Chapter 4

THE INJECTION OF RNA INTO LIVING CELLS: THE USE OF FROG OOCYTES FOR THE ASSAY OF mRNA AND THE STUDY OF THE CONTROL OF GENE EXPRESSION

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I. Introduction

The biologist who wishes to understand how processes that occur inside a normal living cell are organized and controlled often finds it useful to change cell function by deliberately altering the internal environment. This may be comparatively simple to achieve for substances that can pass in and out of cells, but is much more difficult for compo-

nents that are made by the cell itself and which normally remain inside it. One approach is to disrupt the cell, rendering its contents easily accessible. Another is to leave the cell intact, but to inject materials into it. Both have their merits, but the second in particular has the advantage that the concentrations of substances within a cell that is as close as possible to normality can be experimentally altered. In this way the function of macromolecules in a natural cellular environment can be studied, and this article illustrates this principle by describing in some detail how intact amphibian cells can be used for studying the translation of injected eukaryotic mRNA. An *in vivo* assay system of this kind is particularly attractive because translation takes place in a natrual environment so that normal controls affecting translation are likely to operate. It should be possible, for example, to discover whether all mRNA's are translated at the same rate in a certain kind of cytoplasm or whether some are preferentially translated. Factors that restrict or enhance the rate of translation of a particular mRNA may exist, and an *in vivo* assay system should provide an especially useful test of the existence and mode of operation of such substances. On the other hand, there will inevitably be a high background of protein synthesis by the host cell, which is likely to make the system unsuitable for analyzing the detailed biochemistry of the translation process itself.

Embryonic cells seem most likely to provide a cytoplasmic environment suitable for the translation of injected messengers because any restrictions on translation that may exist are less likely to be present in a nonspecialized cell. The cells chosen must also be large enough for microinjection, resilient enough to withstand this operation, amenable to simple biochemical analysis, and easy to obtain. These requirements are admirably met by the oocytes of *Xenopus laevis*, the South African clawed toad. Mature oocytes, which are present in thousands in the adult female, are enormous cells with a diameter of 1 mm or more, and are not harmed by the injection of up to about a hundred nanoliters of liquid, which represents 10–20% of the volume of the oocyte. Purified mRNA preparations can be assayed in this whole cell system, and reference will be made to translation within the oocyte of six species of eukaryotic mRNA; namely, those for duck, rabbit, and mouse globin chains and those for trout protamine, mouse immunoglobin, and calf crystallin. One particular aspect of these experiments shows how the injection of purified mRNA can be used to probe the control systems inside the living oocyte. When sufficient exogenous mRNA is injected, the overall rate of protein synthesis can be increased and translation

of the exogenous mRNA does not suppress endogenous protein synthesis. This shows that the translation systems of the oocyte are not fully occupied, suggesting that the supply of mRNA to the cytoplasm is especially important in setting the rate of protein synthesis. It is unlikely that the existence of spare translational capacity in intact cells could be deduced from experiments with cell-free systems, and this demonstration illustrates one of the advantages of using a whole cell system.

Another important property of the oocyte is that it is a very efficient translation system so that only minute amounts of messenger are required for new protein synthesis to be detectable. Consequently, the oocyte can be used to detect the activity of mRNA in the presence of a large excess of other classes of RNA. The ability of crude RNA preparations to support the synthesis of globin, promellitin, and collagen will be used to illustrate this point. The unpublished work of Knowland and Laskey concerning the collagen-producing activity of crude RNA from mouse fibroblast cells will be discussed in detail. Information concerning the translational and post-translational systems within the oocyte that can be derived from these experiments will also be discussed, and the nature of the regulatory mechanisms governing the expression of genetic information within the oocyte will be considered from the standpoint of the supply of mRNA.

II. The Translation and Assay of mRNA's

In order to identify a mRNA one must demonstrate that it codes for a characteristic protein. This means showing that synthesis of the particular protein is caused by adding the putative messenger to a system derived from cells that neither express nor even contain the genetic information for the protein in question. In practical terms this is most simply achieved by adding mRNA from one species to a translational system derived from another species. The disadvantage of this approach is that translational systems from one species may be restricted in their ability to handle foreign messengers. Restrictions are, however, less likely to exist in a translational system derived from embryonic cells, for these, unlike differentiated cells, give rise to an enormous variety of cell types. In this section we describe the use of the frog oocyte for the translation and assay of heterologous mRNA's.

A. *Purified mRNA*

The use of the oocyte system for the assay of a purified mRNA can be illustrated by reference to the reticulocyte hemoglobin mRNA (Burny and Marbaix, 1964; Lockard and Lingrel, 1967; Lane *et al.*, 1971, 1972a). The RNA to be tested (9 S RNA in this case) is dissolved in buffered saline (88 m*M* NaCl, 1.0 m*M* KCl, 15 m*M* Tris-HCl; pH 7.6) at a concentration of up to 5 mg/ml. After removal from the frog, the oocytes, which are contained in a sheath of follicle cells, are kept

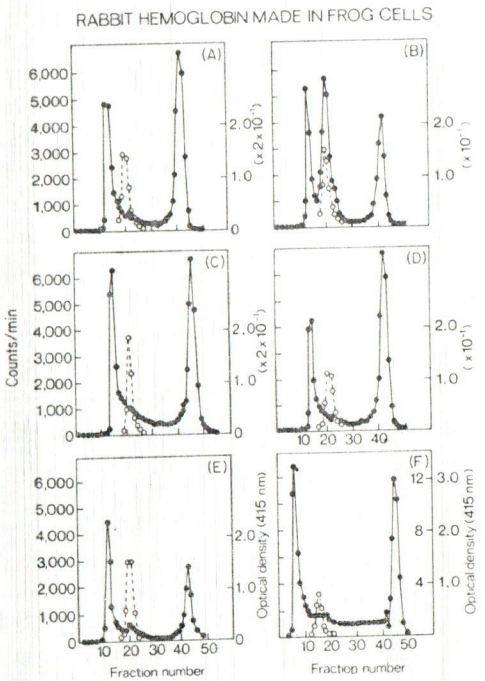


FIG. 1. Hemoglobin synthesis following the injection of various reticulocyte RNA fractions. (A) No RNA, (B) 9 S RNA, (C) 4 S + 5 S RNA, (D) 28 S RNA, (E) 18 S RNA crude, and (F) 18 S RNA purified. Batches of 20 oocytes, injected with hemin and RNA were incubated in [^{3}H]histidine (1 mCi/ml) for 7 hours and were then homogenized with marker rabbit hemoglobin; the resulting supernatant was analyzed on Sephadex G-100 columns (eluted with 0.052 *M* Tris – 0.052 *M* glycine, pH 8.9). The concentration of the RNA solution used for injection was about 1000 μg/ml in each case, yielding an intracellular concentration of about 50 μg/ml. Counts/min refer to material from a single oocyte. Recovery of counts was 95–100% in all cases. (○) Optical density at 415 nm (●) ^{3}H counts/min (Lane *et al.*, 1971). The results with purified 18 S RNA suggest that the small amount of hemoglobin synthesis seen with crude 18 S RNA is the result of contamination with 9 S RNA

in saline (Gurdon, 1968). For injection, they are simply laid moist on a microscope slide, and each oocyte receives up to 100 nl of the mRNA solution. After injection, the oocytes may be cultured for several days in saline. Frog oocytes are freely permeable to amino acids (Lane, 1971), and the products of translation of injected mRNA's can be labeled by simply adding radioactive amino acids to the incubation medium. Figure 1 shows that the presence of 9 S RNA in the injection medium results in the formation of a molecular species that elutes from a Sephadex G-100 column in the same fractions as rabbit hemoglobin. Passage through Sephadex G-100 provides an excellent method for separating hemoglobin from the majority of the newly synthesized proteins. The oocyte system has a high background, but it is reproducible, and Sephadex chromatography enables the amount of hemoglobin made to be measured quite accurately. However, other separation methods are often

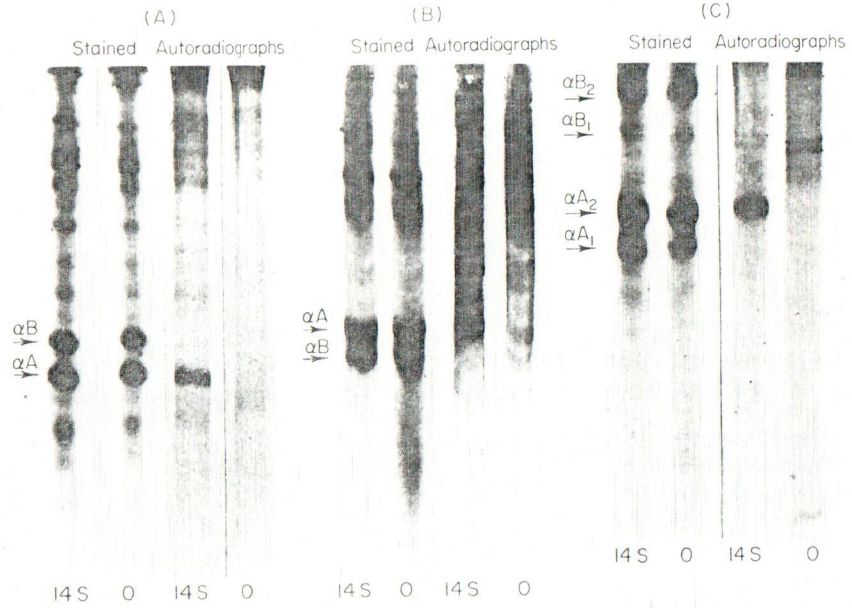


FIG. 2. Calf αA2 crystallin synthesis in 14 S mRNA-injected oocytes. One should note that only the basic urea gel can resolve α-crystallin into its four constituent polypeptide chains. Batches of oocytes were injected with 14 S RNA (isolated from calf lens epithelial tissue) and the frog cells were then incubated overnight in [^{35}S]methionine (0.5 mCi/ml). The cells were homogenized with marker α-crystallin and the supernatant proteins were analyzed on (A) SDS gels, (B) pH 3.0 urea gels, and (C) pH 8.9 urea gels, as described by Berns *et al.* (1972). Gel autoradiographs of newly synthesized proteins for RNA-injected and control oocytes; for comparison the gel staining pattern of total oocyte protein mixed with marker α-crystallin is also shown (Berns *et al.*, 1972).

better suited for the study of other messengers. Figure 2 shows the use of three types of acrylamide gel electrophoresis in the detection of calf αA2 crystallin made in oocytes under the direction of 14 S RNA from calf lens epithelium cells (Berns *et al.*, 1972). Figure 3 shows

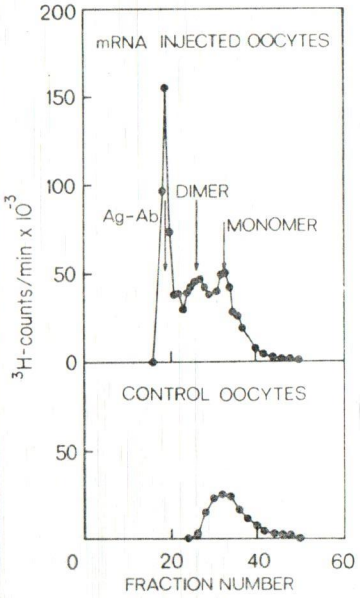


FIG. 3. Synthesis of antibody light chains made in oocytes under the direction of 9–13 S RNA from mouse myeloma cells. The RNA fraction used for the injections was prepared by the method of Stavnezar and Huang (1971). Control and message-injected oocytes were incubated overnight in [^{14}C]leucine before being homogenized in 1 ml of PBS (0.14 *M* NaCl, 1.5 m*M* KH_2PO_4, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4) and centrifuged for 5 minutes at 300 g. Sufficient purified urinary K-41 protein was added as marker to the oocyte supernatant to reach the equivalency point with 0.2 ml of antiserum (as determined by quantitative precipitation assays). Precipitations were performed in the presence of 3% Triton X-100. The antibody precipitates were collected and washed twice in cold PBS, once in 10% TCA for 15 minutes at 85°C, and once each in ethanol and ether. The washed precipitate was dissolved in 1 ml of 6.7 *M* guanidinium hydrochloride, 0.1 *M* Tris-HCl, pH 8.0, and dialyzed against 0.05 *M* glycine-HCl, pH 2.4, to dissociate the antibody–antigen complex. Carrier urinary K-41 protein (2 mg) was added to the dialyzed sample and it was chromatographed on a 1.4 × 70 cm Sephadex G-100 column equilibrated in 0.05 *M* glycine-HCl. The G-100 column resolves precipitated K-41 into undissociated antibody–antigen, dimer-form and monomer-form protein, as shown by the arrows which denote the elution volumes of appropriate markers. That each of these Sephadex fractions contains oocyte-derived light chains has been confirmed by running acrylamide gels under reducing conditions. Control oocytes were treated with injection medium lacking RNA but were treated similarly in other respects; (●) counts per minute.

the use of an antibody precipitation method in the detection of mouse immunoglobin light chains made in oocytes under the direction of 9 S to 131 S mouse myeloma RNA. Frog oocytes contain such a wide variety of proteins that even specific antibodies react unselectively with some of the proteins present; however, as shown in Fig. 3, Sephadex chromatography of the antigen–antibody complex helps to distinguish between the mouse L chains and the slight background of co-precipitable *Xenopus* proteins. Figure 10 shows the tryptic digestion products of antibody precipitable material from myeloma mRNA injected oocytes after analysis on an ion-exchange resin: the resemblance to the tryptic peptides obtained from marker mouse light chains from myeloma cells confirms the nature of the products of translation of injected myeloma 9 S to 13 S RNA.

None of the experiments outlined above formally excludes the possibility that the effect of the injected RNA is to activate quiescent genes present in the frog cell. Figure 4 shows the globin-like species synthesized in oocytes injected with 9 S RNA from duck, rabbit, or mouse reticulocytes. The two major globin species present in the hemoglobin of adult frogs were used to calibrate the carboxymethyl cellulose columns. There is a species-specific response to added messenger; for example, duck mRNA produces only duck globin chains. This shows that the injected RNA acts as a template for the synthesis of the new proteins. The examples cited illustrate the use of the oocyte as a qualitative test system for purified eukaryotic mRNA's.

B. *Use of the Oocyte or Egg to Detect a Single Messenger in a Crude RNA Preparation*

The work described in the preceding sections shows that a variety of purified eukaryotic mRNA's can be translated with high efficiency in *Xenopus* oocytes. In the same way it has been shown that total polysomal RNA from reticulocytes is active in directing the synthesis of hemoglobin. In these cases the mRNA whose translation is assayed constitutes the majority of all mRNA present, but in the following section we show that the oocyte or egg can be used to detect very small quantities of a particular messenger in a preparation of RNA, even if a large excess of other messengers is present.

The sensitivity of such an assay for a particular messenger depends upon the ease of detecting one particular translation product among a wide variety of others and against a background of protein synthesis by the oocyte or egg. A suitable test of the method therefore requires

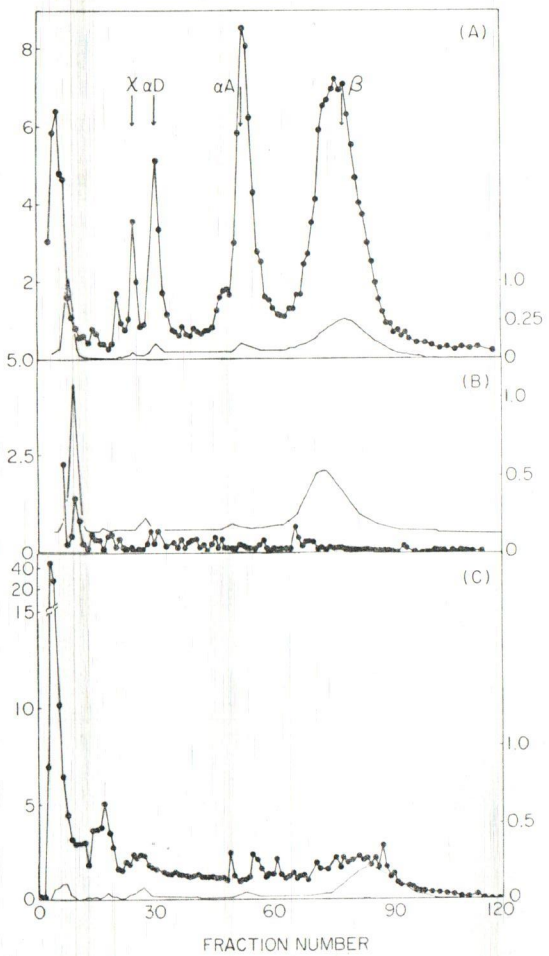
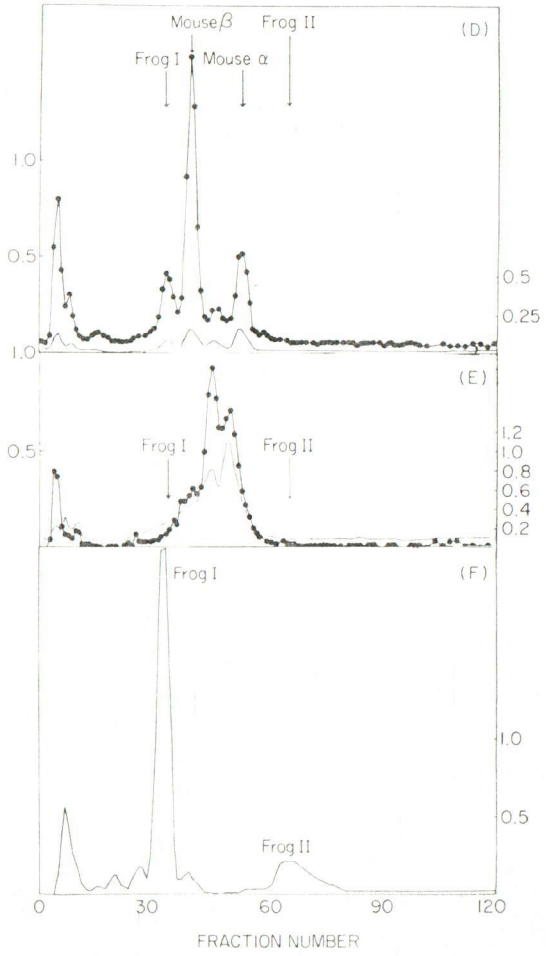


FIG. 4. Species-specific response of frog oocytes injected with globin mRNA's from different animals. The hemoglobin region from the Sephadex elution of oocyte-derived supernatant proteins was (after the addition of further marker hemoglobins) subjected to acid acetone treatment (Rossi-Fanelli *et al.*, 1958). (See Fig. 1 for examples of the Sephadex elution step.) The resulting globin was chromatographed on a Whatman CM-52 carboxymethyl cellulose column eluted with a 2 × 350 ml 8–80 m*M* Na_2HPO_4 pH 6.7 gradient (Lane *et al.*, 1972b). (——) = OD 280 nm. Scale on right side of figure. (A) Globin-like material from a batch of 20 oocytes. The cells had been injected with duck reticulocyte 9 S RNA (dissolved in injection medium at a concentration of 1.25 mg/ml) and incubated in medium containing [^{3}H]-histidine (1 mCi/ml and 50 mCi/mmole). (●——●) = Counts/min × 10^{-2}. Scale on left side of figure. (B) Material from a batch of 20 oocytes handled as in (A) but treated with injection medium alone. (●——●) = Counts/min × 10^{-2}. Scale on left side of figure. (C) CMC chromatography of an acid acetone extract of Sephadex front-peak material and marker duck hemoglobin. The front-peak material came from



the mRNA-injected oocytes described in (A). (●——●) = Counts/min × 10^{-2}. Scale on left side of figure. The oocyte-derived duck globin chains are confined to the hemoglobin region of the Sephadex elution profile. (D) CMC chromatography of globin-like material from oocytes injected with rabbit reticulocyte 9 S RNA (0.5 mg/ml). (●——●) = Counts/min × 10^{-3}. Scale on left side of figure. (E) CMC chromatography of oocyte-derived globin prepared from cells injected with mouse 9 S RNA (1 mg/ml). (●——●) = Counts/min × 10^{-3}. Scale on left side of figure. (F) CMC chromatography of frog globin prepared from a 3% (w/v) saponin lysate of washed frog red blood cells.

RNA from a cell which synthesizes many different proteins, but in which one particular protein is distinctive and easily recognized, although not necessarily present in large amounts. In the work described here (Know-

land and Laskey, unpublished.[1]) RNA from the collagen-producing mouse fibroblast 3T6 was injected into *Xenopus* oocytes and eggs, and collagen synthesis was assayed from the conversion of [5-^{3}H]proline into [5-^{3}H]hydroxyproline in the trichloroacetic acid-insoluble fraction of the injected cells. The ratio of hydroxyproline radioactivity to proline radioactivity provides a measure of collagen synthesis relative to total protein synthesis (Green and Goldberg, 1964), which in the 3T6 cell reaches a maximum of about 7% in stationary phase. This principle was applied to *Xenopus* embryos by Green *et al.* (1968), and no collagen synthesis was detected before gastrulation, suggesting that eggs and oocytes would prove to have an undetectable background of collagen synthesis.

1. Measurement of Hydroxyproline: Proline Ratio

After injection and incubation, oocytes or eggs (de-jellied with 2% cysteine at pH 8) were homogenized in cold 5% trichloroacetic acid (five oocytes or eggs per milliliter). The homogenates were allowed to stand for 10 minutes at 0°C, and the precipitates were collected on glass fiber discs (GF 83) by suction filtration. They were washed thoroughly with cold 5% trichloroacetic acid, then with ether and dried. Each disc was treated with 2 ml of 6 *M* HCl at 108°C for 18–24 hours. Acid and salts were removed from the hydrolysates, and proline and hydroxyproline separated by paper chromatography (Fig. 5). In the system used, as little as 0.002% of the proline radioactivity was found in the hydroxyproline region, setting a lower limit of about 0.005% on the hydroxyproline:proline ratio that could be measured. A typical separation is shown in Fig. 5.

The identity of the hydroxyproline peak shown in Fig. 5 was checked by eluting the material from the corresponding region of a duplicate chromatogram and testing for comigration against 20 amino acids in three different chromatographic systems. The peak material migrated with hydroxyproline in every case, and over 90% of it migrated with authentic [^{14}C]hydroxyproline (Fig. 6).

These results show that the techniques used provide a sensitive and convenient method, well suited to handling multiple samples, for the measurement of hydroxyproline:proline ratios. Using this method the values obtained for *Xenopus* embryos labeled with [5-^{3}H]proline agreed well with those reported by Green *et al.* (1968), using [3,4-^{3}H]-

[1] The authors are particularly grateful to Dr. R. A. Laskey for permission to include a description of this work.

proline and different analytical methods (Table I). An advantage of [5-^{3}H]proline is that no isotope is lost during its conversion to hydroxyproline, and the incorporation of [5-^{3}H]proline observed here was much greater than that found previously with [3,4-^{3}H]proline.

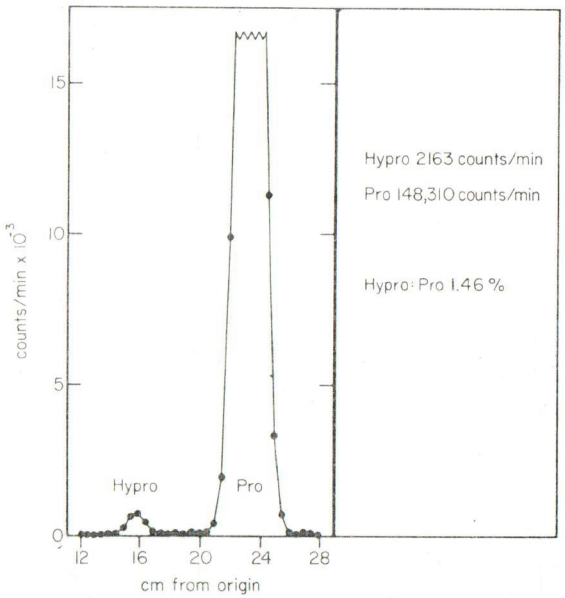


Fig. 5. Separation of proline and hydroxproline. Oocytes were injected with RNA from heavy polysomes as described in Table II. The trichloroacetic acid–insoluble material from 10 oocytes was hydrolyzed with HCl as described in the text. Each hydrolysate was diluted with 5 ml of water containing carrier proline and hydroxyproline and filtered through a Millipore filter to remove the glass fiber and insoluble matter. The filter was washed with 5 ml of water and HCl was removed from the combined filtrates by passage through a column of AG1108 resin (Bio-Rad), following with 10 ml of water. The pH of the effluent was between 8 and 9, and tests using radioactive amino acids showed that both proline and hydroxyproline were quantitatively recovered. The recovery of other amino acids was not tested. Residual salt was removed from the effluent using a column operated according to Dreze *et al.* (1954), except that the amino acids were eluted from this column with 3 *M* acetic acid. After evaporating off the acetic acid, the residue was dissolved in 10% isopropanol and subjected to descending chromatography in butanol:acetic acid:water (12:3:5) with the grain on Whatman 3 MM paper. Six 1-cm wide strips of 3 MM paper were stapled to the bottom of the chromatogram to accelerate the solvent flow toward the end of the run. After locating the spots either by spraying with ninhydrin or with a radiochromatogram scanner the region of the chromatogram spanning hydroxyproline and proline was sliced into 0.5-cm strips. Each strip was placed in a scintillation vial, moistened with 3 drops of 0.01 *M* HCl, covered with water-miscible scintillation fluid, and counted.

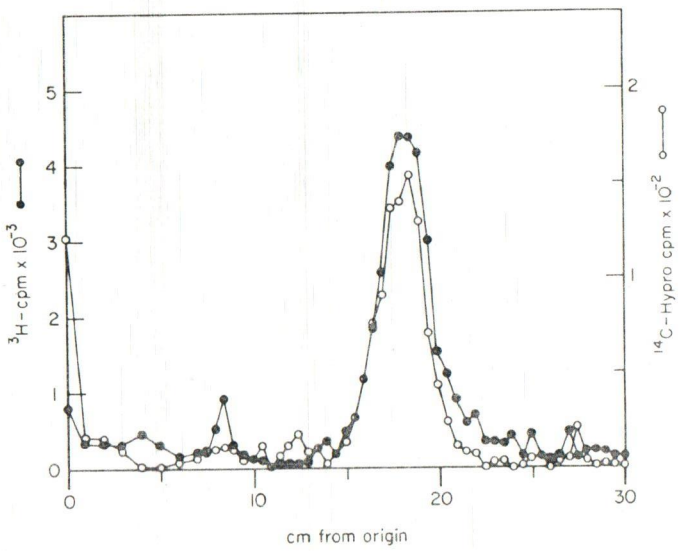


FIG. 6. Co-chromatography of putative hydroxyproline with authentic hydroxyproline. The putative hydroxyproline was eluted from a chromatogram similar to that shown in Fig. 5, mixed with authentic [^{14}C]hydroxyproline and chromatographed in a phenol:ammonia:ethanol solvent (water-saturated phenol, 15 ml; 0.88 ammonia, 1 ml; and ethanol, 4 ml). Strips sliced from the chromatogram were eluted with 0.01 *M* HCl and the radioactivity in ^{3}H and ^{14}C was measured for each eluate. (●——●) = ^{3}H counts/min × 10^{-3}, scale on left side of figure; (○——○) = ^{14}C counts/min × 10^{-2}, scale on right side of figure.

2. Hydroxylation of Protocollagen

The assay for collagen synthesis described above requires the collagen chains to be fully hydroxylated. Protocollagen hydroxylase can be extracted from unfertilized *Xenopus* eggs, and is active *in vitro* on 3T6 protocollagen (Green *et al.*, 1968). It seemed important to examine the activity of the enzyme on protocollagen present in the *intact* egg or oocyte in case protocollagen synthesized after injecting RNA should remain unhydroxylated.

TABLE I

Hydroxyproline: Proline Ratios in *Xenopus* Embryos

Developmental stage	No. of embryos	Counts/min per embryo		Hypro: Pro ratio (%)	Hypro: Pro ratio found by Green *et al.* (1968)
		Hyp	Pro		
10 gastrula	10	9	6,057	0.15	0.08
23 tailbud	5	18	7,056	0.26	0.27
30	5	147	24,591	0.60	0.88
39/40	5	2,219	61,163	3.63	3.38
42	5	4,885	82,397	5.93	5.18[a]

[a] Figure refers to stage 46/47 embryos. Embryos are injected with [5-^{3}H]Pro (10 mCi/ml; 5000 mCi/nmole) and incubated for 3 hours before analysis.

Unhydroxylated 3T6 protocollagen labeled with [5-^{3}H]proline only (Green *et al.*, 1968) was injected into 30 *Xenopus* eggs, and the hydroxyproline:proline ratio was measured after incubating for 3 hours. A value of 0.74 was obtained (hydroxyproline (counts/min, 516; proline counts/min, 700), which compares well with the value of 0.80 for 3T6 collagen (Piez *et al.*, 1963). No hydroxyproline was detectable either in the substrate or in eggs that were frozen immediately after injection. This shows that the intact *Xenopus* eggs can fully hydroxylate injected 3T6 protocollagen.

3. Collagen Synthesis in Oocytes and Eggs after Injection of RNA

When RNA prepared from 3T6 polysomes is injected into *Xenopus* oocytes, the hydroxyproline:proline ratio rises (Table II). RNA from heavy polysomes (190 S to 300 S) is more effective than RNA from light polysomes (80 S to 190 S), in agreement with findings that the majority of collagen synthetic activity is associated with heavy polysomes (Goldberg and Green, 1967; Lazarides and Lukens, 1971), while total RNA has

TABLE II

Stimulation of Collagen Synthesis in Oocytes[a]

Injection	Acid-insoluble hydroxyproline: proline (%)
Saline	0.40
RNA from light polysomes	0.93
RNA from heavy polysomes	2.23
Total RNA	0.45

[a] Polysomes attached to the endoplasmic reticulum were prepared from stationary phase 3T6 cells (Goldberg and Green, 1967) and centrifuged on a 15–60% sucrose gradient. The regions corresponding to 80 S to 190 S (light polysomes) and 90 S to 130 S (heavy polysomes) were pooled, and RNA extracted from them by treatment with SDS (Huez *et al.*, 1967) to dissociate the polysomes and chloroform-isoamyl alcohol extraction to remove residual proteins. The final RNA precipitate was dissolved in and exhaustively dialyzed against injection medium. Approximately 0.25 μg of RNA was used to inject 15 oocytes, which were incubated for 12 hours in medium containing 1 mCi/ml of [5-^{3}H]proline.

only a very slight effect. The fact that not all classes of RNA from a collagen-producing cell are equally effective in stimulating collagen synthesis in the oocyte suggests that the stimulation cannot be a nonspecific response. However, there is a certain background of collagen synthesis in controls, which makes the results difficult to interpret. By removing the connective tissue to which the oocytes, handled experimentally as clusters of 5–10, are attached, the background can be reduced. It can be eliminated by using eggs, in which collagen synthesis is undetectable.

When *Xenopus* eggs are injected with RNA from 3T6 polysomes (190–300 S) and incubated for 2–4 hours, a hydroxyproline:proline ratio of 0.25–0.40% is typically obtained. This value suggests that collagen synthesis represents approximately 0.1% of total protein synthesis in the injected eggs (Green and Goldberg, 1964); a proportion that is not reached during normal development until the neurula stage, and gives some indication of how normal egg function can be altered by injecting RNA.

In the work described above, the collagen messenger activity was detected in total RNA prepared from a mixture of polysomes covering a wide range of sizes. The majority of the RNA preparation is ribosomal, and it is likely that collagen mRNA constitutes only a small proportion of the remainder, but appears to be detectable.

III. Quantitative Studies: The Efficiency of Translation of Injected mRNA

For quantitative work it is essential to determine the form of the relationship between the amount of mRNA injected and the amount of the new protein that is synthesized in response to the messenger. All the mRNA's tested so far have yielded curves of the same form: At first there is a linear response to added messenger, but then the system saturates, and additional messenger has no further effect (Moar *et al.*, 1971; Gurdon and Marbaix, 1972). Thus, for quantitative work one must ensure that the messenger in question is assayed over a concentration range that corresponds to the linear portion of the saturation curve. Figure 7 shows a typical saturation curve for oocytes injected with rabbit reticulocyte 9 S RNA. There is a linear response over the range 0–10 ng 9 S RNA injected per cell. Figure 7 also shows that even at the saturation level injected mRNA does not cause a reduction in endogenous protein synthesis. It is only when much larger quantities of mRNA are injected that a significant reduction in frog protein production occurs (Moar *et al.*, 1971). Thus, the frog oocyte appears to have

a spare translational capacity, which means that the effects of injected messenger are additive until the saturation level is reached. Some 30 experiments with purified rabbit globin messenger have shown that this spare translational capacity varies at the most from 10 to 25 ng RNA per cell between different batches of oocytes. It is not known if the spare capacity is different for different messengers; dissimilarities have been noted (Lane *et al.*, 1972b; Stevens and Williamson, 1972), but

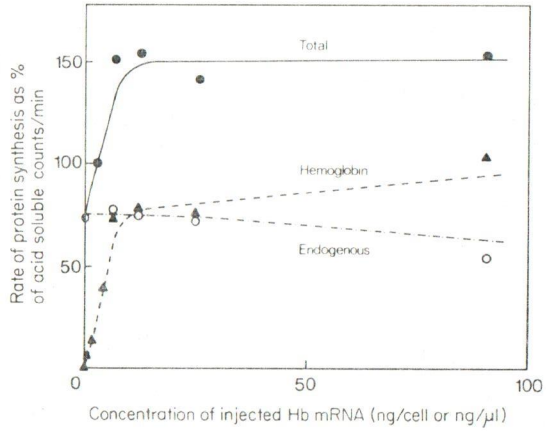


FIG. 7. The relationship between the quantity of 9 S globin mRNA injected into oocytes and the amount of hemoglobin synthesis (---), the amount of endogenous frog protein synthesis (- - -), and the total amount of protein synthesis (——). The values for hemoglobin and endogenous protein counts/min/oocyte were calculated from the results of Sephadex G-100 fractionations, as described by Moar *et al.* (1971). Each point is based on the analysis of about 20 oocytes: Cells were incubated overnight in [^{3}H]histidine. The intracellular concentration of RNA has been calculated assuming a 20-fold dilution of RNA resulting from injection, but leakage has been ignored.

variable amounts of messenger degradation could explain the differences observed. For similar reasons, it is very difficult to prove that one mRNA species is translated more efficiently than another; moreover, the oocyte assay system (or any other messenger assay system) cannot be calibrated in any absolute manner, for no reference RNA preparation containing a known proportion of pure functional mRNA is currently available.

It requires only a little technical expertise to inject cells as large as *Xenopus* oocytes. A standard experiment involves batches of 10–20 cells, and since 100–200 oocytes can be injected per hour, it is possible to test several variables quickly and easily. An important feature of the whole cell system is its sensitivity: mRNA's are often prepared in minute amounts, and it is, for example, possible to detect picogram

quantities of rabbit 9 S RNA using oocytes. The best cell-free systems currently available require much larger amounts of RNA.

Table III shows how the efficiency of translation for messenger-injected oocytes may be calculated. Globin messengers in oocytes are translated once every few minutes, indeed they are read almost as efficiently as are the globin messengers in reticulocytes (at a comparable temperature) (Gurdon *et al.*, 1971). However, the calculation shown in Table III is based on the assumption that the amino acid pool that functions in protein synthesis is equal to the total extractable pool. This assumption is supported by preliminary data obtained by injecting various amounts of labeled histidine into oocytes and which suggest that, for *Xenopus*, the functional pool is approximately the same size as the total extractable

TABLE III

EFFICIENCY OF HB MRNA TRANSLATION IN INJECTED OOCYTES OF MEASURED HISTIDINE POOL SIZE[a]

Measurement	Oocytes of frog A		Oocytes of frog B		
1. Concentration of Hb mRNA in sample for injection (μg/ml)	200	250	125	62.5	31.25
2. Injected Hb mRNA (pg/cell)[b]	14,000	17,500	8,760	4,360	2,180
3. Injected Hb mRNA (pmole/cell)[c]	0.070	0.087	0.043	0.022	0.011
4. Number cells/sample	30		20		
5. Counts/min [^{3}H]histidine incorporated into Hb/cell/hr[d]	4,480	6,428	8,490	6,439	3,155
6. Pool size of histidine (pmole/cell)	23.2		35.9		
7. Specific activity of intracellular histidine pool (counts/min/pmole)[e]	2,845	758	1,392	2,654	1,186
8. Globin chain synthesis[f] (pmole globin/cell/hr)	0.157	0.848	0.610	0.243	0.266
9. Number globin molecules synthesized/hr/9 S RNA molecule (row 8 ÷ row 3)	2.24	9.77	14.0	11.1	24.35

[a] From Gurdon *et al.* (1971).

[b] These values disregard leakage from injected oocytes, though this is believed to occur.

[c] Assumes that the molecular weight of 9 S mRNA is 200,000. Various published and unpublished measurements by different methods have estimated it to be between 175,000 and 225,000.

[d] All samples were labeled by incubation for 10 hours at 19°C in [^{3}H]histidine of specific activity 52.1 Ci/mmole.

[e] Calculated from the histidine pool size and the acid soluble counts/min/cell recovered from columns of Sephadex.

[f] Assumes 9.5 histidine residues per average α- and β-globin chain.

pool (Lane *et al.*, 1972c). Although the total extractable histidine pool varied in amount between batches of cells, it was found for both eggs and oocytes that the functional pool as determined by dilution analysis varied in a corresponding manner. Most results for the functional and extractable pools of oocytes fell in the range 40–80 pmoles of histidine per cell. Both pools seemed slightly higher in eggs. Eppig and Dumont (1972) found that the extractable histidine pool was about 50 pmoles/cell for the *Xenopus* oocyte. Perhaps the simplest way to avoid uncertainties regarding the size of the functional pool is to equilibrate the oocytes with labeled amino acids before injection of mRNA. Unfortunately, the oocyte contains large quantities of many amino acids; thus, this technique requires enormous amounts of radioactivity. Moreover, the background of newly synthesized endogenous protein is raised, and the pre-equilibration method cannot be applied to eggs which are not freely permeable to amino acids.

The sensitivity of the oocyte system does not only depend on the efficiency of messenger translation: equally important is the fact that injected messengers appear to be translated for long periods of time. Thus, pulse labeling at various times following injection shows that after 3 days in oocyte cytoplasm rabbit globin messengers still function with reasonable efficiency. Results obtained using polysomes suggest that messengers retain activity for even longer periods of time.

The products of translation of globin messengers appear to be quite stable within the oocyte, as demonstrated by pulse-chase experiments (Lane, 1972). Figure 8 shows that if incorporation of radioactive histidine into hemoglobin made under the direction of injected messenger is stopped by adding excess unlabeled histidine to the incubation medium, the amount of radioactive hemoglobin present in each oocyte does not decrease over a period of at least 18 hours. Since the incorporation of radioactive histidine into acid-insoluble material stops within an hour, and the oocyte is freely permeable to amino acids the chase is probably effective. The sensitivity of the oocyte system is, therefore, the result of efficient synthesis, over a long period of time, of a stable product.

At the time of writing the oocyte system is 10–100 times more sensitive than the most efficient cell-free systems, (Gurdon *et al.*, 1971); the preparations described by Lockard and Lingrel (1972) and Metafora *et al.*, (1972) are almost as active as the oocyte in the short term, but translation occurs for hours as opposed to days. Nonetheless for many purposes cell-free systems are more convenient; thus, the ascites cell-free system has a low background compared to that of the oocyte and must be preferred to the oocyte as a simple assay system unless only minute amounts of messenger are available. The oocyte does offer certain spe-

cial advantages if one wishes to analyze the control of translation as opposed to merely assaying for mRNA. The work of Laskey *et al.* (1972) showing that the oocyte can translate encephalomyocarditis (EMC)

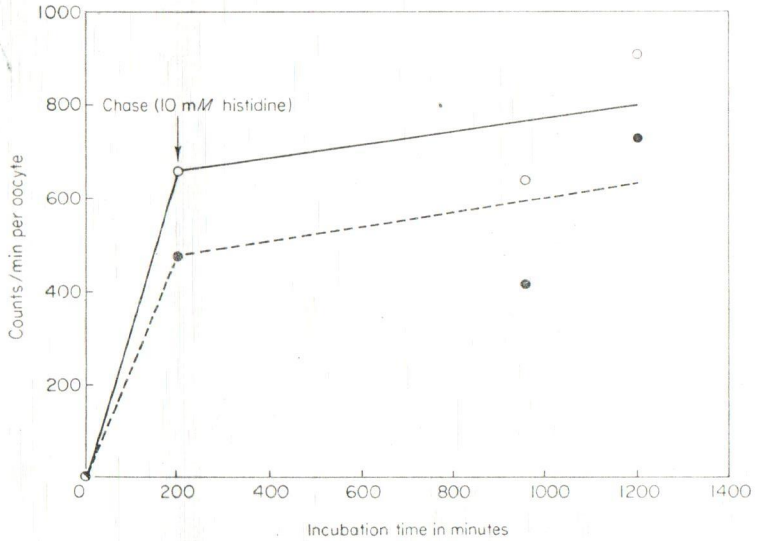


FIG. 8. The stability within the living cell of hemoglobin molecules synthesized in frog oocytes under the direction of rabbit reticulocyte 9 S RNA. Individually separated oocytes were injected with rabbit hemoglobin mRNA, and the cells were then incubated in [^{3}H]histidine for 2½ hours. The oocytes were removed from the radioactive medium, and after washing in Barth-X containing 10 m*M* histidine, the cells were transferred to this latter solution. Batches of 20 oocytes were removed for processing by Sephadex chromatography at 0, 13½ and 18½ hours after the start of the histidine chase. Hemoglobin and endogenous counts/min/cell were calculated from the results of Sephadex fractionation by the method described by Moar *et al.* (1971). The open circles (○——○) refer to hemoglobin counts/min/oocyte, the closed circles (●---●) refer to endogenous frog protein counts/min/oocyte.

RNA illustrates this point; thus, viral products can be detected in oocytes without having to suppress host cell synthesis by adding drugs such as actinomycin D. In addition to correct translation, oocytes are capable of completing the post-translational processing which generates proteins of the same molecular weight as normal EMC proteins. The viral translation process as it operates in a normal cell can be investigated by injecting other factors into the oocyte. Thus, the whole-cell injection system offers certain advantages for the analysis of translation over virus-infected mammalian cells and over cell-free systems derived from them.

IV. The Fidelity of Translation of Injected mRNA's

It is possible to assay and study closely related mRNA species using the oocyte system, for injected messenger appears to be translated with great fidelity. The translation of calf lens 14 S RNA can be used to illustrate this point (Lane and Berns, 1972). Figure 9 shows that after cation-exchange chromatography followed by paper chromatography, the majority of oocyte-derived and lens cell-derived αA2 crystallin tryptic peptides are indistinguishable from each other. The small differences that do exist between the staining and radioactivity patterns can be explained in terms of the known ninhydrin-negative peptides, except in the case of the peptide marked "X" in Fig. 9 which appears in the staining pattern but not in the radioactivity pattern. However, a difference of this general type is not necessarily the result of a translational error (Marbaix and Lane, 1972); for example, the crystallin mRNA and the marker lens crystallin may have been prepared from genetically dissimilar animals.

Duck and rabbit globin mRNA's, and mouse immunoglobin light chain mRNA (Mitch-Smith *et al.*, 1972; Fig. 10), have also been shown by similar means to be translated faithfully in oocytes (Marbaix and Lane, 1972; Lane *et al.*, 1972b). Eukaryotic messengers can also be translated faithfully in cell-free systems, as shown, for example, by Jones and Lingrel (1972) for mouse α- and β-globin mRNA's.

V. The Specificity of the Translational Machinery of the Oocyte

A. *Species Specificity*

The usefulness of the oocyte as a microassay for mRNA's depends, of course, on the specificity of the translational systems within this type of cell. It is probably true that any species of mRNA from a vertebrate can be translated in the frog oocyte. Thus, Table IV shows that messengers from birds, mammals, and fishes are active templates when injected into frog oocytes. Certain viral and insect RNA's are also active, implying that the lack of specificity even extends between phyla.

B. *Cell-Type Specificity*

It is possible that frog oocytes cannot translate messengers from other frog tissues, despite the lack of specificity shown by the oocyte toward mRNA's from the nonovarian tissues of other species; for exam-

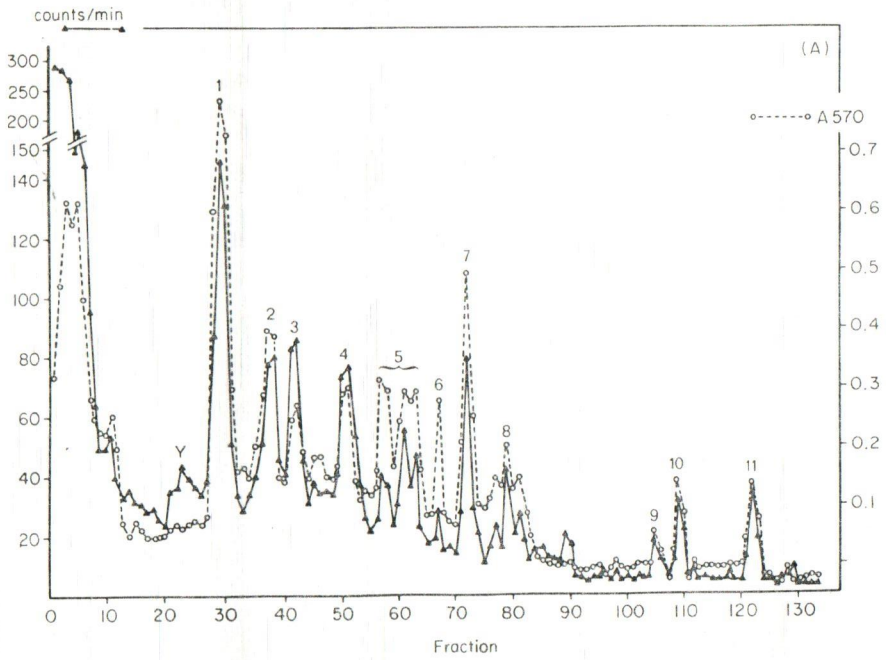
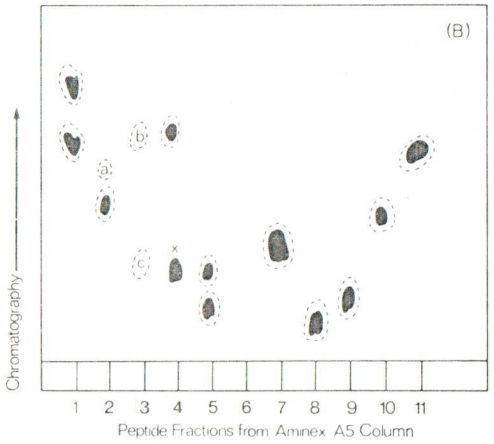


FIG. 9. The fidelity of translation of 14 S crystallin mRNA. αA2 crystallin chains, synthesized in oocytes under the direction of calf lens 14 S RNA were mixed with marker αA2 chains, purified and then digested with trypsin, all as described in Fig. 11. After lyophilization the tryptic digestion products were dissolved in 2 ml of starting buffer (0.2 *M* pyridine-acetate), and 40,000 counts/min of the soluble material were then applied to a Bio-Rad Aminex A-5 column. The column was washed at 50°C under pressure with 15 ml of starting buffer and was then eluted with a 2 × 125 ml linear gradient ranging from 0.2 *M* pyridine-acetate pH 3.1 to 2.0 *M* pyridine-acetate pH 5.0. Counting aliquots from the column eluate showed that the total tryptic digest was separated into 11 fractions by the ion-exchange resin; at this stage there is perfect coincidence between the peptides from the oocyte-derived and lens cell-derived αA2 crystallin chains—with the possible exception of the extra oocyte-derived peptide marked "Y" in (A). An aliquot from each of these 11 fractions was spotted onto paper and the paper chromatogram was run overnight in butanol-acetic acid–water–pyridine mixture. Tryptic peptides originating from the oocyte-derived αA2 chains were located by strip counting and are denoted by broken circles; tryptic peptides derived from added marker calf lens crystallin were identified by ninhydrin staining and are denoted by filled circles. As can be seen there is one peptide [marked "X" in (B)] that is present in the calf lens-derived marker αA2 crystallin but is not present in the oocyte-derived material. There are some peptides of which the converse seems to be true; however, the two labeled peptides marked "a" and "b" were extremely faint and probably represent minor products arising from residual chymotryptic activity. Ninhydrin staining probably lacks the sensitivity to reveal similar quantities of the corresponding marker chymotryptic peptides. Moreover, one at least of the tryptic peptides from αA2 crystallin is



known to be ninhydrin negative. The chromatographic mobility of the other extra radioactive peptide [marked "c" in (B)] suggests that this could be the ninhydrin-negative peptide. Fraction 6 from the ion-exchange column consisted of a small peak containing oocyte-derived and lens-cell derived peptide material. But there was insufficient material from either source for this peptide to be revealed by the counting or staining techniques used on the paper chromatogram.

ple, frog oocytes may be incapable of translating the messengers for frog hemoglobins. If this is the case, then the cell-type specific elements in the translational systems of the oocyte must themselves be species specific. This seems most unlikely.

C. *Untranslatable mRNA*

It is, however, a very difficult question to decide which messengers cannot be translated in the oocyte. Some mRNA's may be inherently incapable of operating within the oocyte, but lack of the expected products might result from, for example, degradation within the oocyte or during analysis. Losses may also result from binding to yolk or some other cell component, or to abnormal post-translational modification. However, the most likely cause of failure is degradation of the mRNA before it is even injected.

Thus, to obtain a meaningful negative result, the same mRNA sample used for injection must be shown to be biologically active in some other type of assay system. Even this test does not circumvent the possibility that the mRNA may have a secondary structure that prevents its translation in the oocyte and that this secondary structure is lost in the alterna-

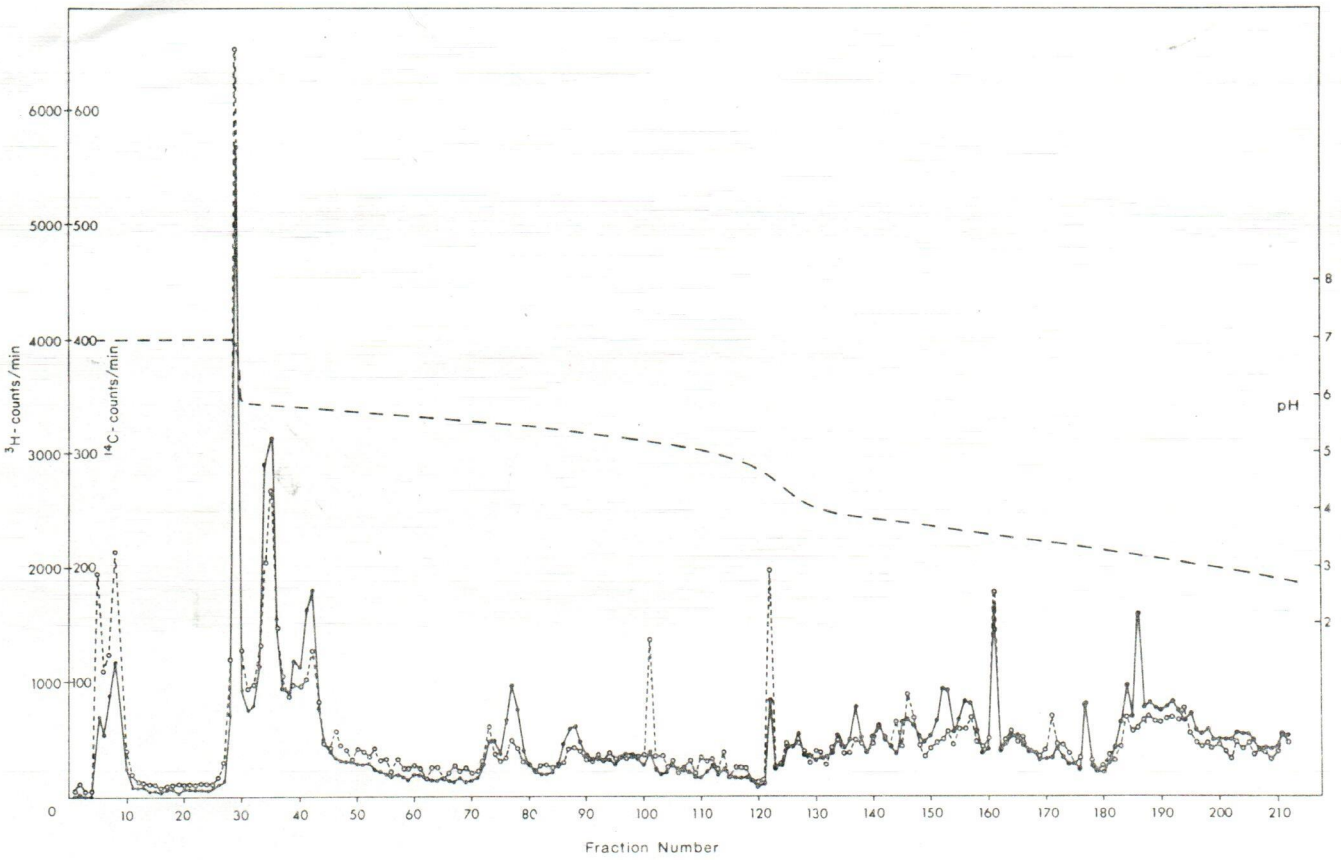


FIG. 10. A comparison between the tryptic digestion products of oocyte-derived and mouse myeloma cell-derived antibody light chains. The Sephadex G-100 fractions corresponding to the [^{14}C]leucine-labeled antigen–antibody complex (derived from mRNA-injected oocytes) were used as a source of partially purified light chains suitable for peptide analysis. (See Fig. 3 and Stavnezar and Huang, 1971.) The samples were dissolved in 6.7 *M* guanidinium hydrochloride and antibody-precipitated K-41 tumor protein, labeled *in vivo* with [^{3}H]leucine, was added. The proteins were reduced with 0.05 *M* dithiothreitol, alkylated with 0.113 *M* iodoacetic acid and digested for 18 hours at 37°C with triphenylchloroketone trypsin. The tryptic products were then applied to a 6 by 120 cm Dowex-1 × 2 column equilibrated with 3% pyridine. The peptides were then eluted at 37°C with a gradient of 0.38 *M* pyridine (200 ml) to 0.1 *M* pyridine, 0.05 *M* acetic acid (130 ml). This was followed by a gradient of 0.1 *M* pyridine, 0.05 *M* acetic acid (150 ml) to 1 *M* acetic acid (75 ml). The broken line denotes the ^{14}C radioactivity (oocyte-derived material) while the continuous line represents tritium-labeled digestion products (mouse myeloma-derived light chain material).

TABLE IV

THE SPECIES AND CELL TYPE SPECIFICITY OF *Xenopus* OOCYTE TRANSLATIONAL SYSTEMS

Nature and source of RNA	Method of preparation	New proteins synthesized	Analytical methods used	References
9 S RNA from rabbit reticulocytes	SDS treatment of polysomes or 14 S mRNP	α- and β-rabbit globin chains	CM-cellulose chromatography; analysis of tryptic peptides by chromatography and electrophoresis	Lane *et al.* (1971) Marbaix and Lane (1972)
9 S RNA from mouse reticulocytes	Phenol extraction of polysomes	α- and β-mouse globin chains	CM-cellulose chromatography	Lane *et al.* (1972b)
9 S RNA from duck reticulocyte	SDS treatment of polysomes or mRNP or phenol extraction	α^A, α^D, and β-duck globin chains	CM-cellulose chromatography; analysis of tryptic peptides	Lane *et al.* (1972b)
14 S RNA from calf lens epithelial cells	SDS treatment of polysomes	αA2 crystallin	Alkaline urea-gel electrophoresis; chromatography and electrophoresis of all tryptic peptides	Berns *et al.* (1972)
9–13 S RNA from cultured K-41 mouse myeloma cells	SDS treatment of polysomes	Antibody light chains	Antibody precipitation followed by Sephadex chromatography, gel electrophoresis, and peptide analysis	Mitch-Smith *et al.* (1972)
4–5 S RNA from trout testes	Gel electrophoresis of polysomal RNA	Protamine	Bio-gel chromatography, gel electrophoresis, peptide mapping	Wu *et al.* (1972)
Polysomal RNA from duck reticulocytes	Phenol extraction of polysomes	Hemoglobin	Sephadex chromatography	Lane *et al.* (1972c)
Polysomal RNA from 3T6 mouse fibroblast cells	SDS treatment of polysomes; isoamyl alcohol/chloroform	Hydroxyproline-containing protein	Measurement of Hypro: Pro ratio in hydrolyzed TCA-insoluble material	Knowland and Laskey (1972)
Total RNA from queen bee glands (putative promellitin mRNA)	Gel electrophoresis	Promellitin-like substance	Butanol/ammonia extraction followed by paper chromatography. Pepsin digestion followed by pH 4.8 electrophoresis; digestion of three peptic products with trypsin or HCl followed by comparison with known promellitin peptides	Kindas-Mugge *et al.* (1972)
High molecular weight RNA from mouse fetal erythroblasts	Phenol extraction of whole cells; sucrose-gradient centrifugation	α- and β-globin chains	Sephadex and CM-cellulose chromatography	Williamson and Drewienkiewicz (1972a,b)
A-rich RNA from nucleus or cytoplasm of 5563 myeloma cells	Phenol extraction at pH 7.6 and 9.0; poly(U) column chromatography	Heavy and light antibody chains	Antibody precipitation and gel electrophoresis	Stevens and Williamson (1972)
EMC RNA from whole virions		6 EMC-specific proteins	Comparison by gel electrophoresis with EMC-infected ascites cells; finger printing of 3 new proteins	Laskey *et al.* (1972)
70 S AMV RNA from whole virions	SDS sucrose gradient centrifugation	None	SDS gel electrophoresis or antibody precipitation using highly pure viral protein-specific antibodies followed by SDS gel electrophoresis	Voght and Lane (1972)
30 S AMV RNA	Heat treatment of 70 S RNA	None		
Whole virions	Prepared from chicken serum	None		
Dissociated virions	Treatment of whole virions with 0.5% NP40 and 5 m*M* dithiothreitol	None		
AMV RNA	As above	None		G. Marbaix, personal communication

TABLE IV (*Continued*)

Nature and source of RNA	Method of preparation	New proteins synthesized	Analytical methods used	References
Rauscher virus RNA (various forms as for AMV above)	From whole virions as for AMV RNA	None	Gel electrophoresis	Bloemendal and Gurdon (1972)
f2 RNA	Cold phenol extraction of phage	None	Gel electrophoresis	Gurdon *et al.* (1971)
Double-stranded f2 RNA and double-stranded poliocarditis RNA	Enzymic synthesis	None	TCA precipitation	Lane *et al.* (1972d)
$AUG(U)_n$	Enzymic synthesis	Inhibition of protein synthesis	Test incorporation of phenylalanine into protein	Gurdon *et al.* (1971); Woodland *et al.* (1972)
AUG	Enzymic synthesis	None	Test incorporation of amino acids into protein	Woodland *et al.* (1972)
poly (A,G,U)		None		
poly (U); poly (A)		None		
Duck reticulocyte tRNA	Isopropanol fractionation	Slight inhibition of protein synthesis	Sephadex chromatography	Lane (1972)
Rabbit reticulocyte 28 S RNA, 18 S RNA, and 4 S + 5 S RNA	Sucrose gradient centrifugation of SDS-treated polysomes	None or small amount hemoglobin	Sephadex chromatography	Lane *et al.* (1971)
Mouse reticulocyte 12 S RNA	Gel electrophoresis SDS-treated polysomes	None	Sephadex chromatography	Lane (1972)
Duck or rabbit reticulocyte polysomes	Centrifugation of reticulocyte supernatant	Hemoglobin	Sephadex chromatography	Lane *et al.* (1971)

tive assay system but not in the oocyte. Moreover, the extraction procedure must be tested by demonstrating that a small amount of the translation product injected into the oocyte can be successfully recovered from it. This latter point is particularly relevant to studies concerning simple synthetic polynucleotides whose translational products are unnatural. Included in Table IV are some of the RNA species that have been injected into oocytes and which do not seem to cause the synthesis of a new type of protein. It remains to be proved, unequivocally, that these RNA species are not translated in oocytes, although some of the viral and synthetic RNA's used were shown to be active templates in cell-free systems.

Thus, much more information needs to be gathered before the specificity of the translational systems within the oocyte can be accurately defined. It seems likely that any type of vertebrate messenger but only some types of viral mRNA can be translated in the frog cell. If the results obtained with polynucleotides such as poly(U) or $AUG(U)_n$ are taken at face value it would appear that certain simple synthetic polymers cannot act as templates in this system. The translation of promellitin mRNA, which is prepared from queen bee glands, suggests that there are even no interphyletic barriers (Kindas-Mugge *et al.*, 1972); thus, it is conceivable that the frog oocytes will translate any mRNA prepared from any species of animal.

VI. The Post-translational Modification of Messenger Products

The frog oocyte presumably contains a wide range of the biosynthetic systems found in normal living cells. Consequently, it is not surprising that the primary translation products made under the direction of added mRNA may undergo normal secondary modification. Figure 11 shows that αA2 crystallin made in oocytes under the direction of 14 S RNA is *N*-acetylated as is the αA2 crystallin of the calf lens epithelium cell. Met-$tRNA_F$ is known to be the initiator tRNA for lens crystallin synthesis (Strous *et al.*, 1972), and the possibility that the 14 S RNA injected also codes for an acetylating enzyme is unlikely (Berns *et al.*, 1972). It appears, therefore, that an enzyme in the frog cell is responsible for the *N*-acetylation of the calf crystallin molecule. Thus, the acetylating machinery of the oocyte appears to be neither cell-type specific nor species specific. Analogous results have been obtained for the phosphorylation of trout protamine made in *Xenopus* oocytes under the direction of injected messenger (Wu *et al.*, 1972), and for the hydroxylation of unhydroxylated collagen injected into *Xenopus* eggs.

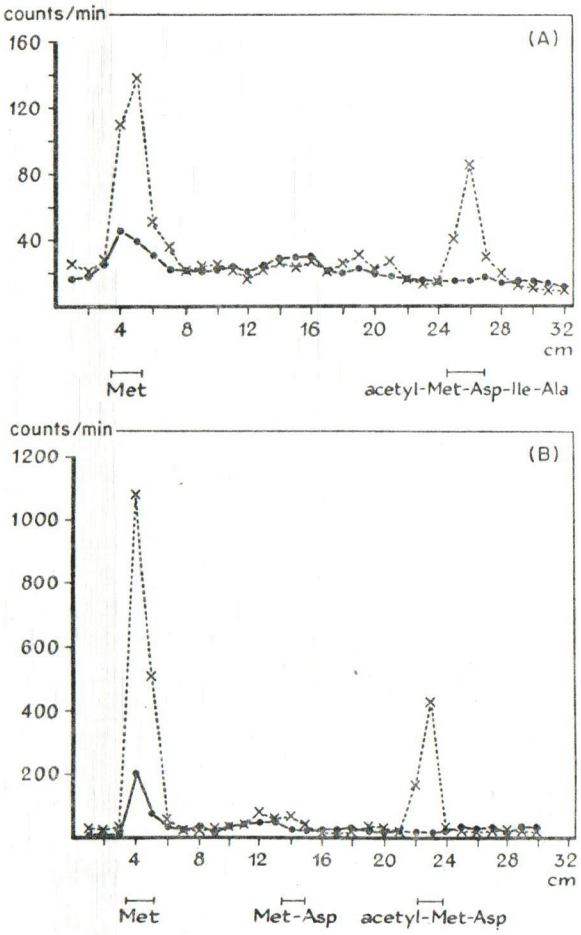


FIG. 11. αA2 calf crystallin chains were separated from the majority of the oocyte proteins by electrophoresis on basic urea gels. The gel segment containing the αA2 band was cut out, minced, dialyzed against, 5 m*M* 2-mercaptoethanol, and, after filtration to remove the gel, the solution was lyophilized. (A) pH 6.5 electrophoresis of the products of subtilisin digestion of this oocyte-derived material. Subtilisin is known to release the N-terminal peptide (acetyl-Met-Asp-Ile-Ala) of αA2 crystallin. (B) The products of pronase digestion of the oocyte-derived material. Pronase digestion proves that the N-terminal peptide is blocked for, as the bars marking the mobilities of synthetic marker peptides show, pH 6.5 paper electrophoresis can resolve the free and blocked N-terminal peptides. The blocking agent is not a formyl group (Berns *et al.*, 1972). The crosses represent labeled peptides from 14 S RNA injected oocytes while the filled circles represent labeled peptides from oocytes injected with buffer only.

Some messengers give rise to a precursor protein which is subsequently cleaved to form the functional polypeptide species (Milstein *et al.*, 1972). The oocyte contains enzyme systems that will, apparently, cleave foreign protein precursor molecules in the expected manner. Thus, the injection of mouse K-41 myeloma mRNA into oocytes causes the synthesis of a molecular species closely resembling the mouse immunoglobulin light chain (Stevens and Williamson, 1972; Mitch-Smith *et al.*, 1972; Mach, 1972); but when mRNA from a similar source is added to a reticulocyte cell-free system the product is a light chain precursor (Milstein *et al.*, 1972; Mach, 1972). Mach has compared the product made in the oocytes with mature light chains, and a comparison has also been made with precursor light chains: the oocyte-derived material is found to electrophorese with the mature light chains when it is run on a gel system that will differentiate between the light chain and its precursor. Milstein *et al.* (1972) have shown that a membrane-bound enzyme is probably responsible for the conversion of precursor to product. Thus, it seems likely that there are enzymes within the oocyte, presumably located on cytoplasmic membranes, that can cleave the light chain precursor in the correct manner.

The translation of EMC viral RNA provides another example of post-translational cleavage of a foreign precursor species within the oocyte. As in the case of the myeloma light chain, cleavage of a large precursor has not been directly demonstrated, but the fact that the six viral proteins found in oocytes were the same size as their counterparts derived from EMC-infected ascites cells (where cleavage is known to occur) (Butterworth *et al.*, 1972) strongly suggests that post-translational cleavage occurs in the oocyte. The cleavage mechanism could be provided by the frog cell or, less likely, specified by the viral RNA. In the case of the myeloma light chain precursor, then, if one assumes that the RNA preparation is homogeneous, the size of the messenger virtually rules out the possibility that the injected RNA codes for the cleavage enzyme. One cannot, of course, apply this argument to EMC RNA which is large enough to code for as yet unidentified viral products. However, the EMC genome, with virus-specific genes distributed throughout it (Butterworth *et al.*, 1972), appears to contain only one initiation site (Smith, 1972); thus, the immediate translation product is likely to be a single polypeptide. This polypeptide must be cleaved to yield the mature proteins, and it is unlikely that cleavage could be performed by the polypeptide itself. Thus, the oocyte may well translate the entire EMC genome and cleave the product correctly, yielding the normal EMC proteins.

It therefore appears that in a number of instances the oocyte can

modify the primary structure of the immediate translational product in order to produce normal protein subunits. It is also possible that the oocyte permits, and perhaps encourages, assembly of subunits into a normal quaternary structure. If oocytes injected with rabbit globin mRNA are labeled by incubation in a precursor of heme rather than globin (δ-aminolevulinic acid), then even in the absence of globin carrier, the radioactivity still elutes in the position of globin chains. However, it is by no means certain that the δ-aminolevulinic acid is labeling heme and not globin; and even if one assumes that the complex is labeled by virtue of its content of radioactive heme, there is no proof that the association of newly synthesized heme and newly synthesized globin does take place in the intact cell and not during subsequent processing of the oocytes. Similar objections may apply to indications that complete globin tetramers ($\alpha_2\beta_2$) or immunoglobin molecules (H_2L_2) can be assembled within the oocyte. Moreover, carrier immunoglobin was added during the analysis, and in the case of oocyte-derived globin, dimers as opposed to tetramers are found if carrier is omitted. Thus, the products of translation of foreign mRNA's can be modified, in a variety of ways, by enzymes present in the recipient frog cell to yield the normal end products, but whether assembly of the components of a multimeric structure is possible remains uncertain.

VII. mRNA Injection as a Means for Analyzing the Translational Systems of the Living Cell

The injection of mRNA into oocytes enables one to investigate the translational machinery and control systems present in the cytoplasm of a normal living cell. This kind of information is difficult to obtain by other means. While cell-free preparations can be used routinely as messenger assay systems, it is much more difficult to obtain accurate information about control mechanisms in normal living cells from such preparations; for example, messenger injection experiments suggest that eggs and oocytes contain nonspecific translational systems. However, similar experiments performed with cell-free systems would be open to the objection that specificity had either been lost or gained during the preparation of the cell extract. The results obtained with a whole cell system are not quite so open to such objections; nonetheless, one can argue that the messenger-injected oocyte is a cell that contains translational machinery that has been forced to function in a nonphysiological manner. One should, perhaps, note that the act of microinjection does not in itself render a cell abnormal, for injected eggs will grow up

into fertile adult frogs (Gurdon and Uehlinger, 1966; Gurdon, 1962). However, whole cell systems complement rather than exclude cell-free systems as a means for analyzing translation.

It is unfortunate, therefore, that there are no well-documented whole cell translation systems other than the *Xenopus* oocyte. Reviewing the field up to 1968, Bhargava and Shanmugam (1971) concluded that there was no proof that living cells could translate exogenous RNA. More recently, Maeyer-Guignard *et al.* (1972) have shown that avian or simian fibroblasts will, in the presence of DEAE-dextran, take up and translate mouse interferon mRNA. One does not know if fibroblasts are capable of translating messengers that code for proteins that are unrelated to the proteins made by the fibroblast itself, for in the example cited above the fibroblasts used were of the type that are normally themselves capable of interferon production. Tuohimaa *et al.* (1972) have noted that crude avidin mRNA is probably taken up and translated by oviduct and shell gland cells. This latter observation implies, but does not prove, that the translational machinery of a highly differentiated cell is, like the systems within the oocyte, nonspecific; for the shell gland does not make avidin. Grässman and Grässman (1971) injected muscle cells with melanoma RNA and found, autoradiographically, an increase in [^{3}H]dihydroxyphenylalanine incorportion; their results also argue for the existence of nonspecific translational apparatus within differentiated cells. However, further studies are required before this type of conclusion is placed on a more than tentative footing.

The results obtained with mRNA-injected oocytes are very clear, and the data obtained (summarized in Table IV) enable one to make three deductions concerning the protein synthetic machinery of the oocyte:

1. There are translational systems within the oocyte that are not species-specific.

2. There are systems within the oocyte that are not cell-type specific (see also Section V,B), and no factors from the donor cell apart from the mRNA are required for the translation of messengers from a variety of cell types.

3. If messenger-specific factors are required for the translation of mRNA's then the factors needed for the synthesis of proteins characteristic of a variety of specialized types exist in the oocyte, a cell which does not normally make these products.

If one takes these results at face value and disregards the possibility (discussed previously) that the injected mRNA is in some artefactual way bypassing the normal cellular control mechanisms, they suggest that the important regulatory systems will be those governing the supply

of free mRNA for it seems that any "free" mRNA (as opposed to one that is masked or unavailable) that reaches the cytoplasm is automatically translated. It is hoped that this extremely naive hypothesis will serve as a useful basis for further experimentation. The idea that specific substances can mask or inactivate free mRNA and thereby prevent translation does not contradict the theory that pure mRNA is automatically translated. Thus, it would seem prudent to focus attention on regulatory mechanisms that could affect the supply of free messenger such as messenger masking, the control of messenger exit from the nucleus and transcriptional control, rather than on mechanisms that must operate at the polysomal level such as tRNA modulation mechanisms or the control of termination.

The naive hypothesis ascribes a central role to the supply of free messenger and one would expect therefore that in the oocyte the rate of protein synthesis should be limited by the amount of free mRNA. The results shown in Fig. 7 suggest but do not prove that this is in fact the case. Figure 7 shows that by injecting rabbit globin messenger one can increase the rate of protein synthesis in the oocyte. Strictly speaking, this only proves that the supply of injected rabbit globin messenger limits the rate of rabbit globin synthesis in oocytes, and it is possible that conclusions drawn from studying the translation of heterologous mRNA injected into the oocyte would not apply to the translation of homologous mRNA injected into the oocyte. It is also possible that conclusions based on the injection of exogenous mRNA, whether homologous or not, would not apply to internally synthesized mRNA.

While not forgetting these reservations, it seems reasonable to generalize to some extent and to suggest that the translational systems that can be occupied by rabbit globin mRNA are limited in their protein synthetic activity by the supply of messenger. It is perhaps unreasonable to assume that this category includes all the translational systems of the frog cells for Fig. 7 shows that over a range of concentrations injected mRNA does not really compete with endogenous messenger. Even a saturating amount of globin mRNA hardly reduces endogenous protein synthesis. Competition can occur at high mRNA concentrations, suggesting that these are not two completely isolated systems. Lack of competition can be explained in terms of compartmentalization, in terms of messenger modification, or in temporal terms—perhaps messengers already engaged in protein synthesis are difficult to displace—but it may also result from the existence within the oocyte of translational machinery that can read only endogenous messengers, for the existence of nonspecific systems does not preclude the existence of specific ones. Until the underlying causes behind the lack of competition have been worked out (and it seems possible to do this), one cannot apply with complete confidence the principles deduced from the injection of exogenous messenger to endogenous messengers. In other words, it remains possible that the rate of endogenous protein synthesis is not limited by the supply of messenger. Consequently, one can only state that the quantitative data yielded by messenger injection experiments is consistent with the hypothesis that messenger supply limits the rate of protein synthesis in frog oocytes.

The limitation of the translation of excess injected mRNA has been studied in a preliminary way (Gurdon and Marbaix, 1972; Marbaix and Gurdon, 1972). As explained previously it is of interest because the lack of competition between endogenous and exogenous mRNA's could result from messenger specificity of the component which limits the translation of injected messenger RNA. The existence of a message-specific translational component would be of interest and potential importance in development; moreover, the existence of a message-specific system would argue against the proposition that because exogenous mRNA limits the rate of foreign protein synthesis, mRNA must also limit the rate of endogenous protein synthesis. It appears that polysome-saturated oocytes can manufacture hemoglobin at three times the maximal rate of hemoglobin synthesis seen in mRNA-saturated oocytes. This result suggests that some component of the polysome preparation other than mRNA is the one that limits the translation of excess mRNA. Consequently, adding more of one of the polysomal constituents should raise the saturation level for mRNA or polysome-injected oocytes. KCl wash factors from polysomes seemed to have little or no effect. However, reticulocyte polysomes contain large quantities of tRNA (10% of the total polysomal RNA) and reticulocyte tRNA was found to raise the saturation level for mRNA or polysome injected oocytes. Thus, if the lack of competition between excess injected messenger and endogenous messenger is the result of a message-specific component, the latter could be tRNA. However, as stated previously there are alternative explanations for the lack of competition.

VIII. The Translation of mRNA-Precursor Molecules

As shown by subsequent development and differentiation the *Xenopus* oocyte and egg contain the genes coding for specialized proteins such as hemoglobin. But the oocyte and egg do not synthesize detectable amounts of such proteins. Thus, if one assumes that any free messenger that reaches the cytoplasm will be automatically translated, the cellular

restrictions on the supply of genetic information must operate at some preceding level. The level at which the flow of information from the hemoglobin genes is restricted could be identified by injecting each intermediate involved in the formation of globin mRNA and seeing which of these intermediates promoted hemoglobin synthesis. Reticulocyte polysomes, 14 S mRNP particles, and 9 S RNA all cause hemoglobin synthesis when injected into oocytes. In the present section we will discuss attempts to extend this series backward by injection of putative mRNA precursors into oocytes.

There is an increasing body of evidence supporting the idea that the immediate gene transcript is a large messenger precursor molecule (Scherrer *et al.*, 1966; Penman *et al.*, 1968; Soiero *et al.*, 1968). Is the oocyte capable of translating or forming a functional messenger from the putative mRNA precursor? In other words, do the restrictions on the expression of genetic information operate at some level other than that of the precursor, for example, at the transcriptional level? If the oocytes were capable of making, for example, hemoglobin after injection of a putative Hb mRNA precursor this result would have to be interpreted cautiously because the oocyte might cleave the precursor or translate it as a result of some nonphysiological process. This argument would not affect the validity of the experiment as a direct demonstration of the precursor product relationship between HnRNA and mRNA. However, if the injected frog cells behave unphysiologically one can gain little or no insight into their intracellular control systems.

In any attempt to identify a precursor of mRNA, it is especially important to prove that the candidate is not contaminated by mature mRNA because it is difficult to purify a putative precursor. The sensitivity of the oocyte assay system makes it very attractive for this kind of experiment.

Most mRNA's and their precursors are thought to contain A-rich sequences (Lee *et al.*, 1971; Darnell *et al.*, 1971a,b: Lim and Canellakis, 1970; Haim *et al.*, 1972; Adesnik *et al.*, 1972). Total nuclear A-rich RNA from mouse myeloma cells apparently supports the production of immunoglobin in the oocyte, suggesting that this RNA contains a precursor of mature, cytoplasmic mRNA (Stevens and Williamson, 1972). However, it is possible that nuclei normally contain mRNA, and it is also possible that in this experiment the nuclear RNA injected was contaminated by mature cytoplasmic mRNA during preparation. Thus, O'Malley *et al.* (1972) showed that hen oviduct nuclei contain A-rich RNA that stimulates avidin synthesis in a rabbit reticulocyte lysate, but these authors assumed that the activity resulted from nuclear mRNA.

Williamson and Drewienkiewicz (1972a,b), in similar experiments, found that the high molecular weight (>35 S) RNA from the erythropoietic fetal mouse liver stimulated the synthesis of mouse α- and β-globins in the oocyte. These authors attempted to exclude contamination by extracting similar RNA from brain tissue in the presence of added mouse globin mRNA. In this case the high molecular weight RNA was inactive, suggesting that the procedure for preparing it eliminated contamination, but this, too, remains uncertain in the absence of a demonstration that the mRNA added to the brain nuclei retained activity. One test for nonspecific aggregation might be to extract high molecular weight RNA from a mixture of mouse fetal liver nuclei and rabbit reticulocyte lysate. Contamination of the high molecular weight nuclear RNA preparation should then be revealed by its ability to direct the synthesis of rabbit globin in addition to mouse globin.

One must conclude by stating that the machinery within the oocyte may well be capable of expressing the information present in mRNA precursors from differentiated cells, and it may well be that in the oocyte restrictions on the expression of genetic information do not operate at the level of free cytoplasmic messenger precursor but at some preceding level; for example, developmentally important mechanisms could involve control of mRNA precursor exit from the nucleus. The exit of mRNA precursors from the nucleus, or their translation within it, cannot easily be examined by injection experiments because it would be hard to ensure that no material injected into the nucleus leaked out into the cytoplasm.

Transcriptional control is perhaps the most obvious possibility, but one must not forget that restrictions involving the masking of messengers are quite compatible with the results obtained from oocyte injection experiments. A large number of investigators have demonstrated the existence of translational control systems (Tomkins *et al.*, 1969; Hunt *et al.*, 1968, 1969; Hogan, Chapter 5, this volume), but as suggested in this article there is little evidence that such systems are primarily responsible for cell differentiation. In the activated sea urchin egg translational systems appear to be responsible for more than the fine control of development (Gross, 1967; Humphreys, 1971); thus, it is possible that further experimentation will reveal the fundamental importance of translational control mechanisms in development, but for the moment such evidence is not forthcoming.

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