Messenger Ribonucleic Acid Specificity in the Inhibition of Eukaryotic Translation by Double-Stranded Ribonucleic Acid†

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ABSTRACT: Double-stranded ribonucleic acid (dsRNA) is a powerful inhibitor of initiation of protein synthesis in rabbit reticulocyte lysates. This paper shows that translation of Mengovirus or Coxsackie virus RNA in a template-dependent reticulocyte lysate is resistant to inhibition by dsRNA in conditions where translation of globin or ascites tumor cell messenger RNA (mRNA) is sensitive. The inhibition of globin mRNA translation by dsRNA is reversed completely upon addition of eukaryotic initiation factor 2 (eIF-2). During dsRNA-induced inhibition of globin mRNA translation, formation of (2′–5′)-oligoisoadenylate was not detectable. The unabated translation of Mengovirus RNA in the presence of dsRNA is dependent on continued initiation: not only is translation of Mengovirus RNA as sensitive as that of globin mRNA to the specific inhibitors of initiation, pactamycin and aurin tricarboxylate, but unlike for globin mRNA the Mengovirus RNA directed incorporation of *N*-formyl[^{35}S]-methionyl-tRNA$_f$ into protein is not inhibited by dsRNA. The resistance of Mengovirus RNA translation to dsRNA is not caused by a lesser dependence on initiation factors, but by a failure of dsRNA to establish inhibition when Mengovirus RNA is used as messenger. The nature of the mRNA being translated is a critical factor in the formation of the dsRNA-activated inhibitor of translation. Mengovirus or Coxsackie virus RNA, it is shown, prevents the formation of dsRNA-activated inhibitor, while globin mRNA does not. Yet, once it is allowed to form, the inhibitor is as effective in blocking translation of Mengovirus RNA as it is in blocking translation of globin mRNA, when assayed in the presence of noninhibitory, high concentrations of dsRNA. During translation of globin mRNA in the template-dependent lysate, the presence of dsRNA stimulates the phosphorylation of the small subunit of eIF-2, as well as a 67 000-dalton polypeptide, that is characteristic of the state of translational inhibition. By contrast, when Mengovirus RNA is used as template, the dsRNA-dependent phosphorylation of either polypeptide is significantly depressed. Moreover, in a ribosomal system containing eIF-2, dsRNA, and ATP, Mengovirus RNA inhibits the phosphorylation of the small subunit of eIF-2 and of the 67 000-dalton polypeptide, apparently in a competitive manner. Globin mRNA does not possess this property. Direct RNA-binding competition studies reveal that dsRNA competes with mRNA for eIF-2, binding this factor more strongly than globin mRNA, but more weakly than Mengovirus RNA. A molecule of Mengovirus RNA exhibits a 30–40-fold greater affinity for eIF-2 than does a molecule of globin mRNA. The progressively greater affinities of globin mRNA, dsRNA, and Mengovirus RNA for eIF-2 are also reflected by the ability of these RNA species to competitively inhibit the binding of methionyl-tRNA$_f$ to eIF-2. These findings demonstrate mRNA specificity in the inhibitory action of dsRNA on eukaryotic protein synthesis. The correlation between the results of translation, inhibitor formation, phosphorylation, and binding competition experiments suggests that the affinity of a given mRNA species for eIF-2 is crucial in determining the sensitivity of its translation to dsRNA. Our results support the concept that the rate-determining event in the establishment of inhibition of translation by dsRNA involves competition between dsRNA and mRNA and that inhibitor formation, phosphorylation, and inactivation of eIF-2 depend on the outcome of this competition.

One of the clearest instances of translational control that can be studied in vitro is the block in initiation of translation in reticulocyte lysates observed in the presence of double-stranded ribonucleic acid (dsRNA)[1] (Ehrenfeld & Hunt, 1971). This block involves the inactivation of an initiation factor (Kaempfer & Kaufman, 1973) identified with eIF-2 (Kaempfer, 1974; Clemens et al., 1975); addition of this factor is sufficient to overcome the dsRNA-mediated inhibition of translation (Kaempfer & Kaufman, 1973; Kaempfer, 1974). The translational block induced by interferon also appears to involve dsRNA-dependent inactivation of eIF-2 as a major mechanism of control (Kaempfer et al., 1979b).

eIF-2 forms a ternary complex with Met-tRNA$_f$ and guanosine 5′-triphosphate (GTP) and promotes binding of Met-tRNA$_f$ to the 40S ribosomal subunit (Levin et al., 1973; Schreier & Staehelin, 1973), yielding a complex obligatory for the subsequent binding of messenger RNA (mRNA) (Darnbrough et al., 1973; Trachsel et al., 1977). In addition to binding Met-tRNA$_f$, eIF-2 itself can bind to mRNA (Kaempfer, 1974; Hellerman & Shafritz, 1975; Barrieux & Rosenfeld, 1977, 1978; Kaempfer et al., 1978a, 1979a; Rosen & Kaempfer, 1979). Specificity in the binding of eIF-2 to mRNA was shown by the finding (Kaempfer et al., 1978a, 1979a) that all mRNA species tested possess a high-affinity binding site for eIF-2, including mRNA species lacking the 5′-terminal cap of 3′-terminal poly(adenylic acid) [poly(A)] moieties, while RNA species not serving as mRNA, such as negative-strand RNA (Kaempfer et al., 1978a), transfer RNA (tRNA), or ribosomal RNA (rRNA) (Barrieux & Rosenfeld, 1977), do not possess such a site. Moreover, eIF-2 also appears to recognize the 5′-terminal methylated cap structure in mRNA: analogues of the cap inhibit the binding of mRNA

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[1] Abbreviations used: dsRNA, double-stranded ribonucleic acid; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Met-tRNA$_f$, methionyl-tRNA$^{Met}_f$; eIF-2, eukaryotic initiation factor 2; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; EAT, Ehrlich ascites tumor; $NaDodSO_4$, sodium dodecyl sulfate; DEAE-cellulose, diethylaminoethylcellulose.

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and of Met-tRNA$_f$ to eIF-2, and addition of eIF-2 relieves the cap analogue induced inhibition of mRNA translation (Kaempfer et al., 1978b).

Recently, eIF-2 was shown to bind specifically to satellite tobacco necrosis virus RNA at the 5′-terminal 44-nucleotide sequence that comprises the ribosome binding site (Kaempfer et al., 1981). Furthermore, eIF-2 binds with high specificity to Mengovirus RNA, protecting three of the four major T1 oligonucleotides that are protected in 40S of 80S initiation complexes (R. Perez-Bercoff and R. Kaempfer, unpublished experiments).

These properties reveal a role for eIF-2 in the recognition of mRNA and its binding to ribosomes, making it a protein uniquely suited for translational control. Indeed, addition of eIF-2 leads to relief of translational competition between α- and β-globin mRNA, both when it is caused by a high mRNA concentration and when it is sharpened by elevated concentrations of salt. A close correlation is obtained between the results of translation experiments and those of experiments measuring directly the binding of globin mRNA to eIF-2. These findings strongly suggest that mRNA interacts directly with eIF-2 during protein synthesis and that α-globin mRNA has a lower affinity for eIF-2 than does β-globin mRNA (Di Segni et al., 1979).

In addition to binding mRNA, eIF-2 also can bind tightly to dsRNA, but the latter binding is not restricted to specific sequences (Kaempfer, 1974; Kaempfer et al., 1978a). This raised the possiblity that a direct interaction between dsRNA and the factor might be critical for the establishment of translational inhibition by dsRNA (Kaempfer & Kaufman, 1973). The finding that dsRNA possesses many binding sites for eIF-2 (Kaempfer et al., 1978a) might then help to explain the effective inhibition by low doses of dsRNA (Ehrenfeld & Hunt, 1971). The mechanism of action of dsRNA is more complex, however, for dsRNA promotes the formation of a macromolecular inhibitor of translation (Hunter et al., 1975; Clemens et al., 1975) and causes activation of a kinase that phosphorylates the small, 38 000-dalton subunit of eIF-2 (Farrell et al., 1977; Levin & London, 1978). Although it was shown that inhibition by dsRNA is concomitant with phosphorylation of eIF-2, it is not yet clear if phosphorylation is the primary cause of inhibition. Taken in its most logical form, the kinase model (Farrell et al., 1977) states that dsRNA induces the phosphorylation of eIF-2, thereby abolishing binding of Met-tRNA$_f$ to 40S ribosomal subunits, and, hence, subsequent binding of mRNA and initiation of translation. If this were so, one would predict that dsRNA should inhibit translation in general, irrespective of the type of mRNA being translated. We report here that this is not the case and demonstrate, instead, mRNA specificity in the inhibitory action of dsRNA on translation.

Specifically, we show that the nature of the mRNA being translated is critically important for determining the inhibitory effect of dsRNA on protein synthesis, for the establishment of inhibition by dsRNA, for formation of the dsRNA-activated inhibitor, for phosphorylation of the small subunit of eIF-2, and for the associated phosphorylation of a 67 000-dalton polypeptide. These phenomena are observed when globin mRNA is used as the template for translation, yet none are seen when Mengovirus RNA is used as template, in spite of the fact that Mengovirus RNA translation is essentially as dependent on continued initiation and on initiation factors as translation of globin mRNA. We show that Mengovirus RNA protects the translation machinery against inactivation by dsRNA. Once inhibition by dsRNA has been established and the dsRNA-activated inhibitor has been allowed to form, however, the translation of Mengovirus RNA is inhibited to the same extent as that of globin mRNA. As judged by direct binding competition experiments, Mengovirus RNA possesses a higher affinity for eIF-2 than does dsRNA, while the opposite is true for globin mRNA. Moreover, Mengovirus RNA, but not globin mRNA, acts as a potent inhibitor of the dsRNA-dependent phosphorylation of the small subunit of eIF-2 and the 67 000-dalton polypeptide.

These results with globin mRNA and Mengovirus RNA reveal a correlation between the affinity of an mRNA species for eIF-2 and the sensitivity of its translation to inhibition by dsRNA.

Experimental Procedures

Reticulocyte Lysate. Lysates were prepared from rabbits made anemic by five successive daily injections of 1 mL/kg of 1.25% 1-acetyl-2-phenylhydrazine in 0.14 M NaCl, 1.5 mM magnesium acetate, and 5 mM KCl. Bleeding was on the eighth day. Lysates were prepared as described by Kaempfer & Kaufman (1972). They were made 50 μg/mL in creatine kinase and 30 μM in hemin and treated with micrococcal nuclease as described by Pelham & Jackson (1976).

mRNA and dsRNA. Globin mRNA was prepared as described (Kaempfer et al., 1978a). Mengovirus and Coxsackie B5 virus RNAs were a gift of Dr. F. Brown, The Animal Virus Research Institute, Pirbright, England. Ehrlich ascites tumor cell poly(A)-containing RNA was prepared similarly to globin mRNA, by resuspending polysomes in 0.5% NaDodSO$_4$ and 0.5 M NaCl and subjecting the suspension to oligo(dT)–cellulose chromatography (Aviv & Leder, 1972). dsRNA from *Penicillium chrysogenum* was a purified fraction containing only the 2×10^6-dalton species and was a gift of Dr. W. Kleinschmidt, Eli Lilly & Co., Indianapolis, IN.

Cell-Free Protein Synthesis. Cell-free translation mixtures of 50 μL contained 30 μL of lysate, 6 μg of mouse liver tRNA (Kaempfer et al., 1978a), 0.6 mM added magnesium acetate, 95 mM added KCl (except in Figures 2 and 10a where 125 and 80 mM were added, respectively), 4 mM creatine phosphate, 20 mM Hepes buffer, pH 7.5, [^{35}S]methionine (Amersham), and mRNA. In the presence of a saturating amount of globin mRNA, translation in micrococcal nuclease treated lysates was 90% or more of that seen in these lysates before digestion. Hot Cl_3CCOOH-precipitable radioactivity was determined in aliquots spotted onto 1-in. square filters (Whatman No. 1 paper).

Preparation of [^{35}S]Met-tRNA$_f$ and N-Formyl[^{35}S]Met-tRNA$_f$ and Assay of Ternary Complex Formation. [^{35}S]-Met-tRNA$_f$ was prepared, and ternary complex formation with eIF-2 and GTP was assayed as described (Kaempfer et al., 1978a). The KCl concentration in the assay was 100 mM. *N*-Formyl[^{35}S]Met-tRNA$_f$ was prepared by including in the charging mixture 0.5 mg/mL calcium leucovorin that had been flushed with H_2 just before addition to the reaction mixture. The extent of formylation was greater than 95% as judged by the mobility of the labeled T1 oligonucleotide during electrophoresis at pH 3.5.

Purification of eIF-2. Rabbit reticulocyte lysate was centrifuged for 3 h at 2 °C in an angle rotor at an average of 150000*g*. The tubes contained a 0.1-mL cushion of 75% enzyme-grade sucrose in 10 mM Tris-HCl, pH 7.4, 2 mM magnesium acetate, and 50 mM KCl. The ribosomal pellet was suspended with the aid of a glass rod into 30 volumes of 10 mM Tris-HCl, pH 7.4, 0.4 M KCl, and 10 mM 2-mercaptoethanol. The suspension was stirred magnetically for 45 min at 4 °C and centrifuged at 150000*g* for 3 h. The

supernatant was precipitated with ammonium sulfate to 50% saturation. The pellet, collected by centrifugation for 20 min at 28000*g*, was dissolved into 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 5 mM 2-mercaptoethanol and dialyzed against 1000 volumes of the same buffer. The dialyzed preparation was centrifuged for 10 min at 2000*g* and stored in aliquots at −70 °C. This ribosomal wash fraction was applied to a 10-volume bed of DEAE-cellulose equilibrated with 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM KCl, 10 mM 2-mercaptoethanol, and 10% glycerol. The column was washed with the same buffer and developed by stepwise elution at 0.1 and 0.22 M KCl. The peak of A_{280} material eluted at 0.22 M KCl was diluted to 0.1 M KCl with buffer A (50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) and applied to a phosphocellulose column about 40% the size of the DEAE-cellulose column. The column was washed with 0.1 M KCl in buffer A and stepwise eluted with 0.4 M KCl in buffer A. A linear gradient of 0.4–0.7 M KCl in buffer A was then applied, and eIF-2 was assayed as soon as the gradient was completed. In this procedure, the only RNA-binding component eluted between 0.4 and 0.7 M KCl off the phosphocellulose column is eIF-2, as judged by the complete coelution of Met-tRNA$_f$ and mRNA-binding activities, and by the ability of Met-tRNA$_f$ to inhibit competitively, and completely, the binding of mRNA (Kaempfer, 1979; Rosen & Kaempfer, 1979).

Phosphorylation of eIF-2 and the 67 000-Dalton Polypeptide. Phosphorylation in whole lysates was in the conditions of translation, in the presence of 30 μCi of [γ-^{32}P]ATP (Nuclear Research Center—Negev; 40–60 Ci/mmol). After incubation for 20 min, reaction mixtures (150 μL) were centrifuged for 2 h at 50000*g* in microcapped polyethylene centrifuge tubes. The supernatant was drained carefully, and the ribosome pellet was dissolved in sample buffer and subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis in 10% gels (Laemmli, 1970), using cross-linked hemoglobin (Sigma) and cross-linked bovine serum albumin (Sigma) as molecular weight markers. The dried gels were subjected to autoradiography.

Phosphorylation in the ribosomal system was in 25-μL reaction mixtures containing 90 mM KCl, 1 mM Mg(OAc)$_2$, 0.1 mM unlabeled ATP, 7.5–15 μCi of [γ-^{32}P]ATP, 20 mM Tris-HCl, pH 7.4, crude initiation factors (40–50% saturated ammonium sulfate cut of 0.5 M KCl ribosomal wash), and crude ribosomes. The ribosomes were prepared by centrifuging 250 μL of micrococcal nuclease treated reticulocyte lysate for 2 h at 50000*g* in a microcapped polyethylene centrifuge tube, removing the supernatant, and dissolving the pellet in 50 μL of 50 mM Tris-HCl, pH 7.4.

Radioiodination of RNA. The method, based on that of Commerford (1971) with some modifications, has been described (Kaempfer et al., 1979a; Kaempfer, 1979; Di Segni et al, 1979). The ^{125}I-labeled mRNA obtained in this procedure is labeled to a relatively low specific activity, (0.5–1) × 10^6 cpm/μg. This low specific activity is important in determining the properties of the labeled RNA. The RNA is fully intact as judged by polyacrylamide gel electrophoresis and autoradiography, as judged for globin mRNA. Both globin and Mengo RNA labeled in our procedure bind to eIF-2 with an affinity equal to that of the unlabeled, native RNA species, as judged by self-competition (Kaempfer, 1979; Kaempfer et al., 1979a).

Binding of mRNA and dsRNA to eIF-2. Reaction mixtures of 50 μL, containing 150 mM KCl, 20 mM Tris-HCl, pH 7.8, 2 mM magnesium acetate, 6 mM 2-mercaptoethanol, eIF-2, and RNA, were incubated for 10 min at 25 °C and then cooled for 10 min at 0 °C before the addition of 1 mL of ice-cold buffer B (20 mM Tris-HCl, pH 7.8, 6 mM 2-mercaptoethanol, 50 mM KCl, and 2 mM magnesium acetate). The samples were passed through 0.45-μm nitrocellulose filters (25 mm diameter, Millipore) at a flow rate of 1 mL/min and washed 3 times with 1 mL of buffer B. Dried filters were counted by γ radiation spectrometry. For a more detailed description, see Kaempfer (1979).

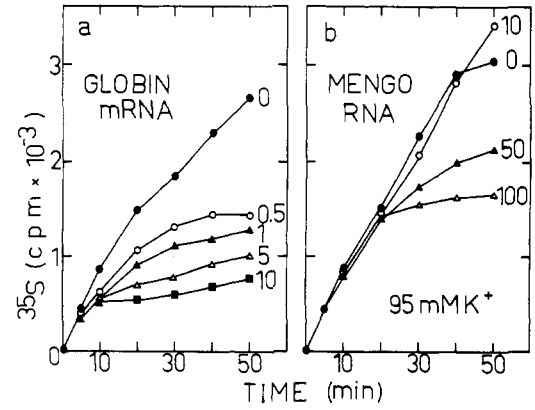

FIGURE 1: Differential sensitivity of Mengovirus RNA and globin mRNA translation to inhibition by dsRNA. A rabbit reticulocyte lysate treated with micrococcal nuclease was incubated with globin mRNA (1.5 μg) (a) or Mengovirus RNA (1.5 μg) (b). DsRNA from *Penicillium chrysogenum* was present at the indicated concentrations, in ng/mL. Translation was at 30 °C in 50-μL reaction mixtures containing 30 μL of lysate, 6 μg of mouse liver tRNA, 95 mM added KCl, and 0.6 mM added Mg^{2+}. This Mg^{2+} concentration was determined as optimal for both mRNA species. Aliquots of 5 μL were sampled at time intervals, and hot CCl_3COOH-precipitable [^{35}S]-methionine was determined. Background without mRNA (300 cpm) was subtracted.

Assay for the Synthesis of (2′–5′)-Oligoisoadenylate. The synthesis of (2′–5′)-oligoisoadenylate in rabbit reticulocyte lysates, both regular and micrococcal nuclease treated, was studied by the method of Hovanessian & Kerr (1978). The lysate (50 μL), containing 30 μM hemin and 50 μg/mL creatine kinase, was incubated for 1 h at 30 °C with 1 mM ATP and varying concentrations of dsRNA. After the addition of 0.45 mL of H_2O and incubation for 5 min at 95 °C, the denatured proteins were removed by centrifugation, and the supernatant was assayed, at a final dilution of 1300-fold, for its ability to inhibit translation of globin mRNA in a micrococcal nuclease treated lysate. In some experiments, the supernatant was loaded onto a DEAE-cellulose column, and inhibitory activity was recovered by step elution at 350 mM KCl (Hovanessian et al., 1977). The extent of inhibition observed ranged from 60 to 80%.

To assay for the synthesis of (2′–5′)-oligoisoadenylate in conditions of protein synthesis, standard translation reaction mixtures containing micrococcal nuclease treated lysate and 1.5 μg of globin mRNA were incubated, with and without 1 mM added ATP, in the presence of varying dsRNA concentrations. After 50 min, the mixtures were assayed for inhibitory activity as described above.

Results

Mengovirus RNA Translation Is Resistant to dsRNA. As seen in Figure 1a, translation of globin mRNA in a template-dependent, micrococcal nuclease treated rabbit reticulocyte lysate is sensitive to inhibition by as little as 0.5 ng/mL dsRNA. Inhibition is essentially complete when 10 ng/mL dsRNA is present and is characterized by cessation of translation after 5–10 min of incubation. These properties

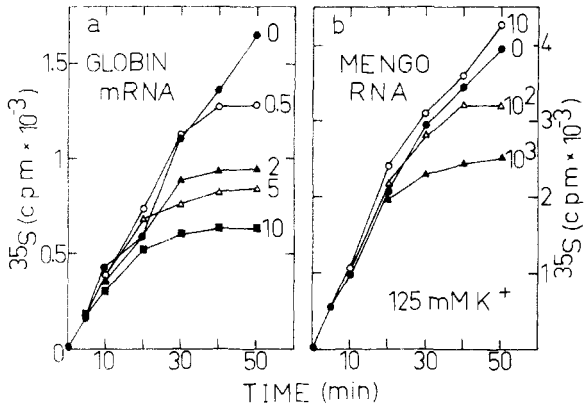

FIGURE 2: Differential sensitivity of Mengovirus RNA and globin mRNA translation to inhibition by dsRNA. Procedure same as described for Figure 1, except that KCl was added to 125 mM. Numbers indicate dsRNA concentration, in ng/mL.

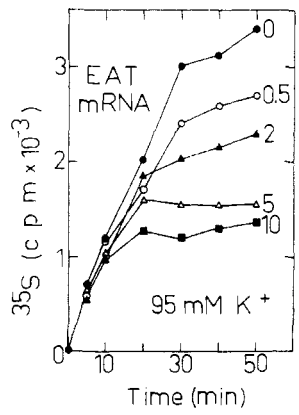

FIGURE 3: Sensitivity of EAT cell mRNA translation to dsRNA. Translation of poly(A)-containing mRNA (1.5 μg) from EAT cell polysomes was same as described in Figure 1, with dsRNA present at the indicated concentrations, in ng/mL.

closely resemble those of a standard lysate (Kaempfer & Kaufman, 1973; Hunter et al., 1975). Mengovirus RNA, as seen in Figure 1b, directs at least as much incorporation as globin mRNA, even though 10 times fewer molecules of RNA are present. In contrast to globin mRNA, however, Mengovirus RNA directed protein synthesis is unaffected by 10 ng/mL dsRNA and is inhibited only partially by 5- or 10-fold greater concentrations. Essentially the same results are obtained when [^{3}H]leucine of ^{14}C-labeled amino acids are used instead of [^{35}S]methionine, or when mouse tRNA is omitted; moreover, addition of Ehrlich ascites tumor cell sap neither stimulates Mengovirus RNA translation in this system nor affects dsRNA sensitivity (data not shown).

In Figure 1, exogenous K^+ ions were added to 95 mM, optimal for globin mRNA translation but slightly less than optimal for Mengovirus RNA. In Figure 2, translation is compared at 125 mM K^+, the optimum for Mengovirus RNA. Again, globin mRNA translation is seen to be sensitive to low concentrations of dsRNA, while Mengovirus RNA translation is resistant. Indeed, 2 orders of magnitude more dsRNA must be added to observe inhibition of Mengovirus RNA directed synthesis, and even then inhibition is only partial. At higher concentrations than were used in Figure 2b, dsRNA loses its inhibitory activity (Hunter et al., 1975). That globin mRNA is not unique in its sensitivity to dsRNA is seen in Figure 3: translation of poly(A)-containing Ehrlich ascites tumor cell mRNA is just as sensitive.

To examine if the continued translation of Mengovirus RNA, observed in the presence of dsRNA in Figures 1 and

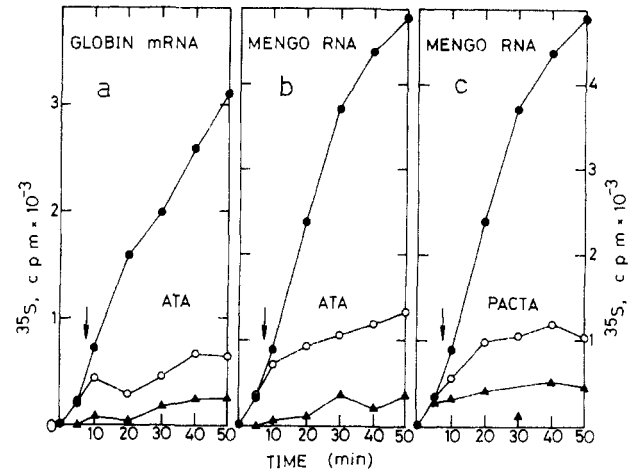

FIGURE 4: Sensitivity of Mengovirus RNA and globin mRNA translation to specific inhibitors of initiation. Translation mixtures containing 1.5 μg of globin mRNA (a) or Mengovirus RNA (b and c) were incubated, as described for Figure 1, in the absence of any inhibitor (●) or with an inhibitor added at 0 min (▲) or at 7.5 min (arrow) after the start of incubation (○). In (a) and (b), the inhibitor was aurintricarboxylic acid (ATA) at a final concentration of 70 μM. In (c), pactamycin (PACTA) was present at a final concentration of 0.8 μM. The equivalent experiment with pactamycin for globin mRNA, done in parallel, gave kinetics very similar to those in panel a, and is not shown in the figure.

2, could be explained by chain elongation independent of further initiation, aurintricarboxylic acid (70 μM) or pactamycin (0.8 μM) was added to reaction mixtures for protein synthesis as used in Figure 1, but containing no dsRNA. At the concentrations used, these two inhibitors block initiation of protein synthesis, but do not affect chain elongation (Lodish et al., 1971; Kaempfer & Kaufman, 1972). Addition of either inhibitor at 7.5 min after the start of incubation led to an almost immediate change in the rate of Mengovirus RNA directed protein synthesis, and by 50 min the amount of synthesis was less than that reached after 15 min in the control (Figure 4b,c). It is seen in Figure 4 that the translation of Mengovirus RNA is essentially as sensitive to initiation inhibitors as the translation of globin mRNA. These results show that Mengovirus RNA translation depends heavily on continued initiation. Hence, the fact that Mengovirus RNA translation continues unabated for 50 min in the presence of 10 ng/mL dsRNA strongly suggests that extensive initiation of Mengovirus protein synthesis can take place in conditions where initiation of globin synthesis is blocked.

Evidence for this concept is provided by the experiment of Figure 5. Here, the number of initiation events was measured directly by the use of *N*-formyl[^{35}S]Met-tRNA$_f$ as donor of labeled methionine. It is seen that the number of initiation events directed by Mengovirus RNA is not significantly affected at dsRNA concentrations that severely inhibit incorporation directed by globin mRNA and that transfer of label from *N*-formyl-Met-tRNA$_f$ into protein closely reflects the incorporation of labeled methionine.

Incubation of reticulocyte lysates with dsRNA and ATP can lead under certain conditions to the formation of an inhibitory substance, (2′–5′)-oligoisoadenylate (Hovanessian & Kerr, 1978). Although formation of this inhibitor could be detected in the reticulocyte lysate by procedures described under Experimental Procedures, none was detected in the reaction mixtures for protein synthesis used in the above experiments, in the presence of dsRNA concentrations ranging from 1 ng/mL to 100 μg/mL. We conclude that (2′–5′)-oligoisoadenylate is not involved in the translational inhibition

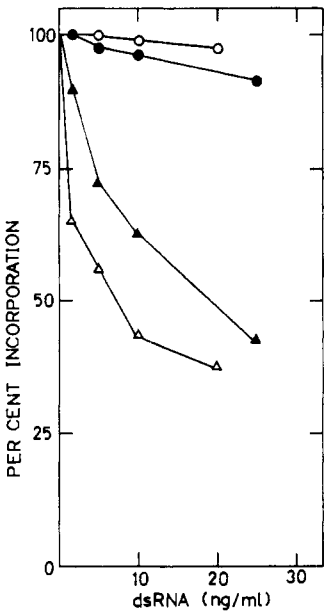

FIGURE 5: Differential sensitivity of Mengovirus RNA and globin mRNA directed utilization of *N*-formyl-Met-tRNA$_f$. Translation mixtures containing 1.5 μg of globin mRNA (△, ▲) or Mengovirus RNA (○, ●) were incubated for 45 min in the presence of *N*-formyl[^{35}S]Met-tRNA$_f$ (▲, ●) or [^{35}S]methionine (△, ○) in the presence of the indicated concentrations of dsRNA. Aliquots of 20 μL were diluted with an equal volume of 1 M NaOH, and after an incubation for 30 min at 37 °C, Cl_3CCOOH-precipitable radioactivity was determined.

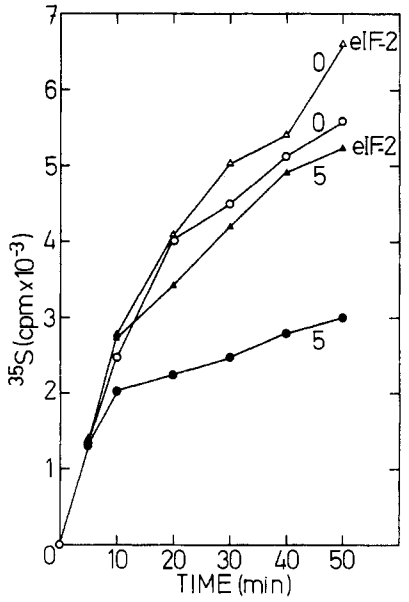

FIGURE 6: Relief of dsRNA-induced inhibition by purified eIF-2. Translation mixtures containing 1.5 μg of globin mRNA were incubated as described for Figure 1, with the indicated concentrations of dsRNA (in ng/mL), in the presence (△, ▲) or absence (○, ●) of 2.2 μg of purified eIF-2. Background without mRNA (300 cpm) was subtracted.

observed in these experiments.

dsRNA is known to cause the inactivation of eIF-2. Indeed, the inhibition of globin mRNA translation observed in Figure 1a can be relieved completely by addition of purified eIF-2 (Figure 6). Since in Figures 1 and 2 fewer molecules of Mengovirus RNA were present than of globin mRNA, one possibility we considered is that translation of Mengovirus RNA requires less eIF-2 and hence can continue in the presence of dsRNA. If that were so, one should expect Mengovirus RNA translation to take place even after globin

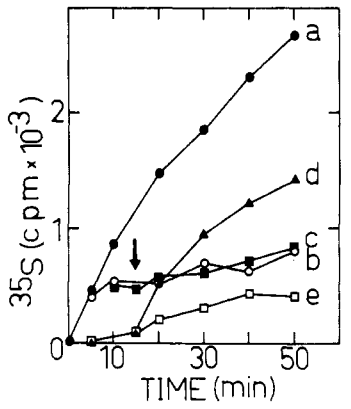

FIGURE 7: Sensitivity of Mengovirus RNA translation to preincubation with dsRNA. Translation mixtures (see Figure 1) contained globin mRNA (1.5 μg) (a–c) and 10 ng/mL dsRNA (b, c, and e). Mengovirus RNA (1.5 μg) was added at 15 min (arrow) in (c–e).

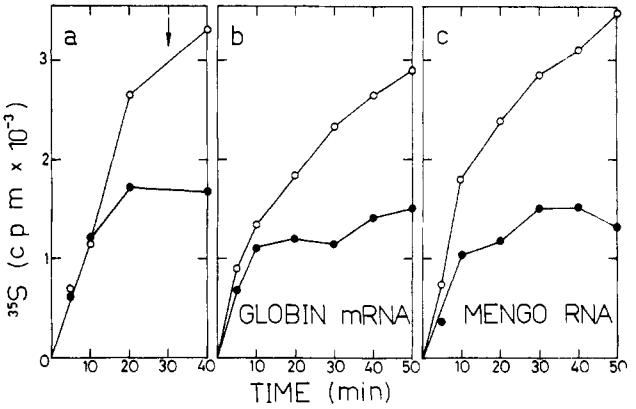

FIGURE 8: Sensitivity of globin mRNA and Mengovirus RNA translation to dsRNA-activated inhibitor. (a) Translation mixtures containing 1.5 μg of globin mRNA were incubated as described for Figure 1, with (●) or without (○) 5 ng/mL dsRNA. After 30 min (arrow), 15-μL aliquots were transferred to 50-μL reaction mixtures for translation, containing 1.5 μg of globin mRNA (b) or Mengovirus RNA (c) and 15 μg/mL dsRNA. Symbols in (b) and (c) correspond to those of the donor lysate in (a).

mRNA translation has been inhibited by dsRNA. In the experiment of Figure 7, ^{35}S incorporation directed by added globin mRNA (a) was shut off essentially completely between 5 and 10 min by 10 ng/mL dsRNA (b). In a sample parallel to (b), Mengovirus RNA was added at 15 min (arrow). This late addition of Mengovirus RNA failed to cause any stimulation of protein synthesis (c), in spite of the fact that 10 ng/mL dsRNA caused no inhibition in Figure 1b. This failure was not due to a general deterioration of the lysate, for when globin mRNA and dsRNA were omitted during incubation, addition of Mengovirus RNA at 15 min elicited excellent incorporation (d). On the other hand, preincubation with dsRNA alone very nearly abolished this incorporation (e).

These results show that Mengovirus RNA, when present from the outset, protects, directly or indirectly, the sensitive component of the protein-synthetic machinery, eIF-2, against inactivation by dsRNA. Yet, when Mengovirus RNA is added after inhibition of translation by dsRNA has been established, its translation no longer can take place. Hence, the resistance of Mengovirus RNA translation observed in Figures 1 and 2 is not caused by a lesser dependence on the components for protein synthesis but by failure of dsRNA to establish inhibition in the presence of Mengovirus RNA.

Mengovirus RNA Prevents Formation of the dsRNA-Activated Inhibitor. Incubation of whole reticulocyte lysates with dsRNA leads to the formation of an inhibitor of protein

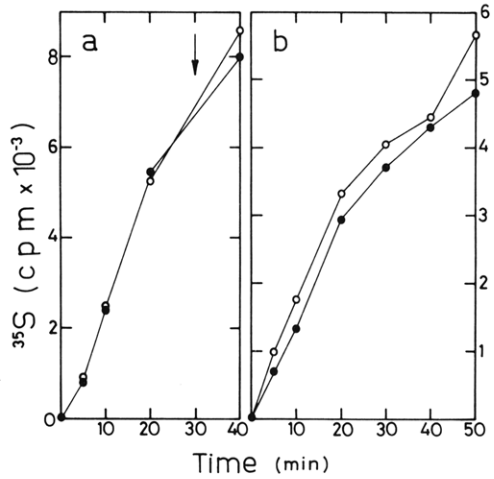

FIGURE 9: Lack of dsRNA-activated inhibitor formation during translation of Mengovirus RNA. (a) Translation was as described in Figure 8a, but with 1.5 μg of Mengovirus RNA as template. After 30 min (arrow), 15-μL aliquots were transferred to 50-μL reaction mixtures for translation, containing 1.5 μg of globin mRNA and 15 μg/mL dsRNA (b). Symbols in (b) correspond to those of the donor lysate in (a).

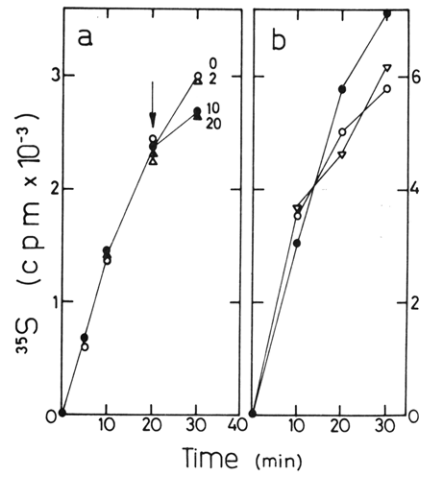

FIGURE 10: Lack of dsRNA-activated inhibitor formation during translation of Coxsackie B5 virus RNA. (a) Translation mixtures containing 1.5 μg of Coxsackie B5 virus RNA were incubated as described in Figure 1, but with 80 mM added KCl (the optimum for translation) and 0 (○), 2 (△), 10 (●), or 20 (▲) ng/mL dsRNA. After 20 min (arrow), 10-μL aliquots were transferred to 50-μL reaction mixtures for translation containing 1.5 μg of globin mRNA and 10 μg/mL dsRNA (b); the symbols (○) and (●) correspond to the donor lysates in (a). A third reaction mixture in (b), denoted by (▽), was identical with that denoted by (○), except that it contained no dsRNA.

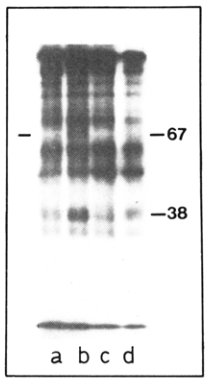

FIGURE 11: dsRNA-induced phosphorylation of eIF-2 and a 67 000-dalton polypeptide during translation of globin mRNA and Mengovirus RNA. Translation mixtures, containing [γ-^{32}P]ATP and globin mRNA (a, b) or Mengovirus RNA (c, d), were incubated with (b, d) or without (a, c) 50 ng/mL dsRNA. The autoradiograms of NaDodSO$_4$–polyacrylamide gels of the ribosomes of these reaction mixtures are shown. The positions of 38 000- and 67 000-dalton polypeptides are indicated.

synthesis (Hunter et al., 1975; Farrell et al., 1977; Levin & London, 1978). Figure 8 shows that formation of a dsRNA-activated inhibitor of translation occurs also in a micrococcal nuclease treated lysate programmed with globin mRNA. In this experiment, aliquots were removed from donor lysates that had been incubated for 30 min in the presence or absence of 5 ng/mL dsRNA (Figure 8a) and transferred to fresh lysates containing, in addition to mRNA, 15 μg/mL dsRNA. At this high concentration, dsRNA is not inhibitory (Hunter et al., 1975), so that the action of an inhibitor can be studied independently of any dsRNA transferred from the donor lysate. Indeed, as seen in Figure 8b, within 10 min the translation of globin mRNA is blocked almost completely by an inhibitor that was activated in the donor lysate by dsRNA. Remarkably, when Mengovirus RNA is used as template in the second lysate, its translation is seen to be just as sensitive to the dsRNA-activated inhibitor (Figure 8c).

The data presented so far lead us to predict that, in contrast to the case with globin mRNA, translation of Mengovirus RNA in the presence of dsRNA does not lead to the formation of a dsRNA-activated inhibitor. This is borne out not only when Mengovirus RNA is substituted as template in the donor lysate (Figure 9a) but also in the presence of RNA from another picornavirus, Coxsackie B5, whose translation is equally resistant to inhibition by dsRNA (Figure 10a): in neither case can dsRNA-activated inhibitor be detected upon transfer to lysates programmed with globin mRNA (Figures 9b and 10b).

Thus, the nature of the mRNA being translated is a critical factor in the formation of the dsRNA-activated inhibitor. Mengovirus or Coxsackie virus RNA prevents the formation of dsRNA-activated inhibitor, while globin mRNA does not. Yet, once it is formed, the inhibitor is equally effective in blocking translation of Mengovirus RNA as it is in blocking translation of globin mRNA.

Mengovirus RNA Prevents dsRNA-Induced Phosphorylation of eIF-2 and a 67 000-Dalton Polypeptide. dsRNA-induced phosphorylation of the small subunit of eIF-2 (a 38 000-dalton polypeptide) and of a 67 000-dalton polypeptide has been shown to occur in fractionated systems containing crude ribosomes and eIF-2 (Farrell et al., 1977; Levin & London, 1978; Lenz & Baglioni, 1978), and phosphorylation of the small subunit of eIF-2 has also been demonstrated in a whole reticulocyte lysate under conditions of protein synthesis (Farrell et al., 1978).

In the following experiment, translation of mRNA in the micrococcal nuclease treated reticulocyte lysate was studied as before but in the presence of [γ-^{32}P]ATP. After incubation, the reaction mixtures were centrifuged to pellet the ribosomes, which were subjected to electrophoresis in NaDodSO$_4$–polyacrylamide gels. As seen in Figure 11, during translation of globin mRNA (a) the presence of dsRNA stimulates phosphorylation of the small subunit of endogenous eIF-2 as well as the 67 000-dalton polypeptide (b). By contrast, when Mengovirus RNA is used as template for translation (c), the dsRNA-dependent phosphorylation of either polypeptide is significantly depressed (d).

Mengovirus RNA Competitively Inhibits dsRNA-Induced Phosphorylation of eIF-2. Since dsRNA-induced phosphorylation of the small subunit of eIF-2 and the 67 000-dalton polypeptide, a phenomenon diagnostic of the state of trans-

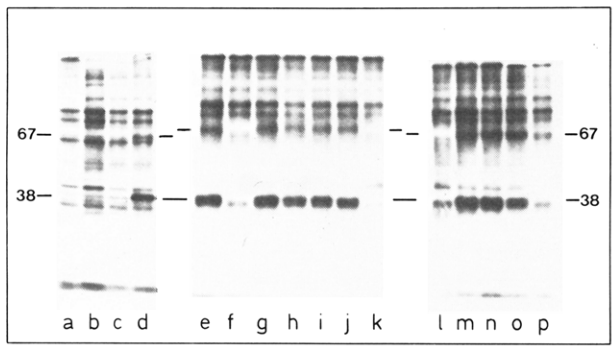

FIGURE 12: Effect of mRNA on dsRNA-dependent phosphorylation of eIF-2 and a 67 000-dalton polypeptide. The ribosomal system, containing [γ-^{32}P]ATP and crude initiation factors, was incubated without dsRNA (a–c, f, and l), with 50 ng/mL dsRNA (d, e, g–j, and m–p), and with 10 μg/mL dsRNA (k). Globin mRNA was present in (b) (0.5 μg), (g) (0.25 μg), (h) (0.5 μg), (i) (1.0 μg), and (j) (4 μg). Mengovirus RNA was present in (c) (0.5 μg), (n) (1.45 μg), (o) (3.6 μg), and (p) (7.2 μg). The autoradiograms of NaDodSO$_4$–polyacrylamide gels of these reaction mixtures are shown. The positions of 38 000- and 67 000-dalton polypeptides are indicated.

lational inhibition, is significantly reduced when Mengovirus RNA is used as template for translation (Figure 11), we have asked if mRNA has the ability to inhibit the phosphorylation of eIF-2 in the standard fractionated system used for this purpose, which contains ribosomes, eIF-2, dsRNA, and ATP. Our finding is that this is indeed the case and that Mengovirus RNA and globin mRNA differ significantly in this property.

Ribosomes isolated from a micrococcal nuclease treated reticulocyte lysate were incubated with [γ-^{32}P]ATP in conditions similar to those described by Farrell et al. (1977), Levin & London (1978), and Lenz & Baglioni (1978). The reaction mixtures were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis and autoradiography (Figure 12). In the absence of dsRNA and mRNA, phosphorylation of the 38 000- and 67 000-dalton polypeptides does not occur (lanes a, f, and l), nor is it observed in the presence of globin mRNA (lane b) or Mengovirus RNA (lane c) alone. Extensive phosphorylation of these two polypeptide species is induced in the presence of low concentrations of dsRNA (lanes d, e, and m) but not with high concentrations of dsRNA (lane k).

The addition of increasing amounts of globin mRNA to reaction mixtures containing dsRNA causes no significant reduction in the extent of phosphorylation of the 38 000- and 67 000-dalton species (lanes g–j). The addition of increasing amounts of Mengovirus RNA, on the other hand, leads to a marked reduction of phosphorylation of both polypeptides (lanes n–p). The sample analyzed in lane p contained 5 times fewer molecules of Mengovirus RNA than the number of globin mRNA molecules present in the sample analzyed in lane j. Clearly, Mengovirus RNA is a far more effective inhibitor than globin mRNA.

Thus, Mengovirus RNA can inhibit the dsRNA-induced phosphorylation of the small subunit of eIF-2, as well as the 67 000-dalton polypeptide, apparently in a competitive manner.

dsRNA Competes with mRNA in Binding to eIF-2. The critical role of the nature of the mRNA in the establishment of inhibition by dsRNA, demonstrated here, raises the possibility that mRNA competes with dsRNA itself, or with a substance generated by dsRNA, in the inactivation process. Since the presence of dsRNA results in the inactivation of eIF-2, and since dsRNA can bind tightly to this factor (Kaempfer & Kaufman, 1973; Kaempfer, 1974; Kaempfer et al., 1978a), we have asked if eIF-2 recognizes dsRNA in a manner resembling mRNA. Here, we show that dsRNA does compete

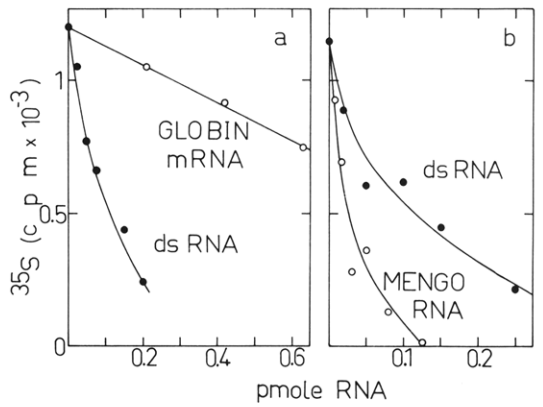

FIGURE 13: Effect of dsRNA, globin mRNA, and Mengovirus RNA on binding of Met-tRNA$_f$ to eIF-2. The assay contained [^{35}S]-Met-tRNA$_f$ [(a) 5000 cpm; (b) 4100 cpm], purified eIF-2 (1.5 ng), and the indicated amounts of dsRNA, globin mRNA, or Mengovirus RNA. Control without eIF-2 [100 cpm in (a) and 80 cpm in (b)] was subtracted.

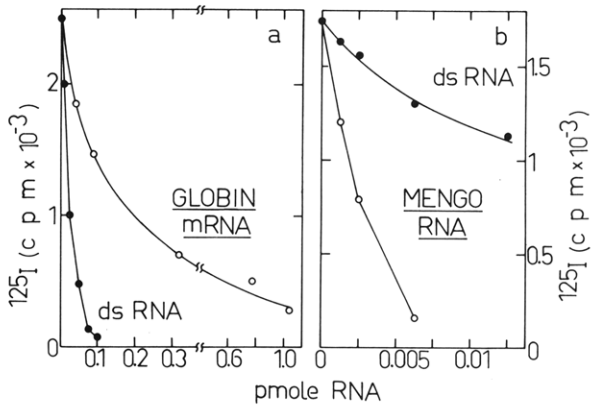

FIGURE 14: Competition of dsRNA, globin mRNA, and Mengovirus RNA for eIF-2. RNA-binding assays in (a) contained ^{125}I-labeled globin mRNA (0.025 pmol; 9500 cpm) and 10.6 ng of eIF-2. In (b), they contained ^{125}I-labeled Mengovirus RNA (0.002 pmol; 4470 cpm) and 0.28 ng of eIF-2. Unlabeled dsRNA, globin mRNA, or Mengovirus RNA was present in the indicated amounts. Control without eIF-2 [110 cpm in (a), 70 cpm in (b)] was subtracted.

with mRNA in binding to eIF-2.

In these experiments, we used eIF-2 purified to about 98%, as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis. Since binding of labeled globin mRNA by this preparation could be inhibited completely by Met-tRNA$_f$, provided GTP was present, but not by uncharged tRNA, it follows that the only mRNA-binding component being studied was eIF-2 (Rosen & Kaempfer, 1979).

The formation of ternary complexes with [^{35}S]Met-tRNA$_f$ and GTP, a highly characteristic function of eIF-2, can be inhibited completely by mRNA (Kaempfer et al., 1978b). As seen in Figure 13, dsRNA also is a competitive inhibitor of ternary complex formation. On a molar basis, dsRNA is a more powerful inhibitor of this function than globin mRNA (a), but a less powerful inhibitor than Mengovirus RNA (b). In each case, inhibition can be overcome by adding more eIF-2 (data not shown). This experiment shows that eIF-2 interacts more strongly with a molecule of dsRNA than with a molecule of globin mRNA, but even more strongly with a molecule of Mengovirus RNA.

That mRNA and dsRNA compete directly for eIF-2 is shown in Figure 14. Here, a limiting amount of purified eIF-2 was incubated with ^{125}I-labeled globin mRNA (a) or Mengovirus RNA (b) and increasing amounts of unlabeled, competing RNA. When dsRNA is used as competing RNA, it

is seen to bind to eIF-2 with about 6 times greater affinity than globin mRNA (a). By contrast, a molecule of dsRNA binds to eIF-2 about 7 times more weakly than Mengovirus RNA (b). It was shown previously that radioiodination does not affect the affinity of mRNA for eIF-2, provided it is to low specific activity: unlabeled globin mRNA or Mengovirus RNA was found to compete with their labeled homologues exactly according to expectation (Kaempfer et al., 1979a; Kaempfer, 1979). In this experiment, the unlabeled and labeled Mengovirus RNA preparations also compete exactly equally, while unlabeled globin mRNA competes somewhat more weakly than its labeled couterpart; however, this difference does not influence the validity of the observation that eIF-2 binds more tightly to dsRNA than to globin mRNA.

Discussion

mRNA Specificity in the Action of dsRNA. The results reported here reveal messenger RNA specificity in the establishment of inhibition of eukaryotic translation by dsRNA. Specifically, they show that translation of Mengovirus or Coxsackie virus RNA in the nuclease-treated reticulocyte lysate is resistant to inhibition by dsRNA, while translation of globin or ascites tumor cell mRNA is sensitive. Because there is evidence that dsRNA specifically affects the function of eIF-2 (Kaempfer, 1974; Clemens et al., 1975), the observed mRNA specificity in the inhibition of protein synthesis by dsRNA, demonstrated here, constitutes independent evidence that is consistent with a direct interaction between mRNA and eIF-2 during translation.

Even though Mengovirus RNA is much longer than globin mRNA, several lines of evidence argue strongly against the interpretation that the resistance of Mengovirus RNA directed protein synthesis is merely due to chain elongation, independent of further initiation. First, Mengovirus RNA translation continues unabated for as long as 50 min in the presence of 10 ng/mL dsRNA, without a perceptible change in rate (Figures 1, 2, and 9). Second, translation of Mengovirus RNA is essentially as sensitive to two different inhibitors of initiation as translation of globin mRNA (Figure 4). Third, there is no significant decrease in the transfer of labeled methionine from *N*-formyl-Met-tRNA$_f$ into protein during translation of Mengovirus RNA in the presence of dsRNA concentrations that are inhibitory to translation of globin mRNA (Figure 5). Fourth, the failure of added dsRNA to inhibit translation of Mengovirus RNA cannot be explained by assuming that the Mengovirus RNA contained some dsRNA and, hence, was already inhibited in the control, for the Mengovirus RNA preparation used was free of dsRNA as judged by the following criteria: linear kinetics of amino acid incorporation for at least 50 min (Figures 1, 2, and 9); absence of dsRNA-dependent phosphorylation of the 38 000- and 67 000-dalton polypeptides (Baglioni et al., 1978) in the presence of Mengovirus RNA, but in the absence of dsRNA (Figure 12, lane c); and failure of high concentrations of dsRNA (20 μg/mL), that eliminate the inhibitory effect of low concentrations (Hunter et al., 1975), to stimulate Mengovirus RNA directed translation over that seen in the control (data not shown). This evidence shows that extensive initiation of Mengovirus RNA translation takes place in the presence of dsRNA.

It cannot be argued that the observed mRNA specificity of the inhibitory effect of dsRNA is related to the use of micrococcal nuclease treated reticulocyte lysates. Our results show that such lysates, when programmed with globin mRNA, behave precisely as whole lysates containing endogenous mRNA with respect to their sensitivity to inhibitory concentrations of dsRNA, the kinetics of inhibition, reversal of inhibition by added eIF-2, formation of the dsRNA-activated inhibitor, phosphorylation of the 38 000- and 67 000-dalton polypeptides, both in whole lysates and in a ribosome system, and reversal of dsRNA-dependent phosphorylation by high concentrations of dsRNA (Hunter et al., 1975; Farrell et al., 1977).

Although incubation of reticulocyte lysates with dsRNA and ATP can lead to the formation of (2′–5′)-oligoisoadenylate (Hovanessian & Kerr, 1978), none was detected under the conditions of translation used in these experiments. Thus, the inhibitory action of dsRNA studied here involves the inactivation of eIF-2 (Figure 6).

Mechanism of Inhibition by dsRNA. In the experiments of Figures 1 and 2, about one-tenth as much Mengovirus RNA was present, on a molar basis, as globin mRNA. Thus, the resistance of Mengovirus RNA translation to dsRNA could, in theory, be caused by a lower rate of utilization of initiation factors. The results of Figure 7 clearly rule out this interpretation, for they show that once inhibition by dsRNA has been established in the presence of globin mRNA, translation of Mengovirus RNA is also blocked. Thus, translation of Mengovirus RNA is resistant to dsRNA not because of a lesser dependence on eIF-2 but because apparently the inactivation of eIF-2 does not occur when Mengovirus RNA is used as messenger.

Indeed, the phosphorylation of the small subunit of eIF-2, as well as a 67 000-dalton polypeptide, that characterizes the state of inhibition of translation by dsRNA, is significantly depressed when Mengovirus RNA serves as template for protein synthesis, even in the presence of 50 ng/mL dsRNA (Figure 11). Even more striking is our observation that dsRNA-induced phosphorylation of these two polypeptides in a ribosomal system can be inhibited almost completely by Mengovirus RNA, but not by globin mRNA (Figure 12).

The possibility that translation of Mengovirus RNA is resistant to the action of the dsRNA-activated inhibitor can be rejected on the basis of the results of Figure 8, showing that Mengovirus RNA and globin mRNA translations are equally sensitive to this inhibitor, when assayed in the presence of noninhibitory, high concentrations of dsRNA.

Any model for the mechanism of inhibition by dsRNA, therefore, must explain our finding that the nature of the mRNA being translated is a critical factor in the *formation* of the dsRNA-activated inhibitor. Indeed, the inhibitor is formed in the presence of globin mRNA, but not in the presence of either Mengovirus RNA or Coxsackie virus RNA (Figures 9 and 10).

These findings lead to the concept that mRNA competes with dsRNA at a stage leading to formation of the translational inhibitor. If that is so, then one might expect these RNA species to compete for a molecular entity that gives rise to the inhibitor. Considering that eIF-2, the component affected by dsRNA, is itself able to bind both to mRNA and to dsRNA (Kaempfer, 1974; Kaempfer et al., 1978a), we have asked if these RNA species can compete in their binding to eIF-2. The results of Figures 13 and 14 show clearly that mRNA and dsRNA do compete for eIF-2 and that a molecule of dsRNA is bound by this factor more tightly than a molecule of globin mRNA but less tightly than a molecule of Mengovirus RNA.

We shall consider two of the simplest models that explain our findings: (1) The key feature of this model is that the rate-determining step in the action of dsRNA involves a competition between mRNA and dsRNA for a proinhibitor. The proinhibitor would exhibit a preference for Mengovirus RNA. Here, activation of the inhibitor by dsRNA would

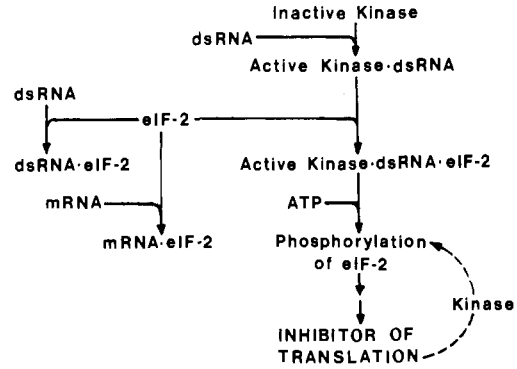

FIGURE 15: Model for the mechanism of action of dsRNA on translation.

occur only when the proinhibitor binds to dsRNA, but not when it binds to mRNA. Conceivably, the subsequent inactivation and phosphorylation of eIF-2 might also involve competition between mRNA and dsRNA for eIF-2, but in this model the latter competition, even if it exists, could not be rate limiting in the inactivation mechanism, because our results show clearly that the nature of the mRNA being translated is critical for the formation of the dsRNA-activated inhibitor rather than for its action. This model fails to explain the observed correlation of the results of the translation and phosphorylation experiments with the RNA-affinity properties of eIF-2.

(2) The essential feature of this model is that the rate-determining step in the inactivation of eIF-2 by dsRNA involves a direct competition of mRNA and dsRNA for eIF-2. Evidence for an active role of eIF-2 in the recognition and binding of mRNA has been presented in the introduction. The present experiments show that dsRNA and mRNA compete in their binding to eIF-2. In this model, binding of eIF-2 to mRNA will lead to protein synthesis, regenerating eIF-2 for further rounds of competition. The sequence of events leading to phosphorylation and inactivation of eIF-2, then, would take place once dsRNA binds to eIF-2. Accordingly, during protein synthesis in the presence of dsRNA, the relative affinities of mRNA and dsRNA for eIF-2, and their effective concentrations, will determine whether eIF-2 binds to mRNA or dsRNA and, hence, whether translation or inhibition will ensue. The striking correlation between the inability of dsRNA to inhibit Mengovirus RNA translation effectively or to cause eIF-2 phosphorylation when Mengovirus RNA is present, and the preferential binding of Mengovirus RNA to eIF-2 on one hand and the reversal of these properties for globin mRNA on the other, is strongly suggestive of this explanation.

Since, as we have shown, the nature of mRNA is critically important not only for the establishment of inhibition but also for the formation of the dsRNA-activated inhibitor, an interesting consequence of this model is that it is the binding of eIF-2 to dsRNA that may lead to the formation of the inhibitor which in turn causes the inactivation of eIF-2.

In the translation experiments, globin mRNA was added in 10^2–10^3-fold molar excess over dsRNA. This excess of mRNA need not be incompatible with the model, for the following reasons. First, a molecule of *P. chrysogenum* dsRNA used here possesses many (>10) binding sites for eIF-2 and can be protected entirely by this factor against ribonuclease digestion (Kaempfer et al., 1978a), while mRNA seems to possess only one site (Kaempfer et al., 1978a, 1979a). Second, as we have shown, a molecule of dsRNA possesses a higher affinity for eIF-2 than a molecule of globin mRNA. Third, not all mRNA molecules may be active or available for eIF-2. Fourth, even though dsRNA can interfere directly with the binding of Met-tRNA$_f$ and of mRNA to eIF-2, binding of dsRNA is by itself not sufficient to inactivate eIF-2, for eIF-2 bound to a dsRNA–cellulose column can be recovered active in binding Met-tRNA$_f$ and in translation (Kaempfer et al., 1978a). This result must mean that binding of eIF-2 to dsRNA cannot be the final step in its inactivation, but must be followed by another event. If this subsequent event, dependent on prior binding of eIF-2 to dsRNA, leads to release of eIF-2 from the dsRNA molecule, then each binding site for eIF-2 on dsRNA would exhibit a turnover that could drastically increase the number of effective sites.

Hunter et al. (1975) and Farrell et al. (1977) found that formation of the dsRNA-activated inhibitor is ATP dependent and does not occur if high concentrations of dsRNA are used but that these levels of dsRNA do not influence the action of the inhibitor. They also showed increased eIF-2–kinase activity in the presence of dsRNA and a correlation between phosphorylation of the small subunit of eIF-2 and translational inhibition. Their findings can be reconciled with those reported in this paper by the following hypothesis that can account not only for the mRNA specificity of dsRNA action but also for the hitherto paradoxical observation that high concentrations of dsRNA are not inhibitory. According to this hypothesis (Figure 15), an inactive eIF-2–kinase is activated by forming a complex with dsRNA. Before this activated kinase–dsRNA complex can phosphorylate eIF-2, this factor must bind to the dsRNA molecule in the complex, forming a ternary complex consisting of kinase, dsRNA, and eIF-2. mRNA can compete with the kinase-bound dsRNA for eIF-2, and when mRNA binds to eIF-2, ternary complex formation does not occur. In the presence of high concentrations of dsRNA, eIF-2 will bind to free dsRNA molecules rather than to those complexed with the kinase, and ternary complex formation also will not occur. Once the ternary complex is formed, ATP-dependent phosphorylation of the small subunit of eIF-2 takes place. The phosphorylation of eIF-2 leads to formation of a translational inhibitor. Since this inhibitor is active in the presence of high concentrations of dsRNA, it may act by making other eIF-2 molecules substrates for an eIF-2–kinase that does not depend on dsRNA, although other explanations are possible.

Acknowledgments

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Mechanism of Inhibition of Enzymatic Deoxyribonucleic Acid Methylation by 2-(Acetylamino)fluorene Bound to Deoxyribonucleic Acid†

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ABSTRACT: Binding of 2-(acetylamino)fluorene (AAF) to C-8 of guanine induces a local destabilization of the DNA helix. A relationship was observed where the degree of DNA modification by AAF was inversely proportional to its methyl acceptor capacity from *S*-adenosyl-L-methionine in the presence of rat brain DNA cytosine 5-methyltransferase. Moreover, substituted DNA (DNA-AAF) behaves as a methylation inhibitor of native DNA. This inhibition is of the mixed type. The substituted DNAs have higher affinities for the enzyme than native DNA. The inhibition is irreversible. Addition of DNA-AAF to the enzyme preincubated with native DNA inhibits methylation, but only after a lag period. This agrees with the model in which the methylase "walks" along the strand to methylate cytosine residues before being detached from the DNA. AAF bound to guanine residues may block the movement of the enzyme along the helix. Single-stranded DNA has an affinity for the methylase 1.6 times lower than that of native double-stranded DNA. On the other hand, single-stranded DNA-AAF is more methylated than double-stranded DNA-AAF. A tentative model taking into account these observations is presented under Discussion. The in vitro hypomethylation of DNA-AAF could explain the in vivo observations made by several authors.

It is presently widely known that chemical carcinogens are either electrophilic reactants or compounds which become activated to electrophilic metabolites (Miller, 1970; Kriek, 1974) able to bind covalently to DNA, RNA, or proteins.

2-(Acetylamino)fluorene (AAF)[1] is a potent liver carcinogen which after metabolic activation binds to liver DNA, RNA, and proteins when administered in vivo [for a review, see Miller (1970)]. 2-(*N*-Acetoxyacetylamino)fluorene (*N*-AcO-AAF), a model ultimate metabolite of AAF, has been shown to react in vitro with DNA to give two adducts, *N*-(deoxyguanosin-8-yl)-AAF and 3-(deoxyguanosin-N^2-yl)-AAF (Kriek et al., 1967; Kriek, 1972; Westra et al., 1976). The first of these adducts represents about 80% of the total. It has been shown

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[1] Abbreviations used: AAF, 2-(acetylamino)fluorene; *N*-AcO-AAF, 2-(*N*-acetoxyacetylamino)fluorene; SAM, *S*-adenosyl-L-methylmethionine; dGuo, deoxyguanosine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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