Inhibition of Cell-Free Protein Synthesis by Low-Molecular-Weight Nuclear Polyadenylate-Containing Ribonucleic Acid Species Isolated from the Lactating Guinea Pig

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(Received 7 September 1979)

1. Poly(A)-containing RNA was isolated from the nuclei of mammary gland, liver and brain of lactating guinea pigs. 2. Total nuclear poly(A)-containing RNA from mammary gland inhibited mRNA-directed protein synthesis by a wheat-germ cell-free system. It also inhibited the endogenous activity of the wheat-germ and other cell-free systems. It did not inhibit a wheat-germ cell-free system directed by poly(U). 3. Total nuclear poly(A)-containing RNA from liver and brain did not inhibit the mRNA-directed wheat-germ system. 4. Fractionation of the nuclear poly(A)-containing RNA revealed inhibitory activity in the <10S fraction from mammary gland as well as that from liver and brain. 5. The mechanism of protein-synthesis inhibition appeared to be at the level of elongation. 6. The inhibitory activity could be reversed in a wheat-germ system by increasing the amount of S-30 supernatant. 7. The mechanism of inhibition of protein synthesis is discussed in relation to other RNA species known to inhibit such systems.

Although the precise nuclear events involved in the synthesis and processing of mRNA species in higher organisms have yet to be established, it is generally accepted that in eukaryocytes mRNA is synthesized in the form of a high-molecular-weight precursor molecule, which is subsequently processed, and then transported to the cytoplasm as the mature mRNA species (Curtis & Weissman, 1976; Strair *et al.*, 1977; Gilmore-Hebert & Wall, 1978; Smith *et al.*, 1978; Roop *et al.*, 1978; Schibler *et al.*, 1978; Bathurst *et al.*, 1979).

During studies to characterize steady-state poly(A)-containing nuclear RNA isolated from the lactating guinea-pig mammary gland, we observed that the addition of this RNA to a variety of cell-free protein-synthesizing systems resulted in the inhibition of mRNA-directed protein synthesis. This was in direct contrast with a variety of reports describing low translational activities of nuclear RNA species (Ruiz-Carillo *et al.*, 1973; Chatterjee & Weissbach, 1974; Grozdanovic & Hradec, 1975*a,b*; Knochel *et al.*, 1975; Rao *et al.*, 1977*a*), though similar to the activity of a low-molecular-weight nuclear RNA

with respect to its effect on cell-free protein synthesis (Rao *et al.*, 1977*b*).

In the present paper we describe the isolation and characterization of poly(A)-containing nuclear RNA species, from the tissues of lactating guinea pigs, which have an inhibitory effect on protein synthesis.

Experimental

Materials

Aurintricarboxylic acid and cycloheximide were purchased from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. L-[4,5-³H]Leucine (40–60 Ci/mmol) and L-[4-³H]phenylalanine (27 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals and solvents were obtained from sources previously described (Craig *et al.*, 1976, 1979) and were of A.R. grade.

Preparation of solutions

All solutions were prepared in deionized double-distilled water. Tris-containing buffers are expressed in molar concentrations of Tris and were adjusted to the required pH at the stated temperature with 4M-HCl.

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All precautions to safeguard against RNA degradation by exogenous ribonucleases were as described previously (Craig *et al.*, 1976; Bathurst *et al.*, 1979).

Scintillation fluid contained 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene.

Isolation of nuclei and the preparation of poly(A)-containing nuclear RNA

Isolation procedures have been described in detail elsewhere (Bathurst *et al.*, 1979). Briefly, nuclei were isolated by using detergents in the presence of spermidine as a ribonuclease inhibitor, digested with proteinase K, and the nucleic acid was extracted with phenol/chloroform. DNA was then removed by digestion with ribonuclease-free deoxyribonuclease, and the poly(A)-containing RNA recovered by selective binding to oligo(dT)-cellulose. The resulting RNA was washed once with 70% (v/v) ethanol containing 40 mM-NaCl and once with ethanol, then dried *in vacuo*, dissolved in sterile double-distilled water, and stored at -70°C .

Preparation of post-nuclear poly(A)-containing RNA

Total RNA was isolated from the post-nuclear supernatant of either the mammary gland or liver of lactating guinea pigs as described by Craig *et al.* (1979). Poly(A)-containing RNA was isolated from this fraction by using oligo(dT)-cellulose as described previously (Craig *et al.*, 1976). Rabbit globin mRNA was isolated as described by Aviv & Leder (1972).

Fractionation of RNA by sucrose-gradient centrifugation in the presence of formamide

Nuclear or cytoplasmic RNA was separated on the basis of size by centrifugation on 5–20% (w/v) sucrose gradients containing 75% (v/v) formamide as described previously (Craig *et al.*, 1979). The relative position of the poly(A)-containing RNA species was then determined by hybridization to [^3H]poly(U) as described by Bishop *et al.* (1974), after dilution of the formamide in each fraction to a concentration of less than 15% (v/v) and at a final concentration of 0.03 M-sodium citrate containing 0.3 M-NaCl (see Craig *et al.*, 1979). RNA marker species (4S, 18S, 28S) were prepared as described by Craig *et al.* (1976).

Cell-free protein synthesis

The preparation of a wheat-germ S-30 cell-free protein-synthesizing system (Roberts & Paterson, 1973) and a modified Krebs II ascites cell-free protein-synthesizing system (Mathews & Korner, 1970) has already been described, as have the optimum conditions for the cell-free translation of milk-protein mRNA in these systems (see Craig *et al.*, 1976). A nuclease-treated rabbit reticulocyte-lysate cell-free

protein-synthesizing system was prepared as described by Pelham & Jackson (1976). Protein was measured by the method of Warburg & Christian (1942).

Analysis of the size distribution of the polyribosome-associated nascent polypeptide present in cell-free protein-synthesizing systems by sucrose-gradient centrifugation

Samples (100–200 μl) of a wheat-germ cell-free protein-synthesizing system were chilled to 4°C and applied to the top of 12 ml gradients, consisting of 10–40% (w/v) sucrose in 50 mM-Tris/HCl, pH 7.6 at 4°C , containing 25 mM-KCl and 5 mM-magnesium acetate. These were centrifuged at 5°C and 100000 g_{av} for 2 h in the SW 40 rotor of a Beckman L5-65 ultracentrifuge. Gradients were fractionated into 25–30 fractions as described previously (Bathurst *et al.*, 1979). The relative position of marker 40S and 60S ribosomal subunits was monitored at 260 nm by using the flow cell of a Unicam SP.1800 recording spectrophotometer, and the polyribosome distribution was determined by analysis of the position of nascent ^3H -labelled peptides associated with the polyribosomes. This was done by precipitation of each fraction with 1 ml of 10% (w/v) trichloroacetic acid in the presence of 100 μg of bovine serum albumin followed by a 20 min incubation at 90°C . Samples were then cooled at 4°C for 20 min, and the precipitated material was collected under suction on glass-fibre discs, washed and processed for scintillation counting as described previously (Craig *et al.*, 1976). Ribosomal-subunit markers (40S and 60S) were prepared from guinea-pig liver as described by Lawford (1969).

Isolation of DNA

DNA was isolated by the method of Kirby & Cook (1967). In order to remove residual RNA, the DNA was dissolved at 1 mg/ml in 0.3 M-NaOH, incubated overnight at 37°C , and then neutralized by the addition of 6 M-HCl. The DNA was recovered by precipitation overnight at -20°C after the addition of 2 vol. of ethanol. The precipitated DNA was collected by centrifugation at 10000 g_{av} for 20 min at -10°C , the supernatant discarded, and the sedimented DNA washed once in 70% (v/v) ethanol/40 mM-NaCl and once in ethanol, then freeze-dried, dissolved in sterile water at 20 A_{260} units/ml, and stored at -70°C until required.

Results

Inhibition of mRNA-directed cell-free protein synthesis by nuclear poly(A)-containing RNA from the lactating mammary gland

We have previously demonstrated that a poly(A)-containing RNA fraction may be isolated from the

post-nuclear fraction of lactating guinea-pig mammary gland which directs the synthesis of all the major guinea-pig milk proteins in various cell-free systems (Craig *et al.*, 1976). As shown in Table 1, the nuclear poly(A)-containing RNA isolated from the same source did not stimulate protein synthesis in a wheat-germ cell-free system, but moreover inhibited the endogenous activity of the system by as much as 50%. Similar inhibition of endogenous protein synthesis was observed when poly(A)-containing nuclear RNA was added to cell-free systems derived from Krebs II ascites cells or rabbit reticulo-

cytes. In contrast, a small but significant (2-fold) stimulation of protein synthesis was observed when poly(A)-containing nuclear RNA isolated from the brain or liver of the lactating guinea pig was added to the wheat-germ cell-free system.

As an initial step towards characterizing this inhibitory activity of the mammary-gland poly(A)-containing nuclear RNA, the nuclear RNA was incubated in the wheat-germ, ascites or reticulocyte cell-free systems in the presence of active mRNA from various sources (Table 2). The results demonstrate that only the nuclear preparation of mam-

Table 1. *Relative ability of poly(A)-containing nuclear RNA species to direct protein synthesis in various cell-free systems*

Cell-free protein synthesis was carried out as described in the Experimental section: 0.005 A_{260} unit of the appropriate poly(A)-containing RNA species was added to each wheat-germ S-30-fraction and reticulocyte-lysate assay, and 0.025 A_{260} unit to each Krebs II ascites-cell S-30-fraction assay. Results are expressed as a percentage of the amount of [^3H]leucine incorporated into protein in the presence of mammary-gland post-nuclear poly(A)-containing RNA (100%). The numbers of individual experiments performed are shown in parentheses. The results represent the means of these determinations, $\pm$ s.d.

Cell-free protein-synthesizing system	Additions	Relative protein synthesis (%)
Wheat germ	None (6)	4.2 $\pm$ 1.7
	Poly(A)-containing cytoplasmic RNA (mammary gland) (6)	100
	Poly(A)-containing nuclear RNA (mammary gland) (6)	2.0 $\pm$ 1.4
	Poly(A)-containing nuclear RNA (liver) (6)	8.4 $\pm$ 2.7
	Poly(A)-containing nuclear RNA (brain) (3)	7.7 $\pm$ 2.5
Krebs II ascites cells	None (3)	28 $\pm$ 1.8
	Poly(A)-containing cytoplasmic RNA (mammary gland) (3)	100
	Poly(A)-containing nuclear RNA (mammary gland) (3)	20 $\pm$ 4.6
Rabbit reticulocytes	None (4)	8.0 $\pm$ 1.8
	Poly(A)-containing cytoplasmic RNA (mammary gland) (4)	100
	Poly(A)-containing nuclear RNA (mammary gland) (6)	3.3 $\pm$ 0.7

Table 2. *Comparative inhibition of mRNA-directed protein synthesis in the wheat-germ cell-free system by nuclear poly(A)-containing RNA isolated from liver, brain and mammary gland of the lactating guinea pig*

Experimental conditions and presentation of data were as described in the legend to Table 1. Assays contained 0.005 A_{260} unit of the appropriate mRNA species, and where indicated 0.005 A_{260} unit of the appropriate nuclear poly(A)-containing RNA species. DNA and poly(A) when present were at final concentrations in the assay of 25 and 20 $\mu\text{g}/\text{ml}$ respectively.

mRNA coding for	Additions	Relative protein-synthetic activity (%)
Milk proteins	None (6)	4.2 $\pm$ 1.7
	mRNA (6)	100
	mRNA and nuclear RNA (mammary gland) (6)	2.2 $\pm$ 1.0
	mRNA and commercial poly(A) (5)	102 $\pm$ 8.5
	mRNA and lactating guinea-pig mammary gland DNA (5)	119 $\pm$ 12.0
	mRNA and nuclear RNA (liver) (3)	104 $\pm$ 3.6
	mRNA and nuclear RNA (brain) (4)	100 $\pm$ 3.5
Rabbit globin	None (4)	13 $\pm$ 2.9
	mRNA (4)	100
	mRNA and nuclear RNA (mammary gland) (4)	11 $\pm$ 2.2
	mRNA and nuclear RNA (liver) (4)	108 $\pm$ 6.9
	mRNA and nuclear RNA (brain) (4)	108 $\pm$ 3.5
Liver protein	None (4)	21 $\pm$ 2.6
	mRNA (4)	100
	mRNA and nuclear RNA (mammary gland) (3)	6.2 $\pm$ 1.6
	mRNA and nuclear RNA (liver) (4)	106 $\pm$ 10

mary origin totally inhibited all exogenous mRNA-directed protein synthesis, whereas preparations from the liver and brain did not. No inhibition of mRNA-directed protein synthesis in the wheat-germ cell-free system was observed as a result of added commercial poly(A) or highly purified DNA, though the latter surprisingly resulted in a small but reproducible stimulation of mRNA-directed protein synthesis. In addition, all nuclear poly(A)-containing RNA preparations failed to inhibit the translation of poly(U)-directed polyphenylalanine synthesis over a range of Mg^{2+} concentration (2.8–10.0 mM) (results not shown).

Fig. 1. Comparison of the inhibiting effect of mammary-gland nuclear RNA, cycloheximide and aurointricarboxylic acid on mRNA-directed protein synthesis in the wheat-germ system

Cell-free protein synthesis in the wheat-germ cell-free system directed by post-nuclear poly(A)-containing RNA isolated from the lactating guinea-pig mammary gland was performed under the conditions described in the Experimental section, but with the following modifications. mRNA ($0.01 A_{260}$ unit) was added to each of four $100 \mu l$ assay mixtures, and protein synthesis allowed to proceed. At 5 min intervals $20 \mu l$ amounts were removed and the incorporation of $[^3H]$ leucine into protein was determined as described previously (Craig *et al.*, 1976). After 10 min incubation (arrow), nuclear poly(A)-containing RNA, cycloheximide or aurointricarboxylic acid was added separately to parallel assays to final concentration of $0.02 A_{280}$ unit/ml, $50 \mu M$ and $50 \mu M$ respectively, and the incubation continued for an additional 15 min, with further sampling at 5 min intervals. ■, mRNA alone; □, mRNA + aurointricarboxylic acid; ○, mRNA + cycloheximide; ●, mRNA + poly(A)-containing nuclear RNA; △, wheat-germ endogenous synthesis.

Subsequent analysis of the mRNA-directed products of the wheat-germ protein-synthesizing system showed that the presence of the mammary-gland poly(A)-containing nuclear RNA species uniformly inhibited the synthesis of all proteins directed by the mRNA for milk protein and globin. Thus the inhibition did not appear to be mRNA-specific (I. C. Bathurst & R. K. Craig, unpublished work).

Identification of the mode of inhibition of poly(A)-containing nuclear RNA

The mechanism by which the mammary-gland nuclear poly(A)-containing RNA inhibited cell-free protein synthesis was investigated by comparing the kinetics of this inhibition with that of a known inhibitor of chain initiation, aurointricarboxylic acid (Lodish *et al.*, 1971), and a known inhibitor of elongation, cycloheximide (Fresno *et al.*, 1976). In these experiments milk-protein mRNA was added to a wheat-germ cell-free protein-synthesizing system, and the incorporation of $[^3H]$ leucine into protein monitored at 5 min intervals. After 10 min incubation, aurointricarboxylic acid, cycloheximide or the inhibitory nuclear RNA was added and the incorporation of $[^3H]$ leucine into protein monitored again at 5 min intervals for a further 15 min. The effect of cycloheximide was immediately to inhibit the incorporation of $[^3H]$ leucine into protein, whereas auro-

Fig. 2. Effect of nuclear poly(A)-containing RNA from mammary gland on the size of polyribosomes bearing nascent peptide chains in a wheat-germ system

Cell-free protein synthesis directed by milk-protein mRNA was carried out as described in the legend to Fig. 1. After 10 min incubation, aurointricarboxylic acid, cycloheximide or nuclear poly(A)-containing RNA was added separately to parallel assays, at final concentrations of $50 \mu M$, $50 \mu M$ and $0.02 A_{260}$ unit/ml respectively. Incubations were then continued for a further 20 min. The assays were then chilled to $4^\circ C$, and the polyribosome distributions determined by sucrose-gradient centrifugation as described in the Experimental section. Ribosomal subunits (40S and 60S) were centrifuged separately on parallel gradients. ■, mRNA alone; □, mRNA + aurointricarboxylic acid; ○, mRNA + cycloheximide; ●, mRNA + poly(A)-containing nuclear RNA.

tricarboxylic acid inhibited after a lag period of 10 min. The nuclear RNA caused immediate cessation of protein synthesis, suggesting that, like cycloheximide, the inhibition was at the level of elongation (see Fig. 1). This was substantiated by a study of the size distribution of wheat-germ polyribosomes bearing nascent protein analysed 20 min after the addition of the inhibitors (Fig. 2). Predictably the

addition of aurintricarboxylic acid resulted in 'run-off', and, in the absence of re-initiation, the loss of large polyribosomes, when compared with the uninhibited control. However, the addition of cycloheximide or of nuclear RNA resulted in the retention of polyribosome profiles similar to those for the uninhibited control, consistent with the inhibition of elongation.

Fig. 3. Size distribution of lactating guinea-pig poly(A)-containing RNA species isolated from the nuclei of brain, liver and mammary gland

The size distribution of nuclear poly(A)-containing RNA species was analysed by centrifugation on 5–20% (w/v) sucrose gradients in the presence of 75% (v/v) formamide. The relative position on the gradient of the poly(A)-containing RNA species was then determined by hybridization of 10% (v/v) of each fraction to $[^3\text{H}]\text{poly(U)}$ as described in the Experimental section. The remaining RNA was recovered by ethanol precipitation. Rabbit reticulocyte 28S and 18S rRNA, rabbit globin mRNA (10S) and *E. coli* tRNA (4S), centrifuged on parallel gradients, were used as molecular-weight marker species (see Craig *et al.*, 1979; Bathurst *et al.*, 1979). Amounts on gradients: (a) 0.61 A_{260} unit of nuclear poly(A)-containing RNA isolated from the mammary gland; (b) 0.59 A_{260} unit of nuclear poly(A)-containing RNA isolated from liver; (c) 0.74 A_{260} unit of nuclear poly(A)-containing RNA isolated from brain. All samples were dissolved in a final volume of 300 μl before centrifugation.

Size distribution of the inhibitory poly(A)-containing RNA species

The size distribution of the nuclear poly(A)-containing RNA species responsible for the inhibition of cell-free protein synthesis was determined by centrifugation on formamide/sucrose gradients of total nuclear poly(A)-containing RNA from the mammary gland. This demonstrated that the distribution of the nuclear poly(A)-containing RNA from each tissue, as determined by hybridization of a portion of each fraction with [³H]poly(U), was quite similar (see Figs. 3a, 3b and 3c). The bulk of the hybridizable material sedimented in the 10–20S region, as determined by RNA marker species centrifuged on parallel gradients, suggesting that minimal degradation had occurred during isolation (see also Bathurst *et al.*, 1979). However, although the profiles were similar, the proportion of poly(A) present in each preparation, as determined from the total poly(A)-containing RNA before centrifugation, was markedly different. Thus for mammary gland, liver and brain, the poly(A) content of each preparation represented 4.9, 4.7 and 1.9% respectively of the nuclear poly(A)-containing RNA populations.

In order to determine the size distribution of the inhibitory RNA species, fractions were pooled into those sedimenting at <10S, 10–20S and >20S, then concentrated by ethanol precipitation. In all cases, 83–88% of the RNA applied to the gradients was recovered in this manner. The recovered RNA was distributed between the <10S, 10–20S and >20S size fractions in the following manner: mammary gland, 25.5, 49.0 and 25.5% respectively; liver, 12.6, 40.3 and 47% respectively; and brain, 20.2, 46.4 and 33.3% respectively. We were surprised by the large amounts of RNA recovered in the <10S fractions, particularly from the mammary-gland and brain preparations.

RNA from each fraction was then added in increasing amounts to the wheat-germ cell-free system, in the presence of a constant amount of milk-protein mRNA. The results (Figs. 4a, 4b and 4c) demonstrate that independent of the tissue of origin the <10S fractions strongly inhibited cell-free protein synthesis, though to differing degrees. Thus the addition of 0.03 μ g of the liver RNA resulted in a 50% inhibition (I_1) of protein synthesis, whereas 0.08 μ g of the mammary-gland preparation and 0.73 μ g of the brain preparation were required to achieve the same degree of inhibition. With the exception of the mammary-gland 10–20S fraction, which was also inhibitory ($I_1 = 0.125 \mu$ g), the >20S and 10–20S RNA fractions either had little effect or alternatively resulted in a slight stimulation of protein synthesis. These effects were not unique to milk-protein-mRNA-directed cell-free synthesis, as the

Fig. 4. Inhibition of mRNA-directed cell-free protein synthesis in the wheat-germ cell-free system by low-molecular-weight poly(A)-containing nuclear RNA (<10S)

Mammary-gland (a), liver (b) and brain (c) nuclear poly(A)-containing RNA species, separated by sucrose gradient centrifugation as described in the legend to Fig. 3 into three fractions (>20S, 10–20S and <10S), were each added in increasing amounts to a standard wheat-germ cell-free protein-synthesizing system containing 0.005 A_{260} unit of lactating guinea-pig mammary-gland cytoplasmic mRNA per assay (see the Experimental section). The relative extents of incorporation of [³H]leucine into protein was then determined for the mRNA-directed assay alone (100% activity) and for different amounts of added nuclear RNA species, from each tissue. O, >20S RNA; $\square$, 10–20S RNA; $\triangle$, <10S RNA.

addition of liver <10S poly(A)-containing RNA fraction to a liver cytoplasmic-mRNA-directed wheat-germ cell-free protein synthesizing system resulted in the same rapid inhibition of protein synthesis. A comparative analysis of the kinetics of inhibition, precisely as described in the legend to Fig. 1, confirmed that all the inhibitory RNA fractions were acting in a similar manner (results not shown).

Reversal of nuclear-RNA inhibition of cell-free protein synthesis

A wheat-germ cell-free system was set up which contained milk-protein mRNA and a quantity of mammary-gland nuclear poly(A)-containing RNA known totally to inhibit cell-free protein synthesis in the presence of the usual amounts of wheat-germ S-30 fraction. As shown in Fig. 5, the inhibitory effect of the nuclear RNA was reversed as more S-30 fraction was added to the incubation.

In an attempt to characterize this phenomenon, and also to investigate whether the inhibitory effect of both the mammary-gland <10S and 10–20S nuclear RNA fractions could be equally reversed, the wheat-germ S-30 fraction was separated by centrifugation (see the legend to Table 3) into a soluble fraction (S-100) and a crude ribosomal fraction (P-100). The ability of either fraction to overcome the inhibitory effect was tested by adding each individually to the wheat-germ cell-free system, either (i) with milk-protein mRNA alone, or (ii) with milk-protein mRNA and either the <10S or 10–20S nuclear poly(A)-containing RNA species.

The results (Table 3) reveal that although neither the S-100 nor P-100 wheat-germ fractions alone supported significant protein synthesis, the addition of

either to a mRNA-directed wheat-germ S-30 system, already inhibited by the presence of a nuclear-RNA fraction, resulted in the resumption of near-

Fig. 5. *Effect of the addition of wheat-germ S-30 extract on the inhibition caused by poly(A)-containing nuclear RNA from the lactating guinea-pig mammary gland in a wheat-germ cell-free protein-synthesis system*

Cell-free protein synthesis in the wheat-germ system was performed as described in the Experimental section: 0.005 A_{260} unit of milk-protein mRNA and 0.01 A_{260} unit of lactating guinea-pig mammary-gland poly(A)-containing nuclear RNA was added to each wheat-germ assay. Increasing amounts of wheat-germ S-30 extract (19.1 mg of protein/ml) were added to the assays containing either both mRNA and nuclear RNA (O) or to the wheat-germ cell-free system alone (●).

Table 3. *Reversal of nuclear poly(A)-containing-RNA inhibition of cell-free protein synthesis by addition of wheat-germ S-30 fractions*

Assays were performed by using the wheat-germ cell-free system S-30 described in the legend to Table 1, in the presence of milk-protein mRNA (0.005 A_{260} unit per assay) and mammary-gland nuclear RNA (10–20S or <10S) (0.01 A_{260} unit per assay) respectively. The wheat-germ S-100 and P-100 fractions were each present at a final protein concentration of 1.9 mg/ml and were prepared by centrifugation of a wheat-germ S-30 preparation for 1 h at 100000 g_{av} and 4°C in the SW56 rotor of a Beckman L5-65 ultracentrifuge. The resulting supernatant fraction (S-100) and the sedimented fraction (P-100) were stored at –70°C after resuspension of the latter in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.6, containing 120 mM-KCl, 5 mM-magnesium acetate and 6 mM-2-mercaptoethanol, at a final protein concentration of 9.5 mg/ml.

Assay content	Relative protein synthesis (%)	
	10–20S RNA	<10S RNA
S-100 alone	0.1	1.5
P-100 alone	0.1	2.3
S-30 alone	4.5	3.2
S-30 + mRNA	100	100
S-30 + mRNA + nuclear RNA	6.6	5.1
S-30 + mRNA + nuclear RNA + S-100	55	58
S-30 + mRNA + S-100	49	60.7
S-30 + mRNA + nuclear RNA + P-100	103	76
S-30 + mRNA + P-100	98	98.7

maximal mRNA-directed protein synthesis. The effect of the P-100 fraction was particularly marked and easiest to interpret, as the P-100 fraction on its own did not significantly affect mRNA-directed protein synthesis in the wheat-germ S-30 preparation. Unfortunately the S-100 fraction, for reasons that we have yet to establish, inhibited normal mRNA-directed protein synthesis by 40–50% [compared with 95% by the nuclear poly(A)-containing RNA species]. However, in spite of this, the addition of S-100 fraction to a mRNA-directed system inhibited by either nuclear RNA species resulted in the resumption of mRNA-directed protein synthesis to the maximal values achieved in the absence of nuclear RNA species, but in the presence of the S-100 fraction. The addition of further mRNA or wheat germ to RNA (2–200 µg/ml in the assay) did not relieve the inhibition of protein synthesis caused by the nuclear RNA species.

Discussion

Numerous published reports describe the inhibition of mRNA-directed cell-free protein synthesis by single- and double-stranded RNA species, of either cytoplasmic or nuclear origin. Characterization of their mode of action demonstrates that some, such as double-stranded RNA, act at the level of initiation of protein synthesis (Darnbrough *et al.*, 1972), whereas others appear to prevent elongation (Rao *et al.*, 1977b), or inhibit the formation of the 80S ribosomal complex (Sarma *et al.*, 1978).

The mechanism of action of inhibition by double-stranded RNA has proved to be of particular interest, as two distinct mechanisms appear to be involved, one resulting in the inactivation of initiation factor eIF2 by a double-stranded-RNA-mediated phosphorylation (Farrell *et al.*, 1977), and the other causing degradation of mRNA by ribonucleases activated by subnanomolar concentrations of the oligonucleotide pppA2'p5'A2'p5A (Kerr & Brown, 1978; Clemens & Vaquero, 1978; Clemens & Williams, 1978; Schmidt *et al.*, 1978).

Our own observations are not consistent with either of the proposed mechanisms for inactivation by double-stranded RNA of protein synthesis, since low-molecular-weight nuclear poly(A)-containing RNA species act at the level of elongation (though a role at initiation cannot be excluded) in an analogous fashion to cycloheximide. Moreover, analysis of the polyribosome distribution shows that this inhibition is not accompanied by polyribosome degradation, thus excluding the participation of an activated nuclease (Clemens & Williams, 1978). These conclusions are substantiated by previous observations by Grill *et al.* (1976), who showed that double-

stranded RNA does not inhibit protein synthesis in the wheat-germ cell-free system.

Although we have established that the inhibitory action is at the level of elongation, several experimental observations remain unresolved and deserve further comment. In particular, it is difficult to rationalize our observation that all the low-molecular-weight nuclear RNA species inhibited protein synthesis, the liver RNA being particularly inhibitory, yet only the total mammary-gland nuclear poly(A)-containing RNA preparation was inhibitory. In fact total poly(A)-containing RNA preparations derived from liver and brain gave rise to a small but significant stimulation of the incorporation of radiolabelled amino acids into protein, of a similar order of magnitude to those described by Rao *et al.* (1977a).

It is possible that the inhibitory effect of the total mammary-gland nuclear poly(A)-containing RNA reflects only the activity of those molecules present in the 10–20S size fraction, as such inhibiting activity was not observed in similar fractions isolated from the brain or liver. Whether or not this proves to be the true situation remains to be resolved. However, the intriguing question remains as to how the inhibitory effect of the liver and brain poly(A)-containing RNA preparation remains 'masked' until after the size analysis has been performed.

Several laboratories have reported the association of low-molecular-weight RNA species hydrogen-bonded to nuclear and cytoplasmic poly(A)-containing RNA (Jelinek & Leinwand, 1978) and to nuclear ribonucleoprotein complexes (Howard, 1978). It is possible that the inhibition that we have observed may be due to low-molecular-weight poly(A)-associated RNA molecules, which have been 'melted' from the parental poly(A)-containing RNA molecules during gradient centrifugation under highly denaturing conditions in the presence of formamide (see Bathurst *et al.*, 1979). Such a conclusion would also justify the unexpectedly high proportion of recovered RNA in the <10S fraction, a fraction which contains a relatively small proportion of the total hybridizable poly(A).

Assignment of a physiological role to the inhibitory RNA species, whether poly(A)-containing or poly(A)-associated, can only be speculative, as, with the exception of the 10–20S species of the mammary gland, their distribution in the guinea pig is not organ-specific, though quantitative differences are apparent. They also appear to be of nuclear origin, though we have yet rigorously to exclude their presence in the cytoplasm. In this respect it is interesting that Jelinek & Leinwand (1978) report the absence of poly(A)-associated low-molecular-weight RNA from mRNA species isolated from polyribosomes. This observation, taken in context with our own

observations *in vitro*, suggest that these low-molecular-weight RNA molecules may play a physiological role at the level of translation. Thus the inhibition that we have observed may simply result from the annealing of these molecules, presumably to a non-coding region of the mRNA, physically preventing the movement of ribosomes along the mRNA. Our observation that these RNA species do not inhibit poly(U)-directed polyphenylalanine synthesis supports this contention, and our observation that the inhibition is reversible suggests that factors exist in the cytoplasm which, if present in sufficient quantity, may 'melt' such hybrid molecules, thereby allowing protein synthesis to proceed.

A low-molecular-weight non-polyadenylated nuclear RNA species, which inhibits cell-free protein synthesis in a similar manner to the guinea-pig RNA species, has been described previously by Rao *et al.* (1977b). It is possible that this molecule, designated U1 and isolated from Novikoff-hepatoma cells, may prove to be very similar to the inhibitory RNA species isolated from the guinea pig, should subsequent work prove conclusively that the inhibitory species are poly(A)-associated and not poly(A)-containing.

Unfortunately, as evidence for nuclear protein synthesis is at best circumstantial (see Goidl & Allen, 1978), it is difficult at present to assign a role in translational control *in vivo*, as has been proposed by Bester *et al.* (1975) and Lee-Huang *et al.* (1977) for inhibitory low-molecular-weight cytoplasmic RNA species.

A similar low-molecular-weight poly(A)-containing RNA species isolated from embryonic-chick heart which inhibits cell-free protein synthesis in the reticulocyte lysate has also been reported (Deshpande *et al.*, 1977). This, in addition, appears to induce changes in stage-4 chick blastoderm cultivated *in vitro*, similar to those seen during embryonic heart differentiation, suggesting a role in gene regulation.

We conclude that guinea-pig tissues contain low-molecular-weight poly(A)-containing or poly(A)-associated RNA species (<10S) located in the cell nucleus, of undefined physiological significance, which reversibly inhibit cell-free protein synthesis at the level of elongation.

We thank the Wellcome Trust for supporting this work.

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