Effects on Protein Synthesis of Injecting Synthetic Polyribonucleotides into Living Cells

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Micro-injection into the oocytes and eggs of *Xenopus laevis* was used to ascertain the effects of synthetic polyribonucleotides on protein synthesis in living cells. Poly(U) and poly(A) were not translated detectably, nor did they change the rate of endogenous protein synthesis. The same was true of poly(G,U), poly(A,G,U), poly(A,C,G,U), G-U-G-(U)_n, A-(U)_n and AUG. In contrast, A-U-G-(U)_n was a potent inhibitor of protein synthesis in the cell. This might be because it is initiated normally but lacks a termination codon, or because it inhibits the translation of other molecules in some way not dependent on its normal initiation. Poly(G,U), poly(A,G,U) and poly(A,C,G,U) inhibited haemoglobin synthesis when they were injected into the oocyte with haemoglobin mRNA. The synthetic polyribonucleotides did not inhibit the translation of the natural mRNA when the two sorts of molecules were injected at different times. It is suggested that the synthetic RNA molecules compete with the natural mRNA for a pre-initiation factor in limited supply.

It has been shown that oocytes and eggs of the toad *Xenopus laevis* are able to translate the mRNA coding for haemoglobin (Lane *et al.*, 1971), a result now extended to a range of other types of mRNA. The efficiency with which the cells utilize injected haemoglobin mRNA is comparable to that observed in the reticulocyte from which it was derived, and is much higher than is commonly observed in cell-free systems (Gurdon *et al.*, 1971). Micro-injection of oocytes and eggs therefore seems to be a suitable technique to apply to other aspects of protein synthesis, and should be particularly helpful in determining the extent to which protein-synthetic systems constructed *in vitro* accurately represent the characteristics of translation in living cells.

Much of our knowledge of the mechanism of protein synthesis has come from the translation of synthetic polyribonucleotides in cell-free systems. In the present paper we describe experiments designed to investigate the effect of some of these substances on protein synthesis in living cells.

Materials and Methods

Materials

Poly(U), poly(A), poly(G,U), poly(A,G,U), poly(A,C,G,U) and rabbit haemoglobin were obtained from Miles Laboratories Inc., Kankakee, Ill.,

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U.S.A. The other synthetic polyribonucleotides were prepared by Dr. J. Brown at the M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge. Rabbit haemoglobin mRNA was a gift from Dr. J. B. Lingrel (University of Cincinnati Medical Center, Ohio, U.S.A.). Mono-, di-, tri- and tetra-phenyl-alanine, polylysine and Tris were obtained from Sigma Chemical Co. Ltd. (St. Louis, Mo., U.S.A.). Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals were manufactured by BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Handling biological material. Details of methods of obtaining, culturing and injecting eggs and oocytes have been described previously (Elsdale *et al.*, 1960; Gurdon, 1967; Gurdon *et al.*, 1971). Eggs were used for experiments in which it was desirable to have no background incorporation from cells other than those injected. Oocytes, surrounded by their follicle cells, were used in the globin mRNA competition experiments since the small amount of protein made in follicle cells did not affect these results. Oocytes have the advantage that they are permeable to amino acids and survive for long periods after injection. For studies of eggs radioactive precursors were injected with the polymers. The precursors were introduced into oocytes by adding them to the incubation medium. Except where stated otherwise, the solution used for injection contained 88 mM-NaCl, 1 mM-KCl and 15 mM-Tris-HCl (pH 7.6).

Analysis of acid-insoluble material. For routine

acid-precipitation eggs or oocytes were homogenized in 2ml of 0.5M-KCl-0.05M-Tris-HCl (pH7.5). Samples (0.1ml) were dried on Whatman GF 83 filters for measurements of total radioactivity in the sample. Further samples (0.25ml) were added to 18% (w/v) trichloroacetic acid containing 0.05% of the appropriate non-radioactive amino acid. The sample was heated at 85°C for 30min, and then filtered on GF 83 filters. These were washed successively with 10ml of 18% trichloroacetic acid containing the appropriate amino acid, 10ml of ethanol and 10ml of diethyl ether. The filters were dried and the radioactivity was determined by scintillation counting in 5ml of a fluor consisting of 4g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre of toluene.

After the incorporation of [^3H]lysine into polypeptides in cells injected with poly(A), the precipitation was modified because polylysine is soluble in trichloroacetic acid solutions. The 0.25ml samples were added to 2ml of 5% (w/v) trichloroacetic acid containing 0.25% sodium tungstate, pH2.0 (Gardner *et al.*, 1962). The sample was filtered on GF 83 filters, which were washed with 10ml of 18% (w/v) trichloroacetic acid at 85°C, and then in the same way as in the usual precipitation procedure.

Purification of phenylalanine polymers. Oocytes or eggs were homogenized in 2ml of 0.5M-KCl-0.05M-Tris-HCl (pH7.5). Trichloroacetic acid (50%, w/v) was added to give a final concentration of 18%, and the sample was then heated at 85°C for 30min. The protein precipitate was isolated by centrifuging at 3000g for 15min. It was washed with 2ml of 20% (w/v) trichloroacetic acid. It was then extracted with 0.1ml of butan-1-ol, saturated with 2M-NH₃, in a boiling-water bath for 10min. The insoluble material was removed by centrifuging at 23°C and 3000g for 5min. The supernatant was spotted on to Whatman no. 1 paper, as were marker mixtures of mono-, di-, tri- and in some cases tetraphenylalanine. The chromatogram was developed by descending chromatography with butan-1-ol saturated with 2M-NH₃ (Felicetti & Lipmann, 1968). The solvent front was allowed to run for about 35cm. Phenylalanine and its polymers were located by ninhydrin-staining of markers run in parallel with the samples. The radioactive chromatograms were cut into 1cm strips, which were then cut into smaller pieces and placed in scintillation vials, to which was added 1ml of water-saturated butan-1-ol to elute amino acids. After agitation for 1h, 14ml of scintillation fluid was added. Each litre of this fluor contained 4g of 2,5-diphenyloxazole, 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 625ml of toluene and 375ml of 2-ethoxyethanol.

Fractionation of proteins from cells injected with haemoglobin mRNA. This was done on columns (1cm × 50cm) of Sephadex G-100 by the methods of

Lane *et al.* (1971) by using commercial rabbit haemoglobin as a marker.

Results

Effects of homopolymers

Two types of homopolymer, poly(U) and poly(A), were injected into oocytes and eggs. Since the former should code for polyphenylalanine and the latter for polylysine, each polynucleotide was injected with [^3H]phenylalanine and with [^3H]lysine, so that a general effect on protein synthesis could be distinguished from the synthesis of the appropriate polyamino acid. The result of a typical experiment is shown in Figs. 1(a)–1(d). Neither poly(U) nor poly(A) raise the rate of incorporation of labelled amino acids into acid-insoluble material. Similar results have consistently been obtained with both oocytes and eggs. In Figs. 1(c)–1(d) small inhibitions of incorporation may be seen. This effect is not very reproducible and therefore may not be significant.

The experiment just described leaves open the possibility that poly(U) or poly(A) are indeed used as templates for protein synthesis, but only to a very small extent. The sensitivity of detection can be raised by applying procedures expected to purify homopolymers. We have attempted to do this for polyphenylalanine.

This is a difficult molecule to characterize because it is very insoluble. One of its properties is a greater stability to acid hydrolysis than is shown by most polypeptides. An attempt was made to detect such material in eggs injected with poly(U), but they contained no more than the controls. Another way to purify polyphenylalanine is by paper chromatography. In our experiments the chromatograms were developed with butan-1-ol saturated with 2M-NH₃. In this system, the longer the chain, the nearer to the solvent front polyphenylalanine runs (Felicetti & Lipmann, 1968). A test of the validity of the procedure is shown in Figs. 2(a) and 2(b). This shows that poly(U) stimulates the synthesis of various sizes of polymers of [^3H]phenylalanine in a cell-free system prepared from oocytes by the methods of Cox *et al.* (1970). The products identified were mainly of small size, whereas the poly(U) was of high molecular weight (>50000). It therefore seemed likely that the cell-free system degraded poly(U). When examined by the same procedure control oocytes and those injected with poly(U) did not differ (Figs. 2c and 2d). It could be that the polyphenylalanine synthesized in living cells was too large to be extracted by the procedure used. Polyphenylalanine may be hydrolysed to a mixture of shorter oligomers by limited hydrolysis with HCl, and these may then be separated by paper chromatography. This method of analysis also fails to show polyphenylalanine synthesis in control and poly(U)-injected cells (Figs. 2g and 2h).

Fig. 1. Incorporation of radioactive amino acids into eggs injected with poly(U), poly(A), or poly(A,G,U)

Eggs were injected with 50 nl of injection medium (○) or 50 nl of a solution of poly(A) or poly(U) at 1 mg/ml in injection medium (●). These solutions also contained radioactive amino acid at 1.25 mCi/ml. After the appropriate length of time batches of 10 eggs were frozen. The points at 0 and 60 min represent the average of two determinations; the points at 30 min and 100 min are from a single determination. In (d) and (f) acid precipitation was by the tungstate-trichloroacetic acid method, whereas in (a), (b), (c) and (e) trichloroacetic acid alone was used. (a), Poly(U) and [³H]phenylalanine; (b), poly(U) and [³H]lysine; (c), poly(A) and [³H]phenylalanine; (d), poly(A) and [³H]lysine; (e), poly(A,G,U) and [³H]phenylalanine; (f), poly(A,G,U) and [³H]lysine.

The evidence summarized in this section therefore indicates that poly(U) and poly(A) either are not used, or are used very inefficiently, as messengers by eggs.

Effects of polymers beginning with putative initiation codons

It is generally thought that only the codons AUG and possibly GUG can act as initiators of mRNA

translation. Two artificial polyribonucleotides beginning with these codons were available to us, A-U-G(U)_n and G-U-G(U)_n (*n* is approx. 35). Both of these substances were known to stimulate phenylalanine incorporation in a Krebs ascites cell-free system at the expected Mg²⁺ concentrations (M. B. Mathews, personal communication).

The effect of A-U-G(U)_n and G-U-G(U)_n on the

Fig. 2. Chromatographic analysis of acid-insoluble material for polymers of phenylalanine

Eggs were injected with poly(U) or A-U-G-(U)_n at 250 µg/ml, together with [³H]phenylalanine at 5 mCi/ml. An acid-insoluble extract was made, and analysed by paper chromatography as described in the Materials and Methods section. The position of mono-, di- and tri- (and sometimes tetra-) phenylalanine markers is indicated. The methodology was tested in the experiment shown in (a) and (b). These diagrams show the products made in control (a) 0.5 ml cell-free systems prepared from *Xenopus* ovary exactly as described by Cox *et al.* (1970), and those to which poly(U) was added (b). The product was treated in the same way as injected eggs. (c) shows the analysis of control eggs. (d) and (e) show a similar analysis of eggs injected with poly(U) and A-U-G-(U)_n respectively. The radioactive protein was also subjected to partial hydrolysis with 6M-HCl for 2.5 h at 100°C to break long phenylalanine chains down to oligomers (Old & Gorini, 1965). The analysis of control eggs is shown in (f), and of eggs injected with poly(U) and A-U-G-(U)_n in (g) and (h) respectively.

incorporation of [³H]phenylalanine and [³H]lysine is shown in Table 1. G-U-G(U)_n has no significant effect, whereas A-U-G(U)_n always produced almost complete inhibition of the incorporation of any radioactive amino acid used. Table 1 also shows that

the inhibition is similar for both [³H]phenylalanine and [³H]lysine incorporation, and that it is produced even by small amounts of A-U-G(U)_n. In the experiment shown here equally complete inhibition of protein synthesis was produced with 12.5 and

Table 1. *Effects of synthetic polyribonucleotides on amino acid incorporation*

Groups of 16 eggs were injected with 50 nl of injection medium containing the synthetic RNA and radioactive amino acid at 5 mCi/ml. After 100 min incubation at 21°C the eggs with quite normal appearance (about 12) were frozen. Each percentage listed below is the average of two measurements, made on the eggs of two females laying simultaneously.

Sample injected	Amount injected (ng/cell)	% of total radioactivity converted into acid-insoluble material		Average of preceding two columns
		[³ H]Phenylalanine	[³ H]Lysine	
Injection medium (control)	—	9	8	9
Poly(U)	12.5	10	10	10
	1.25	7	9	8
A-U-G-(U) _n	12.5	0.5	0.2	0.4
	1.25	0.2	0.2	0.2
GUG-(U) _n	10.0	9	10	10
	1.0	6	9	8
A-(U) _n	10.0	7	—	7
	1.0	5	11	8
AUG	10.0	7	9	8

1.25 ng/egg. The very small amount of protein still synthesized was checked for the presence of [³H]-polyphenylalanine, but none was found (Figs. 2g and 2h).

Effects of other polyribonucleotides

Of the polyribonucleotides so far discussed only A-U-G(U)_n appears to have any pronounced effect on protein synthesis in living cells. The effects of five further polymers were investigated to check how much the inhibition produced by A-U-G(U)_n is dependent on its particular nucleotide sequence. These were A-(U)_n, AUG triplet, poly(G,U), poly(A,G,U) and poly(A,C,G,U).

A-(U)_n was tested to show that the third nucleotide of a molecule of the type A-U-N-(U)_n must be G if an inhibition is to be produced. Table 1 shows that A-(U)_n has no pronounced effect on incorporation of [³H]lysine or [³H]phenylalanine. The small inhibition observed is probably not significant. More accurate results could be achieved by injecting much larger numbers of eggs with each polyribonucleotide. However, it was thought better to test all of the different polymers on eggs laid by the same females on the same day. The size of the batches of eggs injected, and hence the accuracy of the experiment, was therefore limited by the reproductive capacity of the frogs and the stamina of the experimenters. The radioactive protein synthesized by eggs injected with A-(U)_n was tested for polyphenylalanine in the same way as the samples depicted in Fig. 2, but none was found.

The triplet AUG was the second substance tested (Table 1). Again this had no significant effect on protein synthesis, even at 30 pmol/cell, whereas A-U-G-(U)_n is completely inhibitory at 0.05 pmol/cell. It thus appears that the inhibitory effect of

A-U-G-(U)_n depends on the initial triplet being followed by further nucleotides.

The random polymer poly(A,G,U) is important for two reasons. First, one in 27 of the molecules may be presumed to begin with the codon AUG. Secondly, it should also contain termination codons. This is relevant because the inhibitory effect of A-U-G-(U)_n may be ascribed to the absence of a terminator codon (see the Discussion section). Fig. 1 shows that poly(A,G,U) has only a small apparent inhibitory effect on incorporation of [³H]lysine or [³H]phenylalanine. It was injected at 50 ng/egg, and one therefore assumes that sequences beginning AUG were present at about 2 ng/egg. Almost complete inhibition of protein synthesis was produced by A-U-G-(U)_n at 1.25 ng/egg (Table 1). Poly(A,G,U) therefore produces a much smaller effect on protein synthesis than one might predict from the hypothesis that any polyribonucleotide that bears an initial AUG triplet is inhibitory. (This interpretation assumes that the copolymer used was truly random.)

There are three possible terminator codons, all of which should occur in poly(A,G,U). Any particular codon should, on average, occur within 27 codons of an initiator codon in poly(A,G,U). If this polymer directs protein synthesis in the cell the polypeptides made should average eight amino acids if there are three functional terminator codons *in vivo*, 17 if there are two, and 26 if there is one. A search for small peptides of these sizes was made by using Sephadex and Bio-Gel P columns. No differences were found between control eggs and those injected with poly(A,G,U).

Similar experiments were conducted with poly-(G,U) and poly(A,C,G,U). These polymers had no significant inhibitory effect on endogenous protein

synthesis and no products of the expected size were detected. Poly(A,C,G,U) should direct the synthesis of polypeptides averaging 20, 42 or 63 amino acids by the same argument as that applied to poly(A,G,U).

Effects of synthetic polyribonucleotides on the translation of injected mRNA molecules

The experiments described above indicate that the synthetic RNA molecules tested are translated inefficiently, if at all, in amphibian cells. Nor, with the notable exception of A-U-G-(U)_n, do they affect endogenous protein synthesis to any marked extent. We may conclude that they do not compete with, or interfere in some other way with, the translation of mRNA molecules already being translated in the cell. A competitive effect might be expected of molecules possessing some, at least, of the attributes of messengers. In this respect, however, they are no different from an injected natural mRNA, since globin mRNA competes slowly and poorly with endogenous mRNA molecules (Moar *et al.*, 1971), even though it functions as an excellent mRNA in oocytes and eggs (Gurdon *et al.*, 1971). A better way to assay for interference of the expected sort is therefore to inject a mixture of the synthetic

RNA and a natural mRNA, and thus to assay for competition during the period in which the exogenous mRNA becomes incorporated into the protein-synthetic machinery of the oocyte. Experiments of this sort were conducted by using rabbit globin mRNA.

In the initial experiments globin mRNA was mixed with the synthetic polynucleotides before injection. The concentration of globin mRNA chosen was equivalent to 10 ng/oocyte, just sufficient to saturate its haemoglobin-synthetic capacity. Results from a typical series of experiments are shown in Fig. 3. It is evident that all three synthetic polynucleotides tested decreased the translation of globin mRNA. The efficiency of inhibition was in the order poly(A,G,U) > poly(G,U) > poly(A,C,G,U). None of these polymers produced a significant decrease in the synthesis of oocyte protein. We also tested poly(U) and A-U-G-(U)_n in a similar way. Poly(U) inhibited globin mRNA translation to only a small extent, if at all. A-U-G-(U)_n, as might be expected, inhibited globin mRNA translation completely (Fig. 4). It also inhibited endogenous protein synthesis, as expected, although the inhibition was only partial for the reason given in the legend to Fig. 4. (To calculate the inhibition of protein synthesis, approx. 50%, account must be taken of the different pool specific activities in the two types of cell.) Globin synthesis was fully suppressed even when globin mRNA (75×10^{-15} mol/oocyte) was present in slight molar excess over A-U-G-(U)_n (43×10^{-15} mol/oocyte).

These experiments showed that when synthetic polyribonucleotides are mixed with globin mRNA and injected into oocytes they suppress its translation. In contrast they have no effect on endogenous protein synthesis. This difference could result either from a difference between globin and oocyte mRNA molecules, or from a difference between pure, injected mRNA and mRNA already active in protein synthesis. These possibilities might be distinguished by injecting globin mRNA and synthetic polyribonucleotides at different times. The results of such an experiment are depicted in Table 2. Globin mRNA translation is inhibited by poly(A,G,U) when it is mixed with the mRNA (line 2), but not when it is injected separately 2 h later (line 6). This result is complicated by the fact that no inhibition was observed when the poly(A,G,U) was injected only 30 s after the globin mRNA (line 3). There are two possible explanations of this result. The least likely is that poly(A,G,U) forms an inactive complex with globin mRNA when they are mixed in the injection medium (0.1 M-KCl-2.5 mM-MgCl₂ in this experiment). More likely is that, even in short-interval double-injection experiments, the two solutions mix with oocyte cytoplasm before they mix with each other. They therefore independently associate with cytoplasmic components, and do not compete for

Fig. 3. Inhibition of globin mRNA translation by synthetic polyribonucleotides

Batches of 15 oocytes were injected with 50 nl of a solution of synthetic polyribonucleotides as stated and globin mRNA at 200 µg/ml. The oocytes were incubated for 5 h in [³H]histidine at 0.5 mCi/ml. The radioactive protein was analysed on columns of Sephadex G-100. Haemoglobin synthesis is expressed in terms of incorporation into oocyte protein to allow for minor differences in the entry of [³H]histidine into the cells. This method of presentation is justified by the fact that the proportion of total radioactivity incorporated into oocyte proteins was not altered significantly by any of the substances injected. ○, Poly(A,G,U); ●, poly(G,U); ▲, poly(A,C,G,U).

them, as they might if mixed. This is virtually inevitable, partly because diffusion is rapid with the small volumes used, and partly because it is impossible to inject both solutions into exactly the same region of the cell. Injecting solutions at intervals of 30s would not therefore be much different from injecting them at an interval of 2h and a similar inhibition of globin synthesis would be expected. The simplest interpretation of these double-injection experiments is that poly(A,G,U) and globin mRNA compete for some component in the cell present in limited amounts, binding firmly to RNA, and neces-

sary for protein synthesis. This hypothesis is discussed at more length in the Discussion section.

Discussion

Our experiments should be discussed in relation to studies of two types, those which involve the injection of natural mRNA molecules into living cells, and those concerning the behaviour of synthetic polyribonucleotides in cell-free systems.

The most extensive information of the first kind comes from experiments in which globin mRNA

Fig. 4. Inhibition of globin mRNA translation by A-U-G-(U)_n

(a) Oocytes were injected with globin mRNA at 300 µg/ml or 15 ng/oocyte; (b) A-U-G-(U)_n at 12.5 µg/ml or 0.6 ng/oocyte was also added. After incubation with [³H]-histidine the haemoglobin synthesized in 6h was analysed on columns of Sephadex G-100. Peak I is oocyte protein, peak II haemoglobin and peak III is mainly free [³H]histidine. In this experiment, clumps of oocytes were used, and only the largest were injected (see Gurdon *et al.*, 1971). This is the reason why endogenous protein synthesis was only partially inhibited.

Table 2. Effect of time of injection on the inhibition of globin mRNA translation by poly(A,G,U)

Batches of 40 oocytes were injected with 40nl of 0.1 M-KCl-2.5mM-MgCl₂ containing, where stated, globin mRNA at 200 µg/ml and poly(A,G,U) at 2000 µg/ml. In the experiment summarized in line 3 poly(A,G,U) was injected as soon after globin mRNA as possible (approx. 30s). The second injection was performed after 2h and the oocytes were then incubated for 4h in [³H]histidine at 0.5 mCi/ml. Of the oocytes that were unaffected by the operation 20 were used for protein analysis.

	Substances injected		Incorporation into haemoglobin
	At zero time	After 2h	
1	Globin mRNA	None	0.88
2	Globin mRNA + poly(A,G,U)	None	0.17
3	Globin mRNA and then poly(A,G,U)	None	0.81
4	Globin mRNA	Injection medium	1.20
5	Injection medium	Globin mRNA	1.09
6	Globin mRNA	Poly(A,G,U)	1.17
7	Poly(A,G,U)	Globin mRNA	1.16

from mammalian reticulocytes was injected into oocytes and eggs. In these amphibian cells rabbit α - and β -globin was made for many hours at an efficiency comparable with that in intact reticulocytes, each mRNA molecule being used many times (Gurdon *et al.*, 1971, 1973). This high efficiency is very important in assessing the effect of synthetic polyribonucleotides. The maximum stimulation of [3 H]histidine incorporation into globin is produced by injecting oocytes or eggs with not more than 10ng of globin mRNA (Moar *et al.*, 1971). However, no stimulation of incorporation of [3 H]phenylalanine or [3 H]lysine was observed when 50ng or more of poly(U) and poly(A) were injected, nor could the appropriate radioactive polypeptides be identified. Haemoglobin synthesis is best detected by using histidine as the radioactive precursor because it is unusually rich in this amino acid. Even so haemoglobin contains only 6% histidine, whereas the radioactive homopolymers coded by poly(U) or poly(A) would have contained only one amino acid. Since histidine and phenylalanine have pools of comparable size (Eppig & Dumont, 1972), the assay for polyphenylalanine should have been nearly 20 times more sensitive than that for haemoglobin. Globin mRNA stimulates histidine incorporation by as much as twofold. Therefore our failure to distinguish an effect of the synthetic RNA molecules on protein synthesis indicates that they are translated very inefficiently, if at all (at most 1000 times less than natural mRNA).

One reason synthetic polyribonucleotides have no effect on protein synthesis might be that they are rapidly degraded in living cells. Experiments on poly(U) degradation (H. R. Woodland, unpublished work) show that, although some poly(U) is degraded immediately after injection into the oocyte, a substantial amount remains after many hours. For example, when poly(U) is injected at 2mg/ml, or 100ng/oocyte, as much as 31ng/oocyte survives after 22h. If this amount of poly(U) had been active in protein synthesis it would have had a drastic effect on phenylalanine incorporation, since only 10ng of globin mRNA saturates the globin-synthetic capacity of the oocyte.

The conclusion that poly(U) and poly(A) do not function as mRNA in living cells is quite consistent with the results of experiments on cell-free systems. *In vitro* poly(U) and poly(A) are translated only at high Mg^{2+} concentrations (10–15mM; see such reviews as Bretscher, 1969; Haselkorn & Rothman-Denes, 1973). On the other hand, it is believed that the Mg^{2+} concentration is much less (about 3mM) in vertebrate cells. In vertebrate cell-free systems only AUG seems to act as an initiator codon at this concentration (Brown & Smith, 1970). In these systems, as in those of bacteria, ribosomes will also recognize GUG codons as initiators, but only at Mg^{2+} concen-

trations slightly higher than normal (Brown & Smith, 1970). It therefore seems likely that only AUG functions as an initiator codon in vertebrate cell-free systems. This conclusion is strengthened by the observation that in living yeast cells AUG will initiate translation of iso-1-cytochrome *c* mRNA when present at several positions in the mRNA molecule, whereas GUG is not able to perform this function (Stewart *et al.*, 1971).

The experiments reported above are compatible with the view that AUG is a codon of unique properties. The only polymer to have had a significant effect on protein synthesis, albeit an inhibitory one, was A-U-G-(U)_n. G-U-G-(U)_n, A-(U)_n, (U)_n and AUG all had no significant effect. The inhibition by A-U-G-(U)_n can be explained in four ways: (1) the A-U-G-(U)_n preparation could have contained inhibitory impurities; (2) A-U-G-(U)_n could act as an inhibitor of protein synthesis at some step other than initiation; (3) the initiation site of A-U-G-(U)_n could be sufficiently different from natural initiation sites for A-U-G-(U)_n to inhibit the translation of other mRNA molecules, without itself being translated; (4) A-U-G-(U)_n could initiate normally, but, because it lacks a termination codon, inhibit protein synthesis by binding all of some rate-limiting component, and not allowing it to recycle to further mRNA molecules (for example, this component could be tRNA^{Phe}).

The first of these explanations is not easily refuted, but there are three arguments against it. First, the particular preparations that we used stimulated phenylalanine incorporation in the expected way in a cell-free system prepared from Krebs ascites cells (M. B. Mathews & A. E. Smith, personal communications). Secondly, we have shown that the inhibitory agent is ribonuclease-sensitive. Lastly, the A-(U)_n and G-U-G-(U)_n that we used were made by the same procedure as the A-U-G-(U)_n, but neither were detectably inhibitory. It therefore seems likely that the effect produced by A-U-G-(U)_n is a property of its particular nucleotide sequence. Unfortunately the other three possible explanations of the inhibition fit the available data equally well.

Poly(A,G,U) and poly(A,C,G,U) contain both initiation and termination codons and function as messengers in cell-free systems. It is therefore surprising that they have little if any effect on protein synthesis on oocytes and eggs, although poly(A,G,U) may be very slightly inhibitory. Our observations fit the view that the requirements for initiation *in vivo* are more stringent than those in present-day cell-free systems.

The effect of most of the synthetic polymers on the translation of a simultaneously injected mRNA was quite different from that on oocyte protein synthesis. Poly(A,G,U), poly(A,C,G,U) and poly(G,U) all inhibited the synthesis of globin when

injected with globin mRNA. On the other hand globin mRNA behaved like an oocyte mRNA when it was injected separately. It is difficult to be sure why this occurs, but we favour the view that the synthetic polymer competes with globin mRNA for some component of the translational machinery present in limited supply. Lingrel & Woodland (1974) have suggested that the component which limits the capacity of oocytes to translate injected globin mRNA molecules is a pre-initiation factor. It was postulated that this agent binds permanently to mRNA molecules and either enables them to recruit ribosomes or to prevent mRNA degradation. We now suggest that this agent binds to the synthetic polymers, as well as to a natural mRNA. Presumably the affinity of any RNA for this agent would be reflected in the degree of inhibition it produces. The factor would therefore bind to RNA molecules in the following order of decreasing affinity: poly(A,G,U) > poly(G,U) > poly(U) > rRNA. [rRNA is notable in not being inhibitory (Gurdon *et al.*, 1971), a property of obvious importance in the living cell.] Since binding of the postulated factor is permanent once it occurs, it is easy to explain why globin synthesis is not inhibited when the mRNA is injected before the synthetic polymer. When poly(A,G,U) was injected 2h before globin mRNA (Table 2, line 7) there was no inhibition of subsequent globin synthesis. The simplest explanations of this observation are that poly(A,G,U) does not survive for 2h or, more likely, that more of the rate-limiting component becomes available during the incubation period.

The validation of our hypothesis will be greatly assisted by experiments with natural mRNA molecules. However, if we are correct, synthetic polyribonucleotides could prove useful in isolating the postulated pre-initiation factor by using, for example, affinity-chromatography methods.

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References

- Bretscher, M. S. (1969) *Progr. Biophys. Mol. Biol.* **19**, 175-197
- Brown, J. C. & Smith, A. E. (1970) *Nature (London)* **226**, 610-612
- Cox, R. A., Ford, P. J. & Pratt, H. (1970) *Biochem. J.* **119**, 161-164
- Elsdale, T. R., Gurdon, J. B. & Fischberg, M. (1960) *J. Embryol. Exp. Morphol.* **8**, 437-444
- Eppig, J. J. & Dumont, J. N. (1972) *Develop. Biol.* **28**, 531-536
- Felicitetti, L. & Lipmann, F. (1968) *Arch. Biochem. Biophys.* **125**, 548-557
- Gardner, R. S., Wahba, A. J., Basilio, R. S., Lengyel, P. & Speyer, J. F. (1962) *Proc. Nat. Acad. Sci. U.S.A.* **48**, 2087-2094
- Gurdon, J. B. (1967) in *Methods in Developmental Biology* (Wilt, F. H. & Wessels, N. K., eds.), pp. 75-84, Thomas Y. Crowell Co., New York
- Gurdon, J. B., Lane, C. D., Woodland, H. R. & Marbaix, G. (1971) *Nature (London)* **233**, 177-182
- Gurdon, J. B., Lingrel, J. B. & Marbaix, G. (1973) *J. Mol. Biol.* **80**, 539-551
- Haselkorn, R. & Rothman-Denes, L. B. (1973) *Annu. Rev. Biochem.* **42**, 397-438
- Lane, C. D., Marbaix, G. & Gurdon, J. B. (1971) *J. Mol. Biol.* **61**, 73-91
- Lingrel, J. B. & Woodland, H. R. (1974) *Eur. J. Biochem.* in the press
- Moar, V. A., Gurdon, J. B., Lane, C. D. & Marbaix, G. (1971) *J. Mol. Biol.* **61**, 93-103
- Old, D. & Gorini, L. (1965) *Science* **150**, 1290-1292
- Stewart, J. W., Sherman, F., Shipman, N. A. & Jackson, M. (1971) *J. Biol. Chem.* **246**, 7429-7445