Inhibition of Peptide Initiation by a Low Molecular Weight RNA from Rabbit Reticulocytes*

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A heat-stable inhibitor of protein synthesis has been isolated from the postribosomal supernatant of rabbit reticulocytes. Its activity is not susceptible to protease treatment but is destroyed by incubation with alkali. Inhibitory activity can be quantitatively recovered in the aqueous phase after phenol extraction and has the ultraviolet absorption spectrum of a nucleic acid. It is concluded that the inhibitor is RNA. The inhibitory activity sediments in the range of $3\,S$, but it has not been demonstrated whether the inhibitory RNA is a single molecular species. The inhibitory RNA does not affect peptide elongation but rather blocks a step of peptide initiation. It does not interfere with the formation of the ternary complex between initiation factor 2, GTP, and methionyl-tRNA. It and does not activate a protein kinase phosphorylating initiation factor 2. The inhibitory RNA appears to be a novel type of RNA that inhibits polypeptide initiation at a step involving ribosomal subunits.

Cytoplasmic RNA molecules other than rRNA, tRNA, or mRNA have been implicated in translational control of protein synthesis. In almost all cases the control mediated by these molecules appears to be negative, i.e. translation of certain or all mRNA is depressed rather than enhanced. A well documented example of this type of RNA is dsRNA which has been shown to inhibit protein synthesis in interferon-sensitized mammalian cells or in reticulocytes which do not need prior interferon treatment (as reviewed in Ref. 1). dsRNA activates a protein kinase which phosphorylates specifically the $\alpha$ subunit of eIF-2 leading to decreased initiation of peptides on ribosomes.

Two small RNA molecules with inhibitory or stimulatory effects on protein synthesis were isolated from postribosomal supernatants and ribosomal salt wash fractions, respectively, of Artemia embryos (2). The former RNA is a pyrimidine-rich molecule of $M_r = 6000$ which blocks the elongation step of protein synthesis at the level of elongation factor 1-mediated binding of aminoacyl-tRNA to ribosomes. Translational activity is restored in the presence of a separate purine-rich molecule of $M_r = 9000$ derived from the salt wash fraction. The activity of this second RNA type has been found to be higher in developing rather than dormant embryos; therefore, the two RNAs were postulated to play a role in developmental regulation of protein synthesis.

More recently, RNA molecules isolated from small cytoplasmic ribonucleoprotein particles (10-15 $S$) of embryonic chicken muscle were shown to inhibit protein synthesis in vitro (3-5). The inhibitory RNA fraction was comprised of a heterogeneous population of RNAs in the range of 4-5 $S$ that do not appear to contain polyadenylate or oligouridine regions of significant length. Translation of capped messages from various sources and uncapped encephalomyocarditis viral mRNA were inhibited to the same extent (3, 4). Inhibition of protein synthesis appears to be focused at the level of initiation, but the exact mechanism is as yet undefined.

In this paper we report the isolation of an inhibitor of protein synthesis from the postribosomal supernatant of rabbit reticulocytes. The inhibitory species is concluded to be RNA in nature, is heat stable, and inhibits protein synthesis at the level of initiation. The relationship to previously reported inhibitory RNAs is discussed.

**Experimental Procedures**

**Materials**

ATP, creatine phosphate, creatine phosphokinase, sodium dodecyl sulfate, hemin (bovine Type I), dithioerythritol, glutathione, poly(U), GMP-PCH$_2$P, N,N',N'-tetramethylthelyleneediamine, acrylamide, and EGTA were purchased from Sigma. GTP and edeine were purchased from Calbiochem-Behring. [y$^3$P]ATP (2000 Ci/mmol), [3H]leucine (312 Ci/mol), and sucrose, ultrapure, were purchased from Schwarz/Mann. [5S]Methionine (1080 Ci/mmol) was obtained from Amer sham Corp. Cytochexamine (Sigma) was purchased from Nutritional Biochemicals. a-Chymotrypsin was purchased from Boehringer Mannheim. Proteinase K was obtained from Beckman Instruments. Ammonium persulfate and N,N'-methylenebisacrylamide were purchased from Eastman Kodak Co. Microgranular DE-52 cellulose was obtained from Whatman. Millipore filters (HAWG, 0.45 $\mu$m pore size) were obtained from Millipore Corp. (Bedford, MA). Double-stranded RNA (reovirus RNA) was a kind gift from Dr. A. Shatkin (Roche Institute for Molecular Biology, Nutley, NJ). All other chemicals used were reagent grade.

**Solutions**

The solutions used were: Solution A, 20 mm Tris-HCl (pH 7.5), 10 mm 2-mercaptoethanol, 0.02% NaN$_3$, Solution B, 20 mm Tris-HCl (pH 7.5), 100 mm KCl, 10 mm 2-mercaptoethanol; gel electrophoresis sample solution, 50 mm Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate, 0.1 mm dithioerythritol, 0.004% bromophenol blue.

**Methods**

Preparation of the Postribosomal Supernatant

The postribosomal supernatant fraction was prepared from rabbit
Assays of Inhibition Activity

(a) Reticulocyte Lysate System—The procedure used for the preparation of the lysates has been described by Adamson et al. (11). Protein synthesis in lysates was measured in incubation mixtures containing the following in a final volume of 50 µl: 10 mM Tris-HCl (pH 7.5), 90 mM KCl, 1.5 mM MgCl₂, 5 mM dithioerythritol, 0.5 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, creatine phosphokinase at 2.5 units/ml, and 45 µg of 0.5 M KCl-washed reticulocyte postribosomal supernatant by ammonium sulfate precipitation. Proteins that precipitated between 40 and 70% saturation at pH 7.0 were used. This fraction is called the “40–70% AS” fraction.

(b) Fractionated System—The assay was carried out as described previously (7). Protein synthesis was measured in incubation mixtures containing the following in a final volume of 100 µl: 20 mM Tris-HCl (pH 7.5), 80 mM KCl, 4 mM MgCl₂, 5 mM glutathione, 1 mM ATP, 0.4 mM GTP, 12 mM creatine phosphate, 30 units/ml of creatine phosphokinase, 2.5 x 10⁻⁵ M [³²P]leucine (40 Ci/mmol), 5 µg of deacylated tRNAf, 0.1 µg of diphosphopyridine nucleotide, and about 100 µg of poly(U). One unit of inhibitory activity is defined as the amount required to reduce the level of [³²P]leucine incorporation into protein in the reticulocyte lysate system by 50%.

Protein and RNA Determinations

Protein concentrations were determined routinely by the method of Warburg and Christian (15) and were checked occasionally with the procedure involving Amido Schwarz staining as described by Spierer and Weissmann (16). RNA concentrations were determined spectrophotometrically assuming that an absorbance of 1.00 at 260 nm corresponds to a concentration of 41.7 µg of RNA/ml. These values were verified by the orcinol method (17).

Phosphorylation Assay and Analysis by Polyacrylamide Gel Electrophoresis

Reticulocyte polyosomes, 80 µg, prepared as described (6, 7), were incubated in a final volume of 25 µl containing 20 mM Tris-HCl (pH 7.5), 80 mM KCl, 1 mM dithioerythritol, 0.1 mM ATP, 2 mM MgCl₂, 0.26 units of creatine phosphokinase, and 5 mM creatine phosphate. Incubation was for 5 min at 37°C, the reaction was quenched by placing the samples on ice and adding 50 µl of a solution containing 10 mM Tris-HCl (pH 7.5), 90 mM KCl, 2 mM MgCl₂. The samples were layered onto 4.5-ml linear 15–45% sucrose gradients containing 10 mM Tris-HCl (pH 7.5), 90 mM KCl, 2 mM MgCl₂, and centrifuged at 59,000 rpm in a Beckman SW 60 Ti rotor at 4°C for 45 min.

(b) Sedimentation of tRNA—Two A₂₆₀ units of tRNA in 0.1 ml of solution B were loaded onto a 4.5-ml linear sucrose gradient as described. The sample was spun in a Beckman SW 60 Ti rotor at 59,000 rpm for 5½ h at 4°C. Also, 2 A₂₆₀ units of Escherichia coli tRNA and of E. coli S ribosomal RNA were run in parallel tubes as standards.

The UV absorbance profiles were obtained by upward displacement of the centrifuged gradients through an ISCO model UA-5 UV absorbance monitor.

Results

1. Purification of the Inhibitor—An inhibitor of protein
synthesis was partially purified from the postribosomal supernatant of rabbit reticulocytes by two consecutive chromatographies on DEAE-cellulose. Approximately 4 liters of postribosomal supernatant (about 145 g of protein) were loaded onto a column (7.5 x 120 cm) filled with DEAE-cellulose that had been equilibrated in solution A with 20 mM KCl. After extensive washing with the same solution which eliminated 85-90% of the protein, mainly hemoglobin, additional protein was eluted by increasing the salt concentration to 250 mM. Then a fraction (DE 500) was eluted from the column with solution A containing 500 mM KCl. The DE 500 fraction had potent inhibitory activity when added to a reticulocyte cell-free protein-synthesizing system. This fraction contained approximately 1% of the protein applied to the column and a significant amount of nucleic acid as indicated by an A_{260}/A_{280} ratio of about 1.7. The DE 500 fraction was concentrated by ultrafiltration in an Amicon apparatus using a FM-10 membrane, dialyzed against solution B, and then loaded on a DEAE-cellulose column (2 x 70 cm) that had been equilibrated in the same solution. After extensive washing, bound material was eluted with a linear salt gradient, 1800 ml total volume, of 100 mM to 700 mM KCl in solution A. Fractions of 8 ml/10 min were collected, and their absorbance at 260 and 280 nm was determined. An aliquot of each fifth fraction was dialyzed overnight against solution B. Then, inhibitory activity was determined with a 25-μl aliquot of the dialyzed fractions. The results are shown in Fig. 1. Two peaks of inhibitory activity were found for protein synthesis in the reticulocyte lysate system. The first peak was eluted at approximately 200 mM KCl and contained most of the protein from the DE 500 fraction. Activity in this peak was significantly reduced when it was heated at 60 °C for 10 min or when it was pretreated with protease (data not shown). No further experiments are described with this fraction. The second peak was eluted between 380 mM and 420 mM KCl. Pooled fractions, indicated by the bar in Fig. 1, had an A_{260}/A_{280} ratio of about 1.8 and gave a typical UV spectrum of RNA. It is this fraction that has been used for the experiments described in the following sections.

2. Characterization of the Inhibitor—The data of Fig. 1 indicate that inhibitory activity and RNA elute together at about 400 mM KCl. To determine the nature of the inhibitor, the experiments described in Table I were carried out. Pretreatment of the inhibitor with trypsin, chymotrypsin, and proteinase K had no effect on activity as measured with the reticulocyte lysate system. Results for chymotrypsin and proteinase K are shown. The latter enzyme has no side chain specificity in the hydrolysis of peptide bonds and degrades proteins to small peptides and amino acids (20). Inactivation of the proteolytic enzymes was accomplished by heating to 100 °C for 10 min. It had been shown previously that this treatment does not affect the inhibitory activity of the preparation. The inhibitory activity was sensitive to alkali treatment and when extracted with 90% phenol was recovered in the aqueous phase with a reproducible increase in specific activity. These results indicate that the inhibitor is RNA which we call inhibitor RNA (iRNA). The recovery of inhibitory activity during the isolation of iRNA is given in Table II. In routine preparations the pooled fractions from DEAE-cellulose (indicated in Fig. 1) were subjected to phenol extraction before use in the experiments described below.

The sedimentation constant of the inhibitory molecules was estimated by sucrose gradient analysis. About two A_{260} units of the iRNA preparation were loaded on 5-25% linear sucrose gradients. Parallel gradients contained the same amount of either E. coli tRNA^pre or 5 S ribosomal RNA. The results obtained after centrifugation, fractionation, and determination of inhibitory activity are shown in Fig. 2. Individual fractions from the gradient containing the iRNA preparation and individual fractions from a parallel gradient run without an RNA sample were lyophilized, and then H_2O was added to give one-fourth of the original volume before aliquots of these fractions were added to the reticulocyte lysate system. Sucrose itself causes a small decrease of protein synthesis in the reticulocyte lysate system. This nonspecific inhibition was subtracted for each fraction, and the normalized data are shown in Fig. 2. Inhibition due to the iRNA was found in fractions having a lower sedimentation constant than tRNA.

![Figure 1. Elution of inhibitory activity from DEAE-cellulose.](image)

The details of this chromatography are described under "Results." Inhibitory activity (■■) was measured in the reticulocyte lysate system. 100% activity equals 35,407 cpm incorporated in the 50-μl assay system described under "Experimental Procedures." Absorbance at 280 nm (△——△) and 260 nm (△—△) was determined; ———, KCl concentration.

### Table I

**Characterization of the inhibitory activity**

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>% Inhibition</th>
<th>Remaining activity</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not incubated</td>
<td>30</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>100 °C for 10 min</td>
<td>30</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>75</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>+ α-Chymotrypsin</td>
<td>61</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>78</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>+ Proteinase K</td>
<td>79</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>40</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+ 0.3 mM KOH</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

### Table II

**Isolation of iRNA**

The purification procedure is described under "Results." One unit of activity is defined as the amount of iRNA that reduces protein synthesis in the reticulocyte lysate system by 50%.

<table>
<thead>
<tr>
<th>Total units</th>
<th>RNA Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE 500</td>
<td>14,740</td>
</tr>
<tr>
<td>DEAE gradient</td>
<td>7,597</td>
</tr>
<tr>
<td>Phenol extraction</td>
<td>9,972</td>
</tr>
</tbody>
</table>
**Inhibition of Peptide Initiation by a Small RNA Molecule**

**Fig. 2. Sucrose gradient analysis of iRNA.** Inhibitory RNA was isolated as described under “Results” and analyzed by sucrose density centrifugation as given in detail under “Experimental Procedures.” Fractions from the gradient after centrifugation were treated as described under “Results,” and their inhibitory activity was determined. Control values for $[^{14}C]$leucine incorporation into proteins in the reticulocyte lysate system with the corresponding amount of sucrose added ranged from 12,300 cpmp (fraction 3) to 6,250 cpmp (fraction 13). These were taken as 0% inhibition. Absorbance at 260 nm (---); percentage inhibition (•—•).

used as a 4 S standard and also less than the $A_{260}$ absorbance peak. These findings indicate that the inhibitory component of the iRNA fraction is not tRNA and that the iRNA fraction is not homogeneous in the inhibitory component. When material from the DEAE column (cf. Fig. 1) before phenol extraction was analyzed in the same way, inhibitory activity was found in the same position of the sucrose gradient (data not shown). This suggests that the iRNA is not complexed with proteins in the form of a ribonucleoprotein particle molecule that would sediment faster than its free RNA.

3. **iRNA Inhibits Peptide Initiation**—Addition of iRNA to a reticulocyte lysate protein-synthesizing system resulted in a concentration-dependent decrease of $[^{14}C]$leucine incorporation into peptides, measured as material that is precipitable by trichloroacetic acid (Fig. 3A). Total inhibition was not achieved, even when relatively large amounts of iRNA were added. Maximum inhibition is obtained with the addition of approximately 50 μg of iRNA, and no further inhibition is obtained with the addition of up to 200 μg of iRNA. Edeine, a small basic peptide antibiotic that has been characterized as an inhibitor of peptide initiation (10), was used as a control. Radioactive leucine incorporated into protein in the reticulocyte lysate system in the presence of 0.2 μM edeine is due almost exclusively to elongation of nascent peptides that were present on the ribosomes at the time of the preparation of the lysate. Inhibition by iRNA does not go below the level of edeine inhibition indicating that iRNA blocks peptide initiation but not elongation. This conclusion is supported by the observation that the maximum inhibition obtained in the presence of edeine is not increased by the addition of iRNA (data not shown). The effect of iRNA on the time course of peptide synthesis in lysates is given in Fig. 3B. Addition of iRNA to the lysate results in a decreased level of synthesis characterized by a reduced constant rate of synthesis which remains linear as long as the control lysate. The first few minutes of synthesis in the presence of iRNA closely parallels the rate of synthesis in the presence of edeine.

The effect of the iRNA on polysome formation in lysates was studied with the results given in Fig. 4. For these experiments, lysates were incubated for 3 min in the absence or presence of iRNA and then placed on ice and loaded on a sucrose gradient. Centrifugation and analysis of the distribution of ribosomes and polysomes were performed as described under “Experimental Procedures.” As shown in Fig. 4A, the lysate lacking inhibitor has a large proportion of ribosomes in the polysome region with almost equal distribution of polysomes containing 3, 4, and 5 ribosomes. An even higher proportion of polysomes is seen in the presence of cycloheximide (Fig. 4D), which inhibits peptide elongation and thus prevents polysome breakdown (21). In contrast, incubation of the lysate with iRNA (Fig. 4B) causes disaggregation of polysomes with only a few ribosomes left in di-, tri-, and tetraribosomal structures. The released ribosomes accumulate in the 80 S monoribosome region with no detectable increase in the
levels of 40S and 60S ribosomal subunits. The effect of iRNA is very much like that of edeine (Fig. 4C) which also causes a rapid accumulation of 80S monoribosomes.

Further evidence that the site at which iRNA inhibits protein synthesis is at the initiation step comes from two other sources. Most if not all of the enzymatic steps of peptide initiation with natural mRNA are bypassed in poly(U)-directed synthesis of polyphenylalanine (14). When iRNA was added to the poly(U)-directed system in the same or higher amounts that caused inhibition in the lysate assay, no inhibitory effect was observed (Table III). Generally, the iRNA appeared to cause a small increase in polyphenylalanine synthesis. The basis for this apparent stimulation is not known but probably is not directly related to inhibition of peptide initiation seen in the lysate system.

The effect of iRNA also was determined in a "fractionated system" derived from reticulocytes. It is described under "Experimental Procedures." The main feature of this system is limited peptide initiation so that about half of the total [14C]leucine that is incorporated is into the carboxyl-terminal portion of nascent peptides that were initiated in the intact cells. Edeine was used to distinguish between elongation of nascent peptides and de novo synthesis requiring peptide initiation. Data presented in Table IV supports the conclusions that iRNA appears to be an inhibitor of peptide initiation with little or no effect on elongation. The level of [14C]leucine incorporated into peptides decreases with increasing additions of iRNA and approaches the level of inhibition obtained in the presence of 2 μM edeine as was observed with the lysate system. The addition of both edeine and iRNA does not result in additive inhibition. Table IV also presents data comparing the effect of decacylated rabbit liver tRNA which is similar in size to iRNA. The fractionated reticulocyte system as described above is supplemented with 9 μg of tRNA per assay tube, which is optimal under the conditions used. Additional tRNA causes a decrease in peptide synthesis. The inhibition observed with the added tRNA in the data of Table IV is small when compared with the inhibition obtained with an equivalent amount of iRNA.

4. Possible Site of Inhibition—The data presented above indicate that the inhibitor is an RNA molecule and that it inhibits protein synthesis at the level of initiation. As mentioned in the introduction, dsRNA has similar characteristics. The dsRNA-dependent protein kinase present on rabbit reticulocyte polysomes requires dsRNA and ATP for activation and inhibits peptide initiation by phosphorylation of the α subunit of eIF-2 (22). The experiment described in Fig. 5 indicates that iRNA does not increase the low level of phosphorylation seen when polysomes were incubated with eIF-2 and ATP alone. Reticulocyte polysomes containing the dsRNA-sensitive eIF-2α kinase were preincubated with ATP and iRNA or authentic dsRNA, and then eIF-2 and [γ-32P]ATP were added. The extent of eIF-2 phosphorylation was followed by autoradiography after the components had been separated by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. The amounts of dsRNA and iRNA used in the experiment gave the same level of inhibition when assayed in the reticulocyte lysate system. However, the respective levels of eIF-2α subunit phosphorylation in this experiment are quite different. Incubation of the polysomes with dsRNA lead to considerable phosphorylation of eIF-2α, whereas incubation with iRNA had no apparent effect on eIF-2α phosphorylation. The iRNA does not significantly activate the dsRNA-dependent kinase. Considered with the thermal stability of iRNA, these data indicate that it is not dsRNA. For comparison, phosphorylation of purified eIF-2 by the heme-controlled eIF-2α kinase is shown (Fig. 5, Track I). Also shown in Track 1 is a relatively heavy phosphorylation band related to the 100,000-dalton peptide of the eIF-2α kinase preparation and a light phosphorylation band related to the β subunit of eIF-2.

Conceivably iRNA might inhibit peptide initiation by competition with or inactivation of a specific component of the system. We attempted to identify such a component by supplementing a nuclease-treated reticulocyte lysate (23) with

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**TABLE III**

Effect of iRNA on poly(U)-directed polyphenylalanine synthesis

The assay system is described under "Experimental Procedures." iRNA was added, followed by a 10-min incubation at 37 °C. Incorporated [14C]phenylalanine was measured as described.

<table>
<thead>
<tr>
<th>Additions</th>
<th>[14C]Phenylalanine incorporated (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.28</td>
</tr>
<tr>
<td>iRNA, 8.2 μg</td>
<td>3.43</td>
</tr>
<tr>
<td>iRNA, 24.6 μg</td>
<td>3.91</td>
</tr>
</tbody>
</table>

**TABLE IV**

Effects of iRNA or tRNA on protein synthesis in the fractionated reticulocyte system

The assay system is described under "Experimental Procedures." The indicated components were added followed by a 20-min incubation at 37 °C. Incorporation of [14C]leucine was measured as described.

<table>
<thead>
<tr>
<th>Additions</th>
<th>[14C]Leucine incorporated into peptides (cpm x 10^-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.09</td>
</tr>
<tr>
<td>15 μg of iRNA</td>
<td>4.77</td>
</tr>
<tr>
<td>30 μg of iRNA</td>
<td>4.25</td>
</tr>
<tr>
<td>85 μg of iRNA</td>
<td>3.90</td>
</tr>
<tr>
<td>2 μM edeine</td>
<td>3.46</td>
</tr>
<tr>
<td>85 μg of iRNA and 2 μM edeine</td>
<td>3.50</td>
</tr>
<tr>
<td>15 μg of tRNA</td>
<td>5.86</td>
</tr>
<tr>
<td>30 μg of iRNA</td>
<td>5.29</td>
</tr>
</tbody>
</table>

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**Fig. 5. Effect of iRNA on phosphorylation of eIF-2α.** Reticulocyte polysomes, 80 μg, were preincubated with ATP and either dsRNA or iRNA followed by further incubation with eIF-2α and [γ-32P]ATP as described under "Experimental Procedures." Additions to the incubation mixtures were: track 2, none; track 3, 0.5 ng of dsRNA; track 4, 1.0 ng of dsRNA; track 5, 10.4 μg of iRNA; track 6, 36 μg of iRNA. Track 1 is from an incubation containing the heme-controlled eIF-2α kinase, eIF-2α, and [γ-32P]ATP. Samples were electrophoresed on 15% polyacrylamide gels in sodium dodecyl sulfate. The autoradiogram of the dried gel is shown. The amounts of RNA added in 3 and 4 were equal to one unit of inhibitory activity in the reticulocyte lysate assay.
various fractions while maintaining the inhibitor at a constant level. The separate additions of mRNA, elf-2, a ribosomal salt wash fraction, or the 40-70 AS fraction from the postribosomal supernatant did not relieve inhibition in the presence of the inhibitor. Furthermore, iRNA did not change the synthesis (data not shown). Although caution is warranted in interpreting these results, they may indicate that iRNA inactivates the ribosomes rather than any of the components listed above.

The effect of iRNA on partial reactions of peptide initiation was determined. Ternary complex formation between elf-2, methionyl-tRNA<sub>f</sub>, and GTP was not influenced by iRNA (data not shown). Rather surprising results were obtained when the effects of iRNA on binding of methionyl-tRN<sub>A</sub> to 40 S ribosomal subunits were tested. The binding reaction, which is mediated by elf-2, can be carried out with either GTP or its nonhydrolyzable analog, GMP-P(CH<sub>2</sub>)P. The data presented in Table V indicate that iRNA has a different effect on the binding reaction when these two nucleotides are used. Concentrations of iRNA that inhibit the reticulocyte lysate system in the range of 50% or more cause a small but reproducible increase in the amount of Met-tRN<sub>A</sub> bound to 40 S subunits when the binding reaction is carried out with GTP. However, inhibition occurs if GMP-P(CH<sub>2</sub>)P is substituted for GTP in the reaction mixture. The mechanistic basis for this difference is not clear. Previously we observed a differential inhibition by edeine of this reaction when binding was carried out with GTP or its analogue (10). Inhibition by edeine was observed only with GTP. It was suggested that Met-tRN<sub>A</sub> may be bound in different sites or states on 40 S ribosomal subunits depending upon whether or not GTP is hydrolyzed. In contrast to edeine iRNA may decrease the stability of Met-tRN<sub>A</sub> binding into the ribosomal site that is accessible without GTP hydrolysis.

**Table V**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Met-tRN&lt;sub&gt;A&lt;/sub&gt; bound to 40 S pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.53</td>
</tr>
<tr>
<td>iRNA, 17 µg</td>
<td>8.45</td>
</tr>
<tr>
<td>iRNA, 44 µg</td>
<td>8.57</td>
</tr>
<tr>
<td>iRNA, 87 µg</td>
<td>6.15</td>
</tr>
</tbody>
</table>

The size of iRNA described here is in the order of 3 S as judged by sucrose gradient centrifugation. It appears to be similar in size and site of inhibition to the inhibitor RNA from embryonic chicken muscle reported by Sarkar and coworkers (4, 5) and by Sells and his colleagues (3). However, unlike the muscle inhibitor which was associated with protein and was isolated as ribonucleoprotein particles of 10 to 15 S, there is no indication that reticulocyte iRNA is tightly associated with protein. Its sedimentation coefficient was not detectably altered by deproteinization. Analysis of this muscle RNA by polyacrylamide gel electrophoresis in formamide revealed three prominent bands of about 4 S and two minor bands of about 5 S; however, it was not known which, if any, of these components had inhibitory activity. Preliminary chromatographic fractionation of the reticulocyte iRNA are in agreement with the centrifugation data of Fig. 2 in that they indicate inhibitory activity does not correspond to the bulk of the RNA as indicated by absorbance at 260 nm. Although it seems clear that inhibitory activity per unit of RNA can be increased considerably by further fractionation, at this time there is no basis for concluding whether iRNA is a single molecular species or a population of components that have similar sedimentation and chromatographic properties.

It is possible that this apparent difference in iRNA from the two sources may reflect the physiological state of embryonic muscle cells versus reticulocytes. The latter are enucleated cells in a state of terminal differentiation in which globin constitutes about 90% of the protein that is being synthesized. However, the available data do not allow a firm conclusion that the RNA from the two sources are identical or equivalent in either structure or the mechanism by which they cause inhibition of peptide initiation.

Previously, we reported the presence of a heat-stable inhibitor in the postribosomal supernatant of rabbit reticulocytes which was thought to be a low molecular weight protein that we called HS (24). This inhibitor appeared to be activated by heat and was thought to block peptide initiation indirectly with a heat-labile component by activation of an elf-2α kinase. The relation between HS and iRNA is unclear. The iRNA fraction described here is similar to HS in size and chromatographic properties on DEAE-cellulose. However, unlike HS, there is no indication that it is directly or indirectly involved in activation of an elf-2α kinase or that the mechanism by which it inhibits peptide initiation involves phosphorylation of elf-2.

Inhibition by iRNA of elf-2-mediated Met-tRN<sub>A</sub> binding to 40 S ribosomal subunits with GMP-P(CH<sub>2</sub>)P but not with GTP is surprising. Previously we observed a differential inhibition by edeine of this reaction when binding was carried out with GTP or its analogue (10). Inhibition by edeine was observed only with GTP. It was suggested that Met-tRN<sub>A</sub> may be bound in different sites or states on 40 S ribosomal subunits depending upon whether or not GTP is hydrolyzed.

In contrast to edeine iRNA may decrease the stability of Met-tRN<sub>A</sub> binding into the ribosomal site that is accessible without GTP hydrolysis.

**Acknowledgments**—We thank M. Hardesty and M. Rodgers for their excellent technical assistance, M. E. Powers and T. Reeves for preparation of the typescript, and F. Hoffman for photography and art work. We also thank Dr. A. Shatkin for the kind gift of reovirus dsRNA.

**REFERENCES**


**Inhibition of Peptide Initiation by a Small RNA Molecule**

**DISCUSSION**

The size of iRNA described here is in the order of 3 S as indicated by absorbance at 260 nm.
Inhibition of Peptide Initiation by a Small RNA Molecule