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Double-Stranded RNA as an Inhibitor of Protein Synthesis and as a Substrate for a Nuclease in Extracts of Krebs II Ascites Cells

(encephalomyocarditis virus/messenger RNA/ribonuclease)

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ABSTRACT **Concentrations of double-stranded RNA above about 0.1 μg/ml inhibit translation of encephalomyocarditis viral RNA and mouse globin messenger RNA in extracts of Krebs II ascites cells. Protein synthesis initially proceeds at the control rate, then abruptly shuts off in a manner similar to that observed in reticulocyte lysates [Hunt, T. & Ehrenfeld, E. (1971) *Nature New Biol.* 230, 91–94]. Substantially higher concentrations of double-stranded RNA are required to give this effect in ascites extracts.**

Subcellular fractions of Krebs II ascites cells contain a nucleolytic activity capable of digesting several natural and synthetic double-stranded RNAs. This nuclease is most active under conditions of protein synthesis, and part of the activity remains associated with ribosomes upon sedimentation. It is probably because of digestion of double-stranded RNA by this nuclease that higher concentrations of double-stranded RNA are required for inhibition of protein synthesis in Krebs cell extracts than in reticulocyte lysates.

Double-stranded RNA (dsRNA) is involved in several biological processes in animal cells. In particular, dsRNA induces the production of interferon (1–3), can cause tumor regression (4, 5), and has been implicated in the shut-off of protein synthesis in cells infected with poliovirus (6, 7). Several recent reports have demonstrated the binding and uptake of dsRNA by cells in tissue culture (8, 9), and dsRNA has been found in normal rat liver (10) and in nuclei of sea urchin embryos (11).

One of the most striking effects of dsRNA is the ability of very small amounts to inhibit the initiation of globin synthesis in reticulocyte lysates (6, 7, 12, 13). Another cell-free extract that can translate various mammalian mRNAs faithfully is that prepared from Krebs II ascites cells (14). This system has the advantage that it can translate efficiently exogenous RNA of both viral and cellular origin (15). We report here the effect of dsRNA on the translation of encephalomyocarditis (EMC) viral RNA and mouse globin mRNA in Krebs cell extracts. Our results demonstrate that protein synthesis is inhibited by dsRNA in a manner qualitatively similar to that reported for globin synthesis in extracts of rabbit reticulocytes (6, 7, 12), but that substantially higher concentrations of dsRNA are required. During these investigations, we detected a nuclease in extracts of ascites cells that digests dsRNA, and we believe that such an activity accounts for the lower sensitivity of this extract to dsRNA.

MATERIALS AND METHODS

Procedures for handling Krebs II ascites cells and S-30 extracts for protein synthesis have been described (15, 16). Protein synthesis reactions contained, in 25 μl, 7.5 μl of incubated S-30 extracts, 2.5 mM Mg^{++}, and 0.1 M KCl. [^{14}C]Phenylalanine or [^{14}C]aminoacid mixture (475 Ci/mol and 45 Ci/atom, respectively) were obtained from the Radiochemical Centre, Amersham, England; other components of the reactions were as specified (15, 16). EMC viral RNA was prepared as before (15), mouse globin mRNA was a gift from Dr. J. B. Lingrel, College of Medicine, Cincinnati, Ohio, and f2 single-stranded (ss)RNA was prepared from f2 bacteriophage. S-30 extracts were fractionated into ribosomes and S-150 supernatant by centrifugation at 48,000 rpm for 2.5 hr in a 50Ti rotor in a Beckman ultracentrifuge.

The dsRNAs tested as inhibitors of protein synthesis were obtained as follows: reovirus RNA was the gift of Dr. R. T. Hunt, Cambridge University; dsRNA from a virus of *Penicillium chrysogenum* was the gift of Dr. D. Planterose, Beecham Research Laboratories, Betchworth, Surrey, England; phage f2 replicative ensemble, the derivative of the replicating complex from phage f2-infected cells that contains dsRNA, was prepared and purified as described (17). The preparation used here was badly degraded after several years of storage at 4°, and contained dsRNA fragments no larger than 8 S, rather than the original 14S or greater dsRNA.

dsRNAs tested as substrates for nuclease activities were prepared as follows: the homopolymer pair poly(G·C) containing [^{3}H]GMP (1.8×10^6 dpm/μg) was synthesized as before (18) by use of poly(C) and *Escherichia coli* RNA polymerase, kindly provided by Dr. J. Roberts of this laboratory. ^{32}P-labeled phage f2 dsRNA and ssRNA were prepared and purified from phage-infected cells as described (19), and were used at a specific activity of 3 to 6×10^5 dpm/μg. ^{32}P-labeled phage f1 ssRNA and dsRNA (specific activity about 1×10^6 cpm/μg) were synthesized *in vitro* with [^{32}P]CTP (The Radiochemical Centre, Amersham), and purified as described (20).

E. coli ribonuclease III (21, 18) was purified (18) from strain MRE 600 with several modifications of the earlier procedure (Robertson, H. D. & Hunter, A. R., in preparation). The enzyme had an activity of about 5000 units/ml, where one unit solubilizes 1 nmol of poly(AU) nucleotides/hr (18).

Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; EMC, encephalomyocarditis virus.

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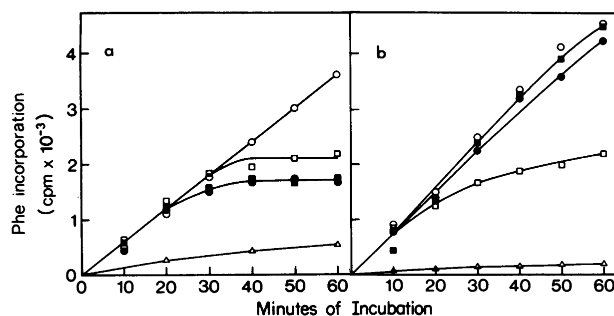

FIG. 1. (*a*) Effect of different concentrations of dsRNA on rate of protein synthesis. 10-μl Samples were removed from 75-μl protein synthesis reactions containing 25 μg/ml of EMC RNA (see *Methods*) for determination of incorporation into hot Cl_3CCOOH-insoluble material. Control, (○——○); with 0.17 (□——□), with 0.4 (■——■), or with 1 (●——●) μg/ml of dsRNA from *P. chrysogenum;* without EMC RNA (△——△). (*b*) Abolition of inhibitory activity of dsRNA by prior incubation with RNase III. dsRNA from *P. chrysogenum* (2 μl of an 880 μg/ml solution) was incubated for 90 min at 37° with 10 μl of RNase III or TM buffer, then for a further 90 min after addition of another 10 μl of RNase III or TM buffer. The incubated dsRNA was diluted 1:40 with water and 9 μl was added to 75 μl of protein synthesis reactions, which were assayed as in Fig. 1*a*. Control (○——○); with dsRNA from *P. chrysogenum* incubated in TM buffer, (□——□); with dsRNA from *P. chrysogenum* incubated with RNase III, (■——■); with an identical amount of fresh RNase III, (●——●); with no EMC RNA, (△——△).

Digestion of dsRNA in ascites subcellular fractions was assayed in a volume of 0.1 ml; the assay mixture contained 75 μl of TM buffer [0.02 M Tris·HCl (pH 7.6)–0.01 M magnesium acetate–0.2 M NH_4Cl–5% sucrose]. Incubations were at 37°, and samples were withdrawn periodically for determination of cold 5% Cl_3CCOOH-precipitable radioactivity (18). When reaction mixtures contained a substantial proportion of an ascites subcellular fraction in the salts of medium K [25 mM Tris·HCl–(pH 7.5)–125 mM KCl–5 mM magnesium acetate–6 mM 2-mercaptoethanol] (15), control incubations were performed with an equal amount of these salts.

RESULTS

dsRNA and protein synthesis

The effect of added dsRNA on the rate of EMC RNA translation by the ascites cell-free system is shown in Fig. 1*a*. The inhibition is similar to that observed in the reticulocyte system (6, 7, 12): after a period during which protein synthesis proceeds at the control rate, there is an abrupt and almost complete cessation of protein synthesis. This shutoff occurs at about 30 min with 0.17 μg/ml of dsRNA, and somewhat earlier (at about 20 min) with higher concentrations (0.4 μg/ml). A further increase in the concentration of dsRNA did not bring about any decrease in the time required to block incorporation.

Fig. 1*b* shows that prior incubation of the inhibitory dsRNA with RNase III, an *E. coli* nuclease specific for dsRNA (21, 18), abolished its action on protein synthesis. It is also apparent that RNase III itself has only a slight effect on protein synthesis at this concentration, though higher activities of this nuclease are highly inhibitory (data not shown).

The effects of various concentrations of dsRNA on translation in the ascites system were also examined. Fig. 2*b* shows that phage f2 dsRNA inhibited the translation of EMC viral RNA by up to 75%. It is evident that about 1–10 μg/ml of partially degraded f2 dsRNA is required to obtain inhibition of EMC translation under these conditions. Using *P. chrysogenum* and reovirus dsRNAs, which are much larger than the f2 dsRNA fragments, we found that substantially lower concentrations (about 0.05–0.1 μg/ml) are sufficient to inhibit translation (Fig. 2*a, e, f*). In no case have we found inhibition by any dsRNA at a concentration lower than 0.02 μg/ml in the ascites system. This result contrasts with the findings of Hunter *et al.* (12), who detected inhibition with concentrations of dsRNA below 0.5 ng/ml in reticulocyte lysates.

It is evident (Fig. 2*a*) that a certain proportion of the incorporation in the ascites system is resistant to inhibition, even at high dsRNA concentrations. Furthermore, the highest dsRNA concentrations tested did not relieve this inhibition, in contrast to the results obtained with rabbit reticulocyte lysates (7, 12). Fig. 2 also shows that the endogenous incorporation of this system is inhibited by dsRNA. Comparable concentrations of ssRNA from phage f2 do not inhibit translation in this system (Fig. 2*c*). In fact, a small stimulation is reproducibly obtained at high concentrations of f2 RNA, both in the presence and in the absence of added mammalian mRNA. Preliminary indications suggest that this stimulation

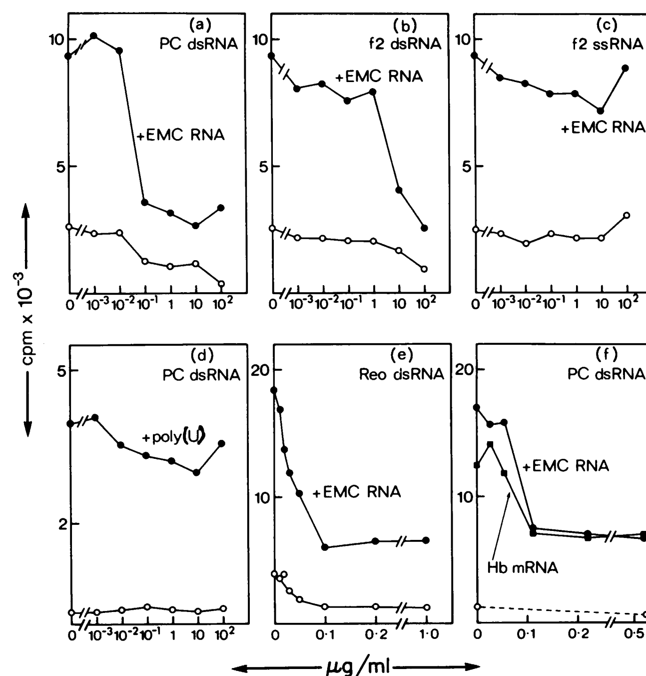

FIG. 2. Effect of several types of dsRNA on translation of various mRNAs. 25-μl reaction mixtures containing [¹⁴C]-phenylalanine (*a–d*) or [¹⁴C]aminoacid mixture (*e, f*) were incubated for 60 min. The Mg^{++} concentration was raised to 5 mM in (*d*). Messenger RNAs that were present in reactions denoted by (●——●) or (■——■), were added at the following concentrations: EMC RNA, 20 μg/ml; mouse globin mRNA, 20 μg/ml; poly(U), 100 μg/ml; reactions with no added mRNA, (○——○). In each case, the potential inhibitory nucleic acid (indicated in the upper right-hand corner of each panel) was present in the concentrations indicated in the abscissa (note log scales in *a–d*, linear scales in *e* and *f*). The ordinate scales refer to the incorporation of ¹⁴C into hot Cl_3CCOOH-insoluble material. *PC, P. chrygenum; Hb,* hemoglobin; *Reo,* reovirus.

is the result of low levels of translation of the f2 RNA under these conditions.

Fig. 2*f* shows that translation of globin mRNA and EMC RNA were inhibited in a similar manner. To discover whether the inhibitory action of dsRNA extends to the incorporation directed by synthetic polynucleotide messengers that lack an initiator codon, we used poly(U). This messenger requires a somewhat higher Mg^{++} concentration for optimal translation. Fig. 2*d* shows that under these conditions protein synthesis was markedly less sensitive to dsRNA.

A nuclease that digests dsRNA in ascites cell extracts

One way to explain the lower sensitivity of the ascites extract to dsRNA relative to the reticulocyte lysate would be to postulate the existence in the ascites extract of a nuclease capable of degrading dsRNA. Hunter *et al.* (12) have already reported the absence of such an activity from reticulocyte extracts. In contrast, the ascites S-30 fraction solubilizes the dsRNA purified from *E. coli* infected with bacteriophage f2 (Fig. 3.)

Using the substrate poly(G·C), we assayed a series of fractions obtained during the preparation of S-30 from ascitic fluid. Table 1 shows that no activity occurs in the ascitic fluid itself or in the final wash of the cells before lysis. This result is important, since Stern (22) has reported the existence in animal sera of a nuclease capable of digesting poly(I·C). Activity against poly(G·C) is observed after homogenization of the ascites cells, and much of this activity remains in the S-30 supernatant. A similar pattern of intracellular localization was obtained for *E. coli* RNase III (18).

Having established that we are dealing with an intracellular activity, we investigated its properties in S-30 extracts in more detail. An extract was separated into ribosomal and S-150 fractions, as described in *Methods*, and activity against equal amounts of phage f1 dsRNA or ssRNA of identical specific activity was measured. In the S-30 fraction, 72% of the dsRNA or 35% of the ssRNA was solubilized in 24 min, and a similar 100% difference in rate was observed with the S-150 supernatant fraction. However, with resuspended ribosomes, 70% of the dsRNA, but only 12% of the ssRNA, was solubilized in this interval. Similar results were obtained with ssRNA or dsRNA prepared from phage f2. In each case, solubilization of dsRNA is inhibited by 50 mM EDTA. These results suggest that a nuclease with preference for dsRNA is located in the cytoplasm of ascites tumor cells, perhaps in association with ribosomes.

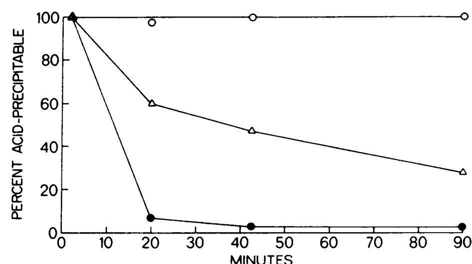

FIG. 3. Digestion of f2 dsRNA by ascites S-30 extracts. ^{32}P-Labeled f2 dsRNA was prepared as described in *Methods*. Each assay included, in a volume of 100 μl: 70 ng of f2 dsRNA (about 20,000 cpm); 25 μl of ascites S-30 or medium K; and 75 μl of TM buffer. 20-μl Samples were withdrawn at the times indicated for determination of cold Cl_3CCOOH-precipitable radioactivity. Control incubation, with no added nuclease, (○——○); reaction containing 10 units of *E. coli* RNase III, (●——●); reaction containing 25 μl of ascites S-30, (△——△).

TABLE 1. *Digestion of dsRNA by ascites-cell fractions*

Additions	pmol of poly(G·C) digested
None	0
Media RS and K	0
RNase III	16.4
RNase III + media RS and K	14.6
Ascitic fluid	0
Supernatant from eighth wash of ascites cells	0
Ascites cell homogenate	24.6
30,000 × *g* pellet	20.3
30,000 × *g* supernatant (S-30)	22.1

The dsRNA substrate, poly(G·C), is described in *Methods* Ascites cells were washed by repeated sedimentation and suspension in medium RS (5.2 mM KCl-130 mM NaCl–7.4 mM $MgCl_2$) and were homogenized and fractionated as described (15). All samples were adjusted to equal volumes before assay. Reactions contained in 50 μl: 10 ng (30 pmol) of poly([^{3}H]G·C), and 5 μl of a buffer containing 0.5 M Tris·HCl (pH 7.6)–0.20 M magnesium acetate–1.0 M NH_4Cl–40% sucrose. Additions were as follows: Medium RS and medium K, 5 μl each; *E. coli* RNase III, 0.2 unit in 10 μl of TM buffer; ascites cellular fractions, 10 μl each. Incubation was at 37° for 30 min, and digestion was assayed as described in *Methods.*

Effects of prior incubation with dsRNA

Hunter *et al.* (12) showed that prior incubation of dsRNA with reticulocyte lysates resulted in a potentiation of the inhibitory effect. It might have been anticipated that observation of this phenomenon in the ascites extract would be obstructed by the nuclease described above. However, as shown in Table 2, prior incubation of the ascites S-30 fraction together with dsRNA indeed led to an increase in the inhibitory action. Addition of dsRNA at the same time as the mRNA inhibited protein synthesis directed by EMC RNA by about 50%, regardless of whether the S-30 itself was preincubated or not. When the S-30 was first incubated for 5 or 15 min with an identical

TABLE 2. *Effect of prior incubation with dsRNA on protein synthesis*

		[^{14}C]Phe incorporation (cpm × 10^{-3})		
		Prior incubation (min)		
EMC RNA	dsRNA addition	0	5	15
+	—	15.2	15.3	13.7
+	After incubation	7.9	7.7	7.4
+	Before incubation	8.5	3.2	2.9
+	Both	4.9	3.2	2.8
−	—	2.2	1.6	0.9

The components of 25 μl of protein-synthesis reactions were incubated for the times shown in a volume of 17.5 μl. dsRNA from *P. chrysogenum* (2.5 ng) was added before or after this incubation, or at both times. EMC RNA (30 μg/ml) was then added, the volume was adjusted to 25 μl, and the reaction mixtures were further incubated for 60 min at 37°.

amount of dsRNA, the inhibition of EMC RNA translation increased to 85–90%. Addition of more dsRNA to an extract that had been incubated with dsRNA produced no further inhibition. This result suggests that the plateau level corresponding to the maximal inhibition is rapidly obtained with lower dsRNA concentrations as a consequence of prior incubation.

This potentiation was observed with prior incubation times as short as 1 min, or as long as 40 min. This result suggests that dsRNA rapidly binds to some component of the protein synthetic machinery, and that this interaction protects the bound portion of the inhibitor from the action of the dsRNA-digesting nuclease. A similar situation was created artificially in the reticulocyte lysate when *E. coli* RNase III was added after incubation of the system with radioactive dsRNA (H. D. Robertson, R. J. Jackson, R. T. Hunt, and A. R. Hunter, unpublished experiments). Under these conditions, the nuclease solubilized up to 90% of the dsRNA without reversing the increased inhibitory effect.

DISCUSSION

Effect of dsRNA on protein synthesis in ascites and reticulocyte cell-free extracts

We have shown that dsRNA inhibits the translation of two natural mRNAs in an ascites cell-free system. This effect resembles that previously observed in reticulocyte lysates (6, 7, 12) in the following respects: (*a*) the rate of protein synthesis in the presence of dsRNA shows a sharp cutoff after an initial period of synthesis at the control rate (Fig. 1*a*). (*b*) Translation of poly(U) is inhibited to a much smaller extent than that of natural mRNA (Fig. 2*d*). (*c*) Single-stranded RNA does not inhibit the ascites system (Fig. 2*c*). (*d*) Prior incubation of an ascites S-30 fraction with dsRNA potentiates the inhibitory activity of dsRNA (Table 2). (*e*) At no tested concentration of dsRNA is the inhibition of translation in the ascites extract complete (Fig. 2). Therefore, this sort of inhibition by dsRNA may be a general property of numerous cell types.

In contrast to these similarities in the response to dsRNA of the ascites and reticulocyte extracts, there are several differences: (*a*) Whereas high concentrations of dsRNA (over 10 μg/ml) do not inhibit translation in reticulocyte lysates (7, 12), dsRNA concentrations up to 100 μg/ml do not relieve inhibition in the ascites system. It is, of course, still possible that even higher dsRNA concentrations might have this effect. (*b*) The proportion of ascites translation that is resistant to dsRNA inhibition (Fig. 2) is significantly higher than that reported for reticulocyte lysates (12). This finding is also reflected by the absence of a progressively earlier shutoff of incorporation in the ascites system as the concentration of dsRNA is raised above a certain limit (Fig. 1*a*). Moreover, a significant proportion of ascites translation continues to be resistant to inhibition even after prior incubation with dsRNA (Table 2), whereas such treatment of reticulocyte lysates virtually abolishes their activity (12). (*c*) The minimum dsRNA concentration at which inhibition can be observed in the ascites system is at least 40-fold higher than that observed with reticulocyte extracts (12). Furthermore, the ascites system displays a greater sensitivity to large dsRNA molecules than to small fragments, while the rabbit reticulocyte system was almost equally sensitive to all dsRNA inhibitors used in these experiments (R. J. Jackson, personal communication).

Properties of cell-free extracts that could account for different effects of dsRNA

These differences could be explained in several ways, especially as the reticulocyte is a differentiated cell that synthesizes a small number of proteins, whereas the ascites cell is a tumor cell that makes numerous proteins. Moreover, the incubated ascites S-30 fraction is largely dependent upon added mRNA (15, 16), whereas the reticulocyte system efficiently reinitiates translation of its endogenous mRNA (23). Several of our observations suggest that the explanation for the higher dsRNA concentrations required to inhibit the ascites system is the dsRNA-digesting nuclease present in ascites extracts. First, ascites S-30 fractions can solubilize dsRNA at a concentration of up to 0.1 μg/ml under conditions of protein synthesis; this is also the dsRNA concentration below which little inhibition of protein synthesis is observed. Second, this explanation is also consistent with the fact that large dsRNA molecules inhibit the ascites system (but not the reticulocyte system) better than small ones (see Fig. 2*a* and *b*). Finally, in an experiment performed in collaboration with Dr. R. J. Jackson, Cambridge University, we found that prior incubation of dsRNA from *P. chrysogenum* (0.2–1 μg/ml) with ascites cell S-30 diminished by over 80% the ability of that dsRNA to inhibit the more sensitive reticulocyte system.

Digestion of dsRNA by a nuclease in ascites extracts

The experiments in Fig. 3 and Table 1 establish the presence of a nucleolytic activity capable of digesting dsRNA in ascites extracts. There have been several earlier reports of enzymes that use helical RNA as substrate. Activities in *E. coli* RNase III cleave large, regular helical RNA molecules (in either dsRNA (21, 18) or DNA:RNA hybrids (24, 20), reducing them to sizes between 10 and 20 base-pairs (ref. 25 and H. D. R., unpublished observations). In eukaryotic systems, in addition to the activity against dsRNA in serum (22), an activity that digests dsRNA in a frog virus has been reported (26). Also, there have been several reports of enzymes, termed RNase H, that digest the RNA of DNA·RNA hybrids with high specificity (27). These enzymes are incapable of digesting RNA·RNA duplexes, and have been found in calf-thymus nuclei (27) and RNA tumor viruses (28). These earlier findings and our observations here demonstrate that higher cells contain nucleases that can metabolize both of the major types of helical RNA.

Possible roles for the recognition of dsRNA in translation

These findings may help to clarify our view of virus-induced shutoff of host-cell protein synthesis, and to suggest some properties of protein synthesis in eukaryotic cells. Our finding (Fig. 2*f*) that globin mRNA and EMC mRNA translation are inhibited about equally by dsRNA does not support the idea that dsRNA may inhibit translation of cellular, but not viral, mRNAs (6, 7). Thus, translation of EMC RNA and globin mRNA may not be initiated by radically different mechanisms. However, this negative result cannot be considered a definitive disproof of this hypothesis, since more subtle aspects of cellular control processes may not be fully effective *in vitro*.

The findings reported here show that two eukaryotic cell extracts display a qualitatively similar response to dsRNA. In addition, it has been recently reported that translation of reovirus mRNA in extracts of mouse L cells is partially

inhibited by dsRNA (29). The experiment shown in Fig. 2*d*, in which translation of poly(U) is not inhibited by dsRNA, suggests that this inhibitory effect of dsRNA may be confined to the proper translation of natural mRNAs.

Recent studies with reticulocyte lysates (13) have indicated that dsRNA specifically inhibited an early step in the initiation of protein synthesis. In light of the numerous similarities between the two systems in their response to dsRNA, it seems reasonable to suppose that initiation is also the step inhibited in the ascites system, although this has not been proved. Inhibition of protein synthesis by dsRNA could be due to a fortuitous recognition of this nucleic acid by some component of the translation machinery whose normal function does not involve recognition of, or binding to, helical RNA. On the other hand, it may become necessary to postulate a step involving recognition of regular helical RNA as part of the initiation process in higher cells.

Hunter *et al.* (12) have proposed that recognition of a length of dsRNA by a cellular component, such as an initiation factor, could be necessary for translation. This helical region would have to be greater than about 20 base-pairs long and quite stable, since prior treatment of dsRNA with RNase III abolishes inhibition (ref. 12 and Fig. 1*b*). It is possible that the recognition of stable dsRNA in protein initiation may be one way in which mammalian and bacterial processes are differentiated. We have been unable to demonstrate any effect of added natural dsRNA upon the translation of phage f2 RNA by *E. coli* S-30 fractions.

The existence of such dsRNA recognition in higher systems might be part of a pathway in which a nuclease of the sort described here could regulate the extent and rate of translation, either by cleaving the mRNA itself or by removing dsRNA of nonmessenger origin.

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