fold (table

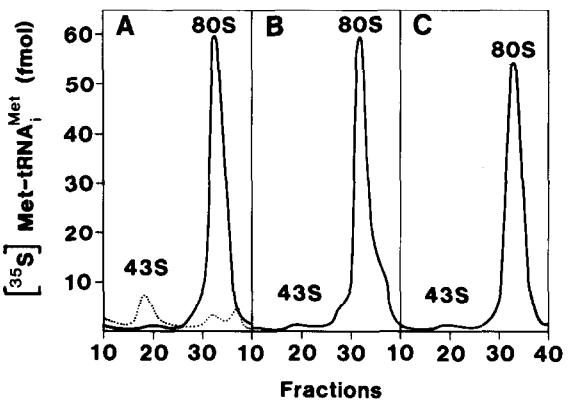

Fig.3. Formation of ribosomal complexes in the presence of oligoribonucleotides homologous to the initiation codon. Cell-free samples (55 μl) contained 240 μM of ApUpG (A), ApUpGpA (B) or ApUpGpG (C). For comparison, a sample without oligoribonucleotide is shown in panel A (dotted line). After 15 min all reaction mixtures received rabbit reticulocyte [^{35}S]Met-tRNA$_i^{Met}$ (3.38 × 10^5 cpm/pmol, 40 nM final concentration) and were further incubated for 30 s. The ribosomal complexes were separated on sucrose density gradients.

Table 2

ApUpG-induced formation of 80 S initiation complexes

Additions	[^{35}S]Met-tRNA$_i^{Met}$ bound (pmol/ml lysate)
ApUpG	41.6
ApUpG + 7-methyl-GTP	54.5
ApUpG + tRNA	65.1
ApUpG + 7-methyl-GTP + tRNA	83.6

The final concentrations were: 200 μM ApUpG, 1.1 mM 7-methyl-GTP and 0.5 mg/ml reticulocyte tRNA. The amount of [^{35}S]Met-tRNA$_i^{Met}$ bound to 80 S complexes was determined as indicated in fig.4A

2). This suggests that ApUpG competes with endogenous mRNA for 80 S ribosomes, since while initiation programmed by globin mRNA is sensitive to 7-methyl-GTP, that by ApUpG is not. In addition, the accumulation of ApUpG-induced 80 S complexes is so massive that the amount formed may be limited by the availability of endogenous Met-tRNA$_i^{Met}$. This was indicated by the fact that the addition of exogenous reticulocyte tRNA increases the formation of these complexes 1.5-fold in the absence or presence of 7-methyl-GTP (table 2). We estimate that about 25% of the

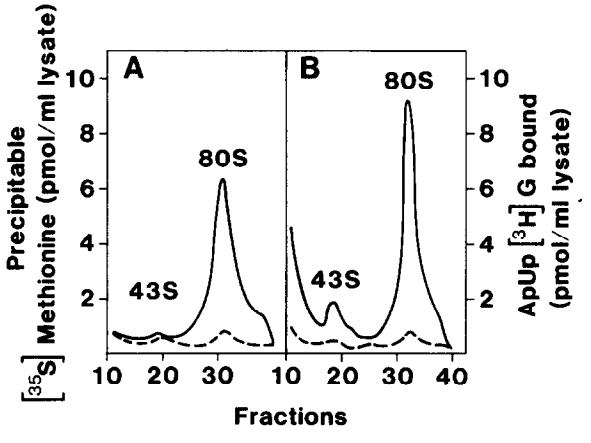

Fig.4. Stoichiometry of ApUpG and Met-tRNA$_i^{Met}$ in ribosomal complexes. Cell-free samples contained L-[^{35}S]methionine (1.75×10^4 cpm/pmol) and 200 μM ApUpG in a final volume of 132 μl (panel A) or 115 μM ApUp[^{3}H]G (1.13×10^3 cpm/pmol) in a final volume of 88 μl (panel B). After 10 min of incubation the ribosomal complexes were separated on sucrose density gradients, and the bound radiolabel was determined. Profiles from samples without added hemin are depicted by the dashed lines.

Table 3

Puromycin reactivity of [^{35}S]Met-tRNA$_i^{Met}$

Additions	Ethyl acetate extractable radioactivity (cpm)
+ Hemin	486
+ Hemin, + ApUpG	2020
− Hemin	186
− Hemin, + ApUpG	28

The final concentration of ApUpG was 200 μM and additional details are given in section 2

endogenous Met-tRNA$_i^{Met}$ becomes bound to these 80 S complexes. Finally the accumulation of 80 S·Met-tRNA$_i^{Met}$·ApUpG complexes does not occur in the absence of hemin (fig.4), demonstrating that their formation requires the proper recycling of active eIF-2 [13].

3.3. *The trinucleotide ApUpG directs Met-tRNA$_i^{Met}$ correctly into the ribosomal P-site*

The amount of [^{35}S]Met-tRNA$_i^{Met}$ bound to the P-site of ribosomes was determined by measuring its reactivity toward puromycin, i.e. the formation of [^{35}S]methionylpuromycin. There is more than a 4-fold increase of this product when polypeptide synthesis in the reticulocyte lysate is inhibited by ApUpG, as compared to the noninhibited control (table 3). Hence, in the presence of the trinucleotide, the methionylated initiator tRNA is correctly positioned in the ribosomal P-site. When ApUpG is added to a hemin-deficient lysate, the radiolabeled puromycin adduct formed is even less than in the noninhibited control, which correlates well with the inability of the lysate to accumulate ApUpG-induced, 80 S complexes under these conditions. The data in table 3 also demonstrate that in the absence of ApUpG there is also considerably less puromycin reactive [^{35}S]Met-tRNA$_i^{Met}$ in the absence than in the presence of hemin, despite the fact that the level of 80 S-bound Met-tRNA$_i^{Met}$ is much greater in the former case [10].

4. DISCUSSION

We have demonstrated that the oligoribonucleotide ApUpG inhibits protein synthesis in the rabbit reticulocyte lysate at the level of

polypeptide chain initiation. The effect is highly sequence-specific, since a large number of other oligoribonucleotides (including ApUpC, ApUpCpC, ApUpCpG., GpApU and GpApUpC) have no inhibitory effect on protein synthesis in this system [8]. As we reported [8] oligoribonucleotides containing the dinucleotide CpA do inhibit protein synthesis, but at the level of polypeptide chain elongation.

We have also tested the effect of the 3′-purine-extended tetranucleotides ApUpGpG and ApUpGpA in this system. If inhibition occurs via complementation of these oligonucleotides to the anticodon of initiator tRNA, thus rendering Met-$tRNA_i^{Met}$ unavailable for proper codon-anticodon interaction with the endogenous mRNA, we expect ApUpGpG to be more inhibitory than ApUpGpA, since the former will form a potential extra base pair in the exposed anticodon loop of the reticulocyte initiator tRNA. This will involve base pairing of the 3′-guanosine of the tetranucleotide with the 5′-cytidine adjacent to the anticodon of the endogenous initiator tRNA. The effect is opposite since ApUpGpA is a more potent inhibitor than ApUpGpG, and therefore, the mechanism of inhibition must be different.

Our results strongly suggest that inhibition by ApUpG, ApUpGpG or ApUpGpA, is mediated by sequestering 80 S ribosomes into deadlocked 80 S initiation complexes, thus rendering the ribosomes unavailable for translating endogenous mRNA. The complexes contain one Met-$tRNA_i^{Met}$ per bound ApUpG, indicating that the initiating triplet alone can serve as an abortive initiation signal. The Met-$tRNA_i^{Met}$ is enzymatically bound to these complexes, i.e. with the active participation of eIF-2, since they do not accumulate under conditions of hemin deficiency, where eIF-2 becomes phosphorylated and its recycling is prevented [14].

The release of an aminoacyl- or peptidyl-residue covalently linked to a tRNA via product formation with puromycin serves as a measure of the extent of binding to the ribosomal P-site of the respective tRNA-species. The increased puromycin reactivity of the methionyl residue attached to the initiator tRNA correlates with the observed build-up of 80 S initiation complexes when ApUpG is present in the reticulocyte lysate and shows that Met-$tRNA_i^{Met}$ is primarily bound to the P-site of the ribosomes. The failure of these complexes to form when the system is made hemin-deficient at the same time correlates well with the lack of methionyl-puromycin formation under the same conditions.

The trinucleotide ApUpG is recognized by the eukaryotic ribosomes as a translational initiation site, indicating that it may be a sufficient determinant for the selection of the start site on mRNAs in eukaryotic protein synthesis. However, ApUpG promotes initiation far less efficiently than natural mRNA (about 1/2000 as well), since approximately 100 μM ApUpG is required to inhibit protein synthesis by 50% in reticulocyte cell-free reactions containing about 50 nM endogenous globin mRNA. In addition, supplementing the reaction mixture with a relatively modest amount of exogenous globin mRNA can largely reverse the inhibitory effect of ApUpG. Thus, flanking sequences about the initiating ApUpG in natural mRNA are clearly very important for its translational efficiency [15]. Nevertheless, the ApUpG-containing oligoribonucleotides generated after micrococcal nuclease treatment of reticulocyte lysate [16] as noted by Kay and Benzie [17] or the addition of a high level of pure ApUpG, as noted here, can interfere with polypeptide chain initiation on natural mRNA.

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