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INHIBITION OF PROTEIN SYNTHESIS BY DOUBLE-STRANDED RNA IN RETICULOCYTE LYSATES: EVIDENCE FOR ACTIVATION OF AN ENDORIBONUCLEASE

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SUMMARY

Double-stranded RNA is a potent inhibitor of protein synthesis in rabbit reticulocyte lysates. Three lines of evidence suggest that at least part of this inhibitory activity is due to activation of a nuclease which degrades mRNA: (1) In the presence of emetine reticulocyte polysomes are partially degraded to structures containing 1-3 ribosomes; (2) 34S Mengo-virus RNA is degraded to fragments sedimenting at less than 18S; (3) The template activity of globin mRNA extracted from the lysates is reduced by 90% when compared to appropriate controls. The ability of double-stranded RNA to activate a nuclease in the reticulocyte system is very similar to that observed in extracts from interferon treated cells and probably involves formation of the unusual oligonucleotide pppA$2'p5'A2'p5'A$.

INTRODUCTION

Protein synthesis in lysates prepared from rabbit reticulocytes is sensitive to inhibition by very low concentrations of double-stranded RNA (1-5). Investigations into the mechanism of this effect have indicated that polypeptide chain initiation is a major target for dsRNA action and addition of stoichiometric amounts of the initiation factor eIF-2 to reticulocyte lysates partially prevents or even reverses the inhibition (4, 6-8). One subunit of eIF-2 is specifically phosphorylated by a protein kinase which is activated by dsRNA and this modification has been correlated with decreased association of initiator Met-$tRNA_f$ with 40S ribosomal subunits (5), a process requiring eIF-2 (9-12).

Abbreviations: dsRNA, double-stranded RNA; SDS, sodium dodecyl sulphate; HCR, haem controlled repressor.

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In contrast to these observations, the action of dsRNA on protein synthesis in extracts from interferon-treated L cells or ascites tumour cells involves effects on one or more post-initiation events (13), although there is evidence for inhibition of initiation as well (14). In particular, degradation of messenger RNA is enhanced in the presence of dsRNA, due to the dsRNA and ATP dependent activation of an endonuclease (14-17). In the light of these observations, we have reinvestigated the effects of dsRNA on reticulocyte cell-free protein synthesis and here we demonstrate that dsRNA also activates a nuclease in this system as it does in interferon-treated mouse cell extracts.

MATERIALS AND METHODS

Materials. Poly rI: poly rC and emetine were from Sigma Chemicals, U.K. Micrococcal nuclease (EC 3.1.4.7) was from P-L Biochemicals Inc. [^{14}C] leucine, final specific activity 32 Ci/mole, was from the Radiochemical Centre, Amersham, U.K. [^{3}H] Mengo virus RNA was a generous gift from Dr R Falcoff, Paris.

Preparation and incubation of rabbit reticulocyte lysates and haem controlled repressor. These procedures were carried out as described previously (18). Incorporation of [^{14}C] leucine (4μCi/ml) into protein was measured in 10μl aliquots of incubation mixtures.

Polysome size analysis on sucrose density gradients. Reticulocyte lysate incubations were diluted with cold buffer (20mM Tris, pH 7.6, 100mM KCl, 5mM Mg acetate) and centrifuged through 20-50% (w/v) sucrose gradients in the same buffer at 50,000 rpm for 35 min in a Beckman SW 50.1 rotor. Absorbance at 260nm was monitored by pumping the gradients through a flow-cell of a recording spectrophotometer.

RNA size analysis on sucrose density gradients. [^{3}H] Mengo virus RNA was centrifuged through 5-20% (w/v) sucrose gradients containing 10mM Tris, pH 7.4, 100mM NaCl, 10mM EDTA, 0.2% SDS at 45,000 rpm for 2h in an SW 50.1 rotor operating at 23°C (14). The gradients were monitored as above.

Extraction of RNA from reticulocyte lysates. Incubation mixtures were diluted 6-fold with 0.2M Tris, pH 8.5 (at 20°C), 50mM KCl, 10mM Mg acetate, 1% Triton X-100; EDTA was added to 2mM and SDS to 1%. RNA was extracted by a phenol:chloroform method (19), ethanol precipitated, washed with 3M sodium acetate and re-dissolved in 10mM Tris, pH 7.6. It was stored at -70°C.

RESULTS AND DISCUSSION

When reticulocyte lysates are incubated with dsRNA under conditions of protein synthesis polysomes become disaggregated (Figure 1b), as previously reported (1, 2). If this effect is

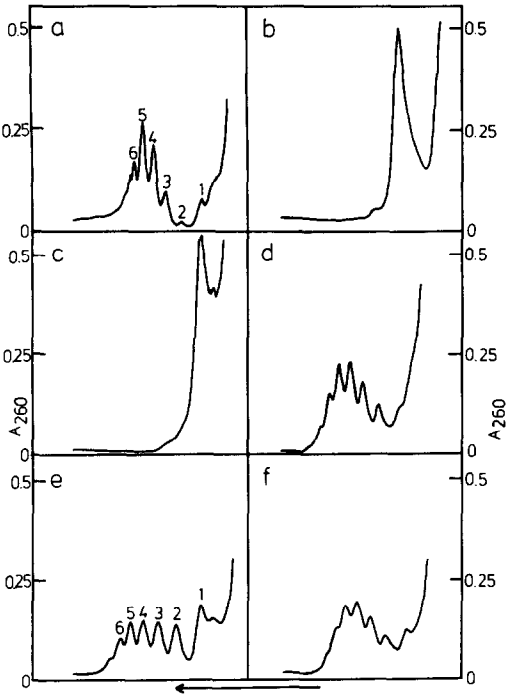

Figure 1. Effect of dsRNA and HCR on reticulocyte polysome size distribution in the presence and absence of emetine.

50µl reaction mixtures were incubated under protein synthesis conditions for 35 min at 30°C with the following additions: (a) None; (b) 10^{-8} g/ml poly rI: poly rC; (c) 260 µg/ml HCR; (d) 10^{-4}M emetine; (e) Poly rI: poly rC plus emetine; (f) HCR plus emetine. The incubations were then diluted with 100 µl of ice-cold gradient buffer and centrifuged through sucrose gradients. Sedimentation was from right to left. The figures in panels (a) and (e) show the numbers of ribosomes in each polysome peak. Measurement of [^{14}C]-leucine incorporation into proteins performed in parallel assays showed inhibition by the dsRNA and by HCR (in the absence of emetine) to be 69% and 76% respectively.

due solely to inhibition of polypeptide chain initiation it should be prevented by blocking ribosome run-off with the inhibitor of chain elongation emetine. In the presence of emetine, however, dsRNA causes limited but significant degradation of polysomes to predominantly monomeric, dimeric and trimeric forms. Only 36% of ribosomes are found in tetramers and larger polysomes (Figure 1e), in contrast to 61% when

emetine alone is added (Figure 1d). It is unlikely that this effect is an artefact due to the presence of emetine, since the antibiotic prevents completely the breakdown of polysomes caused by the haem controlled repressor, a potent inhibitor of initiation in reticulocytes (5, 12) (Figure 1f).

The partial degradation of polysomes induced by dsRNA could be explained by limited endonucleolytic cleavage of the mRNA, amounting to one or two nicks per molecule. We have tested this possibility directly by adding radioactive Mengo virus RNA to reticulocyte lysates and then subjecting the labelled material to size analysis on sucrose gradients in the presence of SDS (14) (Figure 2a). As little as 15 min exposure of the viral RNA to dsRNA in the lysate at 30^{o} strongly enhances the rate of degradation of the 34S material to fragments sedimenting at less than 18S. There is no effect of dsRNA on the rate of appearance of acid soluble degradation products, however, indicating that it is an endonuclease rather than an exonuclease which has been activated (Figure 2b).

The above results provide evidence that both endogenous globin mRNA and exogenous Mengo virus RNA are physically degraded in reticulocyte lysates on incubation with dsRNA. We have also shown that the ability of mRNA to function as a template for protein synthesis is impaired following incubation of lysates with dsRNA. Total RNA was extracted from lysates (18) and its mRNA content estimated by re-translation in a fresh mRNA-dependent cell-free system (20) in which amino acid incorporation is a linear function of the amount of RNA added (Figure 3). RNA from reticulocyte lysates incubated for 60 min with 10^{-8}g/ml dsRNA is only approximately 12% as active as RNA from control lysates in supporting subsequent protein synthesis (Figure 3 and Table 1).

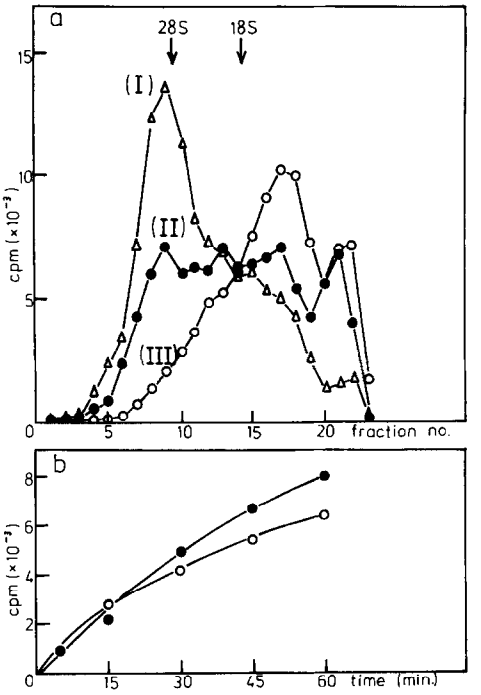

Figure 2. Degradation of radioactive Mengo virus RNA in reticulocyte lysate incubated in the presence and absence of dsRNA.

(a) 24µl reaction mixtures were incubated with 0.5 µg of [^{3}H]-Mengo virus RNA (10^5 cpm) under the following conditions: (I) 0 min; (II) 15 min at 30°C; (III) with 10^{-8} g/ml poly rI: poly rC, 15 min at 30°C. 200µl of buffer containing 10mM Tris, pH 7.4, 100mM NaCl, 10mM EDTA, 0.2% sodium dodecyl sulphate was then added and the samples incubated for 3 min at 30°C. They were then centrifuged through sucrose gradients in the same buffer and 23 fractions per gradient were collected for estimation of radioactivity after addition of 1.5 ml water and 10 ml of "Tritosol" scintillation fluid (31). Sedimentation was from right to left and the arrows show the positions of 18S and 28S r RNA derived from the endogenous ribosomes of the reticulocyte lysate. Panel (b) shows the kinetics of degradation of [^{3}H] Mengo virus RNA to acid-soluble products in incubations identical to the above. Reaction mixtures were incubated at 30°C and 4 µl aliquots removed at the times indicated and precipitated with 100 µl of ice-cold 10% (w/v) trichloroacetic acid. After 1h at 4°C the precipitates were centrifuged for 4 min in a microfuge (Hermle, W. Germany) and 50 µl aliquots of the supernatants counted in 5 ml of "Tritosol" scintillation fluid. ● , control incubation; ○ , with 10-8 g/ml poly rI: poly rC.

This inhibition is not due to the presence of residual dsRNA in the re-extracted material as shown by the fact that RNA from

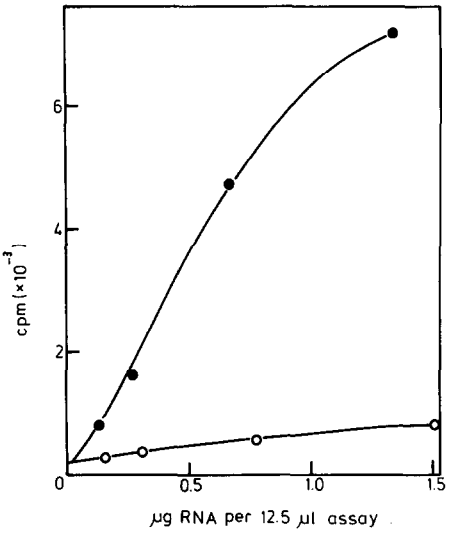

Figure 3. Messenger RNA activity of RNA extracted from reticulocyte lysate incubated in the presence and absence of dsRNA.

1ml reaction mixtures were incubated under conditions of protein synthesis for 60 min at 30°C. They were then diluted and total RNA was extracted as described in Materials and Methods. The unfractionated reticulocyte RNA was translated in the mRNA-dependent reticulocyte lysate prepared by micrococcal nuclease treatment (20), as described previously (18). Incubation was for 60 min at 30°C. ● , RNA from control incubation; ○ , RNA from lysate incubated with 10^{-8} g/ml poly rI: poly rC.

lysates previously exposed to dsRNA does not inhibit endogenous protein synthesis in a fresh lysate (Table 1). Furthermore the dsRNA-induced inactivation of mRNA template activity is time dependent, only 24% inactivation having occurred after 10 min incubation (Table 1). Comparison of the translation of re-extracted RNA samples from lysates incubated with dsRNA or with HCR also establishes that mRNA inactivation is not a secondary consequence of the inhibition of [40S.Met-$tRNA_f$] initiation complex formation (5), or of the breakdown of polysomes, caused by both these substances, since dsRNA impairs mRNA function whereas HCR does not (Table 1).

Our results indicate that endonuclease activity is induced

Table 1. Influence of re-extracted RNA on protein synthesis in mRNA-dependent and native reticulocyte lysates.

(a) Translation in mRNA-dependent lysate

Conditions of incubation before extraction of RNA	Translation of extracted RNA (cpm per µg RNA)	
Control conditions, 60 min	7930	Expt. I
10^{-8}g/ml dsRNA, 60 min	990	
Control conditions, 60 min	7100	Expt. II
10^{-8}g/ml dsRNA, 10 min	5340	
10^{-8}g/ml dsRNA, 60 min	660	
260 µg/ml HCR, 60 min	7900	

(b) Effect on native lysate

Conditions of incubation before extraction of RNA	RNA added (µg)	Protein synthesis (cpm x 10^{-3})
-	0	16.3
Control conditions, 60 min	0.17	17.1
	0.55	18.0
	1.66	12.6
10^{-8}g/ml dsRNA, 60 min	0.12	16.4
	0.41	15.4
	1.22	14.2

RNA was extracted from reaction mixtures incubated under the conditions indicated. Similar incubations were carried out in the presence of [^{14}C]-leucine to monitor the effect of the poly rI: poly rC and HCR on protein synthesis in these initial reaction mixtures. The results showed 68% inhibition and 78% inhibition of incorporation by the dsRNA and HCR, respectively, after 60 min. The re-extracted RNA was added to either a nuclease-treated lysate (20) or a native lysate still containing its own endogenous mRNA and these were then incubated with [^{14}C] leucine for 60 min at 30°C. Panel (a) shows the stimulation of protein synthesis in the mRNA dependent system by the extracted RNA, which was a linear function of the amount of RNA added (Figure 3). Panel (b) shows the effect of increasing amounts of the added RNAs on endogenous protein synthesis in the native lysate. The absence of a significant difference in the latter system between the two preparations used indicates the absence of inhibitory levels of dsRNA after phenol extraction. In the same experiment, authentic dsRNA at 10^{-8} g/ml and 10^{-9} g/ml inhibited endogenous protein synthesis by 69% and 56%, respectively.

in reticulocyte lysates in the presence of dsRNA. It is probable that this endonuclease is activated by the unusual oligonucleotide

$pppA^{2'}p^{5'}A^{2'}p^{5'}A$ which is synthesised from ATP in the presence of dsRNA in reticulocyte lysates (21), as it is in extracts from interferon treated L cells (22-24). We have shown elsewhere (18) that this oligonucleotide activates an endonuclease in reticulocyte cell-free systems in the absence of added dsRNA, and the effects of the two agents on the breakdown of endogenous or exogenous mRNA are indistinguishable (our unpublished observations). It is not clear what relationship the nuclease activation bears to the inhibition of Met-$tRNA_f$ binding to 40S subunits and phosphorylation of eIF-2 reported previously by others (5, 25). Our results (this paper and reference 18) suggest that the two phenomena may be unrelated effects of dsRNA, since under some conditions it is possible to observe one without the other. Their relative importance in causing inhibition of protein synthesis may vary from one lysate preparation to another, and this may account for the variable extent of reversibility of the effect of dsRNA by excess eIF-2, by high concentrations of dsRNA or by various purine derivatives, in some reticulocyte lysates (3-5, 6-8, 26, 27). Once a large proportion of the mRNA in a lysate has been degraded by the endonuclease, however, protein synthesis clearly would not be restored by these agents.

The effects of dsRNA in reticulocyte lysates and in extracts from interferon treated mouse cells have many features in common, including the phosphorylation of specific polypeptides (5, 28-30) and inhibition of Met-tRNA binding to 40S subunits in cell-free systems (14). The presence of nuclease activity in reticulocyte lysates which appears to be very similar to the interferon dependent nuclease (14-17) reveals a further aspect common to the two systems. Interferon may therefore regulate protein synthesis by amplifying normal cellular control mechanisms, such

as that for mRNA degradation, which exist in latent form in reticulocytes.

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