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Translation and stability of ovalbumin messenger RNA injected into growing oocytes and fertilized ova of mice

By KARL M. EBERT[1], BARBARA V. PAYNTON[1], G. STANLEY MCKNIGHT[2], AND RALPH L. BRINSTER[1]

[1]*Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.*
[2]*University of Washington, Department of Pharmacology, D424 Health Sciences Bldg., SK30, Seattle, Washington 98195, U.S.A.*

SUMMARY

Growing mouse oocytes and fertilized ova were injected with chicken ovalbumin messenger RNA ($mRNA_{ov}$) and chicken conalbumin mRNA ($mRNA_{con}$) and cultured *in vitro*. Estimation of $mRNA_{ov}$ and $mRNA_{con}$ stability by hybridization of $cDNA_{ov}$ and $cDNA_{con}$ to extracted mRNA from injected oocytes and fertilized ova indicated a half-life of 147 and 366 h in the oocyte and 5 and 3 h in the fertilized ovum respectively. Stability of $mRNA_{ov}$ was similar in the fertilized and unfertilized ovum. Oocytes injected with chicken ovalbumin mRNA were also labelled with [^{3}H]leucine and ovalbumin synthesis was measured by immunoprecipitation. The amount of ovalbumin synthesized during the initial 7 h was less than during the period of 18–25 or 66–73 h postinjection. The greatest percentage of ovalbumin to total protein synthesis occurred between 66–73 h. Oocytes secreted 12% of the synthesized ovalbumin during each of the 7 h periods (0–7, 18–25 and 66–73 h) indicating a stable mechanism for secretion throughout the culture period. These studies demonstrate: (1) a dramatic difference in stability of injected mRNA between the growing oocyte and the unfertilized or fertilized ovum, and (2) a gradual increase in the translation of injected mRNA by the growing oocyte during *in vitro* culture.

INTRODUCTION

Mouse oocytes from 13-day-old females are unable to grow and mature *in vitro* in the absence of follicular cells (Szybek, 1972; Eppig, 1977; Baran & Bachvarova, 1977). This inability to undergo maturation is thought to involve the lack of sufficient influence from the surrounding follicle cells (via gap junctions) during the growth phase of development (Eppig, 1977). However, during this period the growing oocyte is transcriptionally active and can synthesize detectable quantities of mRNA (Bachvarova; 1981: Brower, Gizang, Boreen & Schultz, 1981). The RNA appears to be extremely stable (Brower, *et al.* 1981; Bachvarova, 1981) and different developmental stages show progressive changes in proteins synthesized (Schultz & Wassarman, 1977; Moor, Osborn, Cran & Walters, 1981; Richter & McGuaghey, 1981;

Kaplan, Abreu & Bachvarova, 1982). The changes in protein synthesis may result from the activation of stored mRNAs during development as suggested by Braude, Pelham, Flach and Lobatto (1979) and supported by the studies on *in vitro* translation of oocyte mRNA (Cascio & Wassarman, 1982). Little is known of this activation of stored mRNA.

In order to investigate the activation process, we have employed direct injection of mRNAs (Brinster, Chen, Trumbauer & Avarbock, 1980; Brinster, Chen, Trumbauer & Payton, 1981; Paynton, Ebert & Brinster, 1983; Ebert & Brinster, 1983) and measured their subsequent translation and decay. Significant differences were found between the oocytes and fertilized egg in mRNA stability; however, the translation of foreign mRNA appeared similar.

MATERIALS AND METHODS

Oocyte collection and mRNA injection

Growing oocytes were collected from 13-day-old C57 × SJL hybrid females. Ovaries were excised, washed in 2 ml of sterile phosphate-buffered saline (PBS) and agitated in an enzyme solution consisting of 0·02% DNase (Sigma; Type III, from Bovine Pancreas), 0·10% collagenase III (Millipore) and 0·10% trypsin (Sigma) in Hanks Balanced Salts Solution (Gibco) for 10 min. The oocytes, free of follicle cells, were washed three times in 2 ml of BMOC-2 medium (Brinster, 1972) followed by a wash in 2 ml of growing oocyte medium. BMOC-2 medium was modified to contain 89 mM-NaCl and 5 mg/ml of bovine serum albumin. Growing oocyte medium consisted of modified BMOC-2 medium supplemented with 5·6 mM-glucose, Basal Medium Eagle essential amino acid solution and Minimum Essential Medium non-essential amino acid solution (Gibco) without leucine plus 10% foetal calf serum (heat inactivated and dialysed against PBS).

Fertilized 1-cell ova were collected from C57 × SJL hybrid females as previously described (Ebert & Brinster, 1983).

Chicken ovalbumin mRNA was partially purified by oligo dt-cellulose chromatography and sucrose gradients (McKnight, 1978). The preparation used for injection was 35% pure as judged by specific cDNA hybridization; the majority of contaminants were ribosomal RNA. This material was injected at an RNA concentration of 9·34 pg/pl. Conalbumin mRNA ($mRNA_{con}$) was purified by immunoprecipitation of polysomes and size selection on sucrose gradients (McKnight, 1978) and was injected at a concentration of 0·68 pg/pl.

Oocytes were injected as described by Brinster and coworkers (1980). An average injected volume of 3·4 pl/oocyte and 7·9 pl/ovum was determined by injection of ^{3}H-GTP. Approximately 90% of the oocytes and 45% of the fertilized ova survived injection. The reason for this difference in survivability is not known but may reflect differences in the structural matrix of the

cytoskeleton and/or the difference in resiliency of the plasma membrane of the two cell types following injection.

Determination of $mRNA_{ov}$ and $mRNA_{con}$ half-lives

Following injection with $mRNA_{ov}$ or $mRNA_{con}$ growing oocytes were cultured for 0, 25 and 73 h and unfertilized and fertilized ova were cultured for 0, 4 and 8 h. Cells were lysed in 25 μl of 1 × SET buffer (1% SDS, 5 mM-EDTA, 10 mM-Tris, pH 7·5) containing 100 μg/ml proteinase K (Beckman). The concentration of the respective mRNA in each sample of cells was determined by cDNA hybridization to aliquots of lysed cells in SET buffer. The percent hybridization was converted to molecules of $mRNA_{ov}$ and $mRNA_{con}$ by comparison to standard curves generated with pure $mRNA_{ov}$ and $mRNA_{con}$ as previously described (McKnight, 1978). The relative molecular mass of $mRNA_{ov}$ and $mRNA_{con}$ was assumed to be $6{\cdot}4 \times 10^5$ and $7{\cdot}9 \times 10^5$ respectively.

Ovalbumin measurements

Oocytes were cultured for up to 73 h *in vitro*. Incubation in the presence of 3 μCi/μl (57·7 nmole) of the [^{3}H]leucine (TRK-510; Amersham) was for 7 h periods corresponding to 0–7, 18–25 and 66–73 h after message injection. Labelling periods of 7 h were necessary to generate enough of a signal to be accurately detected by immunoprecipitation with the limited number of eggs that could be injected per data point. Approximately 50 oocytes were labelled for each data point, washed twice in 2 ml of culture medium and stored −70°C. The cells were solubilized in 100 μl of 0·5% Triton X-100 in PBS and frozen and thawed five times. Samples were centrifuged at 18,000 ***g*** for 30 min and 10 μl aliquots of the supernatant were used to determine the incorporation of [^{3}H]leucine into acid precipitable proteins. Immunoprecipitation of ovalbumin was performed as previously described (Paynton *et al.*, 1983). Each experimental group of cells had a corresponding control group which was treated identically in all experimental phases except injection. Incorporation of [^{3}H]leucine into ovalbumin was calculated by comparing the radioactivity in injected and control immunoprecipitates.

RESULTS

Incorporation of [^{3}H]leucine into acid-insoluble proteins

To study accurately the translation of an injected mRNA throughout a 73 h culture period, total protein synthesis should be unaffected by the culture conditions. Therefore, initial experiments were designed to study total protein synthesis in denuded mouse oocytes cultured for 73 h. Figure 1 shows total [^{3}H]leucine incorporation into acid-insoluble proteins during three different

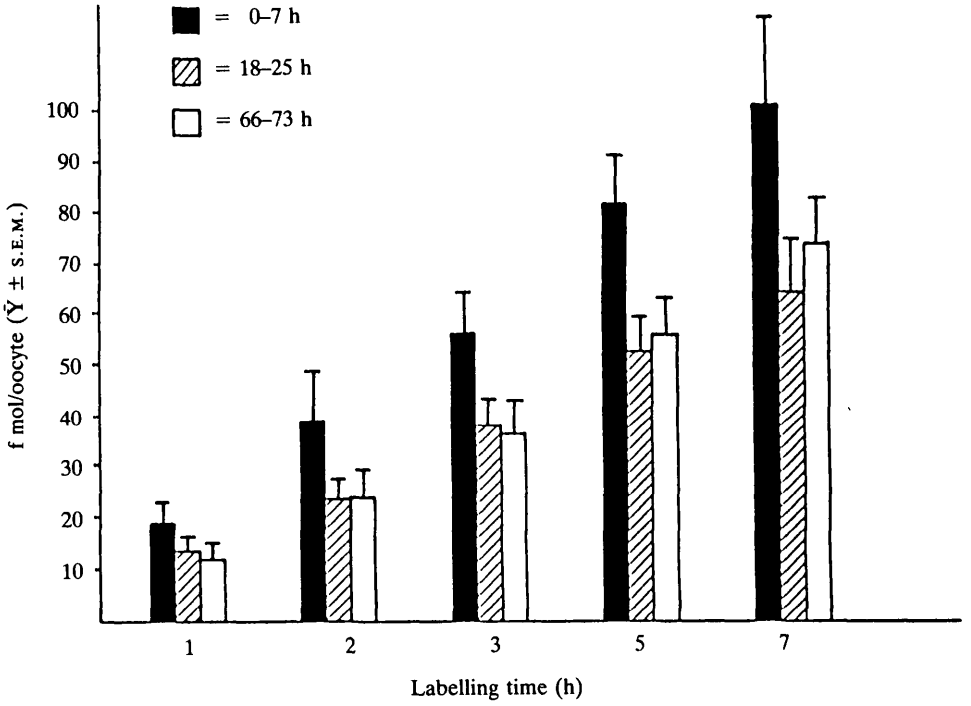


Fig. 1. Incorporation of [^{3}H]leucine into total proteins in the presence of 2·3 μM[^{3}H]leucine as a function of time in growing oocytes from 13-day-old ovaries. Each bar represents a mean ± S.E.M. of three separate experiments. Incorporation during the 0–7 h was significantly ($P<0·001$) higher than during either of the 7 h periods from 18–25 or 66–73 h. There was no change in leucine incorporation between 18–25 and 66–73 h.

7 h periods, i.e. 0–7, 18–25 and 66–73 h of culture. Leucine incorporation was significantly higher ($P<0·001$) during the initial 7 h (101 fmol/oocyte) than in either of the 7 h periods from 18–25 (64 fmol/oocyte) or 66–73 h (74 fmol/oocyte). There was no change in leucine incorporation between 18–25 and 66–73 h. All groups showed a tendency for incorporation rates per h to decrease as the labelling time increased.

Incorporation of [^{3}H]leucine into ovalbumin

Ovalbumin synthesis was calculated as a percentage of total leucine incorporation to reflect the ratio of ovalbumin mRNA to total mRNA translation. The injection of $mRNA_{ov}$ into the growing oocyte did not increase total protein synthesis as the total incorporation of [^{3}H]leucine in acid-precipitable radioactivity by control and injected oocytes was 31·0±2·9 and 26·3±0·9, 14·7±2·1 and 13·2±0·9, 11·7±2·4 and 13·1±2·8 d.p.m./oocyte $\times 10^3$ during

0–7, 18–25 and 66–73 h labelling periods respectively. This is similar to a previously reported finding (Paynton *et al.*, 1983).

Ovalbumin synthesis during 0–7, 18–25 and 66–73 h after the injection of $mRNA_{ov}$ increased from 216 to 403 and 613 d.p.m./oocyte respectively. As shown in Figure 2 the amount of ovalbumin represents 0·82, 3·01 and 4·62% of the total protein synthesis with all percentages being significantly different ($P<0·01$; t-test).

To determine whether the increase in ovalbumin synthesis was due to an increase in the ability of the oocyte to translate a foreign mRNA the longer the oocyte was in culture, oocytes were injected with $mRNA_{ov}$ at 18 and 66 h in culture and labelled for 7 h. The results indicate that oocytes can increase the amount of ovalbumin synthesized during a 7 h period following $mRNA_{ov}$

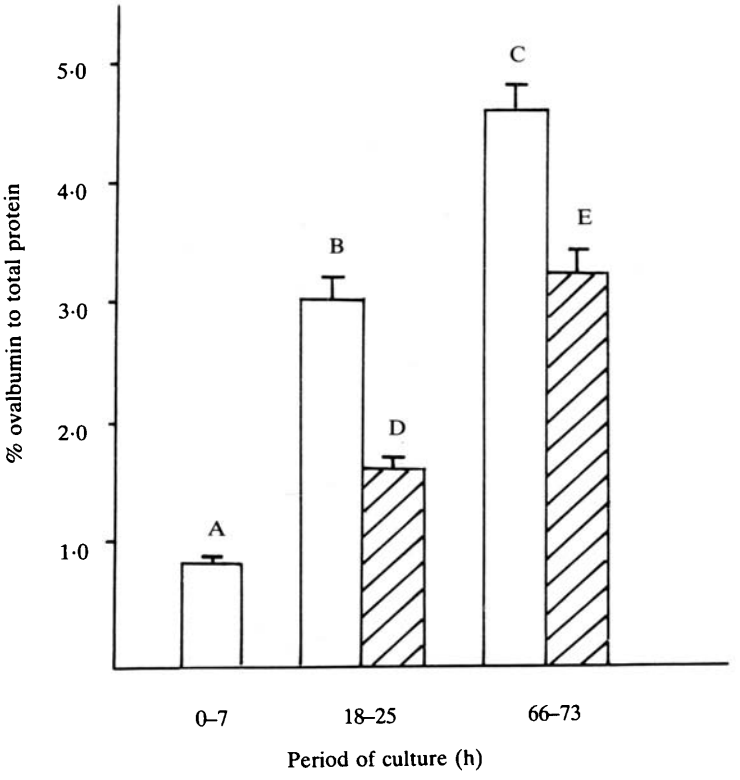


Fig. 2. Ovalbumin synthesis by oocytes from 13-day-old ovaries injected with ovalbumin mRNA as a percent of ovalbumin to total protein synthesis during 0–7, 18–25 and 66–73 h in culture. Approximately 50 oocytes were labelled in the presence of 2·3 μM[^{3}H]leucine and ovalbumin measured by immunoprecipitation. Each bar represents the mean ± S.E.M. for three separate experiments. Solid bars represent oocytes injected at 0 h and labelled at 0, 18 and 66 h for a period of 7 h. Hatched bars represent oocytes injected at 18 and 66 h in culture and labelled for 7 h. Percentages were significantly different from one another with the exception of C–E which had a P value of 0·07 (t-test).

injection the longer the oocytes are exposed to the culture conditions. The amount of ovalbumin synthesized by oocytes injected with $mRNA_{ov}$ at 18 and 66 h in culture represented 1·60 and 3·22% of the total protein synthesis respectively: values that were significantly different from each other ($P<0·05$). This increase in ovalbumin, however, could not account for the total increase in the amount of ovalbumin synthesized by the oocyte when the $mRNA_{ov}$ was injected at 0 h and synthesis determined at 18–25 and 66–73 h postinjection.

The amount of ovalbumin secreted into the medium was approximately 12% of the total amount of ovalbumin synthesized at the three 7 h periods examined (Figure 2) and suggests that the rate of secretion of ovalbumin is constant and that the secretory capacity and machinery of the oocyte is maintained for up to 73 h *in vitro*.

Translation of $mRNA_{ov}$ in growing oocytes and fertilized ova

Differences in total protein synthesis in the growing oocyte and fertilized ovum appear to be related to the size of the cell (Canipari, Pietrolucci & Mangia, 1979). To determine whether there are developmental differences in translation of a specific mRNA, both the fertilized ovum and growing oocyte were injected with $mRNA_{ov}$ and assayed for [^{3}H]leucine incorporation into ovalbumin during an 18 h period. The fertilized ovum and oocyte were injected with 2·7 and 1·2 pg of $mRNA_{ov}$ respectively. Since the average volume of a fertilized ovum is 216 pl and the growing oocyte is 113 pl (average volume for the oocytes injected) the concentration of $mRNA_{ov}$ within the cytoplasm of both cells is nearly equal, i.e. 0·01 pg/pl. The results in Table 1 show that the fertilized ovum incorporated 1044 d.p.m./cell into ovalbumin compared to 545 d.p.m./oocyte and indicate that the fertilized ovum and oocyte synthesize comparable ratios of ovalbumin to total $mRNA_{ov}$ when the concentration of $mRNA_{ov}$ in the cytoplasm is equal. In addition, the rate of secretion of the ovalbumin was identical (29%) suggesting that the rate of secretion of a given protein does not change through this period of development.

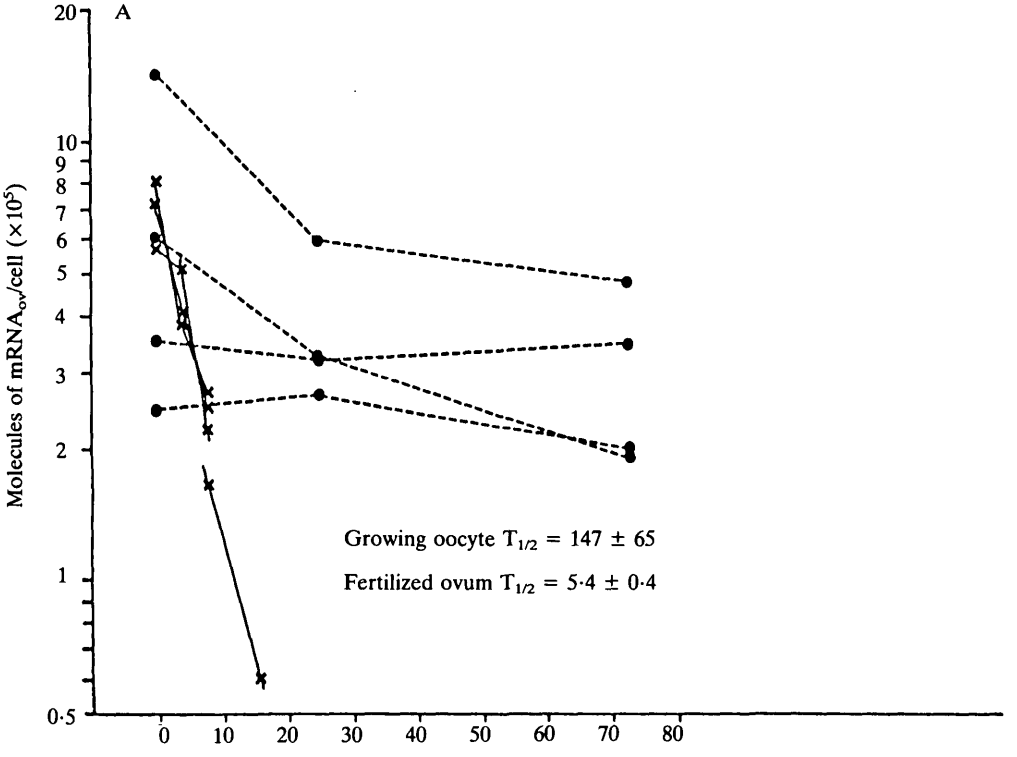
Stability of $mRNA_{ov}$ and $mRNA_{con}$

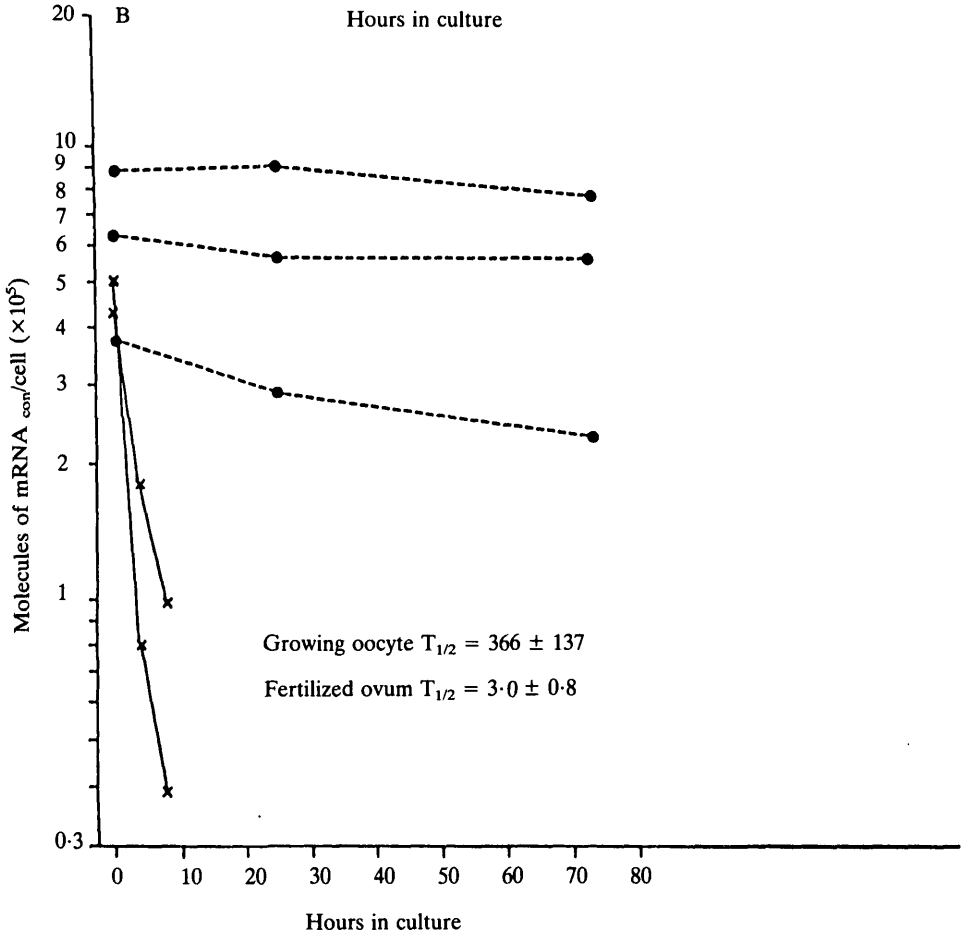
Differences in mRNA stability during the developmental stages of the mouse oocyte may contribute to the lack of need for continuous mRNA production from the mature oocyte to the fertilized ovum (Levy, Stull & Brinster, 1978). To study the stability of a translatable mRNA, oocytes and fertilized ova were injected with $mRNA_{ov}$ or $mRNA_{con}$ and analysed for residual mRNA by its hybridization to their respective cDNA probe. The slopes of the degradation curves in Figure 3A and 3B clearly show a dramatic difference in the half-life of either $mRNA_{ov}$ or $mRNA_{con}$ with an average half-life of 147 h and 366 h for the oocyte and 5·4 h and 3·0 h for the fertilized ovum respectively. This difference was not the result of fertilization since the half-life of $mRNA_{ov}$ in the unfertilized ova was $7·4\pm1·1$ h (n=3).

Table 1. *Incorporation of [^{3}H]leucine into ovalbumin (means ± S.E.M.) by mouse growing oocytes and fertilized ova cultured for 18 h following cytoplasmic injection of ovalbumin mRNA ($mRNA_{ov}$).**

Group	$mRNA_{ov}$/cell (pg)	Incorporation (dpm/cell)			
		Cell	Medium	Total	% Secretion
Oocytes† (n=5)	1·2	392 ± 70	153 ± 24	545 ± 64	29 ± 7
Fertilized ova (n=3)	2·7	738 ± 291	306 ± 125	1044 ± 416	29 ± 1

*The mRNA was injected at a concentration of 0·34 pg/pl in an average volume of 3·4 pl/oocyte and 7·9 pl/ovum.
†From Paynton *et al.*, 1983.


A
Molecules of mRNA$_{ov}$/cell (×10^5)
20
10
9
8
7
6
5
4
3
2
1
0·5
Growing oocyte $T_{1/2}$ = 147 ± 65
Fertilized ovum $T_{1/2}$ = 5·4 ± 0·4
0
10
20
30
40
50
60
70
80
Hours in culture


B
Molecules of mRNA $_{con}$/cell (×10^5)
20
10
9
8
7
6
5
4
3
2
1
0·3
Growing oocyte $T_{1/2}$ = 366 ± 137
Fertilized ovum $T_{1/2}$ = 3·0 ± 0·8
0
10
20
30
40
50
60
70
80
Hours in culture

DISCUSSION

Denuded growing mouse oocytes remain translationally active during 73 h in culture. Protein synthesis during the initial 7 h was higher than at either 18–25 or 66–73 h. Assuming that the efficiency of uptake and internal leucine pools are constant in the growing oocyte during culture, the higher rate of leucine incorporation during the first 7 h may represent a period of time when the oocyte is under the previous influence of the granulosa cells. The presence of follicle cells enhance amino acid uptake by the oocytes (Cross & Brinster, 1974) by intercellular communication (Heller, Cahill & Schultz, 1981). However, no secondary influence from the follicle cells on protein synthesis of cultured denuded oocytes has been reported. During the initial hours in culture the energy substrates supplied by the follicle cells (Salustri & Martinozzi, 1982) may have a residual effect on protein synthesis and the uptake of energy substrates from the medium may not be efficient enough to maintain protein synthesis at an optimal level as the number of hours in culture increases. Although the culture conditions do not seem optimal for oocyte metabolism the fact that leucine incorporation during 18–25 and 66–73 h is essentially the same suggests that during the first 73 h of culture the oocyte is maintained in a stable condition with respect to protein synthesis. Maintenance of protein synthesis by the growing oocyte for an extended period of time in culture allows long-term observation on mRNA utilization.

The growing mouse oocyte was previously shown to translate and secrete ovalbumin following microinjection of ovalbumin mRNA ($mRNA_{ov}$) (Paynton *et al.*, 1983). These earlier experiments were for an 18 h labelling period while the present experiments extended over 73 h. Oocytes labelled for 18 h following $mRNA_{ov}$ injection secreted 30% of the synthesized ovalbumin while oocytes labelled for 7 h in the present study secreted only 12%. Since ovalbumin synthesis is identical during both time periods (d.p.m./ovum/h), the difference in percent secreted reflects the greater amount of ovalbumin in secretory processing (*e.g.* secretory vesicles) during the shorter time period. Ovalbumin secretion during the extended culture in the present study indicates that the secretory mechanism in the oocyte is stable for up to 73 h and allows for further studies on oocyte secretions in the absence of the influence from the follicular cells.

Fig. 3A and 3B. Ovalbumin mRNA (A) or conalbumin mRNA (B) half-lives in the growing oocyte (.---.) and fertilized ovum (x—x) expressed as the number of molecules of $mRNA_{ov}$ or $mRNA_{con}$ per cell (in log scale) that hybridized to their respective cDNA probe as a function of time in culture. Injected ova were analysed for the mRNA at 0, 4 and 8 h while the growing oocytes were assayed at 0, 25 and 73 h postinjection. Each line represents one experiment. The half-life value ($T\frac{1}{2}$) is the mean ± S.E.M. with each experiment generating a value using the equation: $\ln y = a + bx$.

The fertilized ovum synthesized approximately twice as much ovalbumin during 18 h as the growing oocyte. Since the volume of $mRNA_{ov}$ solution injected into the ovum is twice as great as for the oocyte, the amount of ovalbumin synthesized per injected message is about the same for the fertilized ovum and the oocyte, 454 and 387 d.p.m./pg mRNA respectively. Similarly, total protein synthesis is directly related to cell volume in both the growing oocyte and the fertilized ovum (Canipari *et al.*, 1979). The direct relationship of protein synthesis to injected message concentration and of endogenous protein synthesis to cell size suggests that the mammalian egg from the growing oocyte to the fertilized ovum may have similar ability to translate messages (endogenous or injected) per unit volume. Differences in translational efficiency between the oocyte and fertilized ovum cannot be determined from these experiments since they were not designed to measure either: 1) the fraction of injected $mRNA_{ov}$ that binds to the polyribosomes and thus the active translatable component, 2) the internal leucine pool, and 3) the half-life of ovalbumin in the injected cells.

Competition of microinjected mRNA with endogenous mRNA for translation in the fertilized ovum (Ebert & Brinster, 1983) and the growing oocyte (Paynton *et al.*, 1983) is extremely limited. The lack of competition suggests that either the endogenous mRNA has a greater affinity for the ribosomes than foreign mRNA or that the microinjected mRNA is not adequately processed for translation on polyribosomes. The fact that a certain percentage of injected mRNA is available for translation either by competition for ribosomes or other translational co-factors indicates that the major shift in degradation of injected mRNA reflects differences in the cells ability to degrade non-translatable mRNA or naked mRNA not involved in active translation.

Continued synthesis of ribosomes in the early developmental stages of the ovum appears limited since the growing oocyte from the thirteen day old ovary contains approximately 95% of the RNA present in the mature oocyte (Sternlicht & Schultz, 1981) and the turnover of total RNA is extremely long (Brower *et al.*, 1981; Bachvarova, 1981). Increasing the amount of time that $mRNA_{ov}$ is available for ribosomal binding could increase the amount of ovalbumin synthesized if all injected $mRNA_{ov}$ becomes accessible for ribosomal binding. Since $mRNA_{ov}$ half-life is long in the oocyte and the amount of ovalbumin synthesized per $mRNA_{ov}$ molecule increases from 0–7 and 66–73 h post-injection it appears that $mRNA_{ov}$ continues to compete for ribosomal binding sites. However, this is complicated by the fact that the oocyte will synthesize more ovalbumin per molecule of injected $mRNA_{ov}$ the longer the oocyte remains in culture. The inability to adequately determine translational efficiency of injected foreign mRNA makes it difficult to separate the two potentially independent processes.

Richter & Smith (1981) recently reported that messages translated on free polysomes do not effectively compete with messages destined for translation

on membrane-bound polysomes *in vivo* and thus the amount of competition a foreign mRNA will have with endogenous mRNAs is related to the translational binding site of the mRNA. The synthesis and secretion of ovalbumin by the ovum and oocyte indicate that membrane-bound polysomes are available for translation in both cells assuming that ovalbumin mRNA is only translated on membrane-bound polysomes as suggested by Blobel and coworkers (1979). Additionally, the evidence suggests that the oocyte and ovum translate and secrete comparable amounts of ovalbumin when the cytoplasmic concentration of $mRNA_{ov}$ is the same. Therefore the ability to translate mRNAs for secretory proteins does not appear to change during oocyte maturation and fertilization.

The stability of ovalbumin and conalbumin mRNA in the oocyte and fertilized ovum was estimated by measuring the amount of $mRNA_{ov}$ and $mRNA_{con}$ that hybridized to their respective cDNA probes at various time periods following microinjection of the message. The experiments indicate that the half-life of either $mRNA_{ov}$ ($T½ = 147$ h) or $mRNA_{con}$ ($T½ = 366$ h) in the growing oocyte is long and compares favourably with the half-life of total mRNA in the oocyte (Brower *et al.*, 1981). In contrast, the half-life of either $mRNA_{ov}$ ($T½ = 5$ h) or $mRNA_{con}$ ($T½ = 3$ h) in the fertilized ovum was dramatically shorter and is similar to that previously reported for microinjected globin mRNA (Chen & Brinster, 1982). This major decrease in the half-life of the injected mRNA was not the result of fertilization as the unfertilized ovum also had a very short half-life for $mRNA_{ov}$ ($T½ = 7$ h). It should be pointed out that this method of determining the residual amount of injected mRNA in the cell will not separate those fragments of $mRNA_{ov}$ that could bind to the $cDNA_{ov}$ probe but are not the entire mRNA molecule. However, the fact that more ovalbumin was synthesized from injected $mRNA_{ov}$ after 66 h from the time of injection suggests that the $mRNA_{ov}$ left in the oocyte is still composed of translationally active molecules, arguing that the mRNA that hybridized were not completely fragmented into untranslatable molecules. Furthermore, the dramatic difference in the amount of $mRNA_{ov}$ that can bind to the cDNA probe strongly suggests that the mRNA degradation process within these two cell types are remarkably different. The change in the degradation of mRNA may reflect the 'housekeeping' ability of the active one-cell stage to turnover mRNA molecules not bound to ribosomes in preparation for the transcription that takes place during the first few cleavage stages of embryonic development (Levey *et al.*, 1978). The injection of free mRNA into a cell will result in the partitioning of the mRNA into two populations: one that associates with ribosomes and the other not bound to the ribosomes due to the limited amount of unoccupied ribosomes for mRNA binding. Richter, Evers & Smith (1983) have good evidence to show this partitioning of injected mRNA in the *Xenopus* and showed that the population of mRNA in the free form would shift to the membrane-bound polysome

fraction when rough endoplasmic reticulum was also injected. The non-bound injected mRNA may be the population of mRNA that is degraded more efficiently in the mature oocyte than during the earlier growth stages of oocyte development.

The dramatic differences in mRNA stability between the oocyte and the egg clearly suggest a major alteration in how messages are processed and protected at the two stages of development. The differences probably reflect underlying changes in how the endogenous messages are treated between the inactive oocyte and the activated fertilized egg.

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