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Gene expression during mammalian oogenesis and early embryogenesis: quantification of three messenger RNAs abundant in fully grown mouse oocytes

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Summary

Ribonuclease protection assays have been used to quantitatively assess changes in steady-state levels of specific mRNAs during oogenesis and early embryogenesis in mice. The mRNAs encode ZP3 (a glycoprotein that serves as a sperm receptor), LDH-B (heart-type lactate dehydrogenase), and MOM-1 (a protein of unknown function). MOM-1 and LDH-B are expressed in a variety of adult mouse tissues and midgestation embryos, whereas ZP3 expression is restricted completely to oocytes. All three mRNAs are expressed by growing mouse oocytes and accumulate to unusually high levels in fully grown oocytes as compared to somatic cells; 240 000, 200 000 and 74 000 copies mRNA per fully grown oocyte for ZP3, LDH-B and MOM-1, respectively. Steady-state levels of LDH-B and MOM-1 mRNA undergo a modest decline (~20–40 %) during ovulation when fully grown oocytes become unfertilized eggs and, in general, mirror the reported change in poly(A)$^+$RNA levels during this period of development. On the other hand, the level of ZP3 mRNA declines dramatically (~98 %) during ovulation, from ~240 000 copies per oocyte to ~5000 copies per unfertilized egg, and ZP3 mRNA is undetectable in fertilized eggs (<1000 copies per fertilized egg). MOM-1 mRNA is expressed at relatively low levels in morulae (~2000 copies per embryo) and blastocysts (~5000 copies per embryo), whereas ZP3 mRNA remains undetectable (<1000 copies per embryo) at these stages of preimplantation development. These findings are discussed in the context of overall gene expression during oocyte growth, meiotic maturation and early embryogenesis in mice.

Key words: gene expression, mouse oocyte, mouse egg, mouse embryo, oogenesis, mRNA.

Introduction

To a large extent, unfertilized mouse eggs can be viewed as end-products of two developmental processes, growth and meiotic maturation (Jones, 1978; Wassarman, 1988*a*). During each reproductive cycle in mice, a small percentage of the female's pool of nongrowing oocytes (~15 μm in diameter) begin to grow, increasing more than 300-fold in volume within 2–3 weeks, at which time full growth (~80 μm in diameter) is achieved. During this growth period, oocytes accumulate large amounts of all classes of RNA, ribosomes, mitochondria, Golgi and cortical granules, as well as a variety of proteins, including tubulin, actin and lactate dehydrogenase (Wassarman & Josefowicz, 1978; Wassarman, 1983; Bachvarova, 1985; Schultz, 1986*b*; Wassarman, 1988*a*). Growing oocytes also synthesize and secrete glycoproteins that constitute the cell's extracellular coat, or zona pellucida (Wassarman *et al.* 1985; Wassarman, 1988*a*,*b*).

Throughout their growth phase, mouse oocytes are arrested in the dictyate stage of first meiotic prophase (Jones, 1978; Wassarman, 1988*a*). Diffuse chromosomes, contained within a large nucleus, or germinal vesicle (GV), are actively transcribed during this period (Bachvarova, 1985). However, at ovulation, when fully grown oocytes undergo the first meiotic reductive division ('meiotic maturation') and become unfertilized eggs, the nuclear envelope breaks down, chromosomes condense and transcription ceases. The maternal genome is not transcribed again until the 2-cell stage of preimplantation development (Johnson, 1981; Flach *et al.* 1982; Pikó & Clegg, 1982; Pratt *et al.* 1983 ; G. A. Schultz, 1986), about 2–3 days following ovulation.

Meiotic maturation is accompanied by a significant decrease in levels of total RNA and poly(A)$^+$ RNA, as well as by a decrease in rates of synthesis of specific proteins (Wassarman, 1983; Bachvarova, 1985; R. M. Schultz, 1986; Wassarman, 1988*a*). These changes, and those that occur following fertilization and preceding the second cleavage division, take place in the absence of new transcription (Johnson, 1981; G. A. Schultz, 1986) and, therefore, are programmed during oogenesis. The mechanisms by which these changes are programmed and instituted are not clear.

Here we describe the behavior of specific mRNAs during oocyte growth, meiotic maturation and early embryogenesis in mice. All of these mRNAs are abundant in fully grown oocytes. One mRNA encodes ZP3, an 83000 M_r glycoprotein (44000 M_r polypeptide chain; Salzmann *et al.* 1983; Wassarman *et al.* 1985; Ringuette *et al.* 1986, 1988; Kinloch *et al.* 1988; Wassarman, 1988*b*) that serves as the egg's sperm receptor (Bleil & Wassarman, 1980*a*, 1986; Florman & Wassarman, 1985; Wassarman *et al.* 1985; Wassarman, 1987). A second mRNA, called MOM-1, encodes a 76000 M_r protein (R. Roller & P. Wassarman, unpublished results) of, as yet, unknown function. The third mRNA encodes the 36000 M_r subunit of heart-type lactate dehydrogenase (LDH-B), a glycolytic enzyme known to accumulate to very high levels in fully grown oocytes and eggs (Mangia & Epstein, 1975; Mangia *et al.* 1976; Cascio & Wassarman, 1982). All three mRNAs accumulate throughout oocyte growth, and their protein counterparts represent relatively large percentages of total oocyte protein synthesized during oogenesis.

Materials and methods

Collection of mouse oocytes, eggs, and embryos

Growing oocytes, fully grown oocytes, unfertilized eggs and embryos were isolated from female, Swiss albino mice (CD-1, Charles River Breeding Laboratories) as previously described (Schultz *et al.* 1979*a*,*b*).

Purification of oocyte, embryo, and tissue RNA

Oocyte and embryo RNA was purified as described in Kinloch *et al.* (1988). Tissue RNA was purified as described by Feraminsco *et al.* (1982) with some modifications. Following the first ethanol precipitation, the RNA pellet was dissolved in 0·1 M-Tris, pH 7·5, 50 mM-NaCl, 10 mM-EDTA, 0·2 % SDS with 0·5 volumes of the initial homogenate. Proteinase K (100 μg ml^{-1}; Sigma) was added and the solution incubated at 37°C for 1 h. RNA was then extracted twice with phenol/chloroform/isoamyl alchohol and once with chloroform/isoamyl alcohol and precipitated from 0·3 M-sodium acetate, pH 5·2, with 2·5 volumes of ethanol. The final step included precipitation from 3 M-sodium acetate.

Purification of nuclear DNA

Mouse liver nuclei were isolated by a modification of the procedure described in Busch (1967). Briefly, livers were excised, rinsed in cold NaCl (150 mM), minced and homogenized in 6 volumes of 0·25 M-sucrose, 3·3 mM-$CaCl_2$ with a Dounce homogenizer. The homogenate was filtered through cheesecloth and then centrifuged at 600 *g* for 10 min at 4 °C to pellet nuclei and large debris. The pellet was resuspended by repeated pipetting in 9 ml of 2·5 M-sucrose, 3·3 mM-$CaCl_2$, and centrifuged at 45000 *g* for 1 h at 4°C in an SW 50·1 rotor (Beckman) to pellet nuclei. DNA was purified from isolated nuclei by the procedure described by Weeks *et al.* (1986).

Construction and screening of cDNA and genomic libraries

Total RNA from approximately 16000 growing mouse oocytes was primed with oligo-dT and used as a template for cDNA synthesis. Double-stranded cDNA with *Eco*RI linker ends was synthesized and ligated into λgt11 DNA and packaged into phage by methods described in Maniatis *et al.* (1982), except that cDNAs were separated from digested linker fragments by several precipitations from 0·3 M-sodium acetate with 0·6 volumes of isopropanol. The resulting library, containing 80000 recombinants (average insert size of 800 bases), was amplified on *E. coli* LE392.

The cDNA library was screened by using a rabbit antiserum raised against dissolved zonae pellucidae and directed against ZP2 and ZP3 (Greve *et al.* 1982; Salzmann *et al.* 1983). Induction of the fusion proteins and plaque lifts were carried out as described by Young & Davis (1983). A screen of 100000 plaques yielded 2 phage that produced strong, reproducible, IPTG-dependent signals, which proved to carry the same cDNA.

Construction of a *Sau*3A partial-digest library of CD-1 mouse nuclear DNA and isolation of ZP3 genomic clones were carried out as previously described (Kinloch *et al.* 1988).

A mouse LDH-B cDNA clone (mB162) was isolated from a macrophage cDNA library in λgt11 (Clontech) by using a human LDH-B cDNA (hB537) as probe (Sakai *et al.* 1987). The *Eco*RI DNA fragment was subcloned into M13 mp10 phage and its nucleotide sequence determined from both directions.

Preparation of RNA probes

High-specific-activity RNA probes were transcribed with T7 RNA polymerase from template sequences cloned into either pT7-1 or -2 (US Biochemicals) or pGEM-3-Blue (Promega Biotechnology) according to a published protocol (Promega Biotechnology). Reactions contained 40 mM-Tris, pH 7·5, 6 mM-$MgCl_2$, 2 mM-spermidine, 10 mM-NaCl, 10 mM-dithiothreitol, 500 μM each ATP, CTP, GTP, 12 μM UTP, 1 i.u. μl^{-1} RNasin, 100 μCi [α-^{32}P]UTP (3000 Ci mmol^{-1}; Amersham), 0·5–1·0 μg plasmid template, and 40 i.u. T7 RNA polymerase in a final volume of 20 μl. Incubations were at 37°C for 1 h. Following synthesis of RNA, DNA templates were destroyed by addition of 1 μl of RNase-free DNase (Promega Biotechnology) and incubation at 37°C for 15 min. Samples were then extracted with phenol/chloroform/isoamyl alcohol and RNA was precipitated with ethanol.

RNA targets were prepared by the same procedure, except that all unlabeled nucleotides were present at 500 μM and 5 μCi of [α-^{32}P]UTP or [α-^{35}S]UTP was included. Consequently, the amount of target synthesized could be calculated from the percentage incorporation according to the equation:

$$(\text{cts min}^{-1}{}_{\text{incorporated}}/\text{cts min}^{-1}{}_{\text{added}})\ (4 \times 10^{-8}\ \text{mol NTP})$$
$$(330\ \text{g mol}^{-1}\ \text{NTP}) = \text{g RNA synthesized}$$

A synthetic target for MOM-1 probe, used to calibrate the RNase protection assays, was generated from a plasmid containing the entire MOM-1 cDNA insert. The plasmid was linearized with *Sca*I such that T7 RNA polymerase yielded a 2·3 kb sense transcript. Since the plasmid inserts used to generate probe and target were identical over only 394 nt, the

synthetic target protected a 394 nt fragment of the antisense probe in RNase protection assays. MOM-1 mRNA is twice as long as the synthetic target, so that 100 pg of target is equivalent in copy number to 200 pg of MOM-1 mRNA.

Preparation of oligonucleotide probes

Two different 60-mer oligonucleotide probes were used in experiments described here. One of these is complementary to ZP3 mRNA and was prepared on the basis of the sequence of a ZP3 cDNA fragment (Ringuette *et al.* 1986). The other oligonucleotide is complementary to MOM-1 mRNA and was prepared on the basis of the sequence of a MOM-1 cDNA fragment. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA Synthesizer and purified by PAGE. Oligonucleotides were 5′ end-labeled with T4 polynucleotide kinase as previously described (Hay & DePamphilis, 1982).

Ribonuclease protection assays

RNA preparations to be examined were combined with either 1×10^6 disints min^{-1} (MOM-1 and LDH-B) or 2×10^6 disints min^{-1} (ZP3) of probe RNA, lyophilized and dissolved in 10 μl of hybridization buffer (80% formamide, 40 mM-MOPS, pH 6·6, 0·4 M-NaCl, 1 mM-EDTA). RNA was denatured by incubation at 85°C for 5 min and hybridized by incubation at 45°C overnight. Following overnight incubation, unhybridized RNA was digested by addition of 200 μl of a mix containing 10 mM-Tris, pH 7·5, 300 mM-NaCl, 5 mM-EDTA, 40 $\mu g\,ml^{-1}$ RNase A, 2 $\mu g\,ml^{-1}$ RNase T1 and incubation at 37°C for 1 h. Digestion was terminated by addition of SDS (0·7%) and Proteinase K (150 $\mu g\,ml^{-1}$) and incubation at 37°C for an additional 15 min. Protected RNA was purified by extraction with phenol/chloroform/isoamyl alcohol, followed by ethanol precipitation in the presence of carrier tRNA (15 μg). Purified RNA was analyzed on sequencing gels (6% acrylamide/8 M urea; 15 cm long, 0·75 mm thick). Bands were visualized by autoradiography of dried gels. Quantitative analysis was carried out by excising bands from dried gels and subjecting them to liquid scintillation counting in Aquasol (New England Nuclear). In order to correct for background, each experiment included an assay carried out with *E. coli* ribosomal RNA which did not hybridize to any of the probes used. A band corresponding in size to protected fragments was cut from the *E. coli* ribosomal RNA gel lane, counted and used as background for the experiment.

Northern blotting

Preparation, running and blotting of formaldehyde–agarose gels containing RNA were performed as described by Maniatis *et al.* (1982). Blots were prehybridized for 2–3 h at 55°C in 6×SSC, 1·0% SDS, 5×Denhardt's solution, 20 $\mu g\,ml^{-1}$ tRNA, and 50 $\mu g\,ml^{-1}$ denatured salmon sperm DNA. Hybridization was carried out in prehybridization solution in the presence of ^{32}P end-labeled oligonucleotides for 1 h at 55°C. Blots were washed three times in 2×SSC, 1·0% SDS at room temperature, for 5 min each, and twice at 55°C in 1×SSC, 1·0% SDS for 45 min.

In situ *hybridization*

Thin sections of embedded ovaries from 15-day-old mice were placed on subbed slides, dried and fixed in 2% paraformaldehyde. Slides were washed, dried and stored for further use. Hybridizations were carried out essentially as described by Lewis *et al.* (1986). After coating with photographic emulsion (Kodak NTB2 diluted 1:1 with 0·6 M ammonium acetate), slides were exposed for 6–14 days in the dark, developed (Kodak D19) and lightly counter-stained with toluidine blue in sodium borate.

Results

Genomic and cDNA clones used to analyze mouse mRNAs

Isolation and characterization of a ZP3 genomic clone, designated ZP3-G5, is described elsewhere (Kinloch *et al.* 1988). A ZP3-G5 *Eco*RI/*Hin*dIII fragment, 2·6 kb in length, was subcloned and used as probe in experiments presented here. This restriction fragment contains the two 3′-terminal exons (219 and 137 nt) encoding ZP3 (Kinloch *et al.* 1988). ZP3 probe hybridized to a 1·5 kb mRNA on Northern blots of total mouse oocyte RNA and to single bands in Southern blots of mouse genomic DNA digested with a variety of restriction enzymes.

A cDNA clone, designated MOM-1, was isolated as a false-positive during the screening of a λgt11-growing oocyte cDNA library with an antiserum directed against zona pellucida glycoproteins ZP2 and ZP3 (R. Roller and P. Wassarman, unpublished results). It was shown to represent an abundant oocyte mRNA ('major oocyte message-1'; MOM-1) that did not encode any zona pellucida glycoprotein. This 558 nt cDNA contains 483 nt of open reading frame. A 405 nt *Eco*RI/*Msp*I fragment of the cDNA was subcloned and used as probe in experiments presented here. MOM-1 probes hybridized to a 4·6 kb mRNA on Northern blots of total mouse oocyte RNA and to single bands in Southern blots of mouse genomic DNA digested with a variety of restriction enzymes.

A cDNA clone, designated mB162, was isolated from a λgt11-mouse macrophage cDNA library screened with a human LDH-B cDNA probe (Sakai *et al.*), and its complete sequence (534 nt; excluding *Eco*RI linker sequences) was determined. The deduced amino acid sequence of its coding region was confirmed by sequencing tryptic peptides derived from mouse LDH-B. Clone mB162 contains a partial mouse LDH-B cDNA, starting at codon 219 and ending 16 nt after the polyadenylation signal, AATAAA. A 467 nt *Eco*RI/*Sac*I fragment of this cDNA was cloned into plasmid pGEM-3blue to create a plasmid, designated pGEM/LDH-B, that was used to generate RNA probes used in experiments presented here. LDH-B probes hybridized to a 1·4 kb mRNA on blots of total mouse oocyte RNA.

Quantification of mRNAs in fully grown mouse oocytes

Quantification of absolute amounts of MOM-1, ZP3 and LDH-B transcripts in fully grown mouse oocytes was carried out by RNase protection assays, as described in Materials and methods. In such assays, under conditions in which a radiolabeled antisense probe is in large excess over the complementary target sequence, the amount of probe protected from digestion is proportional to the amount of target sequence. By using varying amounts of a synthetic MOM-1 sense RNA (394 nt) as target, a standard curve was constructed relating protected MOM-1 antisense probe (cts min^{-1}) to target RNA (pg) (Fig. 1). Parallel assays were performed using both RNA from 250 fully grown

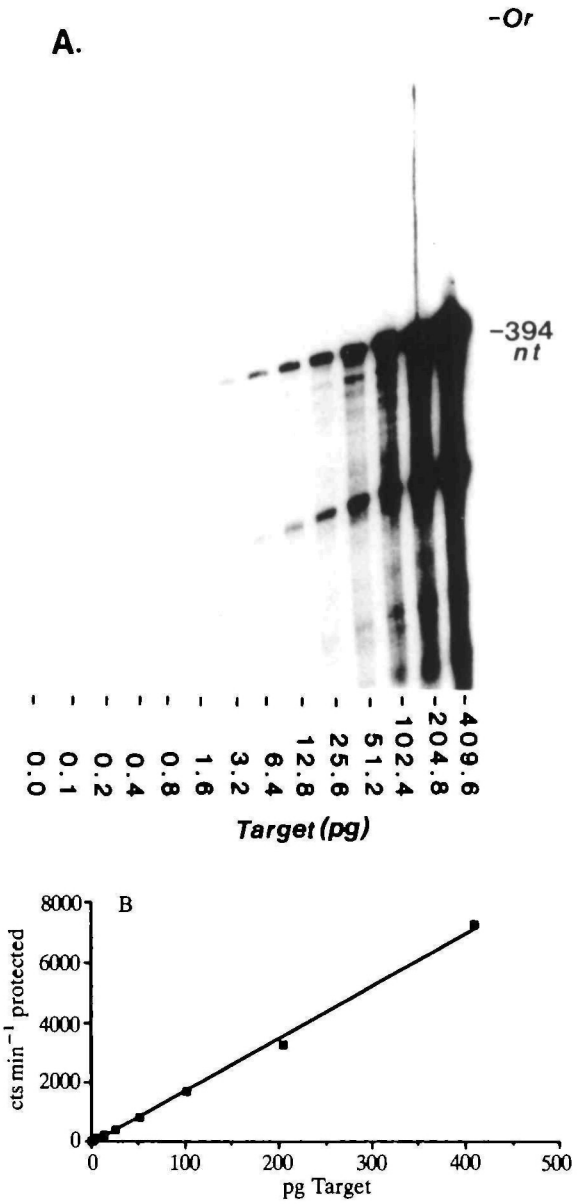

Fig. 1. Calibration of the RNase protection assay using MOM-1 probe and target. RNase protection assays were carried out as described in Materials and methods, using a constant amount of MOM-1 antisense transcript (1×10^6 disints min^{-1}, or ~800 pg), transcribed from *Eco*RI-digested pT7-2/MOM-A-*Msp*B by T7 RNA polymerase, and varying amounts of MOM-1 target transcript (0–409·6 pg), synthesized as described in Materials and methods. (A) Autoradiogram of the gel analysis of RNase protection assays. The position of the 394 nt protected MOM-1 fragment is indicated. Or, origin. (B) Plot of cts min^{-1} associated with the 394 nt protected fragment (panel A) as a function of pg of target transcript added. Bands were excised from the gel (panel A) and subjected to liquid scintillation counting, as described in Materials and methods. The line drawn is derived from a least squares fit of the data.

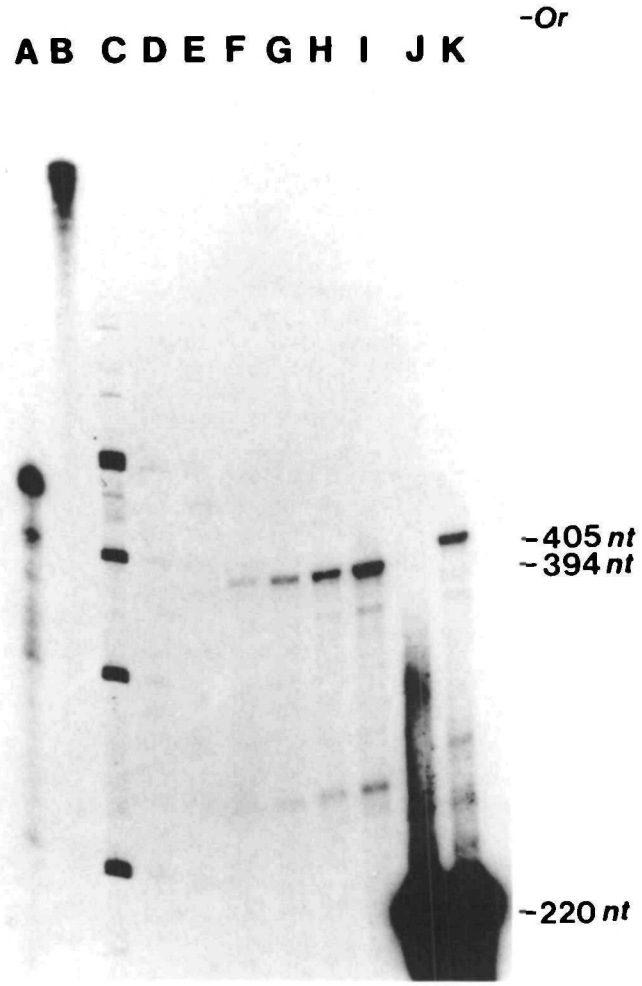

Fig. 2. Quantification of MOM-1 mRNA steady-state levels in fully grown mouse oocytes. Shown is a representative autoradiogram of the gel analysis of RNase protection assays of MOM-1 calibration standards (as in Fig. 4) and RNA from fully grown oocytes, carried out as described in Materials and methods. Positions of the 405 nt fragment protected by oocyte MOM-1 mRNA, the 394 nt fragment protected by the MOM-1 calibration target (see Fig. 4), and the 220 nt fragment protected by the recovery target are indicated. Lane: (A) Undigested probe transcript; (B) undigested calibration target transcript; (C) end-labeled size standards; (D–I) RNase protection assays carried out with 0, 2·5, 5, 10, 20, 40, and 80 pg of calibration target, respectively; (J) *E. coli* ribosomal-RNA plus recovery target; (K) RNA from 250 fully grown oocytes (~125 ng) plus recovery target. Or, origin.

oocytes and varying amounts of synthetic target RNA (equivalent to 0–80 pg MOM-1 mRNA) (Fig. 2). The RNase protection assay exhibited linearity using amounts of the 394 nt target equivalent to nearly 800 pg MOM-1 mRNA (Fig. 1). Protected fragments were resolved on denaturing gels, bands excised and subjected to liquid scintillation counting. As expected, target RNA protected a 394 nt fragment of probe, whereas authentic MOM-1 mRNA protected the entire probe (405 nt) (Fig. 2). To assess recovery of oocyte RNA, a MOM-1 cDNA sense transcript (220 nt) was added to oocytes prior to extraction of RNA and an equivalent amount of the transcript was assayed directly. The ratio of the amount of transcript in oocyte

RNA preparations and the amount assayed directly was used as a correction factor for recovery. In experiments presented here, recovery of oocyte RNA ranged from 70 to 95 %. By using the procedures just described, the amount of MOM-1 mRNA in fully grown oocytes was determined to be 185 ± 15 fg oocyte^{-1} (4 independent experiments), or about $7{\cdot}4 \times 10^4$ copies oocyte^{-1}.

The amount of ZP3 and LDH-B mRNA per oocyte, relative to MOM-1 mRNA, was determined by performing parallel RNase protection assays for each message (i.e. ZP3 *vs* MOM-1 and LDH-B *vs* MOM-1). The amount of ZP3 mRNA was calculated from the ratio of cts min^{-1} associated with the ZP3 protected fragment (219 nt) to cts min^{-1} associated with the MOM-1 protected fragment (405 nt). This ratio was corrected for differences in (a) lengths of protected fragments (MOM-1 fragment is 1·8-times longer than ZP3 fragment), (b) compositions of the probes with respect to the labeled nucleotide (MOM-1 fragment has 1·5-times more uridine per unit length than ZP3 fragment), and (c) sizes of the mRNAs (MOM-1 mRNA is three times the size of ZP3 mRNA). Accordingly, the amount of ZP3 mRNA in fully grown oocytes was determined to be 195 ± 20 fg oocyte^{-1} (4 independent experiments), or about $2{\cdot}4 \times 10^5$ copies oocyte^{-1}. The amount of LDH-B mRNA in fully grown oocytes was determined just as for ZP3 mRNA. As before, corrections were applied for differences in LDH-B protected fragment length (460 nt), probe composition (26·9 % uridine) and message length (1·4 kb), as compared to MOM-1. Two individual experiments gave a value of 150 ± 15 fg of LDH-B mRNA oocyte^{-1}, or about 2×10^5 copies oocyte^{-1}.

Assuming a value of 500 pg of total RNA per oocyte, 20 % as the fraction of poly(A)$^+$RNA per oocyte, and 2000 nt as the average length of poly(A)$^+$RNA in mouse oocytes (Clegg & Pikó, 1983*b*), it can be calculated that fully grown oocytes contain about 9×10^7 poly(A)$^+$RNA molecules. Thus, on a copy-number basis, MOM-1, ZP3 and LDH-B mRNA represent approximately 0·08 %, 0·27 % and 0·22 % of a fully grown oocyte's poly(A)$^+$RNA, respectively (or about 0·6 % collectively; see Discussion).

Specificity of mRNA expression in mouse tissues

Although a great deal is known about LDH-B expression in mouse tissues (Markert *et al.* 1975; Nadal-Ginard, 1978), much less is known about expression of MOM-1 and ZP3. Accordingly, Northern analyses and RNase protection assays were used to examine the tissue specificity of MOM-1 and ZP3 mRNA in mice.

RNA was prepared from fully grown mouse oocytes and 13-day mouse embryos, and from mouse brain, heart, intestine, kidney, liver, muscle, ovary, testis and uterus. Integrity of RNA in these preparations was assessed by inspection of stained formaldehyde–agarose gels, as well as by Northern analysis using a mouse mitochondrial cytochrome b cDNA probe. For Northern analyses of ZP3 mRNA, an end-labeled, synthetic oligonucleotide (60-mer), DNA probe was employed. For RNase protection analyses of both ZP3 and MOM-1 mRNA, radiolabeled antisense transcripts of genomic and cDNA clones, respectively, were used.

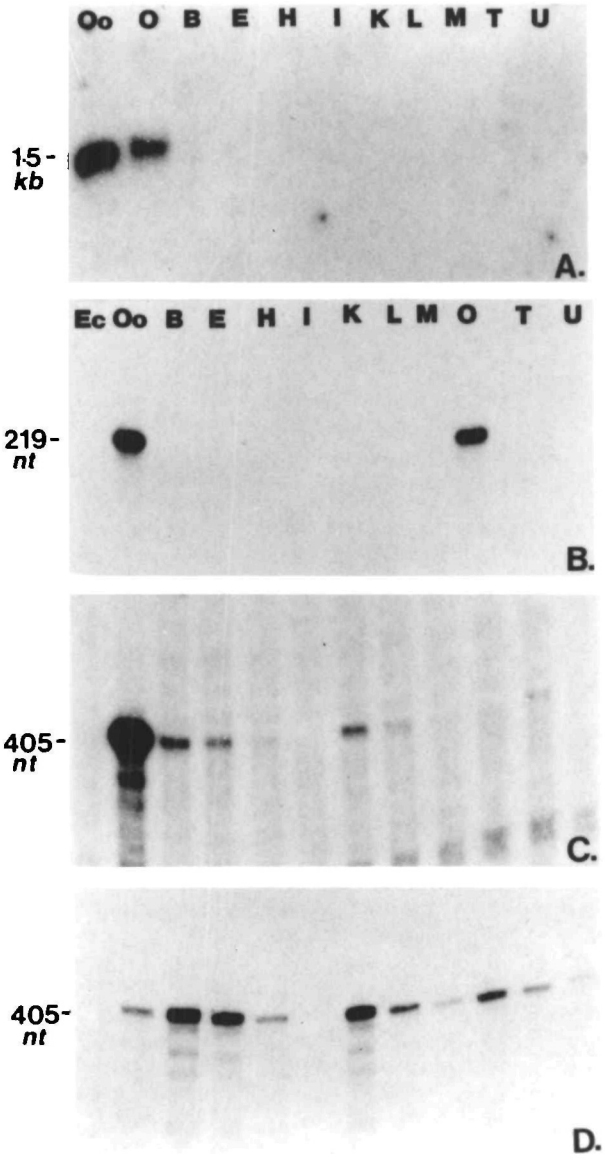

Fig. 3. Northern blot analysis and RNase protection assays of ZP3 and MOM-1 mRNA in mouse tissues and midgestation embryos. (A) Northern blot analysis using a ^{32}P end-labeled oligonucleotide specific for ZP3 mRNA. The oocyte lane contains RNA from 250 fully grown oocytes (~125 ng) and all other lanes contain 10 μg of RNA. (B) RNase protection assays using probe transcribed with T7 RNA polymerase from *Hin*dIII-digested pGEM/G5/R1-A. The oocyte lane contains RNA from 250 fully grown oocytes and all other lanes contain 10 μg RNA. (C) RNase protection assays using probe transcribed by T7 RNA polymerase from *Eco*RI-digested pT7-2/MOM-A-*Msp*I. The oocyte lane contains RNA from 200 fully grown oocytes (~100 ng) and all other lanes contain 100 ng RNA. (D) Same as (C), except that 10 μg of RNA from mouse tissues, embryos, and *E. coli*, rather than 100 ng, was loaded in each lane. Ec, *E. coli*; Oo, oocytes; B, brain; E, 13-day embryos; H, heart; I, intestine; K, kidney; L, liver; M, muscle; O, ovary; T, testis; U, uterus.

Results shown for ZP3 (Fig. 3) were obtained using

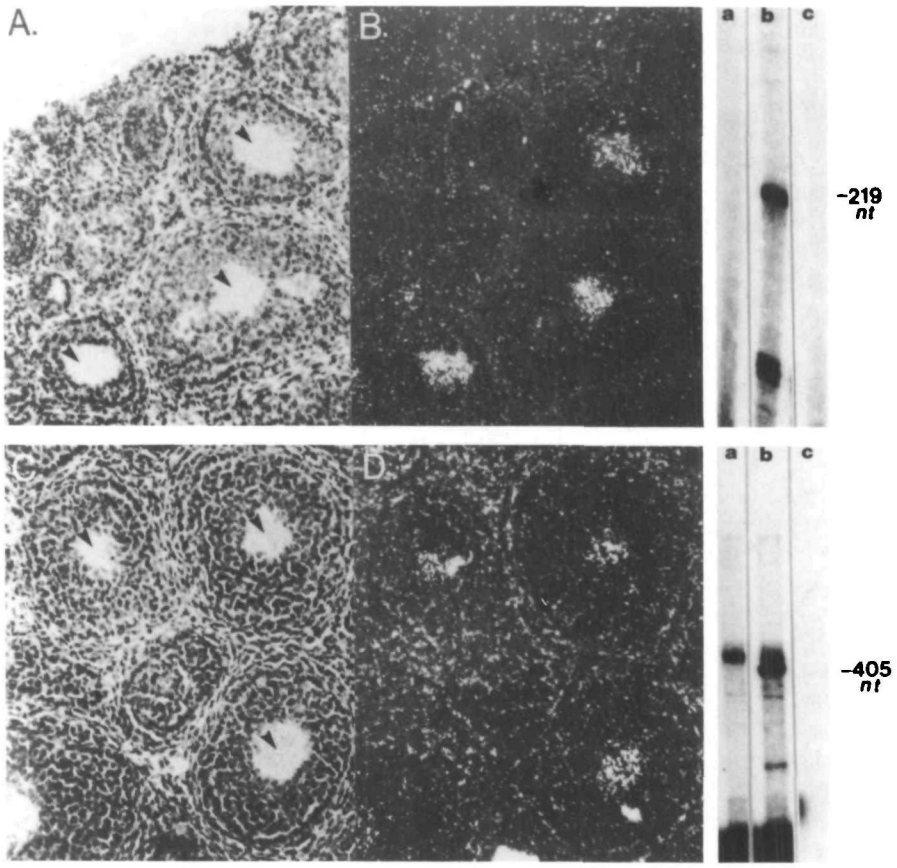

Fig. 4. *In situ* hybridization and RNase protection assays of ZP3 and MOM-1 mRNA in mouse oocytes and follicle cells. *In situ* hybridizations were carried out using radiolabeled 60-mer oligonucleotides specific for either ZP3 or MOM-1 mRNA, as described in Materials and methods. (A and C) Light micrographs of ovarian sections viewed with bright-field optics. The positions of three oocytes, within follicles, in each section are indicated by arrows. (B and D) Light micrographs of autoradiograms of the same ovarian sections seen in (A) and (C), respectively, but viewed with dark-field optics so that silver grains appear as white dots on a black background. Also shown are results of RNase protection experiments carried out with anti-sense probes specific for either ZP3 (Top panel, a–c) or MOM-1 mRNA (Bottom panel, a–c): Lane (a) *E. coli* ribosomal-RNA; (b) RNA from 250 fully grown oocytes; (c) RNA from 100 isolated follicles from which oocytes had been removed (empty follicles), plus recovery target. The protected fragments expected for ZP3 and MOM-1 probes, 219 and 405 nt, respectively, are seen in lane b (oocytes), but not in lane c (empty follicles).

10 μg of each tissue RNA, or about 100 times more RNA than was present in the oocyte samples. However, ZP3 mRNA (~1·5 kb) was detected only in RNA prepared from oocytes and ovaries. Since RNAse protection assays were capable of detecting as little as 0·5 % of the amount of ZP3 mRNA found in oocytes, it follows that mouse tissues, other than ovary, contained no more than 0·005 % the amount of ZP3 mRNA found in oocytes.

Results shown for MOM-1 (Fig. 3) were obtained using 100 ng of each RNA preparation, including oocytes. Although MOM-1 mRNA was found to be highly enriched in oocytes compared to other tissues, it was present in all tissues examined. Estimates of the relative abundance of MOM-1 mRNA were made by carrying out RNase protection assays with 10 μg samples of tissue and oocyte RNA (Fig. 3). Excluding oocytes, brain contained the largest amount of MOM-1 mRNA, followed in decreasing order by 13-day embryos, kidney, ovary, liver, testis, uterus, heart, skeletal muscle and intestine. Based on total RNA levels, brain contained about 2 % and intestine about 0·02 % of the amount of MOM-1 mRNA found in fully grown oocytes.

The relative amounts of ZP3 and MOM-1 mRNA found in isolated oocytes and ovary suggested that these messages were expressed exclusively in oocytes within the ovary. This possibility was examined further by using *in situ* hybridization to localize transcripts within the ovary (Fig. 4) and RNase protection assays with isolated ovarian follicles from which oocytes had been removed (Fig. 4). Results of both analyses suggested that ZP3 and MOM-1 mRNA were found only in oocytes, not in surrounding follicle cells.

Quantification of mRNAs in growing mouse oocytes, ovulated eggs, and early embryos

Since MOM-1, ZP3, and LDH-B mRNAs proved to be abundant transcripts in fully grown mouse oocytes, their steady-state levels during oogenesis and early embryogenesis were determined by using RNase pro-

Fig. 5. Quantification of ZP3, MOM-1 and LDH-B mRNA steady-state levels during oocyte growth, meiotic maturation and early embryogenesis in mice. Shown are representative autoradiograms of RNase protection assays for ZP3 (A), MOM-1 (B), and LDH-B (C) mRNA, carried out as described in Materials and methods. (A) In lanes b–i, RNA from 500 oocytes or ovulated eggs was assayed. Lane (a) *E. coli* RNA; (b) 12–20 μm oocytes; (c) 30–40 μm oocytes; (d) 50–60 μm oocytes; (e) 60–70 μm oocytes; (f and h) 80–85 μm (fully grown) oocytes; (g and i) ovulated eggs. Lane (j) RNA from approximately 500 2-cell embryos. Lane (k) RNA from approximately 500 empty follicles. The position of the ZP3 protected fragment, 219 nt, is indicated. (B) In lanes b–o, RNA from 350 oocytes, eggs or embryos was assayed. Lanes (a and h) *E. coli* RNA; (b) 12–20 μm oocytes; (c) 30–40 μm oocytes; (d) 40–50 μm oocytes; (e) 50–60 μm oocytes; (f) 60–70 μm oocytes; (g and i) 80–85 μm (fully grown) oocytes; (j) ovulated eggs; (k) 1-cell embryos; (l) 2-cell embryos; (m) 4- to 8-cell embryos; (n) morulae; (o) blastocysts. The position of the MOM-1 protected fragment, 405 nt, is indicated. It should be noted that a very faint band at 405 nt was seen in lanes (n) and (o) of the original autoradiogram. (C) Lanes (a and d), *E. coli* RNA; (b) RNA from 250 60–70 μm oocytes; (c and e) RNA from 250 80–85 μm (fully grown) oocytes; (f) RNA from 250 ovulated eggs. The position of the LDH-B protected fragment, 460 nt, is indicated.

tection assays. In each experiment, RNA was prepared from equivalent numbers (250–400) of growing oocytes, fully grown oocytes, ovulated eggs and early embryos. RNase protection assays were carried out using as probes the antisense transcripts of MOM-1 cDNA, ZP3 genomic clone or LDH-B cDNA described above. As before, a synthetic MOM-1 sense-strand target was added to each preparation in order to determine a correction factor for RNA recovery. Some examples of the autoradiographic results of these experiments are presented in Fig. 5.

As seen in Table 1, MOM-1 and ZP3 mRNAs were detected by RNase protection assays at the earliest stages of oocyte growth (~20 μm diameter oocytes) and continued to accumulate to about the mid-to-late stages of oocyte growth (~65 μm diameter oocytes). The steady-state levels of MOM-1 and ZP3 mRNAs did not increase during the final stages of oocyte growth (from 65 μm to 80 μm diameter oocytes). Rather, the level of MOM-1 mRNA remained nearly constant, whereas the level of ZP3 mRNA fell by about 22 %. On the other hand, the steady-state level of LDH-B mRNA exhibited a different pattern, increasing by about 20 % during the final stages of oocyte growth.

The steady-state levels of MOM-1 and ZP3 mRNAs changed markedly during ovulation (Table 1), when fully grown oocytes underwent the first meiotic reduction and became unfertilized eggs. As compared to fully grown oocytes, MOM-1 and ZP3 mRNA levels fell by approximately 50 % (to ~90 fg egg^{-1}) and 98 % (to ~4 fg egg^{-1}), respectively. The level of LDH-B mRNA also fell during ovulation (~20 %), but not to the extent seen with MOM-1 and ZP3 mRNAs. ZP3 mRNA was virtually undetectable in 2-cell and later stage embryos, whereas MOM-1 mRNA, present in 2-cell embryos at about 1 % the level found in fully grown oocytes, became undetectable in 4- to 8-cell embryos, and then reappeared in morulae and increased in blastocysts to approximately 6 % the level (~11 fg embryo^{-1}) found in fully grown oocytes (Table 1).

Discussion

As mouse oocytes grow during a 2- to 3-week period, MOM-1, ZP3 and LDH-B mRNA accumulate to unusually high levels (Table 1). During this period, the absolute rate of total protein synthesis increases about 40-fold (Schultz *et al.* 1979*a*,*b*; Wassarman, 1988*a*). However, the three mRNAs behave differently during the final stages of oocyte growth, as oocytes increase in

Table 1. *Steady-state levels of ZP3, MOM-1 and LDH-B mRNA during oogenesis and early embryogenesis*

	ZP3	MOM-1	LDH-B	Poly(A)$^+$ RNA (no. copies, $\times 10^{-6}$)*
	(no. copies mRNA, $\times 10^{-3}$)			
Growing oocytes				
10–20 μm	9	2	nd	5
30–40 μm	180	30	,,	25
50–60 μm	255	52	,,	54
60–70 μm	300	67	160	85
Fully grown oocytes	240 (0·27 %)†	74 (0·08 %)	200 (0·22 %)	90
Unfertilized eggs	5 (0·01 %)	41 (0·08 %)	160 (0·22 %)	45
Fertilized eggs	<1‡ (<0·003 %)	26 (0·07 %)	nd	40
Embryos				
2-cell	,,	2	,,	27
4- to 8-cell	,,	<1	,,	
Morula	,,	2	,,	
Blastocyst	,,	5	,,	23

* Calculated by using data presented in Bachvarova & DeLeon (1980), Bachvarova (1985), Pikó & Clegg (1982), and Clegg & Pikó (1983*b*).

† Values in parentheses represent mRNA levels as a percentage of total poly(A)$^+$ RNA.

‡ This value represents the lower limit of detection of mRNA by RNase protection assays in experiments described here (i.e. mRNA was undetectable).

nd, not determined.

Table 2. *Summary of ZP3, MOM-1 and LDH-B expression in fully grown oocytes*

	mRNA (no. copies)*	% Poly(A)$^+$ RNA†	% Polysomal poly(A)$^+$ RNA†	% Protein synthesized‡
ZP3	$2{\cdot}4\times10^5$	0·27	1·8	~3
MOM-1	$0{\cdot}7\times10^5$	0·08	0·6	~3
LDH-B	$2{\cdot}0\times10^5$	0·22	1·7	~2

* Determined by using RNase protection assays, as described here (see Table 1).

† Calculated by using data presented here and in Bachvarova & DeLeon (1980), DeLeon *et al.* (1983), and Bachvarova (1985).

‡ Estimated by using data presented in Bleil & Wassarman (1980*b*) and Cascio & Wassarman (1982), and from unpublished results (R. Roller, G. Salzmann and P. Wassarman).

diameter from about 65 μm to 80 μm. During this period, levels of MOM-1, ZP3 and LDH-B mRNA remain constant, decrease (~23 %) and increase (~20 %), respectively (Table 1). Levels of all three mRNAs fall during ovulation (10–12 h), when the absolute rate of total protein synthesis falls by about 20 % (Schultz *et al.* 1978, 1979*a*; Wassarman, 1988*a*) and RNA synthesis falls to undetectable levels (Wassarman & Letourneau, 1976; Rodman & Bachvarova, 1976; Bachvarova, 1985). During meiotic maturation steady-state levels of MOM-1 and LDH-B mRNA fall by about 50 % and 20 %, respectively, whereas the level of ZP3 mRNA decreases by nearly 98 % (Table 1). By the 2-cell stage of embryogenesis, ZP3 mRNA is undetectable and MOM-1 mRNA is present at about 1 % the level found in fully grown oocytes (Table 1). Although transcription of the embryonic genome takes place at the late 2-cell stage of development (G. A. Schultz, 1986*a*), ZP3 mRNA is undetectable in blastocysts, whereas MOM-1 mRNA reappears in morulae and is present in blastocysts at about 6 % the level found in fully grown oocytes (Table 2). Thus, transcription of the ZP3 gene occurs exclusively in oocytes, whereas the MOM-1 gene is also expressed in preimplantation embryos. The fact that ZP3 is expressed only in growing oocytes suggests the presence of specific factors that are involved in regulating the gene. In view of the extreme stability of many transcription complexes (Davidson *et al.* 1983; Fire *et al.* 1984; Klein *et al.* 1985; Sassone-Corsi *et al.* 1985; Wang & Calame, 1986), these activation factors need not be synthesized continuously during oocyte growth (2–3 weeks). Rather, they may be synthesized simultaneously with, or for a brief period just subsequent to, the onset of oocyte growth when ZP3 synthesis is initiated.

Changes in ZP3 mRNA described here parallel changes in ZP3 synthesis reported previously (Bleil & Wassarman, 1980*b*; Salzmann *et al.* 1983; Wassarman *et al.* 1985; Wassarman, 1988*b*), and support and extend results of *in situ* hybridization experiments (Philpott *et al.* 1987). ZP3 synthesis and secretion are first detected when oocytes begin to grow and acquire a zona pellucida. The zona pellucida increases in thickness as oocytes increase in diameter, reaching a final width of about 7 μm. During this period, ZP3 synthesis represents 1·5 % to 2·5 % of total protein synthesis in growing oocytes. As oocytes grow from about 65 μm in diameter onward, there is a small (~20 %) decrease in the relative rate of ZP3 synthesis (consistent with changes in ZP3 mRNA levels), however, synthesis continues even in fully grown oocytes. Only after ovulation of unfertilized eggs does ZP3 synthesis fall to virtually undetectable levels.

The decrease in ZP3 mRNA steady-state levels (~20 %) seen during late stages of oocyte growth, when poly(A)$^+$RNA levels increase slightly (Table 1), may indicate that ZP3 transcripts are relatively unstable compared to other oocyte mRNAs. This possibility is supported by the finding that about 98 % of ZP3 mRNA present in fully grown oocytes is lost during ovulation (10–12 h) when transcription is terminated and about 50 % of poly(A)$^+$RNA is lost (Table 1; Bachvarova & DeLeon, 1980; DeLeon *et al.* 1983; Bachvarova *et al.* 1985). The extensive loss of ZP3 mRNA during ovulation, as compared to other transcripts, could reflect sequence-specific degradation of the mRNA. Regulation of mRNA abundance by such a mechanism has been reported for several mRNAs (Shapiro *et al.* 1987) and, in some cases, the sequences that mediate selective degradation, often located in the 3′-untranslated region, have been characterized (Alterman *et al.* 1985; Simcox *et al.* 1985; Shaw & Kamen, 1986; Graves *et al.* 1987; Capasso *et al.* 1987). In this context, it should be

noted that ZP3 mRNA has an unusually short 3′-untranslated region (16 nt; Kinloch *et al.* 1988) that provides very little recognition sequence for a specific degradation mechanism. Of course, such determinants could be located elsewhere in the mRNA. Finally, the behavior of ZP3 mRNA during ovulation could be explained were the mRNA inherently unstable. Accordingly, high rates of transcription during oocyte growth would be required to accumulate ZP3 mRNA, whereas in eggs, in the absence of transcription, levels of ZP3 mRNA would fall dramatically. However, this explanation is unlikely in view of the enormously high rates of ZP3 transcription that would be required to produce the steady-state levels of ZP3 mRNA found in oocytes (see Footnote* for calculations). Thus, ZP3 mRNA must be much more unstable in ovulated eggs than in growing oocytes.

During meiotic maturation of mouse oocytes, the steady-state level of LDH-B mRNA falls by only 20 % (Table 1), while the relative rate of LDH-B synthesis decreases from about 1·8 % to 0·25 % of total protein synthesis (Cascio & Wassarman, 1982). A similar discrepancy has been noted for changes in β-actin synthesis and mRNA levels during meiotic maturation of mouse oocytes, and has been attributed to deadenylation of β-actin mRNA (Bachvarova *et al.* 1985; Paynton *et al.* 1988). Conversely, polyadenylation of tissue-type plasminogen activator mRNA during meiotic maturation of mouse oocytes is thought to account for activation of translation of the mRNA during this period (Huarte *et al.* 1987). Results reported for a variety of eukaryotic systems strongly suggest that the 3′-poly(A) sequence affects both mRNA stability and translational efficiency (e.g. Palatnik *et al.* 1984; Drummond *et al.* 1985; Galili *et al.* 1988). In this context, we have found that the size of LDH-B mRNA decreases from 1·4 (fully grown oocytes) to 1·3 kb (unfertilized eggs) during meiotic maturation (R. Roller & P. Wassarman, unpublished results), suggesting that the sharp decline in LDH-B synthesis during ovulation may be due to deadenylation of LDH-B mRNA. Thus, synthesis of ZP3 and LDH-B during meiotic maturation may be subject to different types of regulatory mechanisms. In the former case, decreased synthesis is probably due to mRNA degradation and, in the latter, decreased synthesis may be due to deadenylation of mRNA.

Although the translational efficiency of mRNAs may vary several-fold, steady-state levels of MOM-1, ZP3 and LDH-B mRNA in mouse oocytes are underrepresented in the poly(A)$^+$RNA population by about a factor of ten, with respect to their contribution to protein synthesis (Table 2). This suggests that the mRNAs may be represented solely in the oocyte's translated, poly(A)$^+$RNA population. Assuming that 15 % of total poly(A)$^+$RNA is associated with polysomes in fully grown oocytes (DeLeon *et al.* 1983), and that the three mRNAs are restricted to this population, their representation in that population becomes 0·6 %, 1·8 % and 1·5 % for MOM-1, ZP3 and LDH-B, respectively. These values are good approximations of the contributions of the mRNAs to protein synthesis in oocytes (Table 2). In this context, more than 90 % of β-actin mRNA in fully grown oocytes is associated with polysomes (Bachvarova *et al.* 1986). Results of preliminary experiments suggest that an analogous situation applies to ZP3 and MOM-1 mRNA in growing and fully grown oocytes (R. Rein & P. Wassarman, unpublished results).

To carry the suggestion just made a step further, evidence that the pool of untranslated poly(A)$^+$RNA in mouse oocytes is, in fact, untranslatable, is provided by results of *in vitro* translation of RNA purified from oocytes and eggs. It can be argued that the proportion of total protein synthesis directed by a specific mRNA *in vitro* should be roughly proportional to its representation in the total mRNA population, regardless of the *in vivo* situation. In support of this argument, it should be noted that RNA purified from cells that exhibit highly selective translational control *in vivo*, such as sea urchin (Jenkins *et al.* 1978; Moon *et al.* 1982) and surf clam (Rosenthal *et al.* 1980) oocytes and embryos, do not exhibit such selectivity *in vitro*. Accordingly, were all mouse oocyte poly(A)$^+$RNA translatable mRNA, it would follow that translation of LDH-B and β-actin mRNA would be 6- to 10-fold lower *in vitro* than *in vivo*. However, such is not the case for either protein. Actin synthesis represents about 0·3 % of total protein synthesis in isolated fully grown oocytes and about 0·4 % of synthesis using RNA prepared from fully grown oocytes (G. Salzmann & P. Wassarman, unpublished results). Similarly, oocyte LDH-B synthesis represents about 1·8 % of total protein synthesis both *in vivo* and *in vitro* (Cascio & Wassarman, 1983). These findings suggest that the pool of translatable mRNA in mouse oocytes is represented largely by polysomal poly(A)$^+$RNA. Therefore, *bona fide* mRNA probably constitutes only about 3 % of total RNA, or about 15 % of poly(A)$^+$RNA in mouse oocytes. An analogous situation exists in sea urchin and amphibian oocytes, where *bona fide* mRNA constitutes about 30 % of total poly(A)$^+$RNA (Davidson, 1986).

* ZP3 mRNA has a $T_{1/2}$ of ~4 h during ovulation, assuming first-order kinetics of degradation. If ZP3 mRNA had the same $T_{1/2}$ in 60–70 μm growing oocytes, where the steady-state level of ZP3 mRNA is $\sim 3\times10^5$ copies oocyte^{-1}, then $\sim 1{\cdot}5\times10^5$ copies would have to be transcribed every 4 h to maintain the level of ZP3 mRNA. Since the ZP3 locus is about 9 kb (Kinloch *et al.* 1988), this would require synthesis of $\sim 2{\cdot}4\times10^4$ nt s^{-1} copy^{-1}. The rate of elongation by RNA polymerase II is estimated at about 50 nt s^{-1} (Manley, 1984). Thus, the ZP3 gene would need ~480 polymerase molecules per 9 kb, or about one polymerase molecule every 19 nt, an extremely unlikely situation. For comparison, it has been estimated that RNA polymerase molecules are located at 100–200 nt intervals along amphibian lampbrush chromosomes (Hill, 1979).

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