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Evidence for a Competitive-Displacement Model for the initiation of protein synthesis involving the intermolecular hybridization of 5 S rRNA, 18 S rRNA and mRNA

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We have previously shown that a 5'-terminal region of mouse 5 S rRNA can base-pair in vitro with two distinct regions of 18 S rRNA. Further analysis reveals that these 5 S rRNA-complementary sequences in 18 S rRNA also exhibit complementarity to the Kozak consensus sequence surrounding the mRNA translational start site. To test the possibility that these 2 regions in 18 S rRNA may be involved in mRNA binding and translational initiation, we have tested, using an in vitro translation system, the effects of DNA oligonucleotides complementary to these 18 S rRNA sequences on protein synthesis. Results show that an oligonucleotide complementary to one 18 S rRNA region does inhibit translation at the step of initiation. We propose a Competitive-Displacement Model for the initiation of translation involving the intermolecular base-pairing of 5 S rRNA, 18 S rRNA and mRNA.

Translation; Ribosome; Intermolecular RNA:RNA hybridization; rRNA; mRNA

1. INTRODUCTION

The detailed molecular mechanisms involved in the initiation of eukaryotic protein synthesis remain unknown. The Kozak scanning hypothesis [1,2] proposes that the ribosome scans a capped mRNA from the 5' end and recognizes a consensus sequence surrounding the bona fide translational Met start codon. The scanning hypothesis implicitly assumes the existence of some mechanism by which the 40 S subunit can read the mRNA sequence and recognize the correct sequence for initiation. Intermolecular base-pairing of this Kozak mRNA consensus sequence with a complementary sequence of 18 S rRNA in the 40 S ribosomal subunit could provide the mRNA alignment necessary for accurate and efficient initiation of translation. Previous analysis has defined two 5 S rRNA-complementary sequences in 18 S rRNA which base-pair to a conserved sequence found in the 5'-terminal region of 5 S rRNA [3]. Further analysis presented here reveals complementarity of these 18 S rRNA sequences to the Kozak consensus scanning sequence. In vitro protein synthesis in the presence of a DNA oligonucleotide complementary to one of these 5 S rRNA-complementary sequences of 18 S rRNA inhibits protein synthesis suggesting the utilization of this region of 18 S rRNA in the scanning of mRNA and the initiation of protein synthesis. We therefore propose a Competitive-Displacement Model which postulates that a sequential series of intermolecular RNA:RNA base-pairing interactions involving 5 S rRNA, 18 S rRNA and mRNA provide a mechanistic basis for events important in the initiation of protein synthesis.

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2. MATERIALS AND METHODS

2.1. *Accessibility of ribosomes and ribosomal subunits to DNA oligonucleotides*

Pelleted polyribosomes were prepared from mouse ascites cells as previously described [3] and stored at −80°C. Polyribosome pellets were resuspended at 4°C in 40 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, and 70 mM KCl (Buffer A). Aliquots containing 5 A_{260} units of resuspended polyribosomes were diluted to 196 μl with Buffer A and dissociated into subunits by the addition of 4 μl of 0.5 M EDTA to a final concentration of 10 mM [4]. Dissociated subunits were then incubated with 50 000–100 000 cpm's (3×10^7 cpm/μg) of various 5' end-labeled DNA oligonucleotides [5] at 4°C for 1.5 h. Each sample was then loaded onto a pre-chilled 5–30% linear sucrose gradient containing Buffer A with 10 mM EDTA and run for 7 h at 35 000 rpm in a Beckman SW40 ultracentrifuge rotor at 4°C. Gradients were fractionated and analyzed spectrophotometrically using a UV monitor. Fractions of 0.4 ml were collected and the radioactivity determined by scintillation counting.

2.2. *In vitro translation*

Rabbit globin mRNA was translated in a rabbit reticulocyte lysate in vitro translation system (Bethesda Research Laboratories) for 40 min following procedures provided by the manufacturer. Protein synthesis was measured by incorporation of [^{3}H]leucine into TCA-precipitable protein. Reactions analyzing oligonucleotide inhibition of translation contained 1–10 μg of DNA oligonucleotide added prior to addition of globin mRNA or poly(A)$^+$ RNA (0.1 μg/20 μl reaction mixture). Poly(A)$^+$ RNA was isolated from mouse ascites cells as previously described [6,7].

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2.3. Inhibition of mRNA binding to 40 S subunits by DNA oligonucleotides

In vitro translation reactions (30 μl total volume) were prepared as above except that NaF was added to a concentration of 10 mM to allow detection of the 48 S pre-initiation complex [8]. Reaction mixtures were then incubated at 30°C for 5 min before the addition of 10 μg of DNA oligonucleotide followed by addition of isolated 3′ end-labeled [9] globin mRNA (0.05–0.1 μg/30 μl reaction mixture). After incubation for an additional 15 min. at 30°C, reactions were stopped by dilution to 200 μl with ice-cold Buffer A. Reaction components were then resolved on sucrose gradients and fractionated as described above.

3. RESULTS

Previous work in our laboratory [3] has demonstrated the in vitro hybridization of a conserved, 5′-terminally located sequence in 5 S rRNA to two evolutionarily conserved and complementary sequences in 18 S rRNA (see Fig. 1). While the biological function of these 5 S rRNA:18 S rRNA hybrids is uncertain at present, further inspection of these 18 S rRNA sequences has recently revealed that they are also complementary to the Kozak scanning sequence [1,2] surrounding the eukaryotic mRNA translation start site (see Fig. 1). To test the possible involvement of these 18 S rRNA sequences in mRNA translation events, we synthesized DNA oligonucleotides complementary to both 18 S rRNA sequences 1 and 2 and then probed the ribosome to assess the accessibility of these sequences in the 40 S subunit. Experiments revealed that both 18 S rRNA sequences 1 and 2 were accessible to hybridization with the complementary DNA probes (Fig. 2). However, sequence 1 hybridized very strongly with its complementary 18 S rRNA sequence suggesting a very exposed location on the surface of the 40 S subunit. Oligonucleotide hybridization to only the 40 S subunit demonstrated the specificity of each oligonucleotide probe. This specificity was confirmed in oligonucleotide competition experiments with cold homologous and heterologous probes as well as RNase H-digestion of the oligonucleotide:18 S rRNA hybrid and sequencing of the generated 18 S rRNA fragments (data not shown). Control oligonucleotides P1 and P2 [3], used in the primer extension sequencing/ mapping of RNase H-digested oligonucleotide:18 S rRNA hybrids are complementary to sequences approximately 50 nucleotides downstream of 18 S rRNA sequences 1 and 2. The ability of oligonucleotide P1 to also strongly hybridize to the 40 S subunit indicated that this region of 18 S rRNA was very exposed on the subunit surface. The inability of either the sequence 1 complementary or P1 oligonucleotide probe to hybridize with the 80S ribosomal couple suggested that these exposed 18 S se-

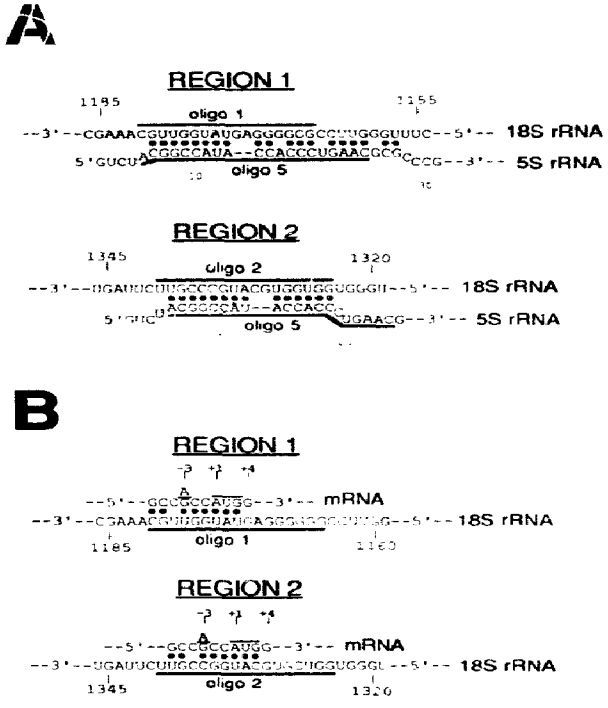

Fig. 1. Structure of 5 S rRNA:18 S rRNA and mRNA:18 S rRNA hybrids. (A) The previously defined [3] hybrid structures between the 5′-terminal sequence of 5 S rRNA and Region 1 and Region 2 sequences of 18 S rRNA. (B) Theoretical hybrid structures that can be drawn between Region 1 or Region 2 of 18 S rRNA and the experimentally-defined optimal translation start site sequence of eukaryotes [1]. The numbers −3, +1, and +4 above the mRNA sequence indicate the position of these nucleotides relative to the A residue of the overlined AUG initiation codon. In both panels, solid lines juxtaposed to the 5 S and 18 S rRNA sequences indicate synthetic complementary DNA oligonucleotides used in these studies.

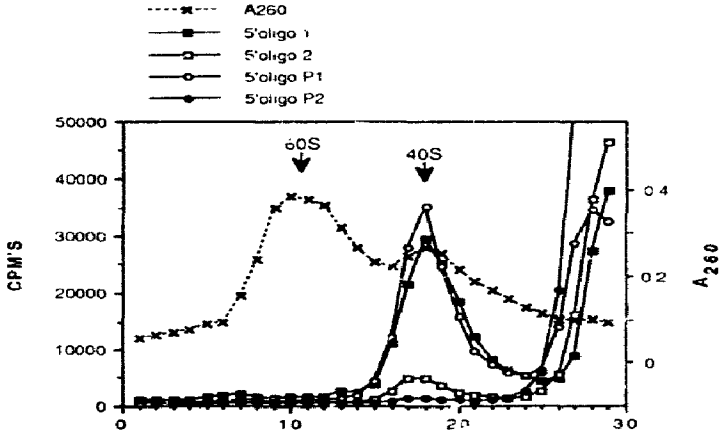

Fig. 2. Accessibility of 18 S rRNA Regions 1 and 2 in the 40 S subunit to complementary oligonucleotide probes. 60 S and 40 S subunits generated by EDTA dissociation were probed with radiolabeled DNA oligonucleotides 1 (Fig. 1), 2 (Fig. 1), P1 (complementary to 18 S rRNA nucleotides 1247–1264) and P2 (complementary to 18 S rRNA nucleotides 1424–1440), resolved on sucrose gradients, and the A_{260} and cpm's of each fraction measured. The positions of 60 S and 40 S subunits in the gradient profiles are indicated by arrows.

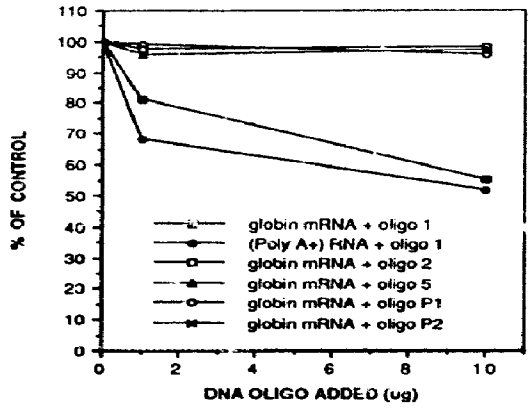

Fig. 3. Specific inhibition of in vitro translation by oligonucleotide 1. In vitro translation reactions using rabbit globin mRNA were carried out in the absence or presence of varying amounts of DNA oligonucleotides 1, 2, 5 (see Fig. 1), P1 and P2, or using mouse poly(A)$^+$ RNA in the absence or presence of oligonucleotide 1. Protein synthesis in each reaction was measured by incorporation of [^{3}H]leucine and was plotted as the percentage of control translation (no oligonucleotide added).

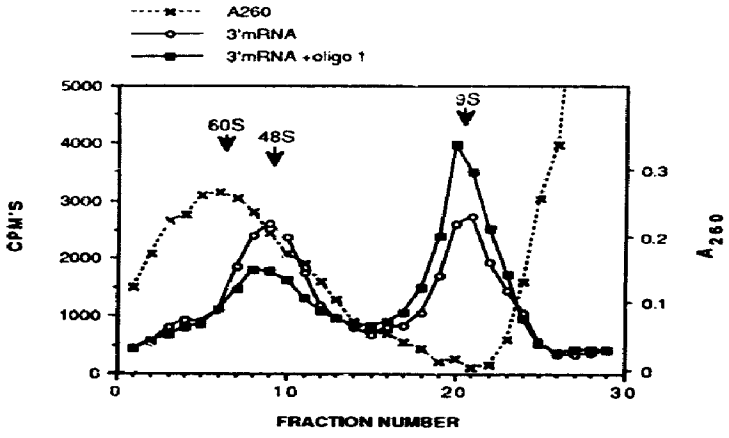

Fig. 4. Specific inhibition of mRNA binding to the 40 S ribosomal subunit by oligonucleotide 1. Rabbit globin mRNA radiolabeled at the 3' end was added to in vitro translation reactions in the absence or presence of 10 μg of oligonucleotide 1. Each reaction contained 10 mM NaF which prevents the 60 S subunit from joining the 40 S subunit, allowing detection of the 48 S preinitiation complex. After incubating at 30°C for 15 min the components of the translation reactions were resolved on sucrose gradients. The gradients were fractionated and the amount of labeled globin mRNA in the 48 S preinitiation complex was determined by scintillation counting. The position of the 60 S subunit, 48 S pre-initiation complex, and free 9 S rabbit globin mRNA are indicated by arrows.

quences were located on the interface between the 40 S and 60 S subunits (data not shown).

The effect of these oligonucleotides on translation was then assessed using an in vitro translation system. Oligonucleotide 1, complementary to 18 S rRNA sequence 1, specifically inhibited the translation of both rabbit globin and total mouse poly(A)$^+$ mRNA (Fig. 3). All other oligonucleotides, including some not indicated here, had no effect on the translation of either rabbit globin or mouse poly(A)$^+$ mRNA. The observed inhibition of translation was not due to oligonucleotide 1 hybridization with added mRNA since neither rabbit α- or β-globin mRNA sequences contain regions of complementarity with this specific oligonucleotide. Additionally, the inhibitory effect of oligonucleotide 1 was most pronounced when it was added to the translation mixture prior to addition of either globin or poly(A)$^+$ mRNa. Presumably, preincubation of this oligonucleotide with the 40 S subunit permitted time for its hybridization to the complementary 18 S rRNA sequence (see RNase H experiments indicated above) to inhibit translation of the subsequently added mRNA. The lack of complete inhibition by oligonucleotide 1 could reflect the low hybrid strength of the short DNA oligonucleotide 1 (17mer) bound to 18 S rRNA (DNA:RNA) compared with the greater strength of the postulated 18 S rRNA:mRNA (RNA:RNA) hybrid (see model below).

Finally, the ability of mRNA to bind to the 40 S subunit in the presence or absence of hybridizing DNA oligonucleotides was assessed using rabbit globin mRNA and the reticulocyte lysate translation system. Addition of 10 mM NaF to the in vitro translation system prevents ribosomal subunit association and allows detection of the 48 S preinitiation complex [8]. Similar to the results observed for the in vitro translation experiments detailed in Fig. 3, oligonucleotide 1 caused a significant reduction in the amount of labeled globin mRNA entering the 48 S preinitiation complex (Fig. 4). Also consistent with the translation results of Fig. 3, oligonucleotides 2, 5, P1 and P2 had no effect on globin mRNA binding to the 40 S subunit (data not shown).

4. DISCUSSION

The work presented here reveals that 18 S rRNA sequence 1 (nucleotides 1190–1153) is exposed on the surface of the 40 S ribosomal subunit and that a specific sequence 1-complementary DNA oligonucleotide inhibits the in vitro translation of mRNA. On the basis of these results and those previously presented [3], we have formulated a model for the initiation of translation based upon a sequential series of intermolecular RNA:RNA base-pairing interactions involving 5 S rRNA, 18 S rRNA, and mRNA (Fig. 5). These interactions provide not only an underlying physical basis for events important in the initiation of protein synthesis but would also provide a temporal order for these events.

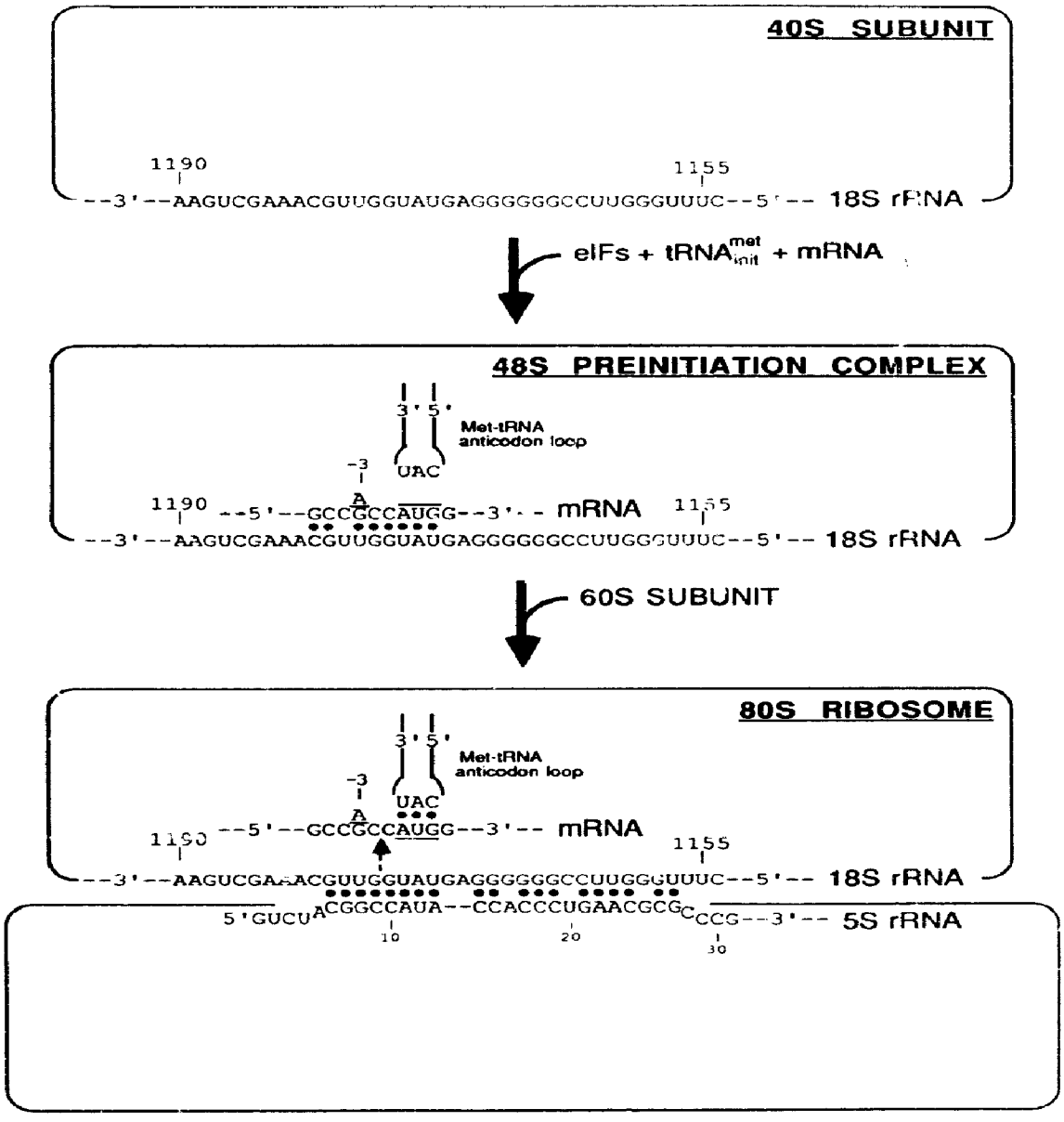

Fig. 5. Competitive-Displacement Model for the initiation of protein synthesis.

The steps proposed in our model are as follows. The 43 S initiation complex containing the 40 S subunit, eukaryotic initiation factors (eIFs), tRNA, and GTP, binds to the 5′ cap of the mRNA. The Region 1 sequence of 18 S rRNA then scans the 5′-untranslated leader of the mRNA until it finds an AUG codon with a proper Kozak consensus sequence with which it can base-pair. At this point scanning stops and the 48 S pre-initiation complex is formed. The mRNA 'locked' by [illegible] then unlocked when the 60 S subunit de[illegible] the hybridization of the 5′-terminal sequence of 5 S rRNA with the Region 1 sequence to displace the base-paired mRNA. The initiator AUG codon in the mRNA is then free to base-pair with the initiator Met tRNA and form the first codon–anticodon interaction to begin protein synthesis. The nature of the displacing intermolecular RNA:RNA interactions and the competition between mRNA and 5 S rRNA for the

same site in 18 S rRNA has prompted us to call this scheme the Competitive-Displacement Model for the initiation of protein synthesis.

Several points about this model are notable. The proposed 18 S rRNA Region 1:mRNA hybrid is G C-rich and has only 1 mismatch out of 9 contiguous base-pairs. Previous mutagenesis of the rat preproinsulin gene has permitted an experimental determination of the optimal consensus sequence for translational initiation in eukaryotes [1]. The mRNA −3 position (A/G) critical for efficient initiation of translation is base-paired to a U in the 18 S rRNA sequence. The ability of a U nucleotide to hydrogen-bond with similar strengths to both A and G nucleotides could explain the relative indifference as to which purine nucleotide occupies this critical position. Although our model only depicts the role of RNA:RNA base-pairing in this process, we do not discount the involvement of ribosomal or accessory proteins in these steps: they must certainly modulate the ability of these RNA sequences to interact. We only propose that the underlying mechanism consists of a sequential series of RNA:RNA interactions. Indeed, our results demonstrating that 5 S rRNA in the 60 S subunit is inaccessible to its oligonucleotide probe (data not shown) despite its location on the surface of the 60 S subunit [10], suggests that the 5′-terminal sequence of 5 S rRNA must undergo a conformational change, perhaps mediated by proteins, before it can base-pair to the Region 1 sequence of 18 S rRNA to displace the mRNA. Lastly, though our model does not indicate it, the initiator Met tRNA may participate in the scanning process through base-pairing of its anticodon sequence to the initiation codon, as has been proposed by others [1,2,11]. In fact, recent experiments in yeast indicate that the initiator tRNA plays a major role in start site selection [11].

Several pieces of available evidence are consistent with our model. First, the base-pairing of the 18 S rRNA Region 1 sequence to both 5 S rRNA and the mRNA start site is very well conserved in the evolution of eukaryotes. Identical hybrid structures can be drawn between this optimal translation start site sequence and 18 S rRNAs from organisms as divergent as mouse, *Xenopus laevis*, and *Saccharomyces cerevisiae* (our unpublished results). Second, several laboratories have reported crosslinking different viral mRNAs to 18 S rRNA in 40 S or 80 S initiation complexes [12–14]. In addition, Erni and Staehelin [15] were able to isolate a stable, base-paired hybrid between 18 S rRNA and globin mRNA from the 40 S initiation complex.

Finally, while there are clearly differences in the mechanisms by which eukaryotes and prokaryotes initiate translation, they may both use similar kinds of intermolecular interactions in this process. The use of a sequence near the 3′ end of 16S rRNA to base-pair to the Shine-Dalgarno sequence of prokaryotic mRNA [16,17] is a direct analogy to our proposed 18 S rRNA:mRNA hybrid. Eukaryotes and prokaryotes may simply use different portions of their small ribosomal subunit RNAs to base-pair to mRNA.

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