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INHIBITION OF PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES BY A DOUBLE-STRANDED RNA COMPONENT IN HELA mRNA

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SUMMARY: Double-stranded RNA (dsRNA) inhibits protein synthesis initiation in rabbit reticulocyte lysates by the activation of a latent dsRNA-dependent cAMP-independent protein kinase which phosphorylates the α-subunit of the eukaryotic initiation factor eIF-2. In this study, we describe a dsRNA-like component which is present in preparations of HeLa mRNA (poly A^+) isolated from total cytoplasmic RNA. The inhibitory species in the HeLa cytoplasmic mRNA was detected by (a) its ability to inhibit protein synthesis with biphasic kinetics in reticulocyte lysates translating endogenous globin mRNA, and (b) by the inefficient translation of HeLa cytoplasmic mRNA in a nuclease-treated mRNA-dependent reticulocyte lysate. The inhibitory component was characterized as dsRNA by several criteria including (i) the ability to activate the lysate dsRNA-dependent eIF-2α kinase (dsI); (ii) the prevention of both dsI activation and inhibition of protein synthesis by high levels of dsRNA or cAMP; (iii) the reversal of inhibition by eIF-2; and (iv) the inability to inhibit protein synthesis in wheat germ extracts which lack latent dsI. By the same criteria, the putative dsRNA component(s) appears to be absent from preparations of HeLa mRNA isolated exclusively from polyribosomes.

INTRODUCTION: Protein synthesis in rabbit reticulocyte lysates is inhibited in response to certain conditions which block protein chain initiation, including the absence of heme (1), or the addition of low levels of dsRNA (1-20 ng/ml) (2,3). These conditions give rise to the activation of separate cAMP-independent protein kinases that specifically phosphorylate the α-subunit (38,000 daltons) of the eukaryotic initiation factor eIF-2 (4). The dsRNA-dependent eIF-2α kinase (dsI) is present in lysates as a latent, inactive form associated with the ribosomes. The activation of dsI requires incubation with dsRNA and ATP and is accompanied by the phosphorylation of endogenous dsI and eIF-2α. This results in an inhibition of protein synthesis with biphasic kinetics; after an initial period of linear synthesis, abrupt shutoff ensues. The dsRNA-dependent (i)activation of dsI, (ii) phosphorylation of eIF-2α, and (iii)inhibition of protein synthesis are all prevented by the addition of high non-physiological levels of dsRNA (20 μg/ml) or cAMP (5-20 mM)(3,5). Additionally, inhibition by dsRNA is reversed by addition of exo-

Abbreviations: dsRNA, double-stranded RNA; eIF-2, eukaryotic initiation factor 2; eIF-2α, α-subunit (38,000 daltons) of eIF-2; dsI, dsRNA-dependent eIF-2α kinase; HeLa $mRNA_{cyt}$ and $mRNA_{rib}$, cytoplasmic and polyribosomal mRNA (poly A^+)

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genous eIF-2 (6), a property which is characteristic of inhibitions caused by eIF-2α kinase activities.

In the present study, we examined the translation of two HeLa mRNA preparations in normal and in nuclease-treated mRNA-dependent reticulocyte lysates. The HeLa mRNA preparations were obtained from (i) total cytoplasmic RNA, and (ii) polyribosomal RNA, by chromatography on oligo dT-cellulose. We describe here the presence of a dsRNA-like component in HeLa cytoplasmic mRNA ($mRNA_{cyt}$) which activates dsI when added to reticulocyte lysates and inhibits protein synthesis with biphasic kinetics. In contrast, the HeLa mRNA fraction isolated exclusively from HeLa polyribosomes ($mRNA_{rib}$) contained no dsRNA.

METHODS: HeLa cells (S3) were grown in suspension in Joklik's minimal essential medium supplemented with 10% calf serum (GIBCO). All RNA preparations were isolated from exponentially growing cultures.
Isolation of HeLa cytoplasmic mRNA (poly A^+) and polyribosomal mRNA (poly A^+). HeLa total cytoplasmic RNA was isolated by the method of Ricciardi et al. (7). For HeLa total polyribosomal RNA, ~10^9 cells were washed twice with PBS and resuspended in 2 vol of hypotonic buffer (20 mM HEPES, pH 7.4/10 mM KOAc/1.5 mM $Mg(OAc)_2$/0.5 mM DTT). After 10 min at 0°, cells were lysed by 10 strokes in a Dounce homogenizer, and KOAc was brought to 70 mM. Nuclei, unlysed cells, membranes, and mitochondria were removed by centrifugation, and polyribosomes were pelleted at 45K rpm (50Ti rotor) for 4 h at 4°C and then resuspended in 100 mM Tris-HCl (pH 9)/100 mM NaCl/1 mM EDTA. Polysomal RNA was extracted as described (8). Poly A^+ mRNA was purified from total cytoplasmic RNA and from total polyribosomal RNA by chromatography on oligo dT-cellulose. Following ethanol precipitation, mRNA was resuspended in sterile glass distilled water and stored at -70°C. The flow-through RNA (poly A^-) was similarly collected and stored.
Protein synthesis assay. Rabbit reticulocyte lysates were prepared as described previously (5). For the experiments in Fig. 2, lysates were made mRNA-dependent by treatment with micrococcal nuclease by the method of Pelham and Jackson (9). Protein synthesis was measured by the incorporation of [^{35}S]methionine (1300 Ci/mmole) into acid-precipitable material as described (5). Reaction volume was 25-50 µl; incubation was at 30°C. Protein synthesis in wheat germ extracts (see Fig. 5) was assayed by the method of Roberts and Paterson (10).
Protein kinase assay. Preparation of reticulocyte Sepharose-6B ribosomes and phosphorylation of ribosomal eIF-2α by endogenous dsRNA-activated eIF-2α kinase, and electrophoresis and autoradiography of SDS gels, were as described (11).

RESULTS: An inhibitor of reticulocyte protein synthesis is contained in HeLa cytoplasmic mRNA (poly A^+). The addition of HeLa mRNA (20 µg/ml) isolated from total cytoplasmic RNA ($mRNA_{cyt}$) to hemin-supplemented reticulocyte lysates resulted in an inhibition of protein synthesis with biphasic kinetics (Fig. 1). By contrast, the addition at similar concentrations of globin mRNA (Fig. 1A), or HeLa mRNA isolated from polyribosomes ($mRNA_{rib}$) (Fig. 1B) had no effect on protein synthesis. When HeLa $mRNA_{cyt}$ was subjected to heating (80°C) and quick-cooling, this mRNA fraction retained its inhibitory properties (Fig. 1A). As shown in Table I, the HeLa cytoplasmic RNA which did not bind to oligo dT-cellulose (poly A^- flow through RNA) was not inhibitory. Moreover, the inhibitor remains with the poly A^+ mRNA through a second cycle of oligo dT-cellulose chromatography.

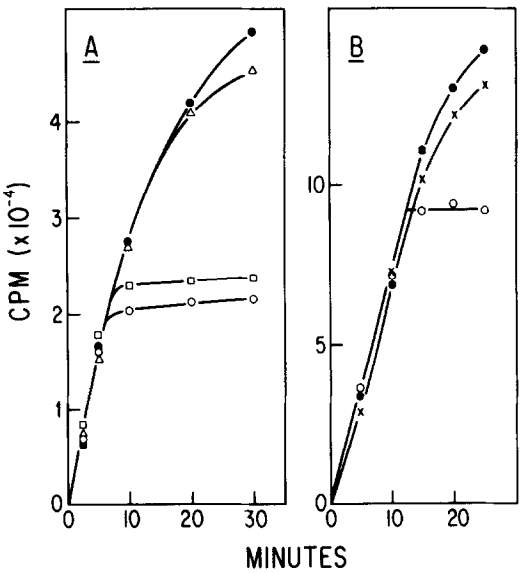

Fig. 1. Inhibition of reticulocyte protein synthesis by HeLa cytoplasmic mRNA. Protein synthesis assays (50µl) were as described in Methods. Additions were as follows: Panel A: (●), no additions; (△), 25 µg/ml of globin mRNA; (o), 5 µg/ml of HeLa cytoplasmic mRNA (untreated) or (■), heated at 80° for 1 min and quick-cooled. Panel B: (●), no additions; (o), 20 µg/ml of HeLa cytoplasmic mRNA; (x), 20 µg/ml of HeLa polyribosomal mRNA.

Additional evidence for the presence of an inhibitory component in HeLa $mRNA_{cyt}$ preparations is the inefficiency of translation of $mRNA_{cyt}$. The titration of globin mRNA, HeLa $mRNA_{cyt}$, and HeLa $mRNA_{rib}$ were examined in lysates made mRNA-dependent by treatment with micrococcal nuclease. The translation of both globin mRNA and $mRNA_{rib}$ (Fig. 2B) were concentration-dependent. Globin synthesis was optimally stimulated by globin mRNA levels of 50-80 µg/ml, values which are in agreement with previous findings (9). By comparison, HeLa $mRNA_{cyt}$ was relatively inefficient as a source of mRNA, and the extent of [^{35}S]methionine incorporation did not increase with mRNA concentration (Fig. 2A). Heat denaturation of $mRNA_{cyt}$ or the addition of tRNA to the lysate did not restore activity to $mRNA_{cyt}$ (not shown). It should be noted that although incorporation of [^{35}S]methionine is low, the products of translation of $mRNA_{cyt}$ resemble the protein profile of HeLa cells labelled in vivo (not shown).

Evidence for the presence of dsRNA in HeLa mRNAcyt. We examined the possibility that the inhibitory component contained in HeLa $mRNA_{cyt}$ was dsRNA. Protein synthesis in reticulocyte lysates is inhibited by low levels of dsRNA (1-20 ng/ml) due to the activation of a dsRNA-dependent eIF-2α kinase (dsI) (4). A unique property of the dsRNA-induced inhibition is the ability of high levels of dsRNA (20 µg/ml) to prevent inhibition by blocking the activation of dsI (3,4). Fig. 3 shows that the inhibition of protein synthesis produced by HeLa $mRNA_{cyt}$

TABLE I. Inhibition of protein synthesis in reticulocyte lysates by HeLa cytoplasmic mRNA (poly A^+)

RNA added	[^{35}S]Methionine incorporation
	(cpm x 10^{-4})
Experiment 1	
None	18.9
HeLa cytoplasmic RNA (total)	18.3
HeLa cytoplasmic RNA (poly A^-)	20.2
HeLa cytoplasmic RNA (poly A^+)	14.0
Globin mRNA (poly A^+)	20.0
Experiment 2	
None	14.1
HeLa cytoplasmic RNA (poly A^+)	
Oligo dT passage 1	9.3
Oligo dT passage 2	8.2

Protein synthesis assays in reticulocyte lysates (Methods) were supplemented with various RNA fractions as indicated in the table. Experiment 1: RNA concentrations were 4 µg/ml. Total cytoplasmic RNA, not chromatographed on oligo dT-cellulose. Poly A^+, retained on oligo dT-cellulose. Poly A^-, not retained. Experiment 2: RNA concentrations were 5 µg/ml. Passage 2 poly A^+ RNA was obtained by rechromatography of passage 1 poly A^+ RNA.

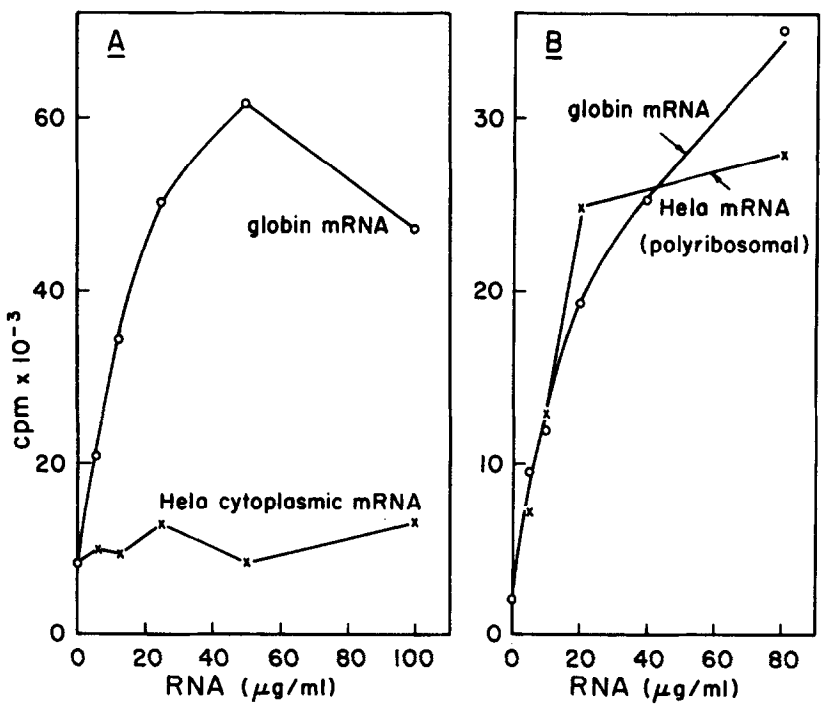

Fig. 2. Titration of HeLa and globin mRNA in micrococcal nuclease-treated mRNA-dependent reticulocyte lysates. Poly A^+ mRNA was added to nuclease-treated lysates (Methods) at the indicated concentrations and assay mixtures were incubated for 30 min at 30°. Panel A: (o), globin mRNA; (x), HeLa cytoplasmic mRNA. Panel B: (o), globin mRNA; (x), HeLa polyribosomal mRNA.

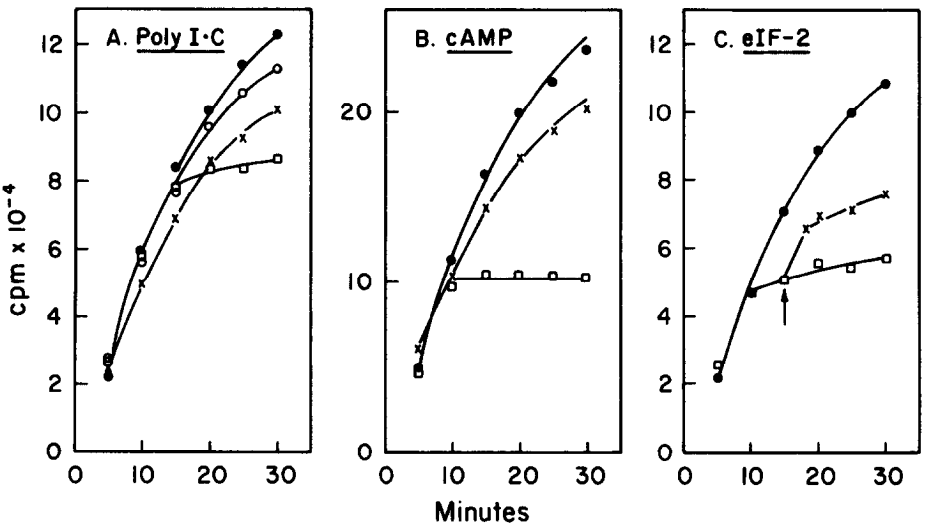

Fig. 3. Inhibition of protein synthesis by HeLa mRNAcyt Reversal by polyI:C, cAMP, eIF-2. Protein synthesis assays in normal reticulocyte lysates were as described in Methods. Additions in all panels: (●), no additions; (■), HeLa cytoplasmic mRNA (5 μg/ml in A, 20 μg/ml in B and C). Other additions were: Panel A: (o), poly I:C (20 μg/ml; (x), HeLa $mRNA_{cyt}$ + poly I:C (20 μg/ml). Panel B: (x), HeLa $mRNA_{cyt}$ + 10 mM cAMP. Panel C: (x), 1 μg (7 pmol) of eIF-2 at 15 min.

in normal lysates was partially prevented by poly I:C (20 μg/ml) (Fig. 3A). Similarly, inhibition by HeLa $mRNA_{cyt}$ was prevented by high levels of cAMP (10 mM) (Fig. 3B) and was reversed by exogenous eIF-2 (Fig. 3C), phenomena which are characteristic of the inhibitions produced by eIF-2α kinases (5). These data provide support for the presence of a dsRNA component in HeLa $mRNA_{cyt}$.

In an effort to quantitate the putative dsRNA contained in HeLa $mRNA_{cyt}$, we compared the inhibitory capacity of HeLa $mRNA_{cyt}$ with purified Reovirus dsRNA (Fig. 4), and found that a 50% inhibition of protein synthesis required approximately 0.3 ng/ml of Reovirus dsRNA or 4 μg/ml of HeLa $mRNA_{cyt}$. Assuming the same relative efficiency, then HeLa $mRNA_{cyt}$ contains about 0.01% dsRNA. However, we emphasize that this is an approximation.

Wheat germ extracts have been shown to translate a variety of exogenous mRNAs with fidelity (10). Since wheat germ contains no detectable dsI (12), we examined the capacity of wheat germ extracts to translate HeLa $mRNA_{cyt}$ (Fig. 5) and found that it is utilized at an efficiency comparable to globin mRNA at concentrations previously reported (10). Hence, in contrast to the effects observed in mRNA-dependent reticulocyte lysates, the dsRNA component in HeLa mRNAcyt does not inhibit the translation of mRNAcyt in wheat germ extracts. In addition, $mRNA_{cyt}$ does not inhibit globin translation in this system (not shown).

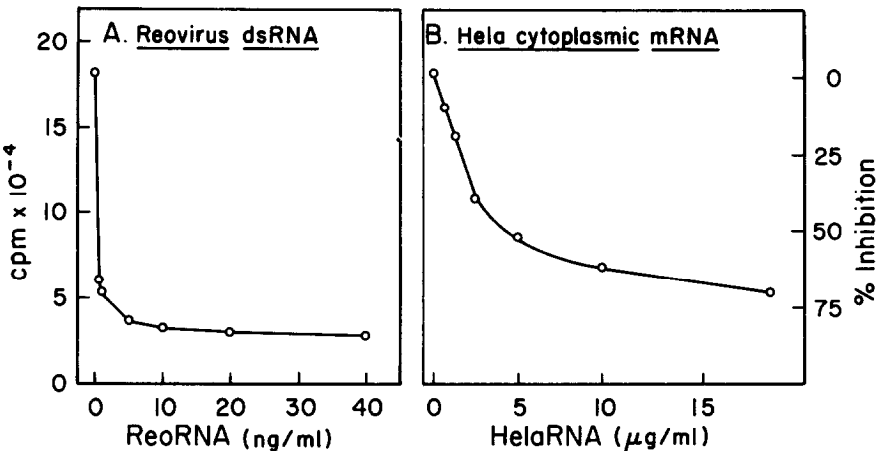

Fig. 4. Titration of Reovirus dsRNA and HeLa cytoplasmic mRNA in reticulocyte lysates. Protein synthesis assays in reticulocyte lysates were as described (Methods). Reovirus dsRNA (panel A) and HeLa $mRNA_{cyt}$ (Panel B) were added at the indicated concentrations. Incubation was 30 min at 30°.

Activation of ribosomal dsI by HeLa mRNAcyt. To confirm that HeLa $mRNA_{cyt}$ contains dsRNA, we examined its ability to activate the latent dsI bound to reticulocyte ribosomes obtained from lysates filtered on Sepharose 6B. As previously shown (11), activation by dsRNA of this ribosomal dsI leads to the phosphorylation

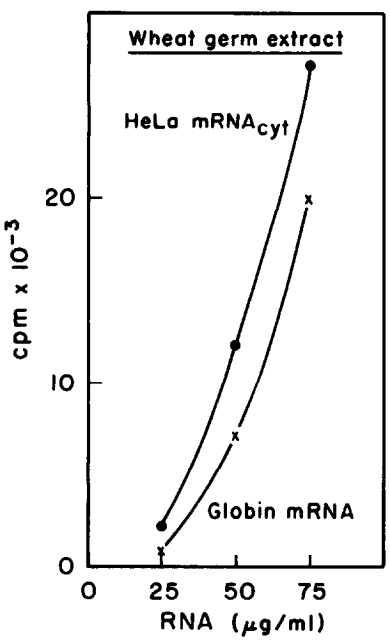

Fig. 5. Translation of HeLa cytoplasmic mRNA and globin mRNA in wheat germ extracts. HeLa $mRNA_{cyt}$ (•) and globin mRNA (x) were added to wheat germ extracts in protein synthesis assays (Methods) at the indicated concentrations. Incubation was at 24° for 30 min. All values are corrected for [^{35}S]methionine incorporated (3 x 10^3 cpm) in the absence of added mRNA.

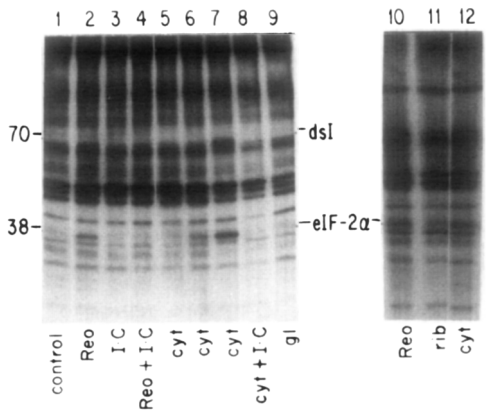

Fig. 6. Activation of reticulocyte ribosome-bound dsI by HeLa cytoplasmic mRNA: ^{32}[P]Phosphoprotein profile. dsI activation assays (20µl) contained 10 mM HEPES-KOH (pH 7.2), 75 mM KCl, 2.5 mM $Mg(OAc)_2$, 1 mM [γ^{32}P]ATP (10 µCi/mmol), and 0.4 A_{260} units of reticulocyte Sepharose 6B ribosomes (Methods). Other additions were as indicated in the figure: Reovirus dsRNA (1 ng/ml), lanes 2,4,10. HeLa $mRNA_{cyt}$, lane 5 (20 ng/ml), lane 6 (2 µg/ml), lanes 7,8,12 (20 µg/ml). HeLa $mRNA_{rib}$ (20 µg/ml), lane 11. Globin mRNA (25 µg/ml), lane 9. Poly I:C (20 µg/ml), lanes 3,4,8. Lane 1 is a control. Incubation was 15 min at 37°. Assays were subjected to SDS-PAGE as described previously(11). In these gels, dsI and eIF-2 migrate as 70K and 38K, respectively.

of endogenous dsI (70K) and of endogenous eIF-2α (38K). Fig. 6 shows that incubation of ribosomes with HeLa $mRNA_{cyt}$ (20µg/ml) results in the phosphorylation of both dsI and of eIF-2α (lanes 7,12). In contrast, HeLa $mRNA_{rib}$ (lane 11) and globin mRNA (lane 9), at similar concentrations, do not activate ribosomal dsI. Lower concentrations of HeLa $mRNA_{cyt}$ produced little or no phosphorylation of these components (lanes 5,6). More significantly, both phosphorylations were blocked when high poly I:C (20 µg/ml) was also present (lane 8).

DISCUSSION: A dsRNA-like inhibitory component, present in very low concentration (<0.01%), has been detected in poly A^+ mRNA prepared from HeLa cytoplasm ($mRNA_{cyt}$). The dsRNA was initially detected by (a) the capacity of HeLa $mRNA_{cyt}$ to inhibit protein synthesis in reticulocyte lysates with biphasic kinetics, and by (b) the low efficiency of translation of HeLa $mRNA_{cyt}$ when added to mRNA-dependent reticulocyte lysates. The inhibitor in $mRNA_{cyt}$ was characterized as dsRNA by its ability to activate reticulocyte dsRNA-dependent eIF-2α kinase (dsI), with the concomitant phosphorylation of endogenous dsI (70K) and eIF-2α (38K). These effects were prevented by high levels of poly I:C (20 µg/ml), which blocks dsI activation by known dsRNA species. Predictably, high poly I:C also blocked the inhibition of protein synthesis by HeLa $mRNA_{cyt}$. From these results we conclude that the inhibitory component in the HeLa $mRNA_{cyt}$ is dsRNA, and that the inhibition of protein synthesis is due to the activation of

dsI. This is further confirmed by the findings that the inhibition is reversed by high levels of cAMP (10mM) or eIF-2, effects which are characteristic of inhibitions produced by eIF-2α kinase activation(5). Significantly, in wheat germ extracts, which lack dsI, the dsRNA component does not interfere with the translation of $mRNA_{cyt}$.

The dsRNA component is not present in the poly A^+ mRNA prepared from HeLa polyribosomes. This suggests that the dsRNA component, which fractionates with mRNA on oligo dT-cellulose, may be associated with mRNA in some non-polyribosomal form in the cytoplasm. Inhibition of reticulocyte protein synthesis by HeLa nuclear RNA that is apparently double-stranded has been reported (13). More recent studies have demonstrated the presence of dsRNA in the nuclei and cytoplasm of Chinese hamster ovary cells (14) and rat liver (15). In both tissues, the dsRNA in the cytoplasm is associated with poly A^+ RNA, but is absent from polyribosomes(14,15).

Although double-stranded RNA is sparsely distributed in nature, certain viruses (such as Reovirus) and mycophage (such as P. chrysogenum) have dsRNA genomes, and many RNA viruses replicate through a dsRNA intermediate (2,16). To diminish the possibility that viral contamination was the source of dsRNA in our HeLa cells, we prepared $mRNA_{cyt}$ from two different HeLa cell stocks. Both preparations inhibit reticulocyte protein synthesis and produce phosphorylation of dsI and eIF-2α. However, we cannot rule out a low level of endogenous viral dsRNA in HeLa cells which copurifies with poly A^+ $mRNA_{cyt}$.

It is possible that the dsRNA we describe is a normal cytoplasmic component of unknown function which has not been previously detected in HeLa due to its low concentration. The sensitivity of reticulocyte dsI kinase activity to low levels of dsRNA (0.1-1 ng/ml) provides a direct assay to monitor for its presence.

This paper is dedicated to the memory of Dr. Vivian Ernst, whose untimely death is a profound loss to her students and colleagues.

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