ABSTRACT  Addition of the human placental RNase inhibitor at 10 μg/ml to a mixture of wheat germ extract and translation components, prior to the addition of mRNA from dog pancreas or influenza virus-infected cells, resulted in a significant increase in the yield of proteins synthesized. Analysis of the translation products by sodium dodecyl sulfate/polyacrylamide gel electrophoresis indicated that the inhibitor preferentially increased the yield of the larger proteins. In the presence of the inhibitor, yields of the preprocarboxypeptidases were increased 4.5-fold and yields of preamylase were increased 15-fold. Incubation of the wheat germ extract or individual translation components with dog pancreas mRNA, with or without the placental inhibitor, indicated significant RNase contamination among the fractions. Two other in vitro protein synthesis systems—the reticulocyte lysate system and the Krebs ascites system—were found to contain latent RNase activity (RNase in complex with the inhibitor) and an excess of RNase inhibitor. The addition of placental RNase inhibitor did not increase the yield in these systems, except in those cases in which the RNase contamination approached the amount of endogenous inhibitor. When used during the isolation of rat liver cell fractions, the placental inhibitor increased the yield (as measured by A260) of rough microsomes and detached polysomes by 24% and 4.6-fold, respectively. Analysis of translation products indicated that detached polysomes isolated in the presence of the inhibitor were intact; those isolated in the absence of inhibitor were degraded.

Efficient in vitro protein synthesis depends upon maintenance of the integrity of mRNA not only during its isolation from the tissue, but also during its translation in vitro. RNase activity has been observed in vitro in the wheat germ system (1, 2) and was thought to be present primarily as a contaminant among the translation components (3, 4). RNase activity would be expected to affect particularly the translation of large mRNAs because they present larger targets for an endonuclease. Mammalian tissues contain a potent inhibitor of their neutral RNase activity (5). Moreover, the endogenous RNase inhibitor, present in the high-speed supernate of rat and chicken livers, has been shown to improve the preparation of intact polysomes from these tissues (6–11).

A method for the purification of the RNase inhibitor from human placenta (K1 = 3 × 10⁻¹⁰ M for bovine pancreatic RNase A) in large yield and stable form has been reported (12). The ready availability of this RNase inhibitor has allowed us to examine its application to in vitro translation studies and to the isolation of rough microsomes and detached polysomes from rat liver.

METHODS
Preparation of In Vitro Translation Systems. The wheat germ extract was prepared essentially as described by Marcus et al. (13) and filtered on Sephadex G-25 equilibrated with 5 mM Tris acetate, pH 7.6/50 mM KCl/1 mM Mg acetate/2 mM dithiothreitol. Rabbit reticulocyte lysate was prepared essentially as described by Palmier (14), except that 2 mM dithiothreitol (cf. ref. 12) was included during lysis of the reticulocytes to ensure full protection of the endogenous RNase inhibitor. Endogenous globin mRNA was degraded with micrococcal nuclease (1). The nuclease-treated lysate was filtered on Sephadex G-25 equilibrated with 20 mM Hepes-KOH, pH 7.5/50 mM KCl/1 mM MgCl₂/0.5 mM ethylene glycol bis(β-aminoethoxy) ether)-N,N,N',N'-tetraacetic acid/2 mM dithiothreitol. Both wheat germ extract and reticulocyte lysate were stored frozen at −80 °C in 0.5-ml samples.

Mouse Krebs ascites cells were originally a gift from J. Goldstein (The New York Blood Center); S9 cell-free extract was prepared according to the procedure of Kerr et al. (15). The pH 5.0 precipitate of a Krebs ascites S9 subnatum fraction was prepared according to Falvey and Staehelin (16) as modified by Freyenstein and Blobel (17).

RNase inhibitor was purified from normal full-term human placenta by affinity chromatography on RNase A-Sepharose by a modification (to be described elsewhere) of an earlier procedure (12). The inhibitor (200 μg/ml) was dialyzed against 20 mM Hepes-KOH, pH 7.6/10 mM KCl/5 mM dithiothreitol/15% (vol/vol) glycerol and stored in 0.5-ml samples frozen at −20 or −80 °C. The protein was fully stable for months; no loss of activity was observed upon repeated freezing and thawing.

Preparation of Dog Pancreas mRNA. The pancreas was homogenized at 1.5 (wt/vol) in 0.25 M sucrose/2 mM dithiothreitol/polyvinyl sulfate at 40 μg/ml (to decrease RNase activity); RNA was extracted from the postnuclear supernate by the ethanol/sodium perchlorate procedure of Wilcockson (18) as modified by Lizardi and Engelberg (19) for use with mammalian tissues. Polyadenylated mRNA was extracted from total RNA by chromatography on oligo(dT)-cellulose (20).

In Vitro Translation Assays. Assays with the wheat germ system were usually performed in a final volume of 100 μl. The mixture contained 40 μl of the wheat germ extract and the following components at the final concentrations shown: 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, 80 μg of creatine kinase per ml, 110 mM K acetate, 20 mM KCl, 2 mM dithiothreitol, 20 mM Hepes-KOH at pH 7.4, and either 14C-labeled reconstituted protein hydrolysate (Schwarz/Mann algal profile) at 40 μCi/ml and supplemented at 0.1 mM with each of five unlabeled amino acids (glutamine, asparagine, cysteine, methionine, and tryptophan) or 15S)methionine (Amersham, 1100 Ci/mmol) at 100 μCi/ml and supplemented at 0.1 mM with each of 19 unlabeled amino acids (1 Ci = 3.7 × 10¹⁰ becquerels). MgCl₂ and spermine were included at their optimal concentrations, which varied slightly with different preparations of wheat germ extract but were usually 0.9 mM.

Abbreviation: NaDodSO₄, sodium dodecyl sulfate.
RESULTS

RNase and RNase Inhibitor in Cell-Free Translation Systems. The amounts of free RNase, RNase-inhibitor complex, free inhibitor, and the inhibitor/enzyme mole ratios found in four in vitro protein synthesizing systems are given in Table 1. The two mammalian systems (rabbit reticulocyte lysate and mouse Krebs ascites S30 supernatant) contained RNase and an excess of RNase inhibitor. Essentially all of the RNase activity was latent—i.e., complexed with inhibitor. This latent RNase activity may be released with the sulfhydryl reagent p-hydroxymercuribenzoate which inactivates both the free and enzyme-bound inhibitor (5, 25). The pH 5.0 precipitate fraction of the Krebs S30 supernatant, which has been used as a source of translation factors (16, 17, 23, 26), contained only the free inhibitor; the RNase-inhibitor complex remained in the pH 5.0 supernate. A similar fractionation occurs in extracts from other mammalian tissues, including the human placental extracts from which the RNase inhibitor used in this study was prepared. In the wheat germ extract, neither free RNase nor inhibition of RNase A activity was detected with the yeast ribosomal RNA assay (12).

Effect of the Placental RNase Inhibitor on the In Vitro Translation of Dog Pancreas mRNA with Wheat Germ Extract. The incorporation of 14C-labeled amino acids into protein synthesized in vitro by the wheat germ system (Fig. 1A) was nearly linear throughout the first 60 min of translation. Then the rate of incorporation decreased and reached a plateau after 90–120 min. In the presence of placental RNase inhibitor at 10 µg/ml, both the rate and extent of protein synthesis were increased. In the experiment shown, an increase of 49% was observed at 120 min. After the termination of translation, the products were separated by gradient polyacrylamide gel electrophoresis in NaDodSO4 and examined by fluorography (Fig. 1B). Increased intensity of the bands was found for those proteins synthesized in the presence of RNase inhibitor, particularly for those of higher molecular weight. This increase in synthesis of larger proteins was found consistently with different preparations of wheat germ extract. This has also been the case in experiments in which the stimulation of incorporation of either 14C-labeled amino acids or 35S-labeled methionine into protein was less than that shown in Fig. 1A.

The relative amounts of three groups of proteins of different sizes synthesized by the in vitro wheat germ system and those observed in cellular synthesis are compared quantitatively in Table 2. The wheat germ system without inhibitor synthesized preamylase (Mr ≈ 55,000) and the preprocarboxypeptidase (Mr ≈ 46,000) poorly. In the presence of RNase inhibitor, there was >10-fold increase in preamylase synthesis

Table 1. Amount of free RNase, RNase-inhibitor complex (RNase-I), and free inhibitor (I) in cell-free protein synthesizing systems

<table>
<thead>
<tr>
<th>Cell-free system</th>
<th>Free RNase (µg/ml)</th>
<th>RNase-I complex, µg/ml</th>
<th>Free I, µg/ml</th>
<th>I/RNase ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocyte lysate</td>
<td>0.01</td>
<td>0.025</td>
<td>2.0</td>
<td>9.1</td>
</tr>
<tr>
<td>Krebs ascites S30</td>
<td>0.01</td>
<td>0.0137</td>
<td>4.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Krebs pH 5 fraction</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>2.6</td>
<td>—</td>
</tr>
<tr>
<td>Wheat germ extract</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>None</td>
<td>—</td>
</tr>
</tbody>
</table>

* Measured during a 30-min incubation at 37°C with yeast ribosomal RNA substrate as described by Blackburn et al. (12).

1 Measured as µg of RNase liberated after p-hydroxymercuribenzoate inactivation of the inhibitor.

2 Measured as µg RNase inhibition and converted to µg of inhibitor based upon a 1:1 enzyme inhibitor complex and a 4:1 ratio of inhibitor to enzyme by molecular weight (12).
and a 3-fold increase in the synthesis of the preprocarboxy-peptidases; the synthesis of the serine proteases (Mr ≈ 28,000) was increased by about 20%. The data in Table 2 demonstrate that the inhibitor does not simply increase the overall rate of protein synthesis; if it had, the distribution of [35S]methionine among the protein groups would have remained unaltered. The combination of increased incorporation of radioactivity, as demonstrated in Fig. 1A, and the improved distribution seen in Table 2 demonstrates an overall 4.5-fold increase in the synthesis of the preprocarboxy-peptidases and a 15-fold increase in the synthesis of preamylase.

We have compared the translation of mRNA from influenza virus A/WSN-infected HeLa cells (gift from Robert Lamb of the Rockefeller University) by the wheat germ system with and without RNase inhibitor. In the presence of RNase inhibitor, we observed an increase (10%) in the incorporation of [35S]methionine into trichloroacetic acid-precipitable protein. Again, the RNase inhibitor also significantly improved the translation of specific viral proteins by the wheat germ system (Fig. 2).

Synthesis of the higher molecular weight proteins—e.g., the hemagglutinin (75,000) and P1, P2, and P3 proteins (85,000–96,000) was markedly increased. Furthermore, by the use of the placentinal RNase inhibitor under the conditions that we have described (31), Robbi and Lazarow (32) have demonstrated a dramatic increase in the translation of rat liver catalase mRNA by wheat germ extract.

**RNase Activity in Individual Translation Components and Effect of RNase Inhibitor.** Each of the components was incubated with or without RNase inhibitor for 30 min at 37°C with dog pancreas polyadenylated mRNA. RNase inhibitor was then added to those vials that did not have it, to terminate any inhibitor-sensitive RNase activity. Residual translational activity of the mRNA in both sets of vials was then assayed by measurement of [35S]methionine incorporation at 24°C with the addition of wheat germ extract and the remaining translation components. Several of the components were found to contain significant amounts of RNase activity. The amount varied from preparation to preparation of the same component. In each case, with the exception of the wheat germ extract, the placentinal RNase inhibitor fully blocked this activity. The RNase inhibitor only partially prevented loss of mRNA activity in the wheat germ extract. This result suggested that the wheat germ extract contained not only a mammalian-type RNase, as a contaminant, but also small quantities of endogenous plant RNase(s). Use of the translation assay in this way allowed the detection of small amounts of nuclease activity that were not detectable by the assay with yeast ribosomal RNA (12) used for Table 1.

The RNase inhibitor was added to the translation mixtures containing the translation products obtained as described for A. Equal volumes of the translation mixtures without (track a) and with (track b) placentinal RNase inhibitor at 10 μg/ml were prepared for NaDodSO4/polyacrylamide gel electrophoresis and fluorography. The Mr values shown at the right (X 10−3) correspond to those of the presecretory proteins preamylase (55,000), preprocarboxy-peptidases (46,000), and the serine proteases (27,000) (23, 27, 28).

**Table 2. Relative synthesis of dog pancreatic proteins**

<table>
<thead>
<tr>
<th>System</th>
<th>Amylase</th>
<th>Procarboxy-peptidases</th>
<th>Serine proteases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular synthesis*</td>
<td>94</td>
<td>9.5</td>
<td>90</td>
</tr>
<tr>
<td>Cell-free synthesis†</td>
<td>&lt;1</td>
<td>5.0</td>
<td>94</td>
</tr>
<tr>
<td>Wheat germ system</td>
<td>10</td>
<td>15</td>
<td>115</td>
</tr>
<tr>
<td>Wheat germ system +1</td>
<td>10</td>
<td>15</td>
<td>115</td>
</tr>
</tbody>
</table>

Equal cpm of [35S]methionine-labeled protein were separated by gradient polyacrylamide gel electrophoresis. Densitometric scans of fluorograms of the polyacrylamide gels were performed. The amounts of protein synthesized in the three molecular weight regions were estimated from the peak areas.

* Cellular protein synthesis was carried out by incubation of pancreatic tissue slices for 1 hr in Krebs–Ringer bicarbonate buffer containing [35S]methionine and physiological levels (29) of 19 unlabeled amino acids. Tissue slices were homogenized in 1% Triton X-100/25 mM Tris-HCl, pH 9.2.

† Synthesis of presecretory proteins (23, 27, 28).
The major source of RNase activity arises not from the wheat germ but from the other components of the translation assay mixture. To demonstrate the cumulative effect of contaminant RNase activities on mRNA stability, a complete mixture of translation components, excluding the wheat germ extract, was incubated with pancreatic polyadenylated mRNA, with or without RNase inhibitor (10 μg/ml). Residual mRNA activity was assayed as described above. The translation products were examined by NaDodSO4/polyacrylamide gel electrophoresis followed by fluorography. Incubation of the mRNA with the combined components amplified the effect of contaminant RNase and led to an almost complete loss of mRNA activity (Fig. 3, track a). With the RNase inhibitor present (track b), this RNase action was inhibited and the mRNA retained full activity. Similarly, when mRNA was incubated with RNase A at 0.25 μg/ml, more than 100 times the total contaminant RNase activity, the mRNA was completely degraded (track c). With RNase inhibitor present (at 10 μg/ml) during incubation (track d) the mRNA retained full translational activity.

Effect of RNase Inhibitor on the Stability of Rough Microsomes and Detached Polysomes from Rat Liver. When placental RNase inhibitor at 10 μg/ml was included in the discontinuous sucrose gradient used to isolate rough microsomes, a 24% increase in their yield (as measured by absorbance at 260 nm) was obtained (Table 3). During the preparation of detached polysomes, a 4.6-fold increase in their yield (based on A260) was obtained.

The isolated rough microsomes and detached polysomes were examined for RNase activity by incubating each cell fraction with or without RNase inhibitor for 30 min at 24°C. The results of this experiment are presented in Table 3. The data show that the presence of RNase inhibitor significantly increased the yield of polysomes and decreased the RNase activity of the polysomes. These results suggest that the presence of RNase inhibitor is essential for maintaining the integrity of the polysomes.

**DISCUSSION**

When added to the wheat germ system during in vitro translation, the RNase inhibitor protects mRNA from degradation by RNase present as a contaminant among the components of the system. The result is a significant improvement in yields of proteins synthesized, as measured by incorporation of radioactivity into protein. Syntheses of high molecular weight proteins are markedly increased. Despite optimal concentrations of K+, Mg++, and spermine (1, 2, 53), the in vitro wheat germ system with added RNase inhibitor did not synthesize dog pancreas secretory proteins with the same distribution as that observed in vivo. Therefore, other factors must also contribute to the efficiency of translation of individual mRNAs in vitro. One factor may be the small amount of inhibitor-insensitive RNase activity found in the wheat germ extract.

**Table 3.** Effect of the human placental ribonuclease inhibitor (I) on the stability of rat liver rough microsomes (RM) and detached polysomes (DP)

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>RM -I</td>
<td>19.0</td>
<td>-I</td>
<td>876</td>
<td>56</td>
</tr>
<tr>
<td>RM +I</td>
<td>23.5</td>
<td>+I</td>
<td>1357</td>
<td>86</td>
</tr>
<tr>
<td>RM -I</td>
<td>0.5</td>
<td>-I</td>
<td>248</td>
<td>7.6</td>
</tr>
<tr>
<td>DP -I</td>
<td>2.3</td>
<td>-I</td>
<td>712</td>
<td>100</td>
</tr>
<tr>
<td>DP +I</td>
<td>2.3</td>
<td>+I</td>
<td>710</td>
<td>100</td>
</tr>
</tbody>
</table>

* Yield is given in total A260 units per gram of rat liver.
† The fractions were preincubated ± RNase inhibitor prior to their translation with reticulocyte lysate, in order to demonstrate the presence of inhibitor-sensitive RNase activity associated with the fractions.
‡ Specific activity is given in [35S]-methionine incorporation (cpm) per A260 unit of RM or DP. Incorporation of [35S]-methionine was measured at 90 min at which time incorporation was complete.
§ Percent based upon activity under optimum conditions.
Addition of the placental RNase inhibitor to either reticulocyte lysate or mouse Krebs ascites systems in general had little effect on in vitro protein synthesis. These systems were found to contain RNase fully inhibited by an excess of endogenous RNase inhibitor. To maintain and utilize this endogenous RNase inhibitor activity, we recommend that these systems be prepared and stored in buffer containing a minimum of 2 mM dithiothreitol (12). However, when the RNase contamination among the components exceeded the amount of endogenous RNase inhibitor in the reticulocyte system, the placental RNase inhibitor had a demonstrable effect, similar to that seen in the wheat germ system.

The choice of an in vitro translation system for optimal synthesis of a particular protein will depend upon the efficiency of translation of its mRNA in that system. The reticulocyte lysate system was found to translate the larger dog pancreas mRNAs more efficiently than did the wheat germ system with added RNase inhibitor. However, the wheat germ system with RNase inhibitor was more efficient than the reticulocyte lysate for the translation of the larger influenza virus mRNAs.

In the application of RNase inhibitor to the isolation of cell fractions that contain mRNA, rat liver was chosen for study because rough microsomes and detached polysomes from this tissue contain considerable quantities of RNase. Initially, the procedure takes advantage of the endogenous RNase inhibitor present in the tissue. Although rough microsomes were found to be relatively stable during isolation, as judged by specific activity, addition of RNase inhibitor to the discontinuous sucrose gradient improved their yield as measured by A_{260}. After isolation, rough microsomes required the presence of placentall RNase inhibitor for retention of full translational activity. Assays for RNase, RNase inhibitor complex, and free RNase inhibitor demonstrated both latent RNase and free inhibitor in the high-speed supernate. This finding accounts for the relative stability of rough microsomes during their isolation. After isolation, however, rough microsomes contained RNase complexed with inhibitor but no free RNase inhibitor. In the absence of free inhibitor, the equilibrium between RNase and its inhibitor would favor dissociation of the complex to yield active enzyme, which would then affect the stability of mRNA associated with the microsomes. The deoxycholate used to solubilize the microsomal membranes for the preparation of detached polysomes appears to release additional RNase from the microsomes. In the absence of excess RNase inhibitor this RNase activity degraded the polysomes during their isolation. This activity could be fully inhibited by the human placental RNase inhibitor.

Based on the studies presented here, we recommend that the RNase inhibitor, a protein readily available from human placenta (12), be used during in vitro translation studies, specifically as an additive to the wheat germ extract prior to the addition of mRNA, to the translation system components prior to their mixture with mRNA, and during the isolation of mammalian cell fractions that contain mRNA–protein.

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