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Evidence for the involvement of the proto-oncogene c-*mos* in mammalian meiotic maturation and possibly very early embryogenesis

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The c-*mos* proto-oncogene exists as a maternal mRNA in mammalian oocytes, in that it has been shown to accumulate in mouse oocytes during the growth phase and to be present at high levels in fully grown oocytes. The function of c-*mos* during the subsequent development of the oocytes and embryos was examined by determining the fate of the oocyte c-*mos* mRNAs by *in situ* hybridization and Northern blot hybridization analysis. A substantial decrease in the levels of c-*mos* transcripts was observed in oocytes undergoing meiotic maturation. By the two-cell stage, levels of c-*mos* transcripts dropped to below the limits of detection using *in situ* hybridization. c-*mos* transcripts remained undectable through the blastocyst stage of embryogenesis. Analysis of meiotic maturation *in vitro* permitted finer temporal resolution of the initial drop in c-*mos* levels. Between ~7 and 17 h of culture, the amount of c-*mos* mRNA fell to 18-43% of the levels found in the fully grown oocyte. This interval corresponds to the progression of meiotic maturation from metaphase I to metaphase II. Our *in vivo* studies showed that ovulation *per se* is not the stimulus for the drop in c-*mos* transcript levels, since preovulatory metaphase II oocytes exhibited this decline to a degree comparable to that of ovulated metaphase II oocytes. The developmental specificity of c-*mos* transcript levels suggests a role of this putative serine kinase in the meiotic maturation of mammalian germ cells.

Key words: cellular oncogene/c-*mos*/*in situ* hybridization/oocyte/meiotic maturation

Introduction

The molecular mechanisms of gamete formation, fertilization and activation of the embryonic developmental program are poorly understood, especially among mammals. Identification of the genes involved in these processes by classical genetic analysis has been complicated by the large size of the mammalian genome and the difficulties in working with mutants with compromised reproductive potential. The small number of cells, especially in the female germ cell lineage and at fertilization and early embryogenesis, presents considerable technical obstacles in molecular approaches to select for genes produced uniquely or abundantly at these developmental stages.

An alternative approach has been to utilize available probes and highly sensitive molecular assays, such as *in situ* hybridization or micro-scale solution hybridization, to examine the expression of more ubiquitously expressed genes in the germ cells and early embryos. Several abundantly expressed 'housekeeping' genes have been examined using this approach, including actin (Giebelhaus *et al.*, 1983, 1985), histone (DeLeon *et al.*, 1983a; Giebelhaus *et al.*, 1983), and U1 RNAs (Lobo *et al.*, 1988).

A category of genes whose expression in the mammalian germ line has recently been demonstrated using this approach is that of the cellular oncogenes. These genes are of particular interest because of their proposed functions in regulating cellular growth, differentiation and proliferation. Among the cellular oncogenes shown to exhibit unique patterns of expression in the male germ line are c-*abl* (Ponzetto and Wolgemuth, 1985), *int*-1 (Shackleford and Varmus, 1987), and several members of the *ras* family (Leon *et al.*, 1987). The proto-oncogene c-*mos* exhibits a particularly intriguing pattern of expression during gametogenesis and embryogenesis. c-*mos* transcripts are detected most abundantly in the gonads of the adult mouse and at low levels in the adult epididymis, day-19 embryo (Propst and Vande Woude, 1985) and in certain somatic tissues (Propst *et al.*, 1987; Mutter and Wolgemuth, 1987). In the testis, expression of c-*mos* is most abundant in the post-meiotic early spermatids (Goldman *et al.*, 1987; Mutter and Wolgemuth, 1987; Propst *et al.*, 1987). In the ovary, c-*mos* transcripts have been shown to accumulate in the cytoplasm of the growing oocytes (Mutter and Wolgemuth, 1987), reaching high levels in fully grown oocytes (Goldman *et al.*, 1987; Mutter and Wolgemuth, 1987).

Following the growth phase, oocytes undergo meiotic maturation, progressing through to the metaphase II stage prior to ovulation. The ovum is arrested at this stage until fertilization, which initiates completion of the reductive division and extrusion of the second polar body. The initial DNA synthesis and first mitotic division occur, followed by cellular divisions eventually yielding the blastocyst with its inner cell mass and trophectoderm. The high levels of c-*mos* mRNAs in the fully grown oocyte suggests the possibility that c-*mos* may be important in subsequent stages of oocyte differentiation or in early embryogenesis.

The extent to which maternal informational molecules contribute to early mammalian embryogenesis is not understood. Analysis of newly synthesized proteins during meiotic maturation, fertilization and very early embryogenesis demonstrates considerable changes in the patterns (e.g. Schultz and Wassarman, 1977a,b; Braude *et al.*, 1979). Since transcriptional activity is low at these stages (reviewed by Bachvarova, 1985), there is a possibility that mRNA accumulated in the growing oocytes might be stored and utilized at these later stages.

In the present study, we sought to investigate the possible function of c-*mos* in the developmental events following oocyte growth by examining the fate of the c-*mos* transcripts found in the fully grown ovarian oocyte. Specifically, we

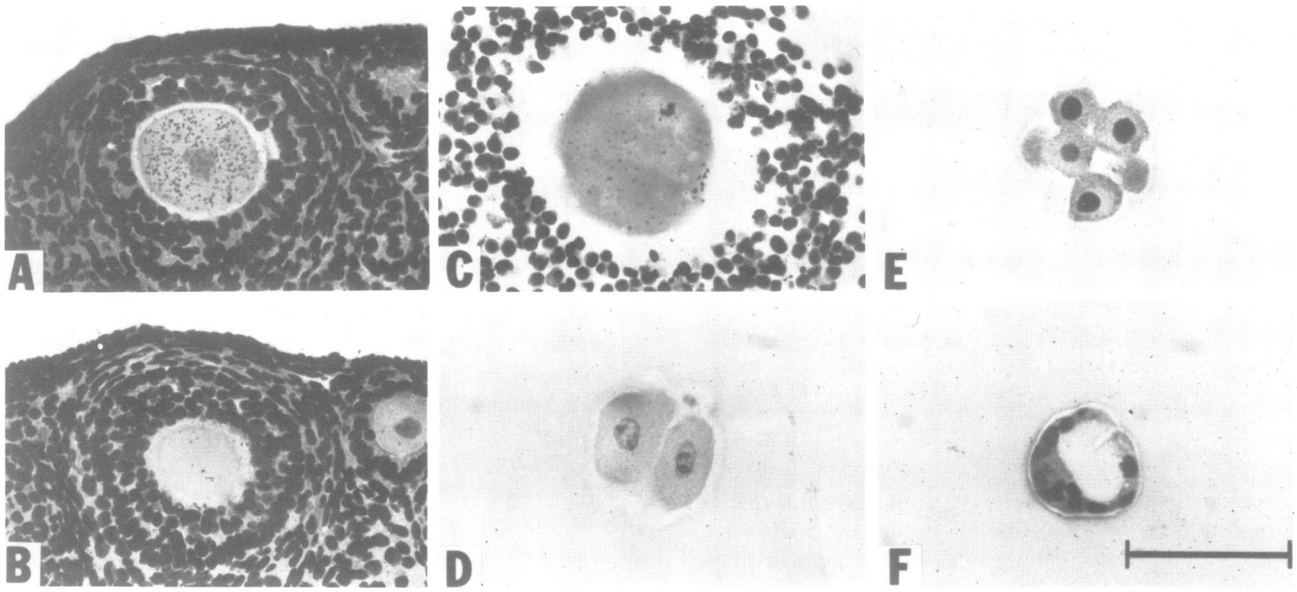

Fig. 1. Photomicrographs of whole ovaries, and agarose-embedded ova and embryos, hybridized *in situ* to c-*mos* probes. Ovulated ova (**panel C**), two-cell embryos (**panel D**), 8–16-cell–early morulae (**panel E**) and blastocysts (**panel F**) were isolated from the oviducts or uteri of hormonally stimulated mice and embedded in agarose as described in Materials and methods. Whole ovaries from unstimulated mice (**panels A** and **B**) were included in the blocks as a positive control tissue. Slides were hybridized with either antisense (**panels A** and **C–F**) or sense (**panel B**) ^{35}S-labeled c-*mos* RNA probes, and developed after 14 days exposure. Autoradiographic c-*mos* signal is visible as punctate dark silver grains, readily seen in growing ovarian oocytes (**panel A**) and to a lesser extent in ovulated ova in M^{II} (**panel C**). The first polar body and metaphase plate are visible in the ovulated ovum. Negative controls hybridized to the complementary strand (sense) *mos* probe show a low grain density (**panel B**). The preimplantation embryos (**panels D–F**) do not contain signal above background. In this composite figure panels A, D and F were in a single slide, as were panels C and E. All slides were hybridized in parallel with an equal number of counts. The scale bar represents 100 μm.

have determined the levels of c-*mos* mRNAs in ovulated eggs and in early embryos through to the blastocyst stage. Particular attention was focused on the peri-ovulatory period of development in which the oocytes resume meiotic maturation.

Results

c-mos transcripts in oocytes, ova and early embryos

To examine the fate of the high levels of c-*mos* transcripts present in fully grown oocytes, we utilized the technique of *in situ* hybridization as our primary experimental approach. The small size (~80 μm) of oocytes and early embryos as well as the difficulties in obtaining large numbers of these cells severely limits the amount of RNA that can be obtained for molecular analyses. To maximize our ability to make semi-quantitative evaluations on the levels of c-*mos* transcripts in oocytes and early embryos in the *in situ* analysis, we were careful to obtain sufficient numbers of cells at different stages of development, all within a single histological section.

In the initial experiments, cells were retrieved at the following developmental stages: ovarian oocytes; ovulated ova; two-cell embryos; four-to-eight-cell embryos; morulae and blastocysts. The cells were embedded in agarose, processed and subjected to *in situ* hybridization analysis using the c-*mos* probe in both anti-sense and sense orientations. As a control for the efficiency of hybridization of the agarose-embedded cells, whole ovaries were fixed and placed in the paraffin block.

Evaluation of the autoradiographic silver grain pattern revealed that the high level of c-*mos* transcripts present in the fully grown ovarian oocytes dropped dramatically by the two-cell stage (Figure 1). Hybridization to embryos in later stages revealed no labeling above background levels in four-to-eight-cell stage, morulae and blastocysts. The low level of labeling was not due to interference from the agarose-embedding procedure, because oocytes which had been manually recovered from ovaries and embedded in agarose showed the typical high levels of c-*mos* transcripts (Figure 2).

More precise examination of the density of autoradiographic silver grains among the various developmental stages suggested that there was a substantial drop in the levels of c-*mos* transcripts in ovulated ova (Figure 2) as compared with isolated ovarian oocytes with an intact germinal vesicle (Figure 2). Quantitation of silver grains indicated that ovulated ova contained only 11–20% the level of c-*mos* transcripts found in fully grown oocytes (Figure 2b).

Northern blot hybridization of c-mos transcripts

The decrease in the level of c-*mos* transcripts was confirmed by Northern blot hybridization analysis of RNA isolated from ovarian oocytes and from ovulated ova. The results are shown in Figure 3. Within each experiment equal numbers of oocytes or ova were used for each sample and equal amounts of carrier RNA were present in the extraction. Oocyte RNA was highly enriched for the 1.4-kb c-*mos* transcript, also seen in RNA from whole ovary (Figure 3, panel A). Densitometry of Northern blot autoradiograms showed that 550 oocytes contained 26% more c-*mos* signal than 20 μg of whole ovary RNA. The level of c-*mos* transcripts is decreased in RNA from ova compared with oocytes, estimated by densitometry to represent 15% of the signal found in oocytes.

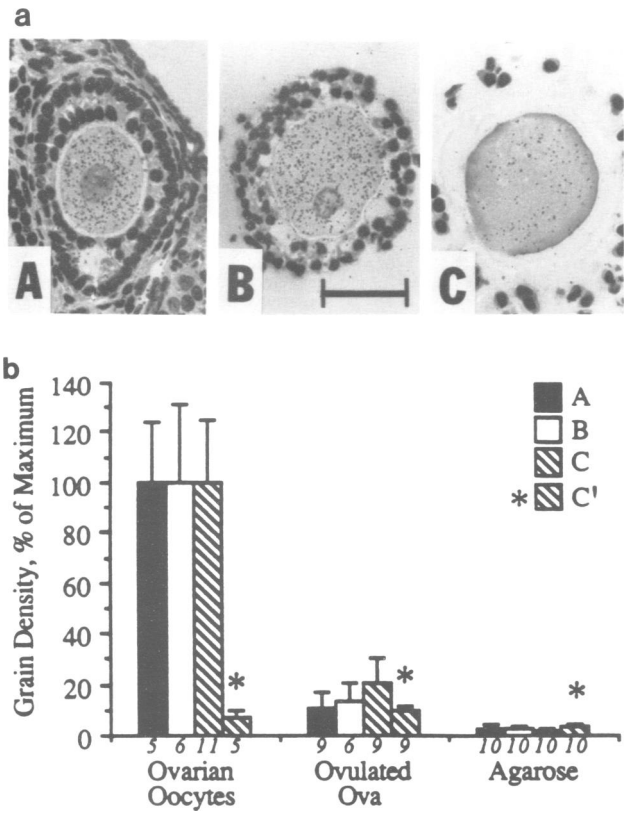

Fig. 2. (**a**) Photomicrographs of ovarian oocytes, and isolated oocytes and ova hybridized *in situ* to antisense c-*mos* probe. Ovaries and isolated oocytes were obtained from mice stimulated with PMS, or ovulated ova recovered from the oviducts of mice stimulated with PMS and hCG. Sections were hybridized with ^{35}S-labeled c-*mos* antisense probe as described and exposed for 14 days. A comparison of panels A and B shows that the process of agarose embedding did not significantly affect the intensity of autoradiographic signals, which is very high in oocytes with an intact germinal vesicle. The quantity of c-*mos* transcripts was reduced during meiotic maturation, as evidenced by a lower grain density in ovulated ova (**panel C**). All panels from a single histological section. The scale bar equals 50 μm. (**b**) Quantitation of c-*mos in situ* hybridization signal in isolated oocytes and ova. Ovarian oocytes and ovulated ova were hybridized to antisense (A–C) or sense (C′) c-*mos* probes. After exposure intervals of 10, 14 and 14 days for slides A, B and C respectively, grain density was quantitated, and the mean grain densities for each group are plotted as bar graphs. To compare data from several slides on a common scale, the maximal grain density for each experimental group (A–C) was designated at 100% and the grain densities for the various cell types within each experimental group was expressed as a percentage of that value. The maximal, or 100%, grain density values for groups A, B and C were 91.6, 101.4 and 111.0 grains/1000 μm^2. In all experiments a negative control probe, sense strand of c-*mos*, was processed in parallel to evaluate nonspecific background levels. One representative sense strand negative control (C′) is shown on the same scale as its companion antisense experimental slide (C). Vertical bars are standard deviations, and numbers below bars are sample size.

In addition, we have repeatedly observed a slight shift in the migration pattern in the c-*mos* transcripts detected in ova, as shown in Figure 3, panel B. The c-*mos* transcript from RNA in ovulated ova migrates at ~1.65 kb in length. The 1.4-kb transcript is detected only in RNA from oocytes and whole ovary. The 1.65-kb transcript, however, is unique to ova that have completed meiotic maturation, a stage of oocyte development poorly represented in unstimulated whole ovaries. It is unlikely that adherent granulosa cells included in the oocyte and ova samples contributed significantly to either of these transcripts, as they are absent in RNA from

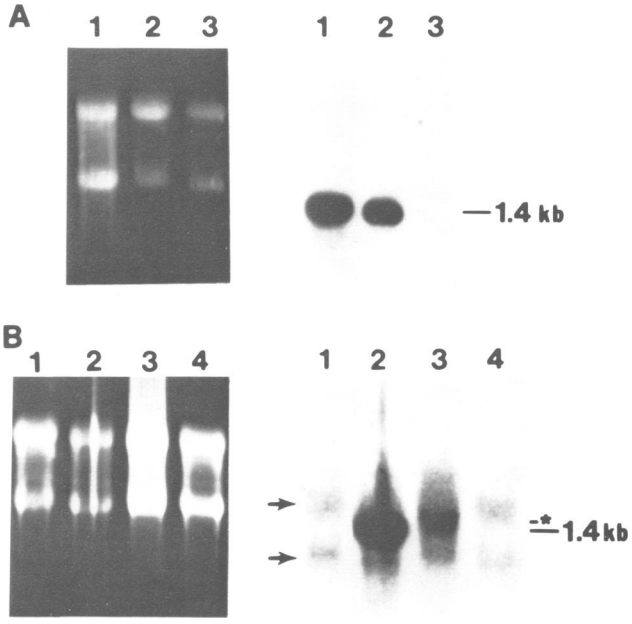

Fig. 3. Hybridization of isolated oocyte and ova RNA to c-*mos* probe. RNA was isolated from whole ovaries, oocytes, ovulated ova and granulosa cells, then electrophoresed, blotted and hybridized to ^{32}P-labeled c-*mos* RNA probe as described in the text. Each panel shows the ethidium-bromide-stained gel on the left, and corresponding c-*mos* autoradiogram on the right. **Panel A**: **lane 1**, RNA from 550 oocytes; **lane 2**, 20 μg total ovary RNA; **lane 3**, 20 μg (carrier) liver total RNA. The 1.4-kb ovary-specific transcript is enriched in isolated oocytes. Exposure time 72 h. **Panel B**: **lane 1**, 40 μg total (carrier) liver RNA; **lane 2**, RNA from 500 oocytes; **lane 3**, RNA from 500 ovulated ova; **lane 4**, granulosa cell RNA. The 1.4-kb ovary-specific transcript is readily seen in oocytes (**lane 2**), whereas ovulated eggs (**lane 3**) contain a smaller quantity of c-*mos*-related transcripts (asterisk) which are ~250 nucleotides (nt) longer. All lanes, including granulosa cells and carrier, have some nonspecific hybridization to the 18S ribosomal band (upper arrow) and a poorly defined shorter species (lower arrow). Exposure time 12 days.

granulosa cells alone (isolated with liver carrier) (Figure 3, panel B). Upon long exposure (Figure 3, panel B) total liver RNA, alone or as a carrier, exhibited faint hybridization at ~1.0 and 1.9 kb, the latter corresponding to the position of the 18s rRNA.

c-mos transcripts in oocytes in the peri-ovulatory period

To evaluate the developmental specificity in the changes in levels of c-*mos* transcripts more thoroughly, three series of experiments were performed, incorporating both *in vivo* and *in vitro* approaches.

To determine if the drop in detectable c-*mos* transcripts preceded or followed ovulation, animals were stimulated with PMS and hCG and ovaries were recovered 10 h after the administration of hCG. These ovaries were enriched for ova just about to be ovulated, as well as oocytes in earlier stages of oogenesis. A substantial decrease in the levels of c-*mos* transcripts was observed in oocytes in stage-8 follicles, as compared with the fully grown oocytes of stage-6 follicles (Figure 4). The single most significant event that occurs between these stages is the resumption of meiosis, with the cells completing metaphase I (M^I) and entering into metaphase II (M^{II}) of meiosis.

To follow meiotic maturation more closely, ovarian oocytes were isolated and allowed to undergo meiotic

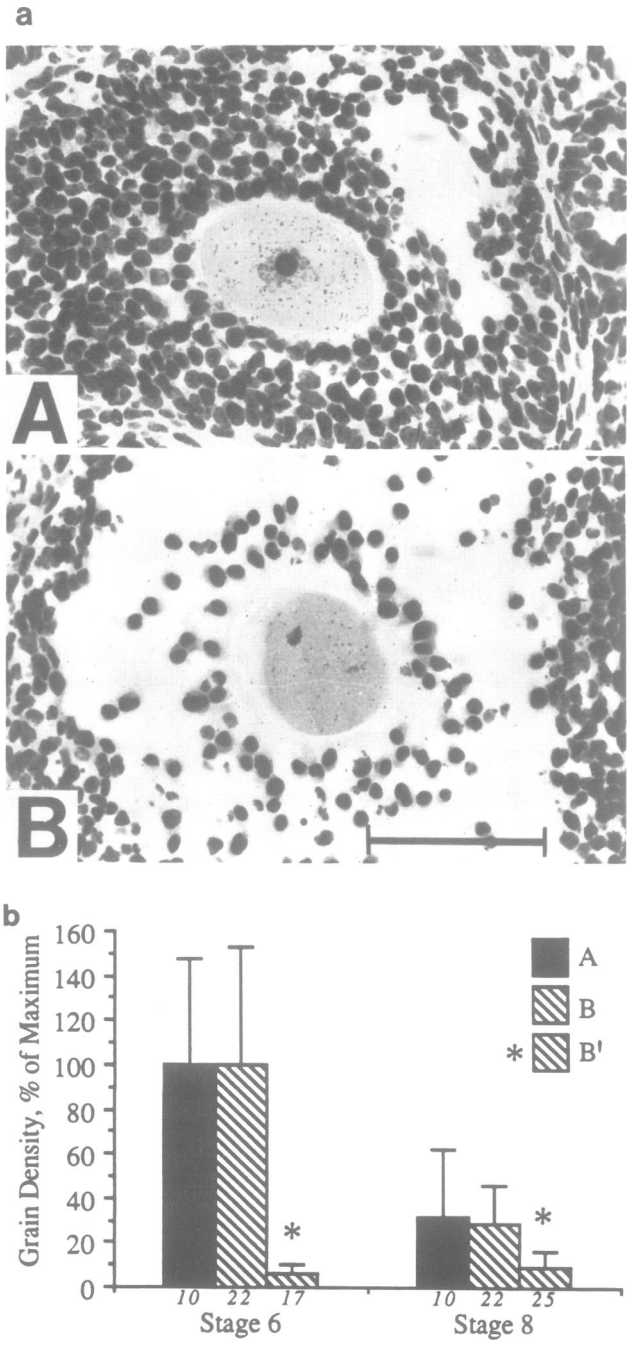

Fig. 4. (**a**) Photomicrographs of antisense c-*mos* probe hybridized *in situ* to ovaries of hormonally stimulated, preovulatory, mice. Mice were administered PMS and hCG, then sacrificed 10 h after hCG injection, at a time when many follicles had undergone final stages of development. The intact ovaries were fixed, sectioned, and hybridized to antisense c-*mos* probe as described. Stage-6 ovarian follicles (**panel A**) contain oocytes with abundant *mos*-related autoradiographic signal. During the later stages of preovulatory development much of this signal is lost, leaving a sparse distribution of silver grains in stage-8 follicle ova (**panel B**). Both panels are from the same histologic section. Exposure time 14 days. The scale bar equals 100 μm (**b**) Quantitation of c-*mos in situ* hybridization signal in ovaries of gonadotropin stimulated mice. *In situ* hybridization results in the experiment illustrated in (a) were quantitated and plotted as bar graphs as described in the legend to Figure 2b. Maximal, or 100% grain density values were 24 and 22 grains/1000 μm^2 for slides A and B. Exposure times were 2 and 4 days respectively.

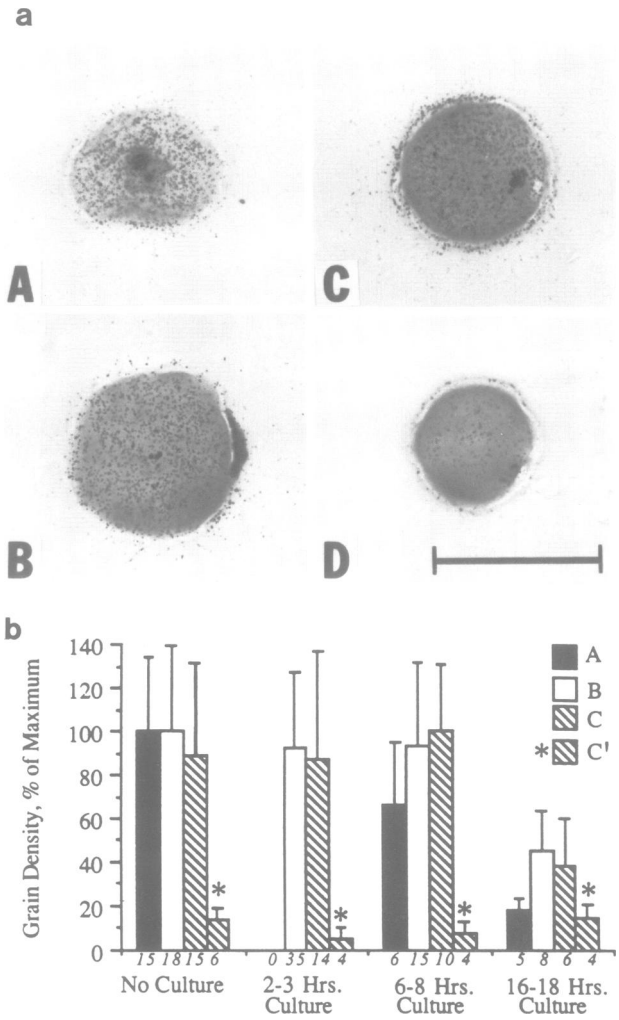

Fig. 5. (**a**) Photomicrographs of oocytes matured *in vitro* hybridized *in situ* to antisense c-*mos* probe. Ovarian oocytes were cultured *in vitro*, processed and hybridized to ^{35}S-labeled c-*mos* probe as described. A constant, high level of autoradiographic signal was seen in the uncultured oocytes (**panel A**, intact germinal vesicle), and in oocytes cultured 2–3 h (**panel B**, germinal vesicle broken down) and 7–8 h (**panel C**, M^{I}). Oocytes cultured 16–18 h (**panel D**, M^{II}) contain demonstrable signal, but greatly reduced compared with other groups. All panels are from a single histological section. Exposure time 19 days. The scale bar is 100 μm long. (**b**) Quantitation of c-*mos in situ* hybridization signal in isolated oocytes matured *in vitro*. The results of the *in vitro* meiotic maturation experiment illustrated in (a) were quantitated and plotted as bar graphs, as described in the legend of Figure 2b. Groups A, B and C were hybridized with antisense probe, C′ with sense *mos* probe. In one slide, B, some oocytes in several groups (6/18 no culture, 7/35 2–3 h, 3/15 6–8 h) had confluent grains unsuitable for direct quantitation (see Materials and methods for discussion). Maximal, or 100% values were 41, 69 and 18 grains/1000 μm^2 for slides A, B and C. Exposure times were 4, 5 and 4 days respectively.

maturation in an *in vitro* system. This approach permits a precise temporal evaluation of the meiotic progression of the oocytes. Oocytes were recovered from unstimulated animals, placed in the *in vitro* maturation culture system, and collected at intervals as designated. The following stages were examined: (i) ovarian oocytes, no *in vitro* culture, cells with intact germinal vesicles and chromosomes in the dictyate stage of prophase I of meiosis; (ii) oocytes after ~2–3 h of culture, in which the germinal vesicle has broken down;

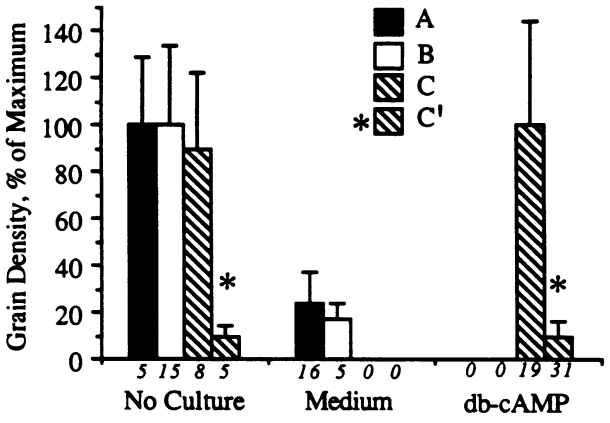

Fig. 6. Effects of dibutyryl cAMP (db-cAMP) on c-*mos* transcript levels during *in vitro* meiotic maturation of oocytes. Isolated ovarian oocytes were cultured for 16–18 h either in medium alone, or medium containing 100 μg/ml db-cAMP. A third group of uncultured isolated oocytes was embedded for comparison to the matured (medium) and arrested (db-cAMP) cells. Embedding, *in situ* hybridization, quantitation and data plotting are as described in Figure 2b. Slides A, B and C were hybridized to antisense *mos* probe, and slide C′ to sense c-*mos* probe. Maximal, or 100%, values for slides A, B and C are 30, 41 and 21 grains/1000 μm^2. All slides were exposed 4 days.

(iii) oocytes after ~6–8 h of culture, M^I of meiosis; (iv) oocytes at ~16–18 h of culture, in which the chromosomes have progressed to M^{II} of meiosis and the first polar body has been extruded. The pattern of changes in c-*mos* transcript levels is readily apparent in the photomicrographs shown in Figure 5a. A similar level of labeling was observed among ovarian oocytes, oocytes which have undergone germinal vesicle breakdown, and in ova at M^I (Figure 5b). In contrast, a 57–82% drop in the level of c-*mos* transcripts was observed in cells which have entered the M^{II} stage (Figure 5b). This drop in c-*mos* transcript levels was similar to that observed in the *in vivo* experiments described above, in which c-*mos* mRNA levels decreased between oocytes in stage-6 follicles and oocytes in later-staged follicles, which may have entered meiosis.

To extend these observations, we performed an experiment in which we manipulated the timing and progression of meiosis *in vitro*. The goal of this experiment was to determine if the drop in levels of c-*mos* transcripts was simply a function of the length of time elapsed in culture following isolation from the ovary or was dependent upon the actual progression of meiosis. To discriminate between these two possibilities, we incubated oocytes in media containing dibutyryl cyclic AMP (db-cAMP), an inhibitor of meiotic maturation (Cho *et al.*, 1974). Oocytes which were incubated with db-cAMP for 16–18 h and which did not therefore undergo germinal vesicle breakdown and meiotic maturation, concomitantly showed no drop in c-*mos* transcript levels (Figure 6).

Discussion

c-*mos* mRNA has previously been shown to accumulate in growing ovarian oocytes (Mutter and Wolgemuth, 1987), being present at high levels in fully grown oocytes (Goldman *et al.*, 1987; Mutter and Wolgemuth, 1987). This study examined the fate of c-*mos* transcripts during subsequent development of oocytes, demonstrating a substantial loss of c-*mos* transcripts in oocytes undergoing meiotic maturation and a further drop to below the limits of sensitivity of detection by the two-cell stage. c-*mos* autoradiographic signal is concentrated within the cytoplasm at all stages, and we have no evidence of bulk c-*mos* mRNA shift from the nucleus to cytoplasm of oocytes, such as has been seen with U1 RNA in mouse oocytes (Lobo *et al.*, 1988).

Meiotic maturation *in vitro* permitted finer temporal resolution of the changes in c-*mos* levels, and showed that c-*mos* mRNA was reduced to 18–43% of the level in fully grown oocytes between about 7 and 17 h of culture, corresponding to the meiotic stages M^I and M^{II} respectively. Ovulation *per se* is not the stimulus for this drop in c-*mos* transcript levels in the ova, since our *in vivo* studies showed that preovulatory M^{II} oocytes exhibit this decline to a degree comparable to that of ovulated M^{II} oocytes. This drop in the levels of c-*mos* transcripts was also seen by Northern blot hybridization analysis of RNA from isolated oocytes and ova.

The magnitude of the decrease in the level of c-*mos* mRNA during meiotic maturation was estimated to be 85% by densitometry of Northern blot autoradiograms, compared with the 80–89% decrease in the number of autoradiographic silver grains detected by *in situ* hybridization (Figure 2b). The similarity in the estimates of decrease of c-*mos* mRNA levels obtained by the two independent methods suggests that, if carefully controlled, quantitation using *in situ* hybridization can provide important information on mRNA levels in cells which are rare or difficult to isolate.

The observed drop in c-*mos* transcripts during meiotic maturation follows a general pattern of mammalian oocyte mRNA turnover (Bachvarova, 1985). mRNA is actively synthesized and maintained in growing oocytes until the preovulatory phase. Meiotic maturation heralds the onset of depletion of approximately half of the poly(A)$^+$ RNA, due to deadenlyation and/or degradation of pre-existing mRNA. This depletion continues progressively and rapidly through the two-cell embryonic stage (DeLeon, 1983b; Piko and Clegg, 1982). Since our methods of measuring c-*mos* transcripts did not depend on the presence of a poly(A) tail, we would suggest that the decrease in the level of c-*mos* transcripts reflected mRNA turnover.

Northern blot analysis of RNA from isolated oocytes and ova revealed quantitative and apparent qualitative changes in c-*mos* transcripts. A single transcript of 1.4 kb was detected in RNA from ovaries and isolated oocytes, while in ovulated ova, a discrete transcript of 1.65 kb was observed. It is unlikely that there was synthesis of new c-*mos* transcripts during maturation, although this possibility cannot be excluded, since oocytes which resume meiosis in culture do incorporate small, but measurable amounts of uridine until the point of germinal vesicle breakdown (Bloom and Mukherjee, 1972). Alternatively, the slightly larger (by ~250 nt) c-*mos* transcripts in ova may be due to polyadenylation of pre-existing oocyte transcripts. The 1.4-kb c-*mos* RNA most likely does contain some portion of a poly(A) tail, as evidenced by the enhancement in detection of c-*mos* transcripts by oligo(dT) column selection of ovarian RNA (Mutter and Wolgemuth, 1987). Lengthening of the poly(A) tail of the 1.4-kb species could occur during maturation, yielding a larger transcript.

Polyadenylation of pre-existing mRNAs during development has been reported for several genes, with a temporal correlation between polyadenylation and translation. Translation of maternal mRNAs in clam (*Spisula*) oocytes and embryos is largely regulated by stage-specific polyadenylation, followed by association with polysomes (Rosenthal *et al.*, 1983; Rosenthal and Ruderman, 1987). In mouse, actin mRNAs accumulate in growing oocytes, are adenylated and become associated with polysomes, and are then deadenylated and degraded at fertilization (Bachvarova *et al.*, 1985). Tissue plasminogen activator (TPA) mRNA is also synthesized in growing mouse oocytes, with polyadenylation and translation occurring during meiotic maturation. Coincident with appearance of the protein product is an increased turnover of TPA mRNA, 80% of which is degraded by M^{II} of meiosis (Huarte *et al.*, 1987).

c-*mos* transcript levels were intimately tied to the process of meiosis, in that they were not detected above background (by *in situ* hybridization) beyond the second metaphase stage, and specifically were not detected in later stages of development, including pre-implantation embryos (two-cell, four-to-eight-cell, morula and blastocyst). That is not to say that c-*mos* transcripts were not present at these stages, but rather that quantities were insufficient for detection by *in situ* hybridization analysis. This is in contrast to observations of mRNA levels for several maternal mRNAs, actin and histone H3, which drop significantly by the two-cell stage, but which rise again after the two-cell stage, presumably due to *de novo* embryonic synthesis (Giebelhaus *et al.*, 1983, 1985; Graves *et al.*, 1985).

Studies on c-*mos* protein have been very limited, due to the fact that the presence of c-*mos* transcripts within normal cells has only recently been demonstrated and because antibodies against the protein have extensive cross reactivity with other cellular proteins (Papkoff *et al.*, 1981, 1983). Nothing is known about c-*mos* proteins in oocytes or early embryos.

It is nonetheless interesting to speculate as to possible roles for the c-*mos* protein during these stages, based upon what is known about changes in patterns of polypeptides in general and the postulated function of *mos* as a protein (serine) kinase (Maxwell and Arlinghaus, 1985; Singh *et al.*, 1986). The proteins essential for germinal vesicle breakdown and condensation of chromosomes are present when meiotic maturation begins (Golbus and Stein, 1976; Schultz and Wasserman, 1977a), but stages beyond metaphase I require proteins synthesized after the germinal vesicle breaks down (Schultz and Wasserman, 1977a,b). Since there is little or no transcription at this time, the newly synthesized proteins would have to originate from stored mRNAs. The patterns of phosphoproteins undergo drastic changes during meiosis. The resumption of meiosis coincides with a drop in cellular cAMP and both phosphorylation and dephosphorylation of several proteins (Schutlz *et al.*, 1983; Bornslaeger *et al.*, 1986a,b). The *mos* protein, as a serine kinase, could play a role in these changes.

Materials and methods

Sources and preparation of recombinant DNA probes

A previously described (Mutter and Wolgemuth, 1987) 960-bp *AvaI*–*Hin*dIII genomic murine c-*mos* insert (Oskarsson *et al.*, 1980) ligated into the linker region of the riboprobe vector plasmid pGEM-3 (Promega Biotech, Madison, WI) was used. Transcripts from the SP6 promoter were in an antisense orientation and recognized the ~1.4-kb c-*mos* ovarian transcript, whereas transcripts from the T7 promoter had a sense orientation and did not recognize c-*mos* transcripts as assayed by blot hybridization analysis.

^{35}S- or ^{32}P-labelled RNA probes were transcribed from linearized pGEM-3 vectors according to protocols suggested by the manufacturer using radiolabeled UTP. Typically 50–90% of the labeled UTP was incorporated, yielding probes with sp. act. of $1.0-1.1 \times 10^8$ c.p.m./μg (^{32}P, Cerenkov counts), or $0.6-2.3 \times 10^8$ c.p.m./μg (^{35}S, liquid scintillation counting).

Sources of tissues and cells

All tissues were obtained from 3- to 6-week-old female B6D2F1 mice (The Jackson Laboratories, Bar Harbor, ME), sacrificed by cervical dislocation.

Superovulation of ova was accomplished by an initial i.p. injection of 5 IU pregnant-mare serum (PMS; Sigma, St Louis, MO) in late afternoon, followed 48 h later by 2.5 IU of human chorionic gonadtropin (hCG; Sigma, St Louis, MO) injected i.p. Ovulated, unfertilized eggs were recovered by puncturing the ampulla of the Fallopian tube the morning after hCG injection. Females to be mated were placed with males the afternoon of hCG injection. Matings were assumed to occur late in the evening. The next morning was designated gestational day 0.5. Preimplantation embryos were recovered by flushing the oviduct or uterus with medium (Rafferty, 1970) after the appropriate gestational intervals as follows: two-cell, 1.5 days; four-to-eight-cell, 2.5 days; morula, 3.5 days; and blastocyst, 4.5 days.

Oocytes for *in vitro* meiotic maturation experiments were isolated from unstimulated ovaries by needle puncture in culture medium (Rafferty, 1970). Oocytes for controls for ovulated ova and embryo experiments were recovered from animals stimulated with 5 IU PMS 48 h before sacrifice. Stage 8 (Pederson and Peters, 1968) ovarian follicles were obtained by injection of 5 IU PMS 48 h before 2.5 IU hCG injection. Ovaries were recovered 10 h after hCG injection.

Culture of oocytes and embryos

Isolated ovarian oocytes or preimplantation embryos were cultured at 37°C, 5% CO_2 in air, for variable intervals, in Ham's F-10 essential medium (K.C.Biological, Lenaxa, KN), supplemented with 75 mg/l streptomycin, 75 mg/l penicillin G, 245 mg/l lactic acid and 2.1 g/l sodium bicarbonate, in the absence of serum. Monitoring and manipulation of cultured cells was accomplished with a Wild M5A dissecting microscope at 50× magnification. Embryo culture was transient (<4 h) pending processing for *in situ* hybridization.

The temporal progression of *in vitro* meiotic maturation following oocyte recovery from the ovary has been defined (Donahue, 1968; Endo *et al.*, 1986). At the time of sacrifice most oocytes are in prophase of the first meiotic division, with an intact germinal vesicle, 80% of which break down by 3 h (Donahue, 1968). Of those cells that exhibit germinal vesicle breakdown, 90–95% progress to metaphase of the first meiotic division at 4.5–9 h of culture (Donahue, 1968). Metaphase of the second meiotic division is more asynchronous, appearing at 11–17 h, and is associated with extrusion of the first polar body. In our experiments, isolated oocytes with intact germinal vesicles were processed as follows to obtain specific meiotic stages: (i) meiotic prophase, no culture, germinal vesicle intact; (ii) germinal vesicle breakdown; oocytes cultured for 2–3 h *in vitro*, selected for nuclear dissolution; (iii) metaphase I of meiosis (M^I); oocytes cultured for 6–8 h, selected for nuclear dissolution; and (iv) metaphase II of meiosis (M^{II}); oocutes cultured for 16–18 h, selected for formation of the first polar body. Oocytes treated with dibutyryl cAMP (db-cAMP) were isolated and cultured in medium containing 100 μg/ml db-cAMP (Sigma, St Louis, MO).

Processing of isolated cells for in situ hybridization

Isolated oocytes and embryos for *in situ* hybridization were embedded in agarose prior to fixation for ease of handling. A 1-mm thick underlayer of 1% standard melting point agarose (LE Agarose, Seakem, Rockland, ME) was prepared in a Petri dish. The oocytes or embryos were micropipetted into 1.2% low-melting-point agarose (gelling temperature, 28°C, BRL, Gaithersburg, MD) made in phosphate-buffered saline with 1% dextran blue 2000 (Pharmacia, Uppsala, Sweden) at 37°C. Each experimental group was micropipetted in a separate agarose drop into the Petri dish, all hardening in a single plane on the surface of the agarose underlayer. When the droplets containing the cells had hardened, an overlay of standard-melting-point agarose was quickly poured and cooled. The 'sandwich' of standard-melting-point agarose, containing cells at the interface, was cut into a rectangular block, sealed by searing the edges with a hot spatula, and immediately fixed. With practice, over 50% of the embedded cells in four different groups of a single block were coplanar in a 4-μm paraffin section.

In situ hybridization

The *in situ* hybridization of ^{35}S-labeled c-*mos* RNA probes to cellular RNAs in paraffin-embedded tissue sections has been described in detail elsewhere (Mutter and Wolgemuth, 1987). All samples were fixed in 4% buffered paraformaldehyde at 4°C overnight before dehydration and paraffin

embedding in a standard Autotechnichon-Duo (Technicon Corp., Tarrytown, NY). Four-micron-thick sections were hydrated, pre-hybridized, hybridized, washed and exposed as described (Mutter and Wolgemuth, 1987). For each experiment, both positive and negative controls were performed as follows. Several normal ovaries were included as a positive tissue control (Mutter and Wolgemuth, 1987). As a negative control, an identical quantity of c-*mos* probe in a sense orientation was hybridized to sections from each block.

In situ analysis

Autoradiographic silver grains in slides developed after 2–14 days exposure were counted visually at 400× magnification with darkfield optics on a Leitz Dialux microscope. The areas of structures in which grains were counted were measured by video image digitalization and calculated using the Bio Quant digitizing morphometry program (R & M Biometrics, Nashville, TN) run on an Apple II computer. Grain density is reported here in absolute values, without subtraction of background grain counts. Densities over the various cell types are expressed graphically as percent of the maximum grain density observed within individual experiments, so that comparisons could be made among experiments. In some experiments, the grain density over oocytes neared confluence. It was difficult to count individual grains above ~100 grains/1000 μm^2. Therefore, this number was used as a best estimate of grain density over cells where labeling neared confluence. This correction likely resulted in occasional underestimation of the actual levels of c-*mos* transcripts in the corresponding cells.

Classification of ovarian follicular stages in tissue sections of whole ovaries was as described by Pederson and Peters (1968), by the number and arrangement of granulosa cell layers as follows: stage 3, a single continuous layer of well-defined granulosa cells; stage 4, two layers; stage 5, three or more layers, no antrum; stage 6, three or more layers with scattered small antra; stage 7, many layers, with a large antrum and cumulus oophorus lacking a distinct stalk; and stage 8, many layers with a large antrum, cumulus oophorus and narrow stalk.

Oocyte RNA isolation

Oocytes in meiotic prophase, with intact germinal vesicles, were retrieved by puncture of ovaries (Rafferty, 1970) of stimulated (5 IU PMS 48 h before sacrifice) mice, in the presence of 100 μg/ml db-cAMP to prevent germinal vesicle breakdown during processing. Matured ova in metaphase of the second meiotic division (M^{II}) were obtained by oviduct puncture (Rafferty, 1970) of previously stimulated (5 IU PMS followed 48 h later by 2.5 IU hCG) mice, ~16 h after hCG injection. Granulosa cells liberated during isolation were collected separately and processed in parallel as a somatic tissue control. Cells in each group were frozen in liquid nitrogen and stored at −70°C. RNA was isolated using the selective LiCl precipitation procedure of Cathala *et al.* (1983). Total RNA from mouse liver (40 μg) was added to each sample during the extraction procedure to serve as carrier.

Northern blot analysis

RNA samples were electrophoresed in denaturing 1.5% agarose:2.2 M formaldehyde gels in 20 mM morpholinopropanesulfonic acid (MOPS) buffer, pH 7.0, at 4°C. The gels were blotted overnight onto Genescreen Plus (NEN Research Products, Boston, MA) according to Maniatis *et al.* (1982) and then crosslinked to the matrix by exposure to a germicidal lamp for 7 min. Hybridization with ^{32}P-labeled RNA probes was performed at 55°C as described by Wahl *et al.* (1979), except that 100 μg/ml *Escherichia coli* tRNA was included in the pre-hybridization and hybridization. Washes were performed at high stringency according to our standard protocols (Ponzetto and Wolgemuth, 1985), except that the final wash employed 0.1 × SSC at 80°C for 40 min. The filters were exposed to X-ray film at −70°C for an appropriate period of time. Autoradiograms were quantitatively evaluated by transmission optical scanning with a Chromoscan 3 Densitometer (Joyce Loebl, Dusseldorf, FRG).

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