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Inhibition of Cellular Protein Synthesis by Double-Stranded RNA: Inactivation of an Initiation Factor

(IF-3/mammalian ribosome cycle/rabbit reticulocytes/interferon inducer)

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ABSTRACT **Inhibition of protein synthesis in rabbit reticulocyte lysates by double-stranded RNA is caused by the inactivation of IF-3, an initiation factor required for the recycling of ribosomes and for their binding to messenger RNA. The evidence for this is that (*i*) the inhibition can be overcome by addition of exogenous IF-3; (*ii*) double-stranded RNA inactivates stoichiometric amounts of IF-3; (*iii*) double-stranded RNA forms a complex with IF-3; and (*iv*) double-stranded DNA, which lacks inhibitory activity, also binds to IF-3, but with a much lower affinity than double-stranded RNA. It is concluded that double-stranded RNA inhibits cellular protein synthesis by tightly complexing with IF-3. It is suggested that IF-3 normally recognizes a double-stranded region in messenger RNA.**

Double-stranded RNA is implicated in the replication of most, if not all, RNA viruses (1). In addition, double-stranded RNA acts as an inducer of interferon, and can elicit cellular immunity against a broad spectrum of viruses (2, 3). Double-stranded RNA, furthermore, is an extremely potent inhibitor of mammalian protein synthesis: its rate of appearance in cells infected with poliovirus parallels the decline in rate of cellular protein synthesis (4), and the initiation of protein synthesis in rabbit reticulocyte lysates is inhibited by very low concentrations of double-stranded RNA (4–6).

We report here that double-stranded RNA specifically inactivates IF-3, a rabbit reticulocyte initiation factor required for the recycling of ribosomes and for their binding to messenger RNA (7). We demonstrate the formation of a complex between IF-3 and double-stranded RNA, and show that the inhibitory activity of double-stranded RNA can be overcome by the addition of exogenous IF-3.

RESULTS

IF-3 Maintains Polysomes in the Presence of dsRNA. Virions associated with the fungus, *Penicillium chrysogenum*, contain *ds*RNA (8, 9). The potent inhibitory activity of this *ds*RNA, used in all our experiments, is illustrated in Fig. 1, which shows the sedimentation distribution of a ^{32}P-labeled rabbit reticulocyte lysate after 20 min of cell-free protein synthesis. While polysomes are efficiently maintained in the presence of added heme (*A;* ref. 10–13), the addition of as little as 0.1 ng/ml of *ds*RNA induces significant accumulation of single ribosomes (S) at the expense of polysomes (*B*); few polysomes remain in the presence of 0.5 ng/ml of *ds*RNA (*C*).

As seen in Fig. 2, the decay of polysomes induced by *ds*RNA in the presence of heme (*d*) resembles the decay observed in the control when heme is omitted (*a* and *b*). Because we originally detected initiation factor IF-3 by its ability to maintain polysomes in the absence of added heme (7), it was appropriate to ask if IF-3 could overcome the effect of *ds*RNA as well. Curve *c* demonstrates that this is indeed the case: even though 10 ng/ml of *ds*RNA was present, added IF-3 gave nearly complete protection of polysomes.

The complete effectiveness of IF-3 in counteracting *ds*RNA is clearly demonstrated in Fig. 3. Even at *ds*RNA concentrations 100-fold greater than are needed to induce the loss of polysomes (see Fig. 1), added IF-3 affords quantitative protection.

IF-3 Maintains Protein Synthesis in the Presence of dsRNA. As seen in Fig. 4, in the presence of added IF-3 aminoacid

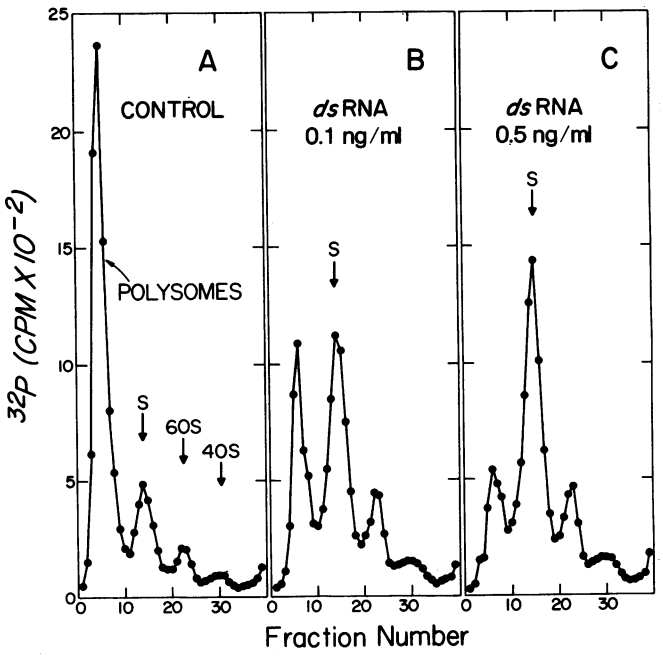

FIG. 1. Decay of polysomes in the presence of *ds*RNA. A lysate of rabbit reticulocytes, labeled with ^{32}P *in vivo* (7), was incubated for 20 min at 25° in a reaction mixture containing 2 mM Mg^{2+} (7) in the presence of 50 μM hemin. Samples *B* and *C* also contained *ds*RNA (preparation: see Fig. 6). The samples were centrifuged through exponential sucrose gradients supported by a 75% (w/v) sucrose cushion (fractions 1–5) as described (7). Peaks from *right* to *left* represent 40S subunits, 60S subunits, single ribosomes (*S*), and polysomes.

Abbreviation: *ds*RNA, double-stranded RNA.

incorporation in a reticulocyte lysate continues for at least 1 hr, whether or not *ds*RNA or heme are present (*e*, *f*, *g*, and *h*). By contrast, without added IF-3, incorporation ceases rapidly, and with the same kinetics, in the absence of heme (*d*) or in the presence of both heme and 50 ng/ml of *ds*RNA (*c*). Less residual synthesis occurs when *ds*RNA is added without heme (*b*). Most of the synthesis in curves *b*, *c*, and *d* is due to continued chain initiation, for when this process is blocked with aurin tricarboxylate, curve *a* is obtained. Apparently, initiation in *b*, *c*, and *d* continues until endogenous IF-3 is exhausted; its inactivation, therefore, must occur slowly.

Stoichiometric Action of dsRNA and of IF-3. The total amount of protein synthesized in 75 min by a reticulocyte lysate lacking added heme is plotted against the amount of added IF-3 in Fig. 5. Beyond a threshold concentration, the protein synthetic capacity increases linearly with added IF-3 (*control*). Thus, stoichiometric amounts of IF-3 are needed to support protein synthesis. The threshold may represent the titration of a translational inhibitor formed in the absence of heme (13).

Addition of *ds*RNA before incubation causes displacement of the dose-response curve toward higher amounts of IF-3; this displacement increases with the *ds*RNA concentration (Fig. 5). Concurrently, there is a progressive decrease in the endogenous capacity to form protein (*initial points*), already noted in Fig. 4, curve *b*. These results demonstrate the limited inhibitory capacity of *ds*RNA, and show that *ds*RNA acts stoichiometrically with respect to IF-3, both of endogenous and exogenous origin.

The displacements in Fig. 5 are not proportional to *ds*RNA concentration. One interpretation supported by our data is

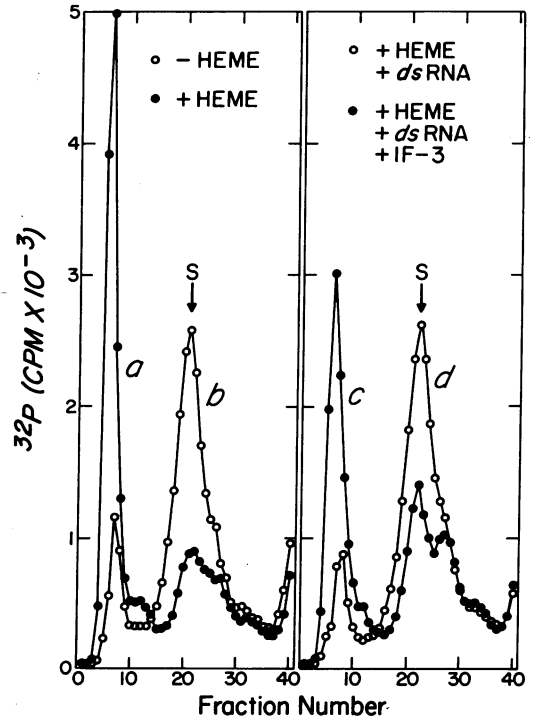

FIG. 2. Effect of hemin and added IF-3 on maintenance of polysomes in the presence of *ds*RNA. ^{32}P-Labeled reticulocyte lysate was incubated as in Fig. 1, with hemin (50 μM), factor IF-3 (50 μg), and *ds*RNA (10 ng/ml) added as shown. The IF-3 preparation was prepared from the 0.4 M KCl ribosomal wash by $(NH_4)_2SO_4$ fractionation (0–50%), DEAE-cellulose chromatography (7), and Bio-Gel A-1.5m filtration (see Fig. 7). Sedimentation profiles are superimposed. Peak on *left* represents polysomes; *S:* single ribosomes.

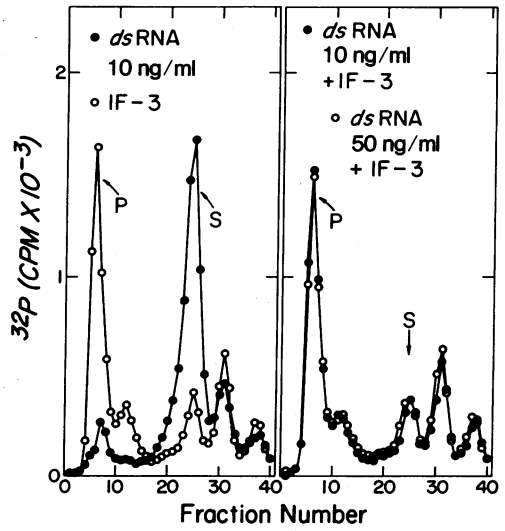

FIG. 3. Protection of polysomes by added IF-3 against decay induced by *ds*RNA. ^{32}P-Labeled reticulocyte lysate was incubated as in Fig. 1. Hemin (50 μM) was present in all samples. Factor IF-3 (see Fig. 2, 88 μg) and *ds*RNA were added as shown. Sedimentation profiles are superimposed. Peaks from *right* to *left* represent 40S subunits, 60S subunits, single ribosomes (*S*), disomes, and polysomes (*P*).

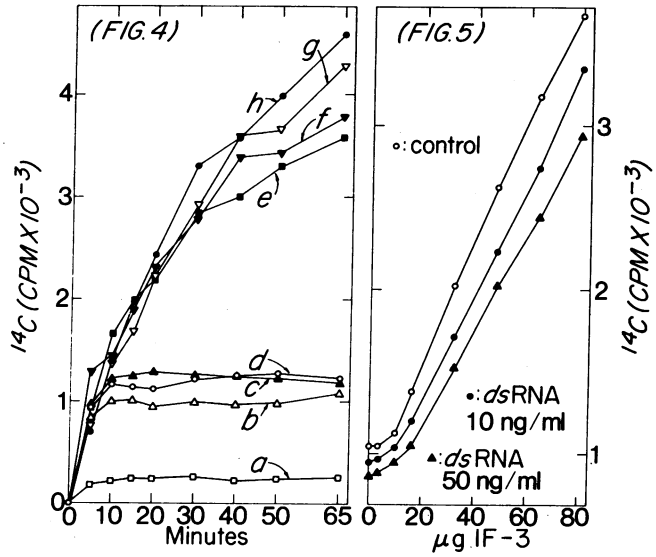

FIG. 4. (*left*) Effects of IF-3 and *ds*RNA on aminoacid incorporation. Reticulocyte lysate (30 μl) was incubated at 30° in a 115-μl reaction mixture (10) containing [^{14}C]aminoacids. Hemin (50 μM) was present in *a*, *c*, *e*, and *f*; *ds*RNA (50 ng/ml) in *b*, *c*, *e*, and *g*; IF-3 (150 μg) in *e*, *f*, *g*, and *h*; and aurin tricarboxylate (70 μM) in *a*. 10-μl samples were assayed for hot Cl_3CCOOH-insoluble radioactivity.

FIG. 5. (*right*) Dependence of protein synthetic capacity on IF-3 and *ds*RNA concentration. Each *point* represents radioactivity in protein after a 75-min incubation at 30° of 15 μl of lysate in a 60-μl reaction mixture (10) that received the indicated amounts of IF-3 and *ds*RNA at 0 min, and [^{14}C]aminoacids at 5.5 min. No hemin was added.

that much of the protein synthetic capacity measured in the presence of *ds*RNA represents escape synthesis, as seen in Fig. 4 (*b* and *d*), and that this escape is relatively insensitive to *ds*RNA concentration.

Complex Formation Between IF-3 and dsRNA. The foregoing results suggest that IF-3 is inactivated by direct interaction with *ds*RNA. To detect the formation of a complex between IF-3 and *ds*RNA, ^{32}P-labeled *ds*RNA of high specific activity was prepared from virions associated with *P. chrysogenum*. Because IF-3 readily complexes with single-stranded RNA from R17 phage (7), it was first necessary to ascertain that the [^{32}P]*ds*RNA preparation was free of single-stranded RNA. This was shown, first, by its salt-dependent sensitivity to RNase, characteristic for *ds*RNA (1), seen in Fig. 6*A;* second, by its full inhibitory activity at 5 ng/ml (data not shown); third, by the absence of detectable single-stranded molecules or single-stranded regions among more than 500 *ds*RNA molecules examined by electron microscopy (J. Wolfson and R. K., unpublished).

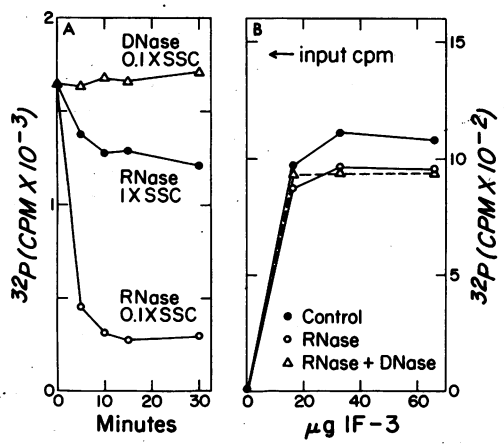

FIG. 6. Nuclease sensitivity of ^{32}P-labeled *ds*RNA and complex formation with IF-3. Spores of *P. chrysogenum* NRRL 1951 were inoculated (10^4/ml) into 1 liter of KC medium (1% bactotryptone–0.5% NaCl–0.3% yeast extract–1.5% glucose–5% lactose) containing 10 mCi of ^{32}P, and shaken for 60 hr at 25°. Mycelia were collected on Whatman no. 1 filter paper, washed with cold H_2O, resuspended in 0.01 M Tris·HCl (pH 7.4)–0.01 M Mg acetate, and passed through a French press at 10–14,000 psi. The extract was clarified, concentrated by $(NH_4)_2SO_4$ precipitation, and centrifuged at 40,000 rpm for 10 hr through a 9-ml preformed linear CsCl gradient (density 1.2–1.5) in 0.15 M NaCl–15 mM Na-citrate (*SSC*). The banded virions were dialyzed against NaCl–citrate. RNA was purified by two cycles of Na dodecyl sulfate–phenol extraction (3 times each) and ethanol precipitation, dissolved in NaCl–citrate, and stored at −20°. Its A_{260}/A_{280} ratio was 2.28. (*A*) Nuclease digestion. ^{32}P-Labeled *ds*RNA (5 μg/ml) was incubated at 30° at the indicated ionic strength, with DNase (4 μg/ml) or pancreatic RNase (5 μg/ml). Samples were taken at intervals and cold Cl_3CCOOH-insoluble radioactivity was measured. (*B*) Binding to IF-3. ^{32}P-Labeled *ds*RNA (0.26 μg) was first incubated for 15 min at 30° in buffer B [0.15 M KCl–2 mM Mg acetate–20 mM Tris·HCl (pH 7.4)–6 mM 2-mercaptoethanol], with RNase (5 μg/ml) or DNase (5 μg/ml) present as shown; the indicated amount of IF-3 was then added, and incubation was continued for another 5 min. The cooled mixture was diluted with buffer B and passed slowly through a 25-mm nitrocellulose filter that was washed with buffer B, dried, and counted. *Arrow* represents input cpm per assay before nuclease treatment.

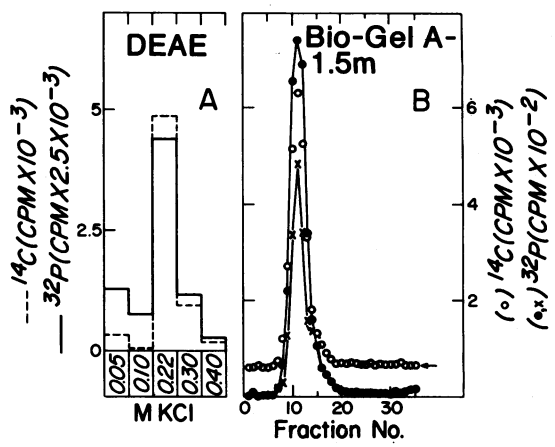

FIG. 7. Copurification of IF-3, *ds*RNA-binding, and DNA-binding activities. Proteins removed from rabbit reticulocyte ribosomes with 0.4 M KCl were fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 0 and 50% saturation was dissolved in buffer A [0.01 M Tris·HCl (pH 7.4)–0.05 M KCl–5 mM 2-mercaptoethanol] and dialyzed against this buffer (7). The material was eluted from DEAE-cellulose (*A*) by two-cycle stepwise elution (7), placed on a Bio-Gel A-1.5m column equilibrated with buffer A (*B*), and eluted at a flow rate of 2.25 ml/hr. (*A*) DEAE-cellulose chromatography. An equal volume of each fraction, dialyzed against buffer A, was assayed for binding of [^{32}P]*ds*RNA as in Fig. 6 (*solid line*), and for its capacity to support protein synthesis as in Fig. 5 (*broken line*); the radioactivity in a control that received buffer A was subtracted. The 0.05-M fraction contains unadsorbed material. (*B*) Bio-Gel A-1.5m filtration. An equal volume of each fraction was assayed for binding of [^{32}P]*ds*RNA as in Fig. 6 (●), for binding of [^{32}P]λ DNA as in Fig. 8 (×), and for its capacity to support protein synthesis as in Fig. 5 (○). *Arrow* indicates amount of aminoacid incorporation in control that received buffer A. In a separate run, markers blue dextran, hemoglobin, and FMN eluted in fractions 11, 24, and 35, respectively.

The formation of a complex between IF-3 and ^{32}P-labeled *ds*RNA is demonstrated in Fig. 6*B*. Although *ds*RNA passes through nitrocellulose filters, it is retained in the presence of IF-3. About 75% of the input radioactivity can be bound in this assay. Significantly, the extent of binding is not much affected by prior incubation of the *ds*RNA with RNase or DNase. We conclude, therefore, that the RNA molecules participating in the complex are truly double-stranded.

The identification of IF-3 with the protein that binds *ds*RNA is based on the observation that the two activities cannot be separated throughout purification. To assay for IF-3, we have utilized the fact that after a brief incubation, aminoacid incorporation in reticulocyte lysates is totally dependent on added IF-3 (Fig. 4; ref. 7). As seen in Fig. 7*A*, the capacity to support protein synthesis and the [^{32}P]*ds*RNA-binding activity are eluted from DEAE-cellulose in the same fraction (0.10–0.22 M KCl); this fraction also contains the activities for polysome maintenance, binding of globin mRNA to ribosomes, and binding of R17 RNA (7). To further purify IF-3, we have taken advantage of its unusual behavior on Bio-Gel A-1.5m columns, where it is voided (Fig. 7*B*). Again, the *ds*RNA-binding activity (*filled circles*) is coincident with the capacity to support protein synthesis (*open circles*).

Binding of DNA to IF-3. DNA of bacteriophages λ and T4 also is retained on filters by IF-3. As seen in Fig. 8*A*, the extent

of binding is similar to that of *ds*RNA (Fig. 6*B*). The DNA-binding activity also cochromatographs with IF-3 (see Fig. 7*B*, *crosses*).

We have confirmed an earlier report (6) that DNA lacks the inhibitory activity of *ds*RNA. At concentrations of 5 μg/ml, *Escherichia coli*, T4, λ, calf-thymus, and chick-blood DNA not only failed to inhibit aminoacid incorporation in reticulocyte lysates, but also did not affect the inhibitory activity of 50 ng/ml of *ds*RNA (data not shown). Thus, it seemed at first surprising that DNA would complex with IF-3. As shown in the competition experiment of Fig. 8*B*, however, DNA binds to IF-3 with much less affinity than does *ds*RNA. The extent of binding of ^{3}H-labeled T4 and λ DNA by a limiting amount of IF-3 decreases drastically when increasing mass equivalents of unlabeled *ds*RNA are present in the assay (*lower two curves*). Conversely, binding of [^{32}P]*ds*RNA is much less affected by increasing mass equivalents of DNA (*upper three curves*); slight differences among various DNA species are observed. Because the two sets of competition curves do not exhibit true reciprocity, possibly because of complexity in the binding interaction, they do not permit quantitative evaluation of the affinity ratio of *ds*RNA and DNA for IF-3.

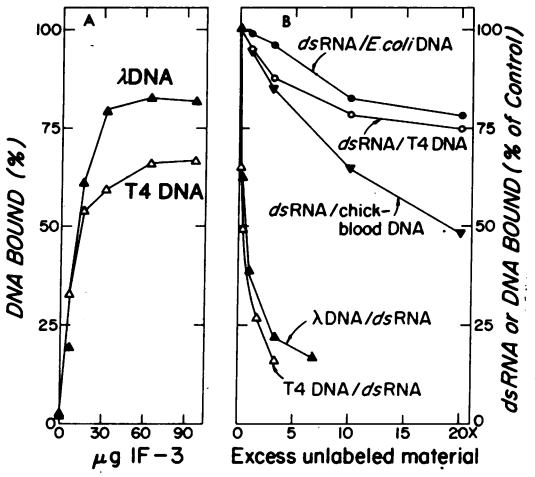

FIG. 8. Complex formation between IF-3 and DNA, and binding competition between *ds*RNA and DNA. (*A*) Binding. ^{3}H-labeled λ DNA (0.32 μg or 3150 cpm/filter) and T4 DNA (0.08 μg or 1930 cpm/filter) were assayed for binding to IF-3 as described for Fig. 6*B*, but nucleases were omitted. Both DNAs were intact as judged by sedimentation on alkaline sucrose gradients. (*B*) Competition. *Upper three curves:* [^{32}P]*ds*RNA was assayed for binding to a limiting amount of IF-3, as described for Fig. 6*B* (without nucleases), in the presence of increasing amounts of unlabeled DNA. *Lower two curves:* ^{3}H-labeled T4 and λ DNA were assayed for binding to a limiting amount of IF-3 as in *A*, in the presence of increasing amounts of unlabeled *ds*RNA. *Abscissa* represents A_{260} ratio of unlabeled to labeled material; *ordinate* shows percent of label bound relative to control in which unlabeled material was absent.

DISCUSSION

Four lines of evidence support the concept that the inhibition of protein synthesis by *ds*RNA is due entirely to the inactivation of initiation factor IF-3: (*i*) Addition of IF-3 completely overcomes the decay of polysomes and the inhibition of protein synthesis induced by *ds*RNA (Figs. 1–4). (*ii*) *ds*RNA inactivates a stoichiometric amount of IF-3 (Fig. 5). (*iii*) *ds*RNA forms a complex with IF-3 (Figs. 6 and 7). (*iv*) DNA is not inhibitory and, though it also forms a complex with IF-3, it does so with much less affinity than *ds*RNA (Fig. 8).

These results support the interpretation that IF-3 is inactivated by direct complexing with *ds*RNA. Any indirect scheme would require more complicated assumptions.

These findings also show that IF-3 recognizes double-stranded nucleic acids in general. This makes the possibility more likely that IF-3 in higher cells normally recognizes a double-stranded region in messenger RNA.

IF-3 was originally detected as a protein factor capable of maintaining rabbit reticulocyte polysomes in the absence of added heme, preventing their decay seen otherwise (7). We termed the factor IF-3 because of its considerable analogy to bacterial IF-3 (see ref. 7). Endogenous reticulocyte IF-3 activity is lost during protein synthesis in the absence of added heme (7). The present findings, therefore, constitute the second example of a specific inactivation of reticulocyte IF-3. We propose that specific inactivation of IF-3 may occur more widely as a mechanism of translational control in higher cells.

The residual protein synthesis seen to occur both in the absence of added heme and in the presence of *ds*RNA (Fig. 4; refs. 4–6), could be explained by the assumption that in both cases IF-3 can be inactivated only when it is exchanging among ribosomal subunits after initiation of polypeptide synthesis, and that several rounds of exchange are needed to exhaust it completely. One *ds*RNA molecule conceivably could complex with a number of IF-3 molecules and thus affect a large number of ribosomes, since IF-3 acts stoichiometrically in maintaining protein synthesis (Fig. 5 and ref. 7). This would explain the effectiveness of low concentrations of *ds*RNA.

The unusual property of IF-3 reported here, of being voided on Bio-Gel A-1.5m (Fig. 7), suggests that IF-3 may aggregate readily without loss of activity, and can be used as a tool in its purification. Whether a single component is responsible for IF-3 activity has not yet been determined. On DEAE-cellulose and Sephadex G-200 (7), IF-3 chromatographs identically with initiation factor IF-M_3 from rabbit reticulocytes (14), and a similar factor from chick embryo (15); an apparently identical factor has recently been reported (16). The relationship of IF-3 to two factors with reportedly different chromatographic and other properties (17, 18) is not clear.

The fact that *P. chrysogenum* is capable of unimpeded growth while supporting *ds*RNA virus replication suggests that, as with some other RNA viruses (1), replication takes place in sacs within the cytoplasm.

Finally, since the toxicity of *ds*RNA (19) has restricted its use as an interferon inducer, the ability of IF-3 to overcome the toxicity of *ds*RNA in cell-free extracts may have wider implications.

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