Effect of Double Stranded RNA on Protein Synthesis in an *in vitro* Wheat Germ Embryo System

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SUMMARY: Incorporation of amino acids into proteins in a cell-free system derived from rabbit reticulocytes was inhibited by either natural (reovirus) or synthetic (poly I:poly C) double-stranded RNAs. The optimal concentration for this inhibition was approximately 0.1 ug/ml. No inhibition was observed following treatment of a cell-free protein synthesizing system derived from wheat germ embryos with double-stranded RNAs at concentrations up to 10 ug/ml. Wheat germ extracts were examined for the presence of a nucleolytic activity capable of digesting double-stranded RNA. No such activity could be detected. A possible explanation for the difference in effect of double-stranded RNA on the two systems is discussed.

Introduction: Double-stranded RNA (dsRNA) in low concentrations is a potent inhibitor of protein synthesis in cells derived from mammalian sources (1, 2, 3). In addition, it plays a primary role in the induction of interferon in vertebrates (4, 5). It has been suggested that these effects of dsRNA play an important role in the replication and course of infection of many RNA viruses - those with double-stranded RNA genomes and those single-stranded RNA viruses which are capable of becoming double stranded during their replication in host cells.

We have examined the effect of dsRNA derived from reovirus and of synthetic dsRNA, poly I: poly C, on the ability of cell free extracts to incorporate amino acids into protein in systems derived from mammalian reticulocytes and wheat germ embryos. Here we report the inability of dsRNA to inhibit protein synthesis in wheat germ extracts. It appears from this and other reports using non-mammalian protein synthesizing extracts (6, 7) that the observed inhibition of protein synthesis by dsRNA may be a phenomenon limited to the mammalian host.

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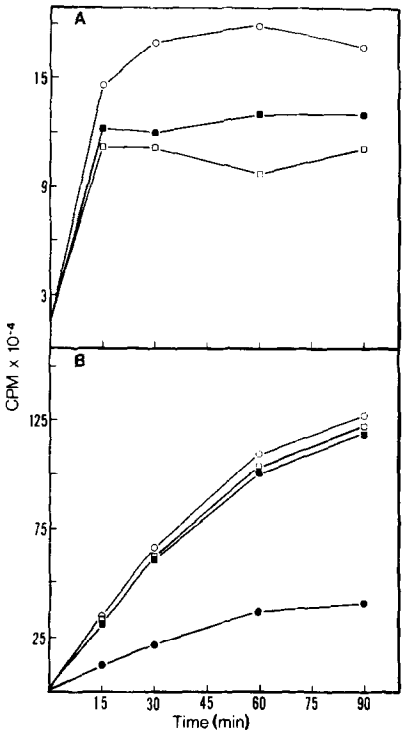

Figure 1. Effect of dsRNA on rate of protein synthesis in cell free extracts. Samples were removed from protein synthesis reactions (see Methods) for determination of incorporation into hot acid insoluble material. (A) Reticulocyte lysates - control (0-0); with 0.1 ug/ml reovirus dsRNA (■-■); with 0.1 ug/ml poly I: poly C (□-□); (B) Wheat germ embryo lysates - control (0-0); with 0.1 ug/ml reovirus dsRNA (■-■); with 0.1 ug/ml poly I: poly C (□-□); without rat liver polysomal RNA (●-●).

Materials and Methods: Rabbit reticulocyte extracts were prepared according to the procedure of Palmiter (8) and stored in small aliquots at -70°C. The reaction mixture contained per ml: 0.4 ml reticulocyte extract, 20 mM Hepes, pH 7.6, 80 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 200 mM GTP, 16 mM creatine phosphate, 80 ug creatine phosphokinase, 30 mM hemin, 0.10 mM 19 amino acids and 75 uCi [^{3}H]-leucine (specific activity 57 Ci/mM). Incubation was at 25°C. Aliquots (25 ul) were absorbed on filter paper discs (Whatman No. 3) and immersed immediately into cold 10% trichloroacetic acid (TCA). The discs were washed twice with 5% TCA, once with hot 5% TCA (90°C), once with cold 5% TCA, once with ethanol ether (1:1, v/v), once with diethylether and dried. Hot acid precipitable radioactivity was counted in a liquid scintillation counter.

Wheat germ extracts were prepared with modifications of the procedure of Marcus et al. (9). One gram of defatted wheat germ embryos (General Mills) was ground with 10 ml of Extract Buffer (1 mM $MgAc_2$, 2mM $CaCl_2$, 90 mM KCl and 20 mM Tris-Ac, pH 7.6). A 27,000 xg supernatant fraction, titrated to pH 7.4, was eluted from a Sephadex G-25 column (25 x 1.8 cm) with Wheat Germ Column Buffer (1 mM Tris-HCl, pH 7.6, 1mM $MgAc_2$, 4mM 2-Mercaptoethanol and 50 mM KCl) and 1 ml fractions were collected. The most visually turbid 8 ml in the void volume were pooled, centrifuged for 10 minutes at 27,000 xg and stored in small aliquots at -70°C. The

reaction mixture contained per ml: 0.2 ml wheat germ extract, 20 mM Hepes, pH 7.6, 10 mM KCl, 65 mM KAc, 3 mM $MgAc_2$, 1 mM ATP, 80 mM GTP, 8 mM creatine phosphate, 40 ug creatine phosphokinase, 2 mM dithiotheitol, 0.06 mM 19 amino acids and 37.5 uCi [^{3}H] leucine (specific activity 57 Ci/mM). Reactions were incubated at 30°C. Aliquots (50 ul) were treated as described for the reticulocyte system.

Rat liver polysomal RNA used as messenger in the wheat germ system was prepared according to the method of Palmiter (10). Reovirus dsRNA was the gift of Dr. A. J. Shatkin, Roche Institute of Molecular Biology, Nutley, N. J. [^{3}H] reovirus dsRNA was the gift of Dr. A. Karu, University of California, Riverside. Poly I: poly C and RNAase A were purchased from Sigma.

Results: Reticulocyte lysates incorporated tritiated leucine into acid precipitable material as shown in Fig. 1A. Addition of either poly I: poly C or reovirus dsRNA to the incubation mixture at a concentration of 0.1 ug/ml inhibited protein synthesis by approximately 35%. This inhibition occurred only after a short lag period ($\sim$ 10 minutes) in good accord with published results (1, 2, 3). Fig. 1B shows translation in a wheat germ cell-free system. Translation of RNA derived from either TMV or rat liver polysomes can be successfully initiated in this system. Linear incorporation over a period of sixty minutes was observed with only low levels of protein synthesis occurring in the absence of an exogenous messenger.

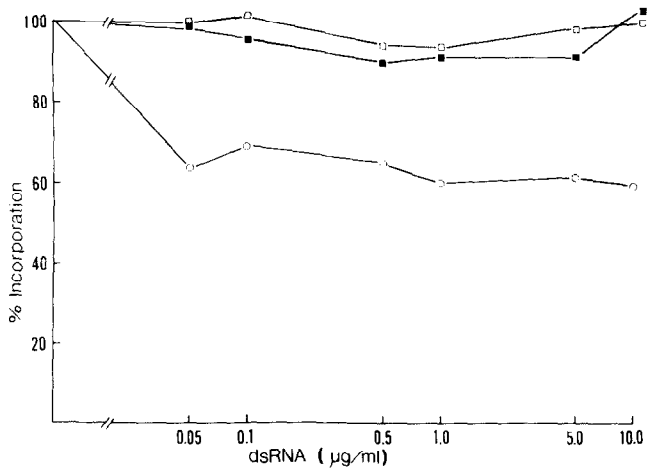

Figure 2. Effect of different concentrations of dsRNA on translation in cell free extracts. Reaction mixtures were incubated for 90 minutes. Samples were removed for determination of incorporation into hot acid insoluble material. Percent incorporation is relative to that in the absence of dsRNA. Reticulocyte system, with reovirus dsRNA (O-O); wheat germ embryo system, with reovirus dsRNA (■-■); with poly I: poly C (□-□).

Unlike the reticulocyte system, incorporation by wheat germ extracts was virtually unaffected for periods up to 90 minutes by the addition of 0.1 ug/ml dsRNA.

Incorporation by reticulocytes and wheat germ extracts was examined in the presence of dsRNA in concentrations up to 10 ug/ml (Fig. 2). A maximal inhibition after sixty minutes in the reticulocyte system of 35 to 40% was observed at concentrations as low as 0.05 ug/ml. Neither reovirus dsRNA nor poly I: poly C had any effect after ninety minutes on the ability of rat liver polysomal RNA to direct protein synthesis in wheat germ extracts.

The inhibition by dsRNA observed in the reticulocyte system has been reported to be potentiated when the extracts are preincubated with dsRNA for short periods of time (1). This preincubation presumably allows for the formation and accumulation of a translational inhibitor. It is possible that for inhibition to occur in the wheat germ system, a similar inhibitor must be accumulated. Components of the protein synthesizing reaction of the wheat germ extract were therefore incubated with either reovirus dsRNA or poly I: poly C for 0, 10 or 30 minutes prior

TABLE 1. Effect of prior incubation with dsRNA on protein synthesis in wheat germ extracts.

	[^{3}H] Leu incorporation (cpm x 10^{-3}) Prior incubation (min)		
dsRNA addition	0	10	30
-	32.7	32.8	28.0
Poly I: poly C	31.7	32.1	27.6
Reovirus	33.7	33.7	31.5

The components of the endogenous protein synthesis reaction were incubated for the times shown in the absence or presence of poly I: poly C or Reovirus dsRNA (0.1 ug/ml). Polysomal rat liver RNA (200 ug/ml) was then added and the reaction mixtures were further incubated for 90 minutes at 30°C.

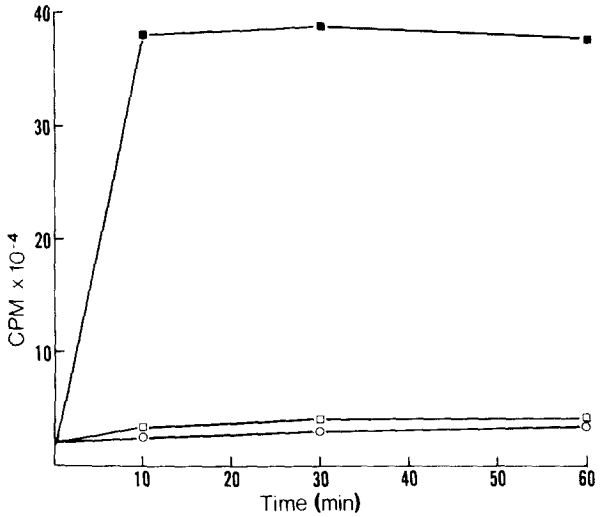

Figure 3. Stability of double stranded RNA in wheat germ embryo lysates. 3.25 ug of ^{3}H- labeled reovirus dsRNA (about 40,000 cpm), native or heat denatured was added to the components of an endogenous wheat germ embryo cell free system in the presence or absence of bovine pancreas RNAase A. Samples were withdrawn at the times indicated for determination of cold acid soluble radioactivity. Wheat germ lysate and native reovirus dsRNA (0-0); wheat germ lysate and native reovirus dsRNA containing 0.3 units bovine pancreas RNAase A (□-□); wheat germ lysate and heat denatured reovirus dsRNA containing 0.3 units bovine pancreas RNAase A (■-■).

to the addition of the messenger, rat liver polysomal RNA. The mixtures were then incubated for an additional ninety minutes. As shown in Table I, preincubation did not result in the observation of an inhibitory phenomenon.

The existence of a nuclease specific for dsRNA could explain the lack of response of the wheat germ system to the added nucleic acid. Such a nuclease accounts for the observation that with ascites cell extracts, high concentrations (1-10 ug/ml) of dsRNA are required to inhibit amino acid incorporation (2). To test for the presence of dsRNA specific nuclease, wheat germ extracts were incubated with labelled reovirus dsRNA (Fig. 3). Bovine pancreas RNAase A was included in the incubation mixture to ensure that the reovirus RNA was maintained in a double-stranded form. No appreciable increase in acid soluble counts was observed over a period of sixty minutes. When the reovirus RNA was heat denatured

to the single-stranded form prior to incubation with the wheat germ extract and RNAase, virtually all the RNA was solubilized. Thus no nucleolytic activity capable of digesting dsRNA could be detected in the wheat germ extract.

Discussion: Our results show that the synthesis of protein in a reticulocyte lysate is sensitive to dsRNA, while synthesis in a wheat germ extract appears to be unaffected even at concentrations up to 100 fold that which is maximally effective in the reticulocyte system. At present there are a variety of cell extracts which have been examined for their response to dsRNA. Inhibition has been reported in all cells derived from mammalian sources (1, 2, 3). In addition to the wheat germ system reported here, cell free synthesis in chick embryo (6), bacterial systems (7) and incorporation directed by poly U in mammalian systems (6) are not affected by dsRNA.

The lack of response of the wheat germ system to the nucleic acid may be explained by several hypotheses:

1) Wheat germ extracts contain dsRNA nucleolytic activity. Ascites cell free systems contain a nuclease which can specifically solubilize dsRNA (2). Nevertheless, the addition of dsRNA at concentrations comparable to those employed in our experiments results in inhibition of ascites extracts. We have been able to demonstrate that no nuclease with properties similar to that observed in the ascites extract is present in the wheat germ system.

2) Improper initiation is occurring in the wheat germ extract. The effect of dsRNA on lysates is postulated to be at the level of initiation (11, 12). This clearly explains why poly U incorporation which bypasses the normal initiation process is unaffected by dsRNA. We can show that proper initiation is occurring in our wheat germ system, since ATA, an inhibitor of initiation, inhibits incorporation with the appropriate kinetics (unpubl. results). In addition, we have used total polysomal RNA from rat liver as well as TMV RNA as message. Therefore the lack of inhibition of protein synthesis in the wheat germ system is not due to the type of messenger, plant virus versus mammalian, being translated.

3) Inhibition results only after the accumulation of a translational inhi-

bitor. In both the reticulocyte and ascites systems inhibition of incorporation is not observed immediately, but only after a lag period of 10-20 minutes. Preincubation of the cell extracts with dsRNA prior to initiation of synthesis results in the abolishment of this lag period (1, 2). During this time (the lag or preincubation) an inhibitor of translation is accumulating. We have examined the wheat germ system to determine whether time is required in order to accumulate such an inhibitor. Preincubation for periods up to 30 minutes in the absence of exogenous mRNA has no affect on incorporation after addition of messenger. These results are consistent with the finding by Ranu et al., that isolated reticulocyte inhibitor, formed by incubation of reticulocyte lysates in the absence of hemin, is ineffective in inhibiting wheat germ lysates (13).

4) Initiation factors from wheat germ differ from mammalian factors. Since initiation factors derived from bacterial cells differ significantly from the eucaryotic factors, it is not surprising that these systems respond differently to dsRNA. Although bacterial systems are not inhibited, it has been shown that their initiation factors bind to dsRNA but with a much lower affinity than with single stranded RNA (7). Therefore the lack of observed inhibition may reflect different affinities of initiation factors for the two types of nucleic acid. Wheat germ systems are extremely efficient at translating exogenous messenger RNAs. This, too, may reflect a very strong interaction between initiation factors in wheat germ and single stranded RNA.

It is interesting to note the recent report of Zehavi-Willner and Pestka who found that the initiation step of protein synthesis in wheat germ extracts is sensitive to temperature (14). Reticulocyte factors can replace the sensitive factors from wheat germ, implying that initiation factors of wheat germ and reticulocytes are different. Possibly these variations may account for the differences in response to dsRNA.

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