RETICULOCYTE 5 S RIBOSOMAL RNA INHIBITION OF CELL-FREE PROTEIN SYNTHESIS

Novel responses in ribosomal behaviour

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1. Introduction

Several reports have recently appeared showing direct RNA involvement in the regulation of protein synthesis. Low molecular weight RNA (4–5.5 S) isolated from nuclear ribonucleoprotein particles of adenovirus-infected HeLa cells was found to inhibit protein synthesis [1]. In addition, RNA species recovered from a ribosomal high salt wash have also been found to have inhibitory activity [2]. Ochoa's group have isolated 2 small (< 10 000 daltons) oligonucleotides from ribosomal salt washes, one of which inhibits protein synthesis, whereas the other fraction binds and inactivates the inhibitory molecule [3]. Similarly, a low molecular weight RNA fraction isolated from rat calvaria was found to inhibit protein synthesis in a mRNA-dependent wheat germ embryo system [4].

Whilst these reports show that certain RNA species can inhibit protein synthesis, the role of integral ribosomal RNA species in the regulation of protein synthesis is largely unknown. It was recently shown that 5 S rRNA, isolated and purified from rabbit reticulocyte ribosomes, severely inhibits translation of a mRNA-dependent cell free system [5]. Here these findings are extended to the mammalian cell free system derived from rabbit reticulocytes and it is shown that isolated 5 S rRNA, in nanogram quantities, potently inhibits cell free protein synthesis. This 5 S rRNA-induced inhibition has been analysed and it is found to elicit novel responses in the behaviour of both polysomes and 80 S monosomes. In addition, the 5 S rRNA, in the presence of ATP and polysomes, provoked considerable polysomal breakdown. These results are discussed in the light of the possibility that 5 S rRNA may represent the natural in vivo equivalent of double-stranded RNA.

2. Methods

Reticulocyte ribosomes were prepared from rabbit reticulocytes as in [6]. The cell-free protein synthesis system consisted of isolated ribosomes, the S-100 supernatant fraction and a mix fraction, and protein synthesis was performed exactly as in [6]. The S-100 supernatant fraction represented the supernatant of a 2 h 100 000 × *g* spin of a reticulocyte lysate.

The 5 S ribosomal RNA used in all the experiments was isolated using the proteinase K method as in [5]. The milder method for 5 S rRNA extraction included 10 mM $MgCl_2$ in all buffers (including ethanol used to precipitate the RNA), and excluded SDS from all the solutions.

Ribosomal populations were analysed using 15–40% isokinetic, exponential sucrose gradients made up in 80 mM KCl, 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$ and 0.1 mM EDTA. The 5 ml tubes (MSE 6 × 5 ml rotor) were spun for 60 min, 48 000 rev./min at 4°C in an MSE Superspeed 65 centrifuge. The sucrose gradients were pumped through a Unicam SP 500 spectrophotometer using a flow cell with a 0.1 cm light path, and the A_{260} profile was simultaneously recorded on an external recorder.

3. Results

Reticulocyte 5 S ribosomal RNA was added to a cell-free protein synthesizing system derived from rabbit reticulocytes and its effect on translation investigated. Figure 1 shows that the addition of small quantities of isolated 5 S rRNA to the cell-free system severely inhibited protein synthesis. Fifty percent inhibition was effected by the addition of 0.4 μg of 5 S rRNA, which is equivalent to a final concentration of 8 μg/ml. This represents a weight ratio of 5 S rRNA:ribosomes of ~1:50. Assuming the average molecular weight of ribosomes to be 3×10^6 and that of the 5 S rRNA to be 100 000, the molecular ratio is in the region of 1 molecule of 5 S rRNA/2 ribosomes. This demonstrates that the 5 S rRNA is a highly potent inhibitory molecule, capable of inhibiting protein synthesis at very low concentrations indeed.

In order to learn more about the manner in which the 5 S rRNA was inhibiting protein synthesis, the ribosomal profiles of control and inhibited systems were analysed. Analytical, exponential, isokinetic sucrose gradients were used, such that both the poly-

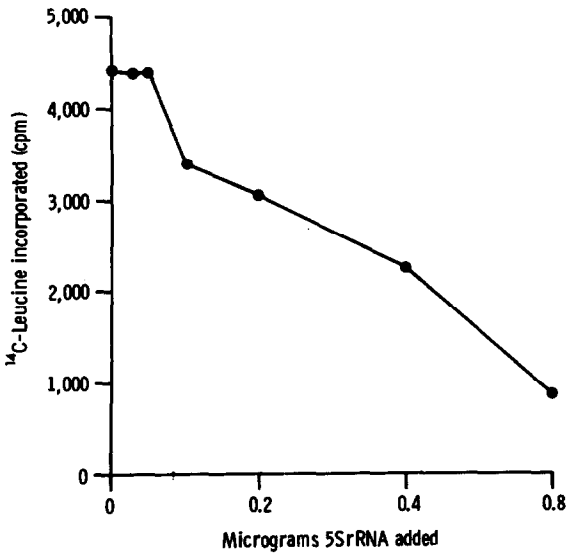

Fig.1. 5 S rRNA was isolated from reticulocyte ribosomes as described in section 2 and added to the cell-free protein synthesizing system (see section 2). Final volume was 50 μl. The tubes were incubated for 40 min at 37°C, placed in ice and then assayed for hot TCA-precipitable radioactivity.

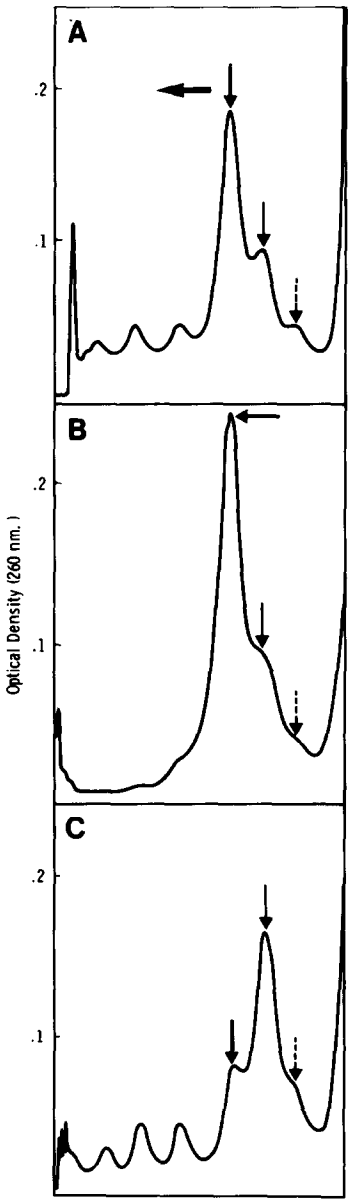

Fig.2. 5 S rRNA was added to the complete cell-free protein synthesizing system and the tubes (final volume 200 μl) were incubated for 10 min at 37°C. The tubes were placed in ice, the contents transferred to 15–40% sucrose gradients and analysed as described in section 2. Sedimentation is from right to left. (A) Complete cell-free system kept in ice; (B) as A but incubated for 10 min at 37°C; (C) as B plus 4 μg 5 S rRNA. The thick arrow indicates the position of 80 S monosomes, the thin arrow shows the 60 S subunit position and the dotted arrow denotes 40 S subunits.

somal as well as the monosome and ribosomal subunit regions were highly resolved. Figure 2A shows that whereas the ribosomal profile of the complete cell free system kept in ice displayed the typical polysomal region as well as 80 S monosomes, 60 S and 40 S subunits, the same system incubated for 10 min at 37°C (fig.2B) had far fewer polysomes and a highly increased 80 S monosome peak. This obviously represents movement and translation of the ribosome on the endogenous mRNA with subsequent run-off and no recycling. This finding correlates with the known poor reinitiation in the fractionated reticulocyte cell-free system. The addition of 5 S rRNA to the cell-free system with subsequent 37°C incubation caused changes in the ribosomal pattern that sharply contrasted with those findings described above. Firstly, the polysomal region (fig.2C) showed that the 5 S rRNA had effected almost 'total freezing' of the polysomal population. This contrasted with the expected movement and subsequent run-off of ribosomes (c.f.: polysomal regions of fig.2B, control; fig.2C, plus 5 S rRNA). Secondly, it was obvious that the addition of 5 S rRNA had also effected the change of 80 S monosomes into 60 S and 40 S subunits. Both these subunit populations were highly exaggerated in the 5 S rRNA-treated system (fig.2C) when compared to the control (fig.2B).

These 5 S rRNA-induced changes in the ribosomal pattern were further investigated to see whether they could also occur in the absence of protein synthesis. Figure 3A shows the ribosomal profile obtained after incubating, at 37°C, isolated ribosomes in the absence of both the mix and S-100 supernatant fractions (see section 2). The prominent presence of polysomes (fig.3A) indicates that neither the movement of ribosomes along the endogenous mRNA nor subsequent run-off had taken place. This is to be expected, as the ribosomes were not supplied with the conditions necessary for protein synthesis. In contrast, the optimal system composed of ribosomes, mix fraction and S-100 displayed the typical ribosomal run-off pattern (fig.3C). The effect of adding 5 S rRNA to both these systems is shown in fig.3B,3D. The 5 S rRNA, even in the absence of protein synthesis, still induced the change of 80 S monosome to 60 S and 40 S subunits (fig.3B). However, the ribosomal profile in the 5 S rRNA-supplemented complete cell-free system (fig.3D) showed highly exaggerated 60 S and

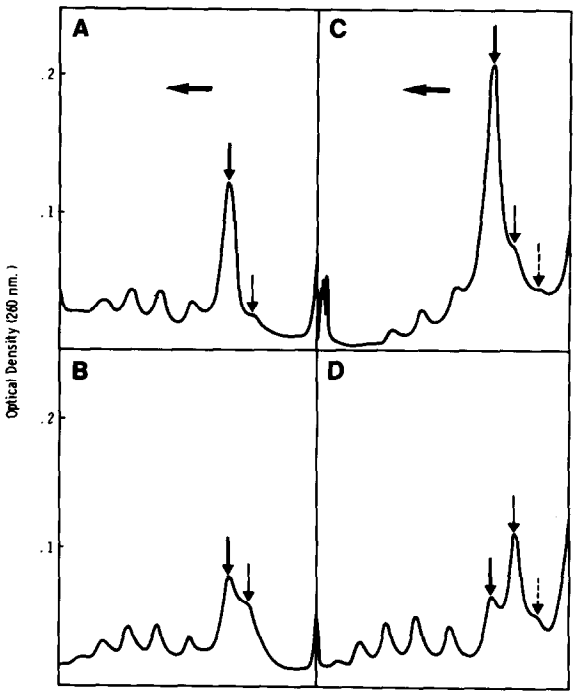

Fig.3. Reticulocyte 5 S rRNA was added either to the complete cell-free system or to ribosomes alone. When incubated with ribosomes alone, final ionic conditions were made up to 50 mM KCl, 20 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$. The tubes (final volume 200 μl) were incubated for 12.5 min at 37°C and then analysed on sucrose gradients as described in section 2. Sedimentation was from right to left. (A) Ribosomes, alone, incubated for 12.5 min at 37°C; (B) as (A) plus 2.8 μg of 5 S rRNA; (C) complete cell-free system incubated for 12.5 min at 37°C; (D) as (C) plus 2.8 μg of 5 S rRNA. Definition of arrows as described in fig.2 legend.

40 S monosome peaks, in addition to the 'frozen' polysomes. When the 5 S rRNA-induced 80 S change to subunits observed in the absence of the necessary requirements for translation (fig.3B) was compared with that obtained under conditions optimal for protein synthesis (fig.3D), it was obvious that the 80 S to subunit transformation was significantly enhanced in the presence of optimal conditions.

It was possible that either the S-100 supernatant or the mix fraction, was responsible for promoting the 5 S rRNA-induced transformation of 80 S to 60 S and 40 S subunits. In order to analyse these possibilities, 5 S rRNA was added to ribosomes either (a) in the presence of S-100 alone, or (b) in the presence

of the mix fraction. Whilst the addition of 5 S rRNA to the complete cell-free system displayed the typical polysomal 'freezing', in addition to the 80 S transformation to 60 S and 40 S subunits (c.f.: fig.4A, control complete system; fig.4B, complete system plus 5 S rRNA), the addition of 5 S rRNA to ribosomes in the presence of S-100 alone showed only slight 80 S change to 60 S and 40 S (fig.4C). This result was very similar to 5 S rRNA-induced change found in the absence of both S-100 and mix fractions (c.f.: fig.4B,3B). However, in contrast to the above, the addition of 5 S rRNA to ribosomes, supplemented only with the mix fraction, caused 80 S transformation to 60 S and 40 S subunits, almost identical to that obtained in the complete cell-free system (c.f.: fig.4D, 5 S rRNA added to ribosomes supplemented with mix fraction; fig.4B, 5 S rRNA added to the complete cell free system).

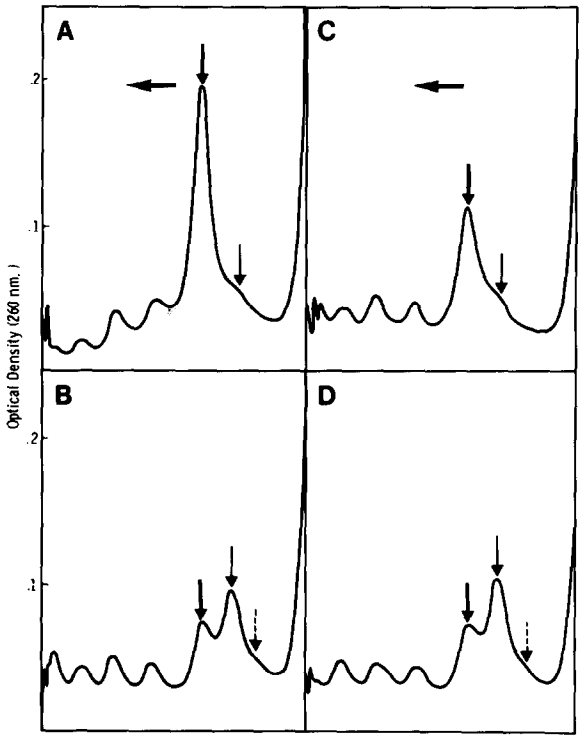

Fig.4. The effect of 5 S rRNA on ribosomes in the presence of only the S-100 supernatant or mix fraction was checked. Tubes (final volume 200 μl) were incubated for 10 min at 37°C and then analysed for the ribosomal profile. Sedimentation is from right to left. (A) Complete cell-free system; (B) as A plus 2.8 μg of 5 S rRNA; (C) 5 S rRNA added to ribosomes in the presence of S-100; (D) 5 S rRNA added to ribosomes in the presence of the mix fraction. Definition of arrows as described in fig.2 legend.

Thus, the mix fraction itself contained a component(s), that stimulated the 80 S transformation to 60 S and 40 S subunits. A simple elimination procedure was adopted in order to identify the component(s) responsible for this stimulation. The ribosomes were separately supplemented with each of the individual constituents of the mix fraction, 5 S rRNA was added and the tubes were incubated at 37°C. The ribosomal profiles were then analysed on sucrose gradients. Figure 5A shows the results obtained when 5 S rRNA was added to ribosomes in the presence of the mix fraction, whereas fig.5F displays the ribosomal profile after incubation of 5 S rRNA with only isolated ribosomes. The addition of dithiothreitol to the 5 S rRNA-treated system did not cause any change in the ribosomal profile (fig.5D, plus dithiothreitol; fig.5F, in its absence), whereas phosphoenolpyruvate in the presence of pyruvate kinase only slightly stimulated the 5 S rRNA-induced change (c.f.: fig.5E, plus phosphoenolpyruvate and pyruvate kinase; fig.5F). However, the addition of GTP significantly stimulated the 80 S transformation to 60 S and 40 S subunits (c.f.: fig.5C, plus GTP; fig.5F), although it did not reach the levels obtained with the full system (c.f.: fig.5C, plus GTP; fig.5A, ribosomes, mix and 5 S rRNA). The most surprising result was obtained when ATP alone was added to ribosomes supplemented with 5 S rRNA. After incubation, analysis on a sucrose gradient showed almost total disappearance of the polysomal population with a concomitant increase in the 80 S, 60 S and 40 S region (fig.5B). The addition of ATP not only stimulated the transformation of 80 S to 60 S and 40 S subunits, but also provoked polysomal disappearance.

It was difficult to reconcile this result with those obtained in the 5 S rRNA supplemented systems composed of either ribosomes and the mix fraction, or ribosomes, mix and S-100 fractions. The mix fraction thus must have contained, in addition, a component(s) antagonistic to the ATP-provoked polysomal disaggregation. These component(s) would only block ATP-dependent, 5 S rRNA-induced polysomal breakdown and presumably not interfere with the

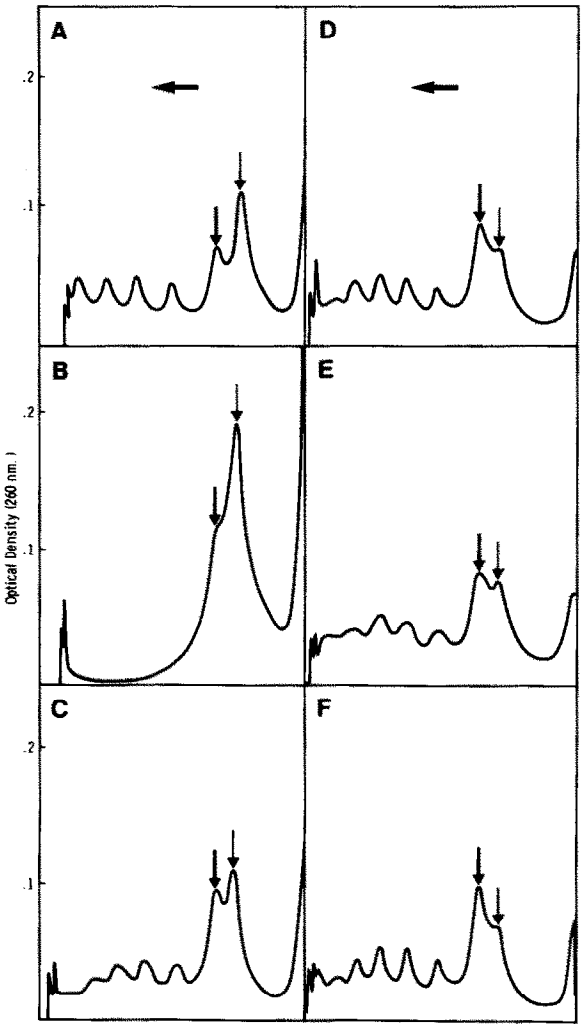

Fig.5. The effect of each constituent on the 5 S rRNA action on ribosomes was checked. Every tube (final volume 200 μl) contained 2.4 μg of 5 S rRNA, 84 μg of ribosomes and final ionic conditions were set at 50 mM KCl, 20 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$. The tubes were incubated at 37°C for 10 min, then analysed for the ribosomal profile. Sedimentation is from right to left. (A) Ribosomes and 5 S rRNA in the presence of the complete mix fraction; (B) ribosomes, 5 S rRNA and ATP at 1 mM final concentration; (C) ribosomes, 5 S rRNA and GTP at 0.2 mM final concentration; (D) ribosomes, 5 S rRNA and dithiothreitol at 1 mM final concentration; (E) ribosomes, 5 S rRNA and phosphoenolpyruvate at 4 mM final concentration and 10 μg pyruvate kinase; (F) only ribosomes and 5 S rRNA. Definition of arrows as described in fig.2 legend.

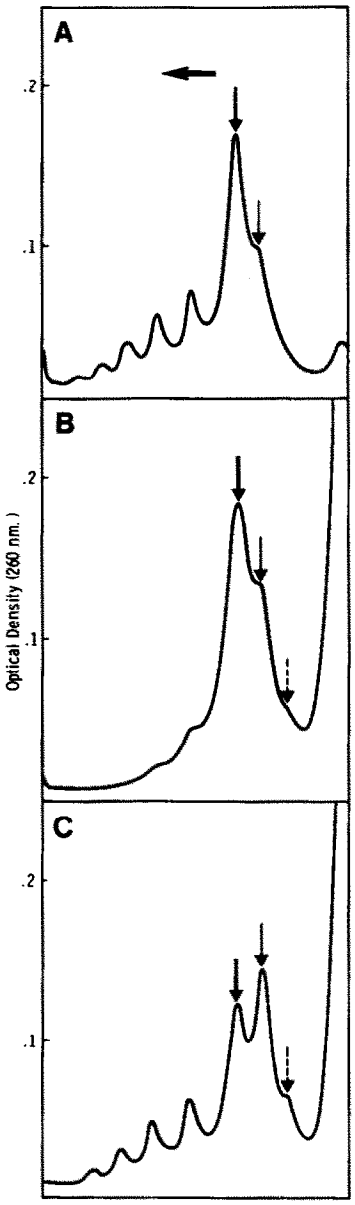

Fig.6. Tubes, with a final volume of 200 μl, were set up containing 80 μg of ribosomes and 2 μg of 5 S rRNA. Incubation was at 37°C for 12.5 min, after which the contents were analysed for the ribosomal profile. Sedimentation is from right to left. (A) Ribosomes, 5 S rRNA and final ionic conditions at 50 mM KCl, 20 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$; (B) as (A) plus ATP and phosphoenolpyruvate at final concentrations of 1 mM and 4 mM respectively; (C) as (B) plus 15 μg of pyruvate kinase. Definition of arrows as described in fig.2 legend.

ATP-promoted, 5 S rRNA-induced transformation of 80 S to the 60 S and 40 S subunits.

The results presented in fig.6 indeed give support to the above propositions. Figure 6A shows that whereas 5 S rRNA added to ribosomes alone induced the appearance of a prominent 60 S peak due to the transformation of 80 S to 60 S and 40 S subunits, the addition of both ATP and phosphoenolpyruvate to the above system caused the typical polysomal breakdown, with stimulation of the 80 S to 60 S and 40 S change (fig.6B). However, addition of pyruvate kinase, to the system described in fig.6B above, not only prevented the ATP-dependent 5S rRNA-induced polysomal breakdown, but also significantly stimulated the transformation of 80 S to 60 S and 40 S subunits.

4. Discussion

Recently it was shown that the addition of either isolated reticulocyte 28 S rRNA or 18 S rRNA, to a reticulocyte lysate system, resulted in a maximum of 50% inhibition in protein synthesis [7]. This maximum inhibition occurred when the individual rRNAs were present at a final concentration of 1200 μg/ml. The results presented here demonstrate that 5 S ribosomal RNA, isolated and purified from rabbit reticulocyte ribosomes, is a potent inhibitor of cell-free protein synthesis in final concentrations as low as 4–8 μg/ml. These results extend and correlate with previous findings [5] that showed 5 S rRNA also to be a potent inhibitor of 9 S mRNA-dependent translation in the wheat germ embryo system.

This potency of 5 S rRNA is somewhat reminiscent of double-stranded RNA-induced inhibition of reticulocyte lysate systems [8]. Indeed the possibility that 5 S rRNA may be the natural biological equivalent of double-stranded RNA, in regulating protein synthesis has already been proposed [5] and is, moreover, further reinforced by findings presented here.

The 5 S rRNA-inhibited cell free system was found to exhibit 2 novel characteristics: (a) a 'freezing' of the polysomes on mRNA; and (b) change of the 80 S monosome to give 60 S and 40 S subunits. 5 S rRNA was found to elicit the 80 S change also in the absence of conditions necessary for protein synthesis, but the presence of either GTP or phosphoenolpyruvate and pyruvate kinase promoted the 80 S transformation. Paradoxically, the addition of ATP alone not only stimulated the 5 S rRNA-induced 80 S transformation, but also provoked a remarkable breakdown of polysomes. This ATP-dependent, 5 S rRNA-induced breakdown of polysomes could have occurred by any one of 3 possibilities: (a) run-off of ribosomes; (b) subunits 'dropping off' the mRNA with immediate formation of monosomes; or (c) endonucleolytic cleavage of the polysomes. Although the results presented here are not adequate to allow one to choose any of the above possibilities, there are indications (work in progress) that in fact the third possibility is occurring. It is interesting then to view this result with Kerr's postulated mediator of double-stranded RNA-induced, ATP-dependent inhibition of protein synthesis [9]. This mediator, a trinucleotide synthesized from ATP, activates an endogenous endonuclease (Professor Michel Revel, personal communication). If the 5 S rRNA-induced, ATP-dependent breakdown of polysomes reported here, acts by a similar mechanism, then inclusion of an energy-regenerating system would restrict the formation of the trinucleotide mediator, thus limiting the action of the putative endonuclease. Figure 6 shows that in the case of 5 S rRNA-induced, ATP-dependent polysomal degradation this was indeed true.

However, it is obvious (fig.2) that 5 S rRNA can inhibit protein synthesis not only by causing polysomal breakdown. There are thus two important phenomena to consider: (a) the transformation of 80 S to 60 S and 40 S subunits with a concomitant 'freezing' of polysomes; and (b) the 5 S rRNA-induced, ATP-dependent breakdown of polysomes.

Preliminary results show that the molecular configuration of the 5 S rRNA molecule is important for determining which effect will be dominant. It has already been shown that isolated prokaryotic 5 S rRNA in aqueous solution can change from the native form to the 'A' form, which displays slightly less double-stranded structure [10]. This conformational change from the native form to the 'A' form occurs easily and even without exposure to denaturing conditions [10]. Whether a similar phenomenon occurs in the mammalian 5 S rRNA molecule is unknown. However, there are results that show a strict relationship between reticulocyte 5 S rRNA structure and its functions described in this paper.

When 5 S rRNA was isolated under conditions optimal for the conservation of hydrogen bonds, the major effect on reticulocyte ribosomes was that of ATP-dependent polysomal breakdown, whereas isolation of 5 S rRNA prepared under slightly harsher conditions showed that the transformation of 80 S to 60 S and 40 S subunits dominated (D.H.W., unpublished results) [11].

In this regard it is interesting to note that double-stranded RNA-induced inhibition of protein synthesis requires both a properly matched base-pair structure [12], as well as a minimum effective size [12].

In conclusion, reticulocyte 5 S rRNA in low concentrations was found to severely inhibit cell free protein synthesis. This 5 S rRNA inhibition was accompanied by freezing of polysomes on endogenous mRNA and transformation of 80 S monosomes to 60 S and 40 S subunits. In addition, 5 S rRNA in the presence of ribosomes and ATP, was found to elicit profound polysomal breakdown. These results possibly support the proposition that 5 S rRNA is indeed the natural in vivo equivalent to synthetic double-stranded RNA.

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