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INTRODUCTION

One of the more important phenomena involved in a developmental program is the interaction between those cellular components which carry developmental information and those parts of the cell that process it; and the most fully studied aspect of this process is the interaction between nucleus and cytoplasm. In the animal which we have studied, the frog *Xenopus laevis*, all the available evidence indicates that the nuclear genetic information is the same in different types of cells (Gurdon, 1962; Gurdon and Laskey, 1970; Laskey and Gurdon, 1970). Thus the production of differences between cells must initially involve heterogeneity in the processing apparatus. In order to understand how the capacity to follow the developmental program is inherited it is therefore necessary first to understand the characteristics of the processing system.

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Genetic activity in early development may be classified under three headings, DNA synthesis, RNA synthesis and protein synthesis. The work reported here is orientated towards finding out as much as possible about the nature and control of these processes. Much biochemical work must, perforce, utilize cell-free systems, but there must always be doubt as to the relation of results obtained in this way to events occurring in living cells. To avoid such doubts the method we have used involve the micro-injection of cell components, or other substances, into living oöcytes or eggs. These two types of cells are biochemically interesting because they are metabolically very different from each other, particularly as regards their nucleic acid metabolism; they are embryologically interesting, because they represent the parent cell of the embryo in its formative and mature phases (for our purposes the sperm can be regarded as of minor importance, and in numerous species it is dispensible); and lastly they are very convenient because of their large size. In this paper we summarize what we know so far about the three classes of genetic activity which occur in these cells, and compare our results with what is known from studies of cell-free systems. We have not attempted a comprehensive review of work done *in vitro*, but confine ourselves to those aspects of *in vitro* work which are directly relevant to that done *in vivo*, and wherever possible that which uses the same or closely related animal species.

DNA SYNTHESIS

Characteristics of Normal Oöcytes and Eggs

The oöcyte of *Xenopus* is a growing, nondividing cell, intensely active in RNA synthesis and protein accumulation. It does not synthesize DNA, except for a short while at the beginning of oogenesis when rDNA* is amplified (Gall, 1969). The full grown oöcyte is stimulated by hormone to undergo the first meiotic reduction division. Various other changes also occur at this time (Smith and Ecker, 1970), after which it is called an egg. The stimulus of fertilization (or artifical activation) leads to the completion of meiosis and the formation of a female "pronucleus." The egg and sperm pronuclei replicate their DNA between 20 and 40 min after fertilization (Graham, 1966). The cell divides after about 90 min and then enters a phase of rapid and frequent cell division, in which the rate of DNA synthesis is faster, and the duration of the S-phase shorter, than found even in rapidly growing bacteria (Graham and Morgan, 1966; Gurdon, 1968b). The egg makes little, if any, nuclear RNA (Gurdon and Woodland, 1969) and is therefore a cell involved primarily in DNA synthesis, in contrast to the oöcyte which makes only RNA. These two cells, one of which may be converted *in vitro* into the other by hormone treatment, are therefore eminently suitable for the study of factors which cause DNA synthesis to begin.

DNA Synthesis Studied in Living Cells by Micro-Injection

Enzymes of DNA synthesis. The basic method we have used in this study is to inject a DNA template and a radioactive deoxynucleotide or deoxynucleoside into living cells. The template has been in the form either of intact nuclei or purified DNA. This contrasts with the conventional method of investigation, which is to attempt to extract the enzymes and then to assay their activity *in vitro*. The kinds of complexities which this latter approach can lead to is well exemplified by recent studies of the DNA polymerases of *Escherichia coli* (e.g., De Lucia and Cairns, 1969; Knippers and Strätling, 1970). The aim of these in-

*Abbreviations: rRNA, ribosomal RNA; mRNA, messenger RNA; Hb, haemoglobin; SDS, sodium dodecyl sulfate; dCTP, dTTP, dGTP, dATP = deoxynucleotide triphosphates of cytidine, thymidine, guanosine and adenosine respectively; rDNA, ribosomal DNA; SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate).

jection experiments is to study the control of DNA synthesis under conditions as near as possible to those of normal cells. This can be achieved by conducting the experiments in the living cell, or eventually by developing *in vitro* systems with similar properties.

In order to start with experiments as simply related to the normal situation as possible, intact nuclei were injected into cells. It was found that they behave in the same way as the resident cell nucleus, for they synthesize DNA in eggs, but not in oocytes. These experiments are discussed more fully by Gurdon and Woodland (1968; 1970). They lead to the general conclusion that the initiation of DNA synthesis is under cytoplasmic control, the cytoplasm becoming competent to induce DNA synthesis when the germinal vesicle breaks down during maturation of the oöcyte. In an attempt to simplify the rather complex interaction which occurs in experiments of this sort Gurdon, Birnstiel and Speight (1969) injected pure DNA into eggs. They showed that injected native and denatured DNA stimulated the incorporation of ^{3}H-thymidine into DNA. This DNA has the same buoyant density as that injected, both when examined in a double-stranded (Gurdon, Birnstiel and Speight, 1969) and a single-stranded form (Ford, C. C. and Woodland, H.R., unpublished data). When denatured DNA is injected. about half of the radioactive DNA extracted behaves as native DNA on neutral CsCl gradients. Thus unless single stranded molecules, parts of which are newly synthesized, become annealed with themselves, the denatured DNA-stimulated incorporation appears to represent replication.

In an analogous study, DNA was injected into oöcytes and in initial experiments, no replication was observed (Gurdon and Speight, 1969). In more recent experiments in which the extraction procedure included extensive protein digestion (Ford, C. C. and Woodland, H. R., unpublished data), it was found that in oocytes, denatured DNA markedly stimulates DNA synthesis (Table 1 and Fig. 1). With the same extraction methods stimulation was not observed when native DNA was injected (Table 1). The DNA synthesized in these ex-

TABLE 1

DNA synthesis *in vivo* in response to injected templates

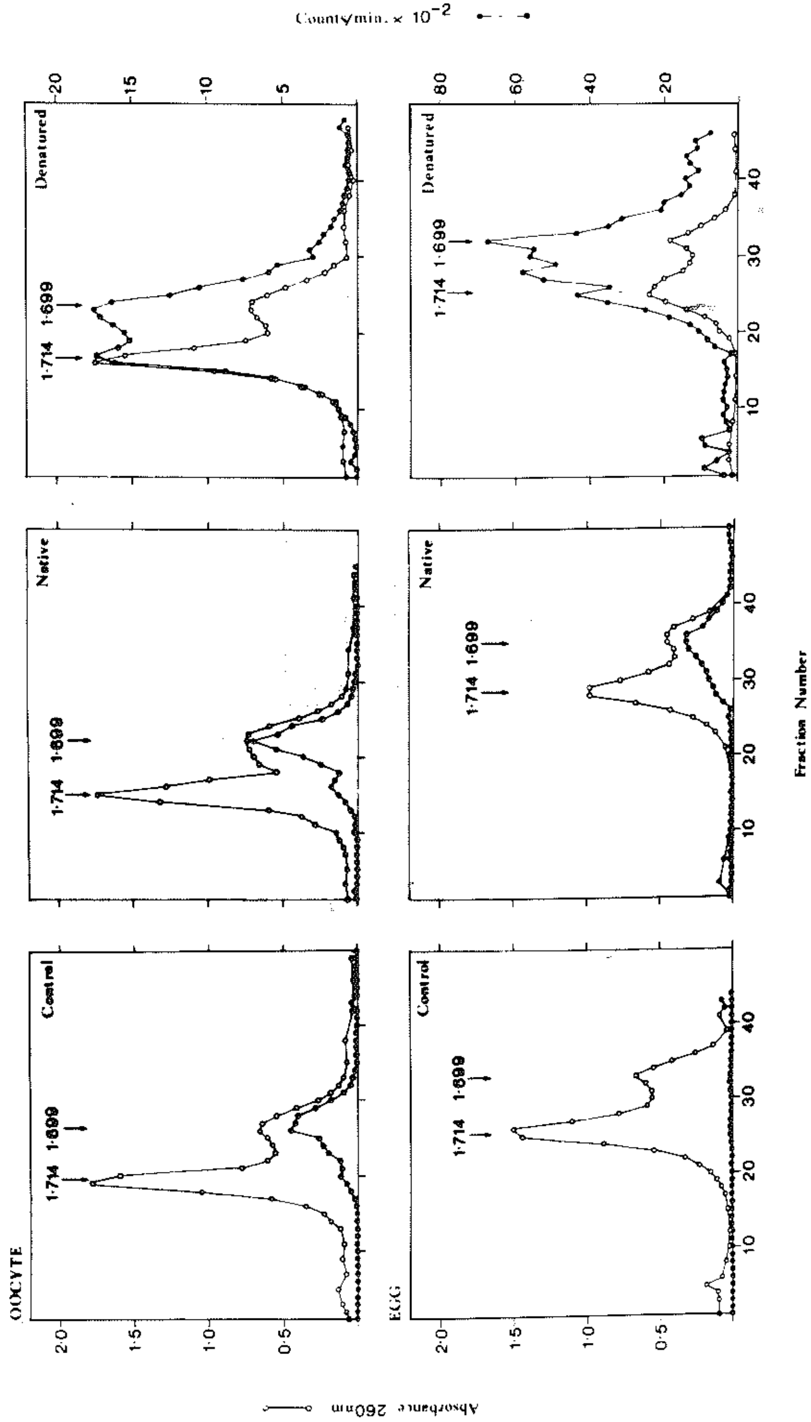
Type of cell	Radioactive precursor	DNA counts/min / nucleotide counts/min: Native DNA template (%)	Denatured DNA template (%)	Denatured / native
Oöcytes*	^{3}H-dATP	0.03	24.8	830**
Oöcytes†	^{3}H-thymidine	0	10.3	∞**
Unfertilized eggs	^{3}H-thymidine	1.10	9.4	8.6

Batches of 30 oöcytes or eggs were injected with DNA (60 μg/ml) and radioactive precursor as described by Gurdon, Birnstiel and Speight (1969) and incubated for 90 min (eggs) or 6 hr (oöcytes). DNA was extracted by procedures described elsewhere (Ford, C. C. and Woodland, H. R., unpublished data). These methods give recoveries of over 50% judged by absorbance. Phosphorylation levels were measured by the methods described by Woodland (1969). Acid insoluble counts within the OD marker on CsCl gradients were adjusted for recovery to give the total DNA counts/min. Values for control samples have been subtracted.

*Full grown oocytes from a female that had not laid for several months.

**The high value of control samples does not allow detection of small amounts of native primed synthesis (i.e., that which would have produced a denatured:native ratio greater than 25:1).

†Full grown oöcytes from a female that had laid eggs three days previously.



periments has been characterized on CsCl isopycnic gradients, and it is found that the product behaves as if similar in base composition to the injected denatured DNA and it includes both radioactive native and denatured types of DNA (Fig. 1). The reason why this denatured component was not observed previously (Gurdon, Birnstiel and Speight, 1969) was probably that a different extraction and purification procedure was used.

Since we know the precursor pool sizes in these cells (see next section) it is possible to calculate the mass of DNA synthesized and hence the proportion of the injected DNA which is replicated (Table 2). It can be seen that in respect to denatured DNA-dependent synthesis, eggs and oöcytes are comparable. The capacity of eggs to replicate both denatured and native DNA falls off quite markedly as the amount of injected DNA is increased.

The results outlined so far show that the natural replication of DNA, characteristic of eggs but not oöcytes, can be copied by injecting nuclei, and also by injecting purified native DNA. Clearly it is now of interest to know if a cell-free system can be prepared with the same properties as the living cell. Initially the aim has been to move in small steps from *in vivo* conditions, and therefore the DNA synthetic

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Figure 1. (Opposite page.) CsCl gradient centrifugation of DNA synthesized after injection of DNA and labeled precursor into oöcytes and eggs. DNA was extracted as described elsewhere (Ford, C. C. and Woodland, H. R., in preparation). Native and denatured calf thymus DNA were added as optical density markers. Samples were dissolved in 1/10 SSC and CsCl added to give a final refractive index of 1.400 and a final volume of 5.0 ml. These were centrifuged for 94 hr at 43,000 rpm in an MSE 10 x 10 ml angle rotor. Fractions were collected by downward delivery and, after refractive index and absorbance measurements were taken, the fractions were precipitated with carrier DNA in 5% TCA. Precipitates were collected on Millipore filters, washed, dried and counted in liquid scintillant.

TABLE 2

Percent of template DNA replicated *in vivo* and *in vitro*

Assay	Type of cell	ng Injected per cell or added per cell extracted	% Replication: Native DNA template	% Replication: Denatured DNA template
	Oöcyte	30	0	3.3
*In vivo**	egg	30	0.6	5.0
	egg	2	3.4	7.7
	egg	0.2	10.0	12.3
*In vitro***	Oöcyte	1000	0.025	0.611
	egg	1000	0.161	1.52

*DNA injection, extraction, and estimation of total counts per minute were as Table 1. Pool sizes were taken from values in Table 4. Values for control samples have been subtracted.

**Extracts were prepared and assays performed as described elsewhere (Ford, C. C. and Pestell, R. Q. W., in preparation). Control values have been subtracted. Incorporation into DNA was measured as acid insoluble counts (shown to be 97% sensitive to DNase). Apart from this, percent replication values were calculated in the same way as the *in vivo* values. ^{3}H dTTP was assumed to have remained 100% phosphorylated.

characteristics of crude extracts of eggs (Ford, C. C. amd Pestell, R. Q. W., in preparation) and oöcytes (Ford, C. C., unpublished data) have been investigated. as one might expect, the egg extract shows incorporation of deoxynucleotide triphosphates into DNA, dependent both on the presence of DNA and the four deoxynucleotide triphosphates, two of the criteria normally applied to identify DNA polymerases *in vitro*. Incorporation is stimulated both by native and de-

natured DNA, but as in many such extracts (Keir, 1965) the latter is much more effective (Table 3). This re-

TABLE 3

DNA polymerase activity in oocytes and eggs, assayed *in vitro*

Source of extract	pMoles dTMP/μg protein per 20 min/10 μg DNA: Native DNA	pMoles dTMP/μg protein per 20 min/10 μg DNA: Denatured DNA	Denatured / native
Oöcyte	25	812	32.5
Egg	60 ± 23	388 ± 203	6.5

The procedures used were the same as for Table 2, except that the values are derived from 20 min incubations. Denatured:native ratios calculated from Table 2 differ from those shown in this table because denatured DNA-primed synthesis is not linear over 1 hr, whereas native-primed synthesis is linear. The large standard error for egg extracts may reflect variability between eggs of different females.

sult correlates well with that obtained *in vivo* at fairly high DNA concentrations (Tables 1 and 2). In preliminary studies the same procedures have been used to study oöcyte extracts and similar results were obtained, except that the preference for denatured DNA was even higher (33:1, Table 3). In oöcytes the stimulation of incorporation by native DNA is so low compared to that by denatured DNA that it probably does not represent synthesis on a native template. It is more likely that the native DNA contained or acquired sufficient nicks to have allowed a denatured DNA-dependent activity to have produced this amount of synthesis. The denatured DNA-dependent activity is present in both oöcytes and eggs in roughly similar amounts (Tables 2 and 3). This high denatured DNA-dependent activity of both oöcyte and egg extracts, contrasted with high native DNA-dependent activity in eggs but not oöcytes, parallels the results obtained with living cells (Table 1). The results obtained *in vitro*, although of a preliminary nature, are therefore in agreement with those obtained *in vivo*.

The value of the assay systems which we have outlined depends on their accurately producing the pattern of DNA synthesis observed in normal cells. For the following reasons we believe this is true for cells injected with DNA.

1. A similar amount of DNA synthesis takes place if the same quantity of DNA is injected in the form of intact nuclei or as purified DNA (Gurdon, Birnstiel and Speight, 1969). If only one nucleus is injected the DNA synthesis observed enables nuclear division and development to proceed in a normal fashion. The implication is that pure DNA and whole nuclei behave similarly as regards the amount of synthesis they stimulate.
2. When native DNA (but not denatured) is injected into eggs there is a lag of about 20 min before a stimulation of DNA synthesis is observed (Gurdon, Birnstiel and Speight, 1969). This lag correlates well with the observation that the normal egg and sperm nuclei begin to synthesize DNA 20 min after fertilization (Graham, 1966).
3. Native DNA, in contrast to denatured DNA, does not stimulate DNA synthesis in oöcytes (Ford, C. C. and Woodland, H. R., in preparation). This observation fits well with the absence of DNA synthesis in normal oöcyte nuclei and in nuclei injected into oöcytes (Gurdon, 1967).

It therefore seems a reasonable hypothesis that the microinjection assay for DNA synthesis gives results which are relevant to the control processes which operate in normal cells. Since the *in vitro* assays give rather similar results with native DNA, it seems possible that they also reflect events which happen in normal nuclei. The significance of the results using denatured DNA is not immediately obvious, but it seems that the synthesis dependent on this type of molecule

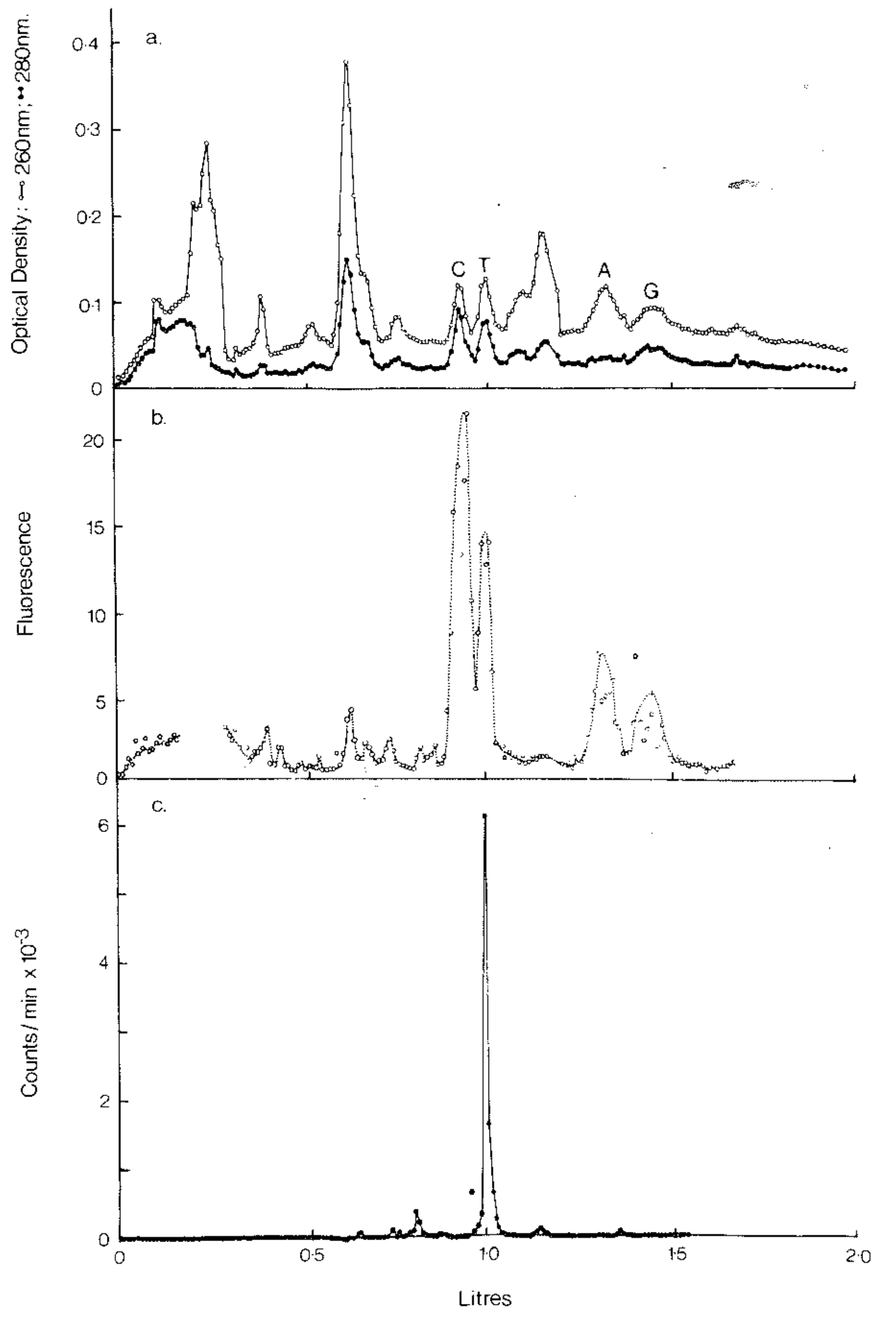
is regulated in a different way from that which governs the replication of normal nuclei. Again this conclusion is supported by both *in vivo* and *in vitro* assays. It therefore seems likely that both the living and the test tube assays will prove useful in studying the control of DNA synthesis.

DNA Precursors

The ability of a cell to support DNA synthesis relies on its containing the four common deoxynucleotide triphosphates. These molecules are therefore capable in principle of controlling DNA synthesis. We have estimated the amounts of these molecules in eggs and oöcytes both by a conventional type of method and by an *in vivo* assay.

The total deoxynucleotide triphosphate content of eggs and oöcytes has been determined by Woodland and Pestell (in preparation). The procedure used is summarized in the legend to Figure 2. It can be seen from this figure that eggs yield four major peaks of deoxyribose-containing material. One co-elutes with ^{3}H-dTTP, the others are in the positions expected of the other three common deoxynucleotide triphosphates and contain the appropriate bases. The data presented in Table 4 indicate that there are similar amounts of each in the unfertilized egg. These amounts are sufficient to enable the synthesis of about 2,500 nuclei. If a similar estimation is carried out on oöcytes, somewhat less pyrimidine and no purine deoxynucleotide triophosphates are detected (Table 4). Although one can never prove a substance to be absent, at face value this result suggests that precursors might play some role in the regulation of DNA synthesis.

We have already described experiments involving the injection of denatured DNA, which indicated that this conclusion is not justified, for oöcytes are able to support DNA synthesis stimulated by denatured DNA (Tables 1 and 2). It is believed that the newly synthesized product has the same base composition as the denatured injected DNA (see above), and therefore it must represent the incorporation of the three deoxynucleotide triphosphates other than the radioactive



one. Since the same result is obtained with ^{3}H-dATP and ^{3}H-thymidine as precursors, all four deoxynucleoside triphosphates seem to be present, and they are not rate limiting, for the injection of further deoxynucleoside triphosphates does not raise the amount of DNA synthesis observed (Ford, C. C. and Woodland, H. R., in preparation). The amount of incorporation into DNA in these experiments indicates that oocytes contain enough DNA precursors to synthesize at least 125-150 diploid nuclei. This amount of purine triphosphate would have been less than that detectable by the chemical methods of estimation employed.

It might be that enzymes other than those which operate on denatured DNA need much higher levels of precursors than the enzyme which replicated denatured DNA. But it is found that injecting further precursors fails to stimulate both native DNA-dependent (Ford, C. C. and Woodland, H. R., in preparation) and nuclear (Woodland, H. R. and Gurdon, J. B., unpublished data) DNA synthesis.

The experiments using living cells therefore argue strongly against a regulatory role of DNA precursors

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Figure 2. (Opposite page.) Dowex-1 chromatography of an extract of 24,000 unfertilized eggs. The extract was first treated with periodate and methylamine, a process which destroys all ribonucleotides with unsubstituted 2'- and 3'-OH groups (Pestell, R. Q. W. and Woodland, H. R., in preparation). The degradation products were then washed through the column with water. This part of the elution profile is not shown. Nucleotides were separated by eluting with a 2 liter linear gradient from 0-1.0 M NH_4HCO_3. (a) UV absorption at 260 nm (○) and 280 nm (●); the positions of dCTP, dTTP, dATP and dGTP are indicated by C, T, A and G respectively. (b) Fluorescence in arbitrary units resulting from the deoxyribose assay of Kissane and Robins (1958), preceded by bromination to labilize the pyrimidine glycosidic bond. (c) Radioactive counts per min of 0.5 ml aliquots from each fraction, derived from ^{3}H-dTTP added to the original mixture in order to estimate deoxynucleotide recoveries (from Pestell, R. Q. W. and Woodland, H. R., in preparation).

TABLE 4

Deoxynucleotide content of eggs and oöcytes

Type of cell	Method of estimation	pMoles/cell			
		dATP	dCTP	dGTP	dTTP
Eggs*	Absorbance at 260 nm	13	19	12	12
	Fluorescence	13	16	11	9
Oöcyte** Expt. A	Fluorescence	<2	8	<1	7
Oöcyte† Expt. B	Fluorescence	<1	2	<1	7

In the experiment with eggs there was sufficient UV absorbance to calculate the content of the deoxynucleotide peaks both by UV absorption as well as by fluorescence. This was not possible in the experiments with oöcytes. (From Woodland, H. R. and Pestell, R. Q. W., in preparation.)

*These results are from the chromatography of an extract of 24,000 unfertilized eggs as shown in Figure 1.

**These results are from the chromatography of an extract of 18,900 large oöcytes taken from two female frogs 3 days after they laid eggs.

†These results are from the chromatography of 12,300 large oöcytes taken from one frog a week after it laid eggs.

in the appearance of the cytoplasmic state which induces DNA synthesis when oöcytes mature to eggs. They also show how microinjection can provide a valuable assay for the availability of substances under the conditions which exist inside normal cells.

RNA SYNTHESIS

RNA Synthesis in Normal Eggs and Oöcytes

Oögenesis is a phase of development involving intense RNA synthesis, in particular rRNA, but also of the other main classes of RNA (Brown, 1967; Gurdon, 1968b; Davidson, 1968). Towards the end of oögenesis the rate of rRNA synthesis is apparently reduced (Crippa, 1970), although quite active RNA synthesis by the nucleoli may be detected by autoradiographic (Gurdon, 1968a; Smith and Ecker, 1970) and biochemical methods. In contrast, the egg makes only minute amounts of RNA, none of which may be detected in the nucleus (Gurdon and Woodland, 1969). Oöcytes and eggs are therefore favorable types of cells for the study of RNA synthesis, in just the same way as they are for the study of DNA synthesis.

RNA Synthesis Studied by Microinjection into Living Cells

Many of the experiments designed to study DNA synthesis in oöcytes and eggs have their exact parallel in the study of RNA synthesis. Thus, we began our study by injecting nuclei of various sorts into these cells. This type of work has shown that in eggs and oöcytes, injected nuclei conform to the activities of the endogenous nuclei, no matter what they did before injection (Gurdon and Woodland, 1968; Gurdon and Woodland, 1970). For example, blastula nuclei, which lack nucleoli and do not synthesize RNA at a detectable rate, are induced to synthesize RNA in oöcytes, and to form typical nucleoli at the same time. On the other hand, neurula nuclei, which are intensely active in RNA synthesis, make no RNA in eggs and their nucleoli vanish (Gurdon, 1968a). These results, which are discussed more fully elsewhere (Gurdon and Woodland, 1968; Gurdon and Woodland, 1970), lead to the general conclusion that the transcription of nuclear DNA is under cytoplasmic control in eggs and oöcytes of *Xenopus*, and that the injected DNA in an intact nucleus is transcribed normally by the host cell.

The interaction between nucleus and cytoplasm is likely to be very complex. Studies involving transcription *in vitro* have indicated that it is the protein component of chromosomes which is responsible for the regulation of genetic activity. We have therefore attempted to establish the feasibility of analyzing transcriptional control in slightly simpler experiments in which purified DNA and nuclear proteins are injected separately.

When purified DNA and ^{3}H-uridine are injected into oöcytes, a stimulation of incorporation is observed, an effect not seen after injection into eggs (Gurdon and Woodland, 1970). This extra incorporation is RNase sensitive and heterogeneous in size (Knowland, Ph.D. thesis). It is not yet certain that it is synthesized on the injected template, but the difference between oöcytes and eggs seems to reflect normal cell function. Preliminary indications are therefore that the injection of DNA into eggs and oöcytes may provide a living assay system for RNA polymerase activity which would prove useful in studying the regulation of transcription. These experiments also seem to indicate that only the DNA component is necessary for the nucleus to make the appropriate response to the cytoplasm in its transcriptional as well as its replicative function.

Of the nuclear proteins, we have investigated the effect only of histones. It appears that in both oocytes and eggs, molecules of this type rapidly accumulate in nuclei, but do not immediately reduce the synthesis of RNA (Gurdon, 1970). This finding is consistent with the inability of added histones to inhibit completely the template function of isolated nucleoli *in vitro* (Liau, Hnilica and Hurlbert, 1965). The indication from *in vitro* experiments is that the nonhistone proteins, possibly with associated RNA, are the important agents of genetic regulation (Bekhor, Kung and Bonner, 1969; Huang and Huang, 1969; Paul, 1970). Although the RNA synthesized *in vitro* on a chromatin template resembles in some respects that made *in vivo* (Paul and Gilmour, 1966; Bekhor, Kung and Bonner, 1969; Huang and Huang, 1969) its identity is not known, and exactly how the events studied *in vitro* bear on those *in vivo* is therefore not clear.

The study of living cells by microinjection provides an opportunity to circumvent this problem, as is well illustrated by the experiments of Crippa (1970) on an inhibitor of rRNA synthesis. These experiments depend on the observation that full-grown oöcytes are less active in rRNA synthesis than immature oöcytes, suggesting that they might contain an inhibitor. Crippa was able to isolate from full-grown oöcytes a protein which bound specifically to rDNA, inhibited rRNA synthesis when injected into growing oöcytes, and which was absent from this latter type of cell. How this protein acts is now known, but it clearly has the properties of a natural rRNA synthesis inhibitor. The great attraction of this agent is that its assay occurs in the environment of a normal cell, and involves changes of genetic activity which are easily identifiable. The study of rRNA synthesis in *Xenopus laevis* presents other advantages, for the ribosomal genes may be purified quite easily, their structure is understood better than any other eukaryote gene, a system for their transcription *in vitro* has already been described (Reeder and Brown, 1970), and the enzyme responsible for their transcription *in vivo* has possibly been isolated (Tocchini-Valentini and Crippa, 1970b).

The study of eukaryote RNA polymerases is at present in a rudimentary state, indeed the investigations of eukaryote chromatin transcription *in vitro* usually utilize prokaryote enzymes. In recent years various factors which form part of bacterial RNA polymerase, and exert a positive control over transcription, have been identified (Burgess *et al.*, 1969). As yet it is not known if such factors exist in eukaryotes, but there is an indication that oöcytes are likely to prove useful in the identification of agents of this type. This comes from an experiment of Tocchini-Valentini and Crippa (1970a) in which the *E. coli* sigma factor was injected into oöcytes. A two-fold stimulation of RNA synthesis was observed. While it is difficult to interpret, this result suggests that oöcytes may be helpful in identifying agents which affect the activity of RNA polymerase.

PROTEIN SYNTHESIS

Protein Synthesis in Normal Eggs and Oöcytes

The characteristics of protein synthesis in embryonic cells of amphibia have been reviewed by Smith and Ecker (1970), but unfortunately our knowledge relates mainly to *Rana pipiens*. In this species the nonhormone stimulated oöcytes seem to be relatively inactive in protein synthesis. The rate of protein synthesis rises several-fold at maturation of the oöcyte, and the same elevated rate is maintained through fertilization and early cleavage (Smith and Ecker, 1969). The proteins synthesized by oöcytes and eggs seem to differ qualitatively, as judged by gel electrophoresis in the presence of SDS. Clearly these changes make the protein synthetic systems of oöcytes and eggs es-especially interesting for study, but as yet it is not known if such changes occur in *Xenopus*. In contrast to those of *Rana*, the oöcytes of *Xenopus* seem to be very active in protein synthesis (Moar *et al*., in press). In various respects the reproductive biology of the two anurans is rather different. The oöcytes of *Rana* grow during the summer and autumn and lie dormant during the winter; the ovary then consists mainly of fully grown oöcytes, which are ovulated and laid in the spring. In contrast the ovary of *Xenopus* seems to contain all sizes of oöcytes at all seasons; laying can be induced at all seasons and can occur at short intervals. In *Rana* the mature ovulated eggs are stored in an ovisac, where they may remain dormant for many days, but in *Xenopus* there is no ovisac and immediately after ovulation the eggs pass down the oviduct and into the external medium. Fertilization therefore always occurs within several hours of ovulation. These differences in the growth of the oöcytes and in events after ovulation make it dangerous to assume *a*

priori that the characteristics of protein synthesis in *Rana* and *Xenopus* are similar.

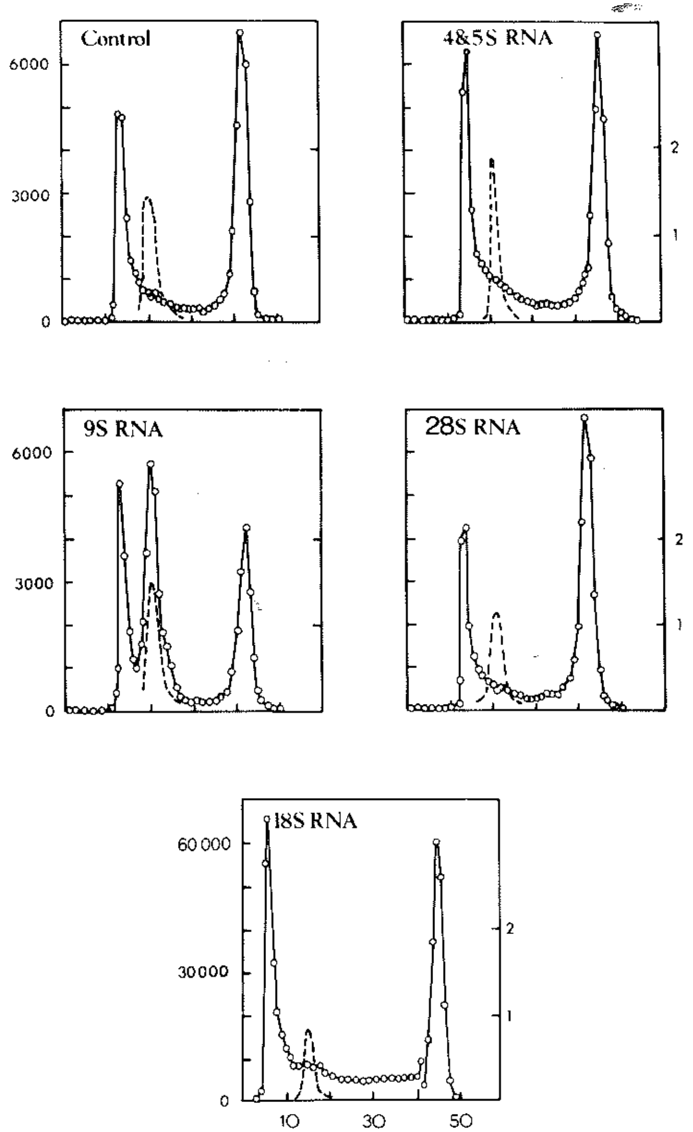
Protein Synthesis Studied in Living Cells by Microinjection

Translation of natural mRNA molecules. Most of our work on natural mRNA has used the putative Hb mRNA, which is the purest RNA of this sort available in large amounts (Chantrenne, Burny and Marbaix, 1967). In the first experiments the main classes of RNA found in rabbit reticulocytes were injected into oöcytes, and as expected only the 9S fraction produced any detectable change in protein synthesis (Lane, Marbaix and Gurdon, in press). This it did by stimulating the synthesis of a ^{3}H-histidine-labeled protein which was characterized as Hb by the following criteria:

1. co-elution with marker Hb on G-100 Sephadex columns (Fig. 3);
2. co-electrophoresis with Hb on acrylamide gels;
3. after removal of haem the dissociated radioactive subunits co-elute with rabbit α- and β-globin chains from CM-cellulose columns;
4. peptides prepared from purified α- and β-chains labeled with ^{3}H-histidine in the frog oöcyte, co-elute from an ion-exchange column with those prepared from globin chains labeled with ^{14}C-histidine (Fig. 4).

The evidence that the 9S RNA of rabbit reticulocytes is the only RNA able to direct Hb synthesis in living cells is therefore fairly conclusive.

Reticulocyte 9S RNA has also been translated successfully *in vitro*. Perhaps the best system so far developed to do this is the reticulocyte lysate described by Lockard and Lingrel (1969). This system is already making haemoglobin at a rapid rate, so it has limitations in the study of translational control. Experiments in which mRNA is translated by the protein synthetic apparatus of another cell have been reported by Heywood (1969; 1970). It was found that myosin mRNA could only be translated by a reconstituted cell-free system of reticulocytes if certain factors were washed off the blood cell ribosomes and replaced by similar factors from the ribosomes of muscle cells. Two results obtained *in vitro* which seem to differ from these are that Hb mRNA can be translated by an ascites cell-free system (Mathews, in press), and that a putative immunoglobulin mRNA from myeloma cells can



be translated by the reticulocyte lysate (Stavnezer and Huang, 1971), in both cases with no added ribosome wash. These results may be abnormal because both the ascites cell-free system and the myeloma RNA are derived from cancerous cells, but they agree with the ability of oocytes and eggs to translate both an Hb mRNA and the immunoglobulin mRNA (Gurdon *et al.*, in press). It may be that this catholic taste for mRNAs displayed by oöcytes and eggs reflects a difference between normal differentiated cells and undifferentiated or cancerous cells. One explanation of the difference between Heywood's results and ours may be that oöcytes and eggs contain Hb mRNA-specific initiation factors. If this were so, they can only be of primary importance in red blood cell differentiation if they are localized in some particular region of the egg.

In order to compare the various systems for mRNA translation in a meaningful way, it is of obvious importance to know how efficient they are. We have attempted to estimate the rate of Hb synthesis in eggs and oöcytes, but unfortunately this type of measurement presents many problems (Gurdon *et al.*, in press). In order to calculate the specific activity of the radioactive amino acid injected into the cell we were forced to assume that the extractable amino acid pool, measured by amino acid analysis, was the same as the actual pool used for protein synthesis. If this were not so our estimates would be a maximum of six times too high. We were also forced to make assumptions which might have led to our having *under*estimated the

Figure 3. (Opposite page.) Batches of 20 oöcytes were injected with haemin and the RNA indicated at 1000 μg/ml (50 mμg/cell) and incubated in ^{3}H-histidine (1 mCi/ml) for 7 hr. The protein synthesized was analyzed on G-100 Sephadex columns in the presence of marker rabbit Hb. The ordinates are counts/min (left) and absorbance at 415 nm (right). The right hand radioactivity peak is reduced x 10^{-3} in the 18S RNA result. The abscissae represent fraction number. Only the 9S RNA produces a significant stimulation of Hb synthesis. (--), absorption at 415 nm; (●), counts/min (redrawn from Lane, Marbaix and Gurdon, in press).

rate of synthesis, per injected mRNA molecule, by about two-fold (Gurdon *et al.*, in press). The efficiency of translation of Hb mRNA, calculated on the basis of these assumptions, is presented in Table 5, together with data from other systems for comparison. It can be seen that each injected mRNA is translated several times per hour, and since synthesis continues for over a day, each must have been translated several hundred times in total. The table also shows that the translation of Hb mRNA in living cells is more rapid

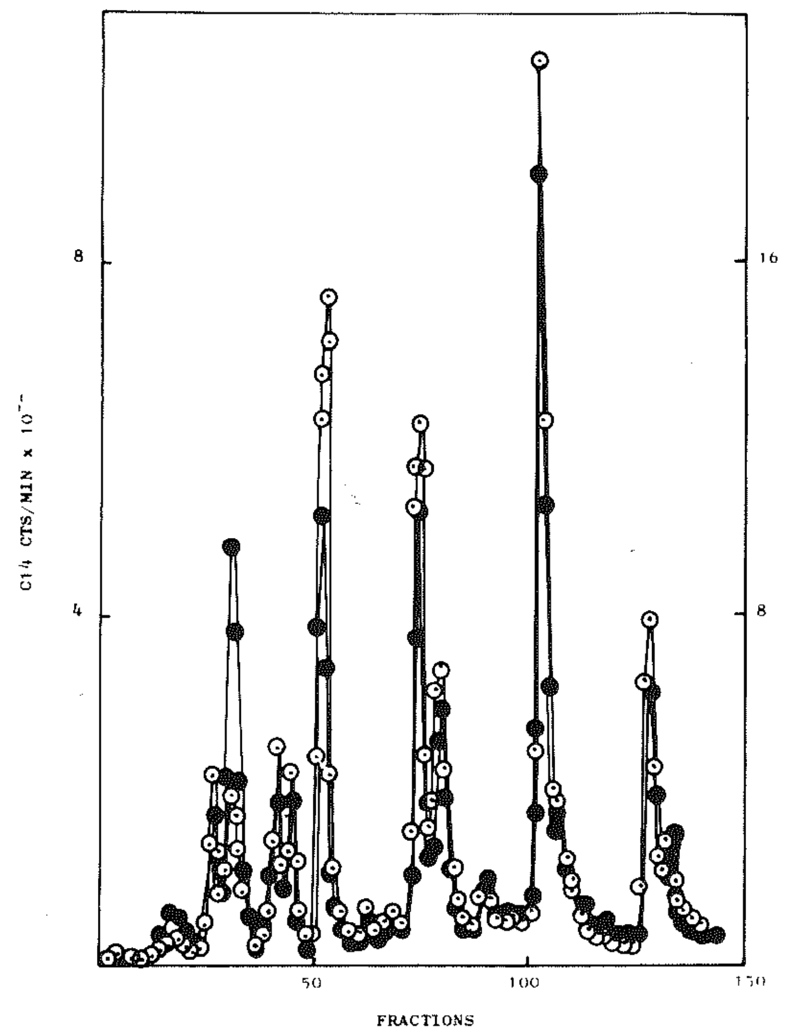


TABLE 5

Efficiency of Hb mRNA translation in various systems

Translational systems	Temperature of incubation (°C)	# of globin chains synthesized/mRNA molecule/hr	Half-life of translational system
Cell-free system (lysate using added mRNA)*	25	0.8	1 hr
Rabbit reticulocytes in culture (using endogenous mRNA)**	20	80	2-3 hr (at 37°)†
Injected oocytes	19	24††	26 hr

*Calculated from data from Lockard and Lingrel (1969).

**Hunt, Hunter and Munro, 1969.

†Armentrout, Schinkel and Simmons, 1965.

††This high value is obtained by the injection of only 0.01 ng of Hb mRNA into the cell. Lower values may be obtained when more is injected.

than in the best cell-free system available, and it compares favorably with that observed in intact retic-

Figure 4. (Opposite page.) Peptide analysis of a tryptic digest of purified β-chains synthesized in oöcytes of frogs and labeled with ^{3}H-histidine (○), and of purified β-chains synthesized in intact rabbit reticulocytes labeled with ^{14}C-histidine (●). The synthesis of β-chains in oöcytes was stimulated by rabbit Hb mRNA injection. The separation is achieved on a 75 x 0.6 cm Technicon type P Chromobead column. Similar results are obtained when the purified α-chains are analyzed in this way (Lane, C. D. and Marbaix, G., in preparation).

ulocytes. This high efficiency of translation, coupled with the long life of Hb mRNA in this cell, gives the oöcytes the peculiar advantage of great sensitivity in the assay of mRNA; quantitites of mRNA in the ng-pg range may readily be identified (Lane, Marbaix and Gurdon, in press). The high efficiency also has an advantage in the study of the translation process, because data derived from systems working at sub-optimal rates may be misleading.

One respect in which the behavior of reticulocyte 9S RNA differs in the test tube from its behavior in living cells is that in the former it generally lowers the overall rate of protein synthesis (J. Lingrel, personal communication), whereas in eggs and oöcytes the rate is stimulated (Moar *et al.*, in press). This stimulation may be as much as 100%, the endogenous proteins being made at the same rate as in controls, and the extra being Hb. It therefore seems likely that the inhibition *in vitro* represents an interaction of an abnormal type, produced by some nonphysiological characteristic of the cell-free system. It is not yet known if the inhibiting agent is the mRNA itself or some untranslated component of the RNA preparation added.

The stimulation of the overall rate of protein synthesis by injected mRNA *in vivo* provides us with information concerning the living cell. It suggests that the amount of protein made in the unmanipulated cell may be limited by the availability of mRNA. Injecting increasing amounts of Hb mRNA has revealed that the translational capacity of the cell is not unlimited, and that it becomes saturated by about 10 ng Hb mRNA per cell (Fig. 5). The cellular component which is limiting is not yet known. These experiments also reveal the surprising fact that the injected message and the endogenous message do not compete for the components limiting translation (except at extremely high mRNA concentrations). This is evident from Figure 5, for the ratio of synthesis on endogenous mRNAs to that on Hb mRNA reaches a plateau, and it is known that the endogenous incorporation is not decreased at these mRNA inputs. The reason for this lack of competition is a matter for conjecture at present, but

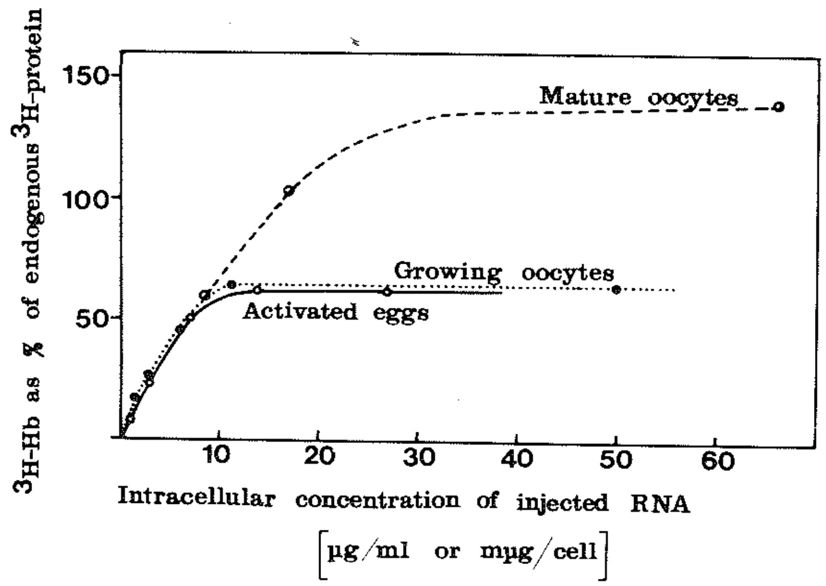


Figure 5. The effect of injecting increasing amounts of Hb mRNA into oöcytes and eggs. The cells were incubated in ^{3}H-histidine for 10 hr and the proteins synthesized were analyzed as described in the legend to Figure 3. It is not yet certain that the growing and mature oöcytes always differ by the amount shown here. "Endogenous" protein is that made on endogenous mRNAs (Moar *et al.*, in press).

the experiment is especially important for the purpose of this article in that it shows that the use of living cells can reveal important phenomena which have not been observed in cell-free systems. It is in revealing phenomena of this type and enabling their further study that injected eggs and oöcytes are likely to be of advantage in the study of the control of translation.

Effects of artificial RNA molecules in living cells. The second problem that we have investigated relates to the characteristics of polyribonucleotides which confer upon them the ability to be recognized as a message. In prokaryotes, genetic evidence and work using cell-free systems supports the view that the translated part of mRNA molecules begins ApUpGp, or GpUpGp (Clark and Marcker, 1968; Bretscher, 1969). A similar conclusion has been reached regarding eukaryotes through the use of mammalian cell-free systems, although the evidence of GpUpGp as an initiator is much weaker than that for ApUpGp (Smith and Marcker, 1970; Brown and Smith, 1970). We have attempted to find whether these conclusions are also valid for living cells (Woodland, H. R. and Ayres, S. E., in preparation). Polyribonucleotides were injected into eggs or oöcytes, together with the appropriate radioactive amino acids. As might be expected, polymers which did not begin with an initiator codon did not stimulate the incorporation of the appropriate amino acid (Table 6): e.g., (Up)n did not stimulate phenylalanine incorporation, nor did (Ap)n stimulate lysine incorporation, and homopolymers of the amino acids coded by these polymers could not be detected. (Up)n might fail to produce an effect because it is rapidly degraded, but this seems not to be the reason because its destruction has been shown to be very slow in oöcytes and eggs. Much more surprising is the result obtained with ApUpGp$(Up)_{\bar{n}}$, which is the best known artificial mRNA in the test tube (Brown and Smith, 1970). It is found that this molecule is an extremely effective inhibitor of protein synthesis, even when small amounts are injected (Table 6). The small amount of residual phenylalanine incorporation does not include detectable amounts of polyphenylalanine synthesis. Unless polyphenylalanine is completely degraded immediately as it is synthesized, ApUpGp$(Up)_{\bar{n}}$ does not therefore seem to be acting as a message. In addition, its inhibitory effect on endogenous protein synthesis contrasts markedly with the behavior of natural mRNA, which fails to compete with endogenous mRNA. Table 6 also shows that GpUpGp$(Up)_{\bar{n}}$ has neither a marked stimulatory nor an inhibitory effect. There is therefore no indication that it is acting as an mRNA, although its effect is clearly different from that of ApUpGp$(Up)_{\bar{n}}$.

TABLE 6

The effects of various polynucleotides on protein synthesis in unfertilized eggs

Sample injected	Inhibition produced with ^{3}H-Phe as precursor (%)	Inhibition produced with ^{3}H-lys as precursor (%)
ApUpGp$(Up)_{\bar{n}}$ 12.5 ng/cell	95	99
ApUpGp$(Up)_{\bar{n}}$ 1.25 ng/cell	98	99
(Up)n 12.5 ng/cell	32	-5
(Up)n 1.25 ng/cell	-14	6
GpUpGp$(Up)_{\bar{n}}$ 10 ng/cell	6	-6
GpUpGp$(Up)_{\bar{n}}$ 1 ng/cell	32	5
ApUpGp 10 ng/cell	24	7

The percentage inhibition is computed from the ratio of acid insoluble to total radioactivity in samples of 10-15 eggs injected with polymers, as compared with those injected with radioactive amino acids alone. In small samples of cells such ratios are very variable, even in controls. An inhibition or stimulation of as much as 30% is therefore not significant. (Woodland, H. R. and Ayers, S. E., in preparation.)

The the results obtained from living cells are quite different from those predicted from experiments conducted *in vitro*, and they provide another illustration of the advantages of using a system which is altered from the normal cell as little as possible. The reason that living cells and test-tube systems do not behave in the same way is obscure at present, but small differences in the way in which bacterial ribosomes react with natural mRNA and with ApUpGp have been reported (Revel, Herzberg and Greenspahn, 1969; Brawerman, 1969). It is known that these bacterial virus messages have ApUpGp initiators within the RNA molecule, so it seems that other nucleotide sequences are important in the initiation of protein synthesis. Our results are consistent with this interpretation being applied to eukaryotes as well as prokaryotes. Since living cells seem to show more stringent initiation requirements than existing cell-free systems they would seem peculiarly suitable for the further investigation of this problem.

CONCLUSIONS

In this paper we have described some experiments designed to study DNA, RNA and protein synthesis in living oöcytes and eggs of amphibia. Even though most of the lines of investigation followed are in their preliminary stages, they indicate that this type of approach will prove of some value. It should assist in the development of cell-free systems which parallel as accurately as possible phenomena occurring in living organisms. Cell-free systems should be especially useful in studying the detailed mechanism of processes occurring in living cells, whereas the use of oöcytes and eggs may have a direct application in the study of regulatory phenomena.

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