

Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis

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SUMMARY

Mouse oocytes were fertilized *in vitro* and the precise timing and sequence of morphological and molecular events occurring during the first cell cycle were investigated. The timing of development through the first cell cycle was found to be initiated by an event associated with sperm penetration rather than with germinal vesicle breakdown. DNA replication is initiated randomly in either pronucleus of a given egg, beginning approximately 11 h post insemination (hpi), and S phase lasting 6–7 h in both. Careful study of polypeptide synthetic profiles revealed three classes of changes in polypeptide synthesis during the first few hours of development: fertilization-independent, fertilization-accelerated, and fertilization-dependent. Pulse-chase experiments and *in vitro* translation of extracted mRNA showed that the changes in polypeptide synthetic profile result from differential mRNA activation, differential polypeptide turnover and post-translational modifications. These results support the notion that following ovulation, development is controlled at two levels. An endogenous (oocyte) programme, set in train by the terminal events of oocyte maturation, may regulate the 'housekeeping' functions of the egg, while sperm penetration activates a further endogenous (fertilization) programme, which may serve to initiate subsequent embryogenesis.

INTRODUCTION

In the mouse egg, as in other animal species, the early events that take place following fertilization are largely, if not exclusively, under maternal control (reviewed by Davidson, 1976, and Johnson, 1981; Woodland, Flynn & Wylie, 1979; Rosenthal, Hunt & Ruderman, 1980). The mouse egg inherits tRNA, rRNA and ribosomal proteins from the oocyte (Bachvarova, 1974; Young, Sweeney & Bedford, 1978; LaMarca & Wassarman, 1979) together with a considerable amount of Poly A⁺ RNA (estimated at 23–25 pg, which represents 7–8% of total RNA; Bachvarova & DeLeon, 1980; Piko & Clegg, 1982). During the 1-cell stage of mouse embryogenesis there is little or no detectable RNA synthesis (Young *et al.* 1978; Piko & Clegg, 1982). The first direct evidence of transcription from the embryonic genome is detected at the early to mid 2-cell stage (Young *et al.* 1978; Levey, Stull & Brinster, 1978). Indeed, neither physical nor chemical enucleation affects any of the molecular changes that have been detected before the very early

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2-cell stage (Braude, Pelham, Flach & Lobatto, 1979; Petzoldt, Hoppe & Illmensee, 1980; Schultz *et al.* 1981; Van Blerkom, 1981; Flach *et al.* 1982), suggesting that these changes may be regulated exclusively at the post-transcriptional level, presumably utilizing maternal components inherited from the oocyte. Thus, the mature, ovulated oocyte can be regarded as a highly specialized cell, primed and ready to implement the post-transcriptional control mechanisms that govern development to the early 2-cell stage. These processes may be initiated by fertilization, and produce the series of morphological and molecular changes leading up to and including the first cleavage division and activation of the embryonic genome.

In this study, the use of *in vitro* fertilization has made it possible to investigate this early phase of development in populations of mouse embryos which show a high degree of developmental homogeneity. The timings of DNA replication, of protein synthetic changes and of morphological changes that occur in the developing 1-cell embryo have been described here with more precision than previously. We also show that the changing pattern of protein synthesis is achieved by a combination of selective mRNA utilization, extensive post-translational modification and differential turnover of polypeptides. Thus, the length of time that embryos are incubated in [³⁵S]methionine, and the duration of "chase" periods in pulse-chase experiments, become critical in examining the nature, and the time course, of changes in patterns of polypeptide synthesis. We have focused our analysis on three polypeptide complexes (the 35×10^3 , 30×10^3 and 45×10^3 relative molecular mass complexes hereafter called 35 kD, 30 kD and 45 kD complexes), and the temporal changes in their patterns of synthesis have been examined to provide insights into the molecular control mechanisms that are operating.

MATERIALS AND METHODS

Recovery and fertilization of oocytes

For *in vitro* fertilization, which was used in all experiments unless stated otherwise, oocytes were recovered from 4 to 5-week-old (C57B1.10 × CBA)F₁ mice (F₁ mice), bred in the laboratory from Olac-derived parents. Female mice were superovulated with 5 i.u. pregnant mare's serum gonadotrophin (PMS; Intervet) followed 48 h later by 5 i.u. human chorionic gonadotrophin (hCG; Intervet). The mice were killed at 12.5 h post hCG, and a maximum of 10 cumulus masses were placed in each 1 ml drop of pre-equilibrated Whittingham's medium (Whittingham, 1971) containing 30 mg.ml⁻¹ bovine serum albumin (BSA; Sigma) and incubated under liquid paraffin (BDH). All incubations were carried out under liquid paraffin at 37°C in an atmosphere of 5% CO₂ in air.

Spermatozoa were collected from the cauda epididymides of male HC-CFLP mice (Hacking and Churchill) and incubated in 0.5 ml drops of pre-equilibrated Whittingham's medium containing 30 mg.ml⁻¹ BSA under liquid paraffin. The sperm suspension was incubated for 1.5 h to allow for capacitation (Fraser & Drury, 1975; Fraser, 1983).

At 13.5 h post hCG 100 μl of sperm suspension was added to the medium containing the oocytes, giving a final sperm concentration of $1-2 \times 10^6$.ml⁻¹. Fertilized eggs were separated from the spermatozoa at 4 h post insemination (hpi) unless stated otherwise, washed twice in pre-equilibrated medium 16 containing 4 mg.ml⁻¹ BSA (M16 + BSA; Whittingham & Wales, 1969) and cultured further in this medium.

For oocytes, mice were killed at 13 h post hCG and the oocytes freed from cumulus cells by brief

exposure to 0.1 M-hyaluronidase (Sigma) in phosphate-buffered saline. Eggs were then washed twice in pre-equilibrated M16 + BSA and cultured further in this medium. Care was taken to exclude any oocytes showing signs of parthenogenetic activation. Some comparative studies were done using oocytes from MFI female mice (Olac).

For *in vivo* fertilization, F₁ females were caged with HC-CFLP males immediately following the same injection regime described above, and the presence of a vaginal plug was taken as an indication of successful mating. 1-cell embryos were flushed from the oviducts at 15 h or 26 h post hCG using phosphate-buffered medium 1 + 4 mg.ml⁻¹ BSA (Whittingham & Wales, 1969). Embryos were collected and cultured in drops of M16 + BSA.

Collection of synchronized populations of 2-cell embryos

1-cell embryos derived from fertilization *in vitro* were cultured in M16 + BSA and examined at intervals of 1 h. Any 2-cell embryos formed within the previous hour were collected and cultured separately, and subsequent development was timed in hours post pick-off (hppo; Bolton, Oades & Johnson, 1984).

Since the times of ovulation, insemination and fertilization vary considerably after fertilization *in vivo*, embryonic development was timed in h post hCG (which induces ovulation between 11–13 h post hCG). For fertilization *in vitro*, embryonic development was timed in terms of hours post hCG (h post hCG) in order to provide a direct comparison with embryos produced by fertilization *in vivo*, in hours post insemination (hpi), or in hours post pick-off after division to the 2-cell stage (hppo) (which occurs over the period 16–20 hpi).

Scoring

To determine the timing of the morphological events following fertilization, F₁ embryos derived from fertilization *in vitro* were observed by inverted phase light microscopy. We have found that embryos at this early stage are particularly sensitive to fluctuations in temperature; these were minimized by handling small groups of embryos, and by making brief observations using a heated microscope stage set at 37°C.

DNA quantitation

At various times after insemination oocytes were collected, fixed on microscope slides and stained using Schiff's basic stain for the Feulgen reaction, exactly as described by Bolton *et al.* (1984). The nuclear DNA content of individual embryos was measured using a Vickers M86 scanning microdensitometer from absorption at 560 nm (method adapted from Dietch, Wagner & Richardt, 1967).

One- and two-dimensional SDS polyacrylamide gel electrophoresis

Embryos were incubated for 1 h or 3 h in a dilution of 3 µl [³⁵S]methionine (1000–1400 Ci.mmol⁻¹, Amersham International Ltd) in 50 µl M16 + BSA. Equal numbers of embryos (5–10 for one-dimensional, 50–60 for two-dimensional separation) were washed three times with protein-free M2 (Fulton & Whittingham, 1978) and placed in 5 or 10 µl double-strength SDS sample buffer (Laemmli, 1970) and stored at –70°C. For one-dimensional analysis proteins were separated on 10 % SDS polyacrylamide gels as described previously (Flach *et al.* 1982). For quantitative comparative analysis, 10 embryos were collected at 1 h or 2 h intervals, and the polypeptides from each sample were separated on adjacent tracks (see Bolton *et al.* 1984 for discussion of this point). For two-dimensional analysis polypeptides were separated in the first dimension according to isoelectric point in cylindrical 4 % acrylamide gels, and in the second dimension according to relative molecular mass on 10 % polyacrylamide gels (O'Farrell, 1975). Following electrophoresis, gels were processed as described by Bonner & Laskey (1974) and exposed for 1–4 weeks to preflashed Fuji RX X-ray film (Laskey & Mills, 1975) for fluorography at –70°C. Films were evaluated as described by Johnson & Rossant (1981). For each two-dimensional gel, a set of reference polypeptides, which are synthesized throughout this period was established, and these were used for localization (Pratt, Bolton & Gudgeon, 1983).

Pulse-chase experiments

Embryos were pulsed for 1 h in M16 + BSA containing diluted, lyophilized [³⁵S]methionine (10–14 Ci.mmol⁻¹) supplemented with unlabelled methionine; they were then chased for specified periods in M16 + BSA supplemented with unlabelled methionine, phenylalanine and leucine, each at a concentration of 100 μM. These procedures were carried out exactly as described by Bolton *et al.* 1984. In one series of pulse-chase experiments newly ovulated oocytes were labelled from 12.5–13.5 h post hCG in Whittingham's medium containing diluted, lyophilized [³⁵S]methionine supplemented with unlabelled methionine. The oocytes were washed three times in Whittingham's medium containing unlabelled methionine, phenylalanine and leucine. Some of these labelled oocytes were retained in culture as ageing oocytes, others were placed into a 100 μl drop of Whittingham's medium supplemented with the unlabelled amino acids and inseminated with 10 μl of sperm suspension (prepared as described earlier).

RNA extraction and translation in vitro

Total RNA was extracted from about 1500 oocytes at 16 h and 37 h post hCG, and from 1000 early 2-cell embryos at 32 h post hCG, and translated *in vitro* in the presence of [³⁵S]methionine in a message-dependent rabbit reticulocyte lysate exactly as described by Bolton *et al.* 1984. Samples (10 μl) of translation mixture were added to an equal volume of double-strength lysis buffer (O'Farrell, 1975) in preparation for resolution by two-dimensional SDS polyacrylamide gel electrophoresis. Fewer polypeptides are translated *in vitro* than *in situ*, which may be partly due to a deficiency of post-translational modifying enzymes in the *in vitro* translation system. When comparing a series of gel separations, allowances were made for local gel distortions or slight variations in exposure time.

RESULTS

Morphological events following fertilization

Extrusion of the second polar body was completed in all embryos between 1 hpi and 3 hpi, confirming previous observations of the timing of this event (Sato & Blandau, 1979; Maro, Johnson, Pickering & Flach, 1984). The time course of pronuclear formation was established by making hourly observations, starting at 4 hpi. Results from three experiments showing pronuclear formation in 192 eggs are shown in Fig. 1. The first, larger pronucleus forms further from the second polar body than the second, smaller pronucleus, and is therefore assumed to be the male pronucleus. The male pronucleus has formed in all embryos between 4 hpi and 8 hpi, and the female pronucleus between 5 hpi and 9.5 hpi. Both pronuclei form near the periphery of the embryo, but by 8–10 hpi they have migrated and lie adjacent to each other in the centre of the embryo. Typically, the first polar body degenerates during the first cell cycle in embryos derived from F₁ oocytes, although in 60–70% of embryos derived from MF₁ oocytes it underwent division before degenerating.

The second polar body is positioned consistently along an axis through both pronuclei. At anaphase, when the two sets of homologous chromosomes separate to opposite poles of the embryo, the second polar body is aligned in the plane of the developing cleavage furrow (see Fig. 5, particularly 5.10), and hence is always positioned between the two blastomeres following the first cleavage division.

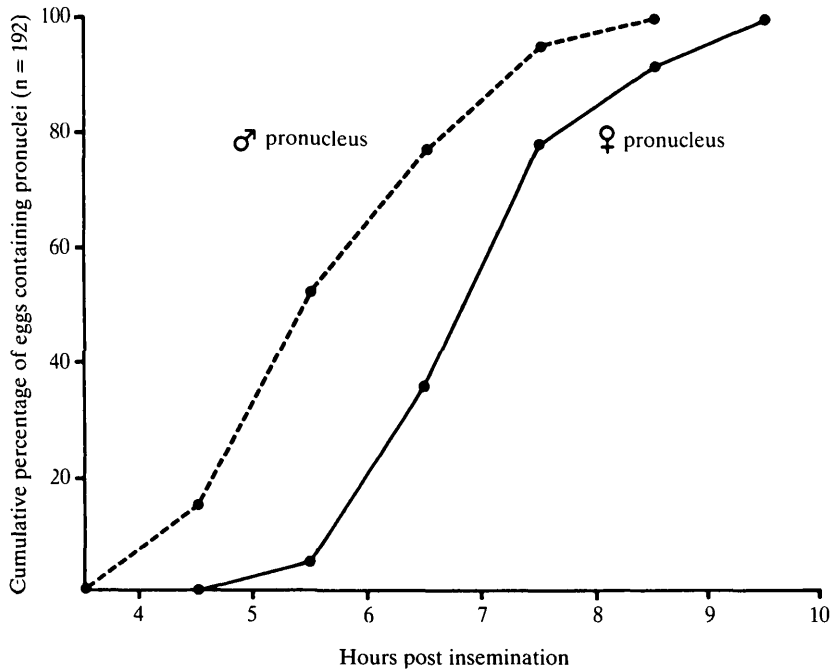


Fig. 1. Timing of appearance of male and female pronuclei after fertilization *in vitro*, expressed as a cumulative percentage of eggs observed serially for formation of male pronucleus (●—●) and female pronucleus (●—●). Time axis is expressed in h post insemination (hpi). n = number of embryos scored.

Synchrony of the first cleavage division

1-cell embryos derived from fertilization *in vivo* show considerable variation in the timing of first cleavage division (Bolton *et al.* 1984). Following fertilization *in vitro*, first cleavage occurs in most embryos within a 3 h period, with the first embryos cleaving at 18 hpi (solid line; Fig. 2). Synchrony is improved by minimizing temperature fluctuation during experimental manipulation.

In order to establish whether the observed asynchrony in the time of first cleavage resulted from variation in the time of sperm entry and oocyte activation, or from an intrinsic variation within the population, individual eggs were observed at 1 h intervals over the period 3–9 hpi. The appearance of the male pronucleus in individual eggs was recorded, and the time of cleavage to two cells noted. Cleavage times were then plotted in relation to time of insemination (solid line; Fig. 2), and to time of male pronucleus formation (dotted line; Fig. 2). These results reveal no evidence of greater synchrony at first cleavage whether insemination, or the post-fertilization event of male pronucleus formation is taken as the starting point. Fertilization occurs in the majority of oocytes within 1–2 h of insemination, as indicated by disappearance of dense clusters of cortical granules from the cytoplasm, formation of a fertilization cone, and extrusion of the second polar body in >90 % of eggs. Formation of the second polar body marks completion of meiosis,

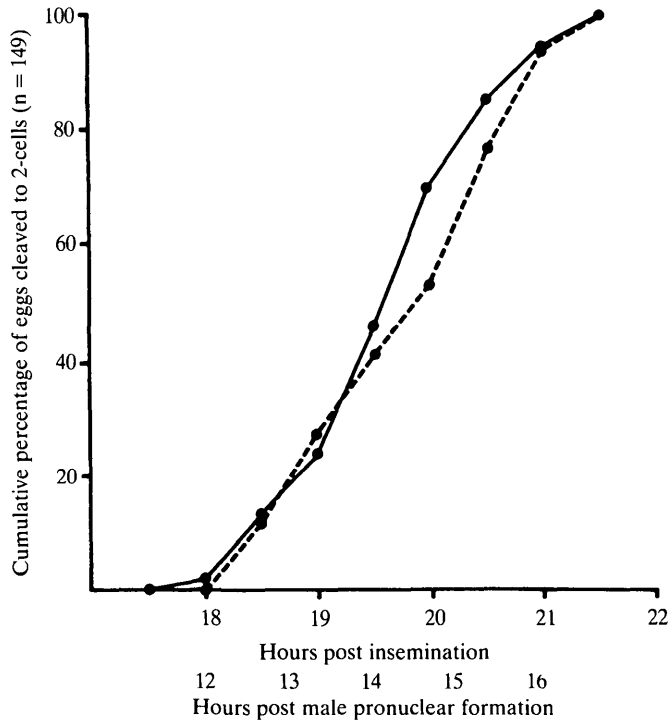


Fig. 2. Timing of first cleavage division expressed as a cumulative percentage from a total of 149 eggs observed serially. Time axis is expressed in h post insemination (●—●) and h after formation of the male pronucleus (●---●). n = number of embryos scored.

and the beginning of the first cell cycle. Therefore the 3 h asynchrony at first division is more likely to be due to variations intrinsic to each oocyte than to variation in time of sperm entry.

Eggs may vary in their developmental rate to first cleavage (a) because they possess unequal maternal- and/or sperm-derived resources, or (b) because the initiation of the first cell cycle is associated with some intrafollicular event such as germinal vesicle breakdown (GVBD), with fertilization merely representing a permissive event during this phase of development. To distinguish between these alternatives, a series of experiments were undertaken in which the time of insemination was delayed by 4 h (i.e. performed at 17.5 h rather than 13.5 h post hCG; late group). The results are recorded in Fig. 3, and indicate that a 4 h delay in the time of insemination appears to make no difference to the length of the first cell cycle. Therefore, it would appear that the oocyte responds to sperm penetration as the trigger for the events of the first cell cycle, culminating in division to two cells. Variation in the length of the first cell cycle thus reflects real differences among individual oocytes.

Timing and duration of DNA replication

In order to establish the time course of DNA replication during the first cell cycle, groups of embryos derived from fertilization *in vitro* were fixed at 2 h intervals after insemination, stained, and the DNA content of each embryo was assayed by microdensitometry. Mouse liver cell nuclei provided 2C and 4C reference values. The combined results from three experiments are shown in Fig. 4, where the DNA content of the two pronuclei combined, and that of the second polar body are plotted against time. DNA replication commences between 10 hpi and 12 hpi and lasts about 7 h. There was no evidence to suggest that one of the pronuclei consistently entered S phase before the other. The second polar body, which inherits a haploid chromatin complement identical to that of the female pronucleus, remains highly condensed throughout the period of study. It was found to undergo partial DNA replication, achieving a value of $1.7C \pm 0.3$ by the late 2-cell stage (Fig. 4).

The morphologies of the stained embryos are illustrated in Fig. 5. During the 2–3 h preceding pronuclear membrane formation, the interphase chromatin is diffuse within the fertilized egg cytoplasm and is consequently poorly stained. Thereafter, the pronuclear DNA can be seen and assayed readily and two pronuclei are distinguishable. The female pronucleus appears smaller, more densely stained, and is positioned closer to the second polar body than the male pronucleus (Figs

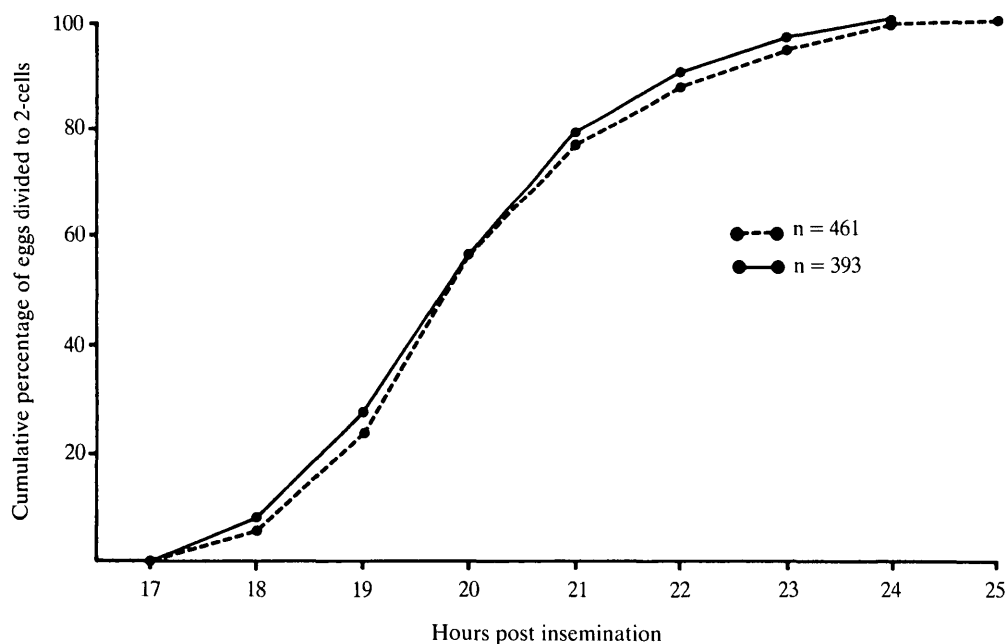


Fig. 3. Timing of first cleavage division expressed as a cumulative percentage from eggs inseminated at 13.5 h post hCG (●---●) or 17.5 h post hCG (●—●). Time axis is expressed in h post insemination (hpi). n = number of embryos scored.

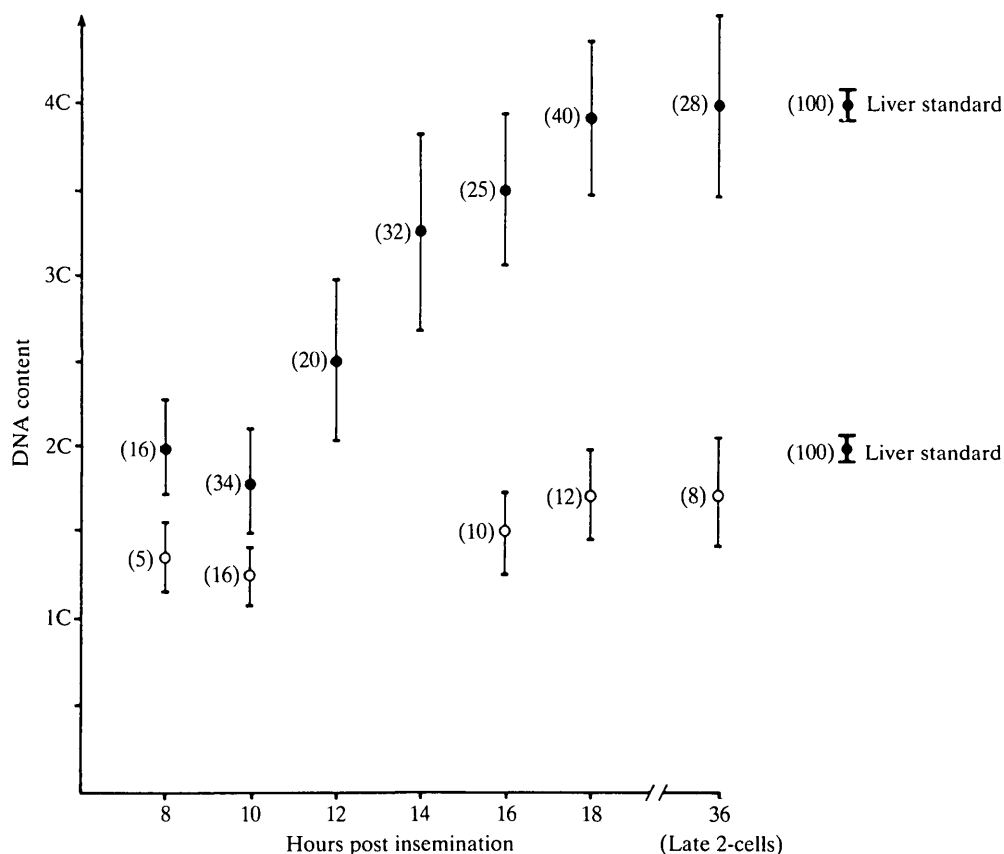


Fig. 4. Time course of DNA replication in the combined pronuclei (●) and second polar body (○) of 1-cell embryos. DNA content was assayed after Feulgen staining by microdensitometric analysis at a wavelength of 560 nm. Each point represents the mean, and bars indicate the standard deviation of several nuclei (number of eggs per sample indicated beside each point). Diploid (2C) and tetraploid (4C) liver cell nuclei provide standards (●).

5.2–5.5). At metaphase, the degree of condensation of the male- and female-derived chromatin has become identical (5.8). Chromosomes in various stages of mitosis were found from 16 to 20 hpi (5.6–5.11).

Patterns of polypeptide synthesis

In order to examine the changes in patterns of polypeptide synthesis of developing embryos, and in order to determine if any such changes are fertilization-dependent, developing embryos and ageing oocytes were labelled for varying periods (1 h or 3 h) in [³⁵S]methionine, and the polypeptide synthetic profiles analysed by one- and two-dimensional polyacrylamide gel electrophoresis. In addition, the role of post-translational modifications and polypeptide stability in the changing patterns of protein synthesis was examined by pulse-chase experiments, with chase periods

varying from 4–8 h. Finally, in order to determine whether selective utilization of stored mRNA is important in protein synthesis, polypeptide synthetic profiles were examined in two dimensions after RNA extraction and translation *in vitro*. Preliminary analysis showed that the pattern of polypeptides synthesized during the 20–24 h period following ovulation remains remarkably constant, at the level of one-dimensional resolution, both in developing embryos (Fig. 6) and in ageing oocytes (Fig. 7). Among those polypeptides that do exhibit changes, three sets (35 kD, 30 kD and 45 kD complexes respectively) were analysed in detail, and the temporal changes in their patterns of synthesis were compared between developing embryos and ageing oocytes (bars; Figs. 6 & 7). In this way, we have established a series of defined molecular events that can be used in a systematic analysis of the underlying regulatory mechanisms operating during this period of development.

(i) *The 35 kD complex*

The 35 kD complex may be resolved into three bands on one-dimensional gels (upper, middle and lower bands; Pratt *et al.* 1983). The composition of this polypeptide complex both changes with time and differs between fertilized eggs and ageing oocytes (u, m, l; Figs. 6, 7 & 8). Although some features of the temporal transitions have been reported (Braude *et al.* 1979; Cullen, Emigholz & Monahan, 1980; Van Blerkom, 1981; Cascio & Wassarman, 1982; Pratt *et al.* 1983), their timing has not been described precisely over the whole cell cycle. For one-dimensional analysis, 1 h labelling periods at 1 h or 2 h intervals have enabled a relatively accurate description of the time course of these changes.

The earliest change that can be detected in the pattern of synthesis of the 35 kD complex polypeptides takes place within 1 h of fertilization. Thus, while newly ovulated oocytes synthesize all three bands of the 35 kD complex, synthesis of 35 kD lower band is no longer detectable within 1 h of insemination (14.5 h post hCG; track 1 F, Fig. 8). If the oocyte remains unfertilized, synthesis of this polypeptide band continues for a variable period of between 3 and 5 h (until 16.5–18.5 h post hCG; see track 3, Fig. 7; tracks 3 U & 4 U, Fig. 8), after which it ceases to be detectable. Therefore, this early cessation of 35 kD lower band synthesis represents an example of a *fertilization-accelerated* polypeptide synthetic change.

From 1 h to 4 h post fertilization (14.5 h to 17.5 h post hCG), synthesis of upper band predominates (tracks 1–4 F, Fig. 8), and appears to be greater than the level of synthesis in the newly ovulated oocyte (cf tracks 0 & 4, Fig. 6). Subsequently, developing embryos show a progression through marked synthesis of both upper and middle bands, to predominant synthesis of middle and lower bands, to predominant synthesis of lower band. Thus, by 10 hpi (23.5 h post hCG) synthesis of 35 kD upper band is negligible (track 10, Fig. 6); by 6 hpi (19.5 h post hCG) synthesis of 35 kD middle band increases (track 6, Fig. 6), reaching a maximum at 8–10 hpi (21.5–23.5 h post hCG; tracks 8 & 10, Fig. 6), and declining thereafter, becoming undetectable by 20 hpi (33.5 h post hCG; track 20, Fig. 6); at approximately 6 hpi

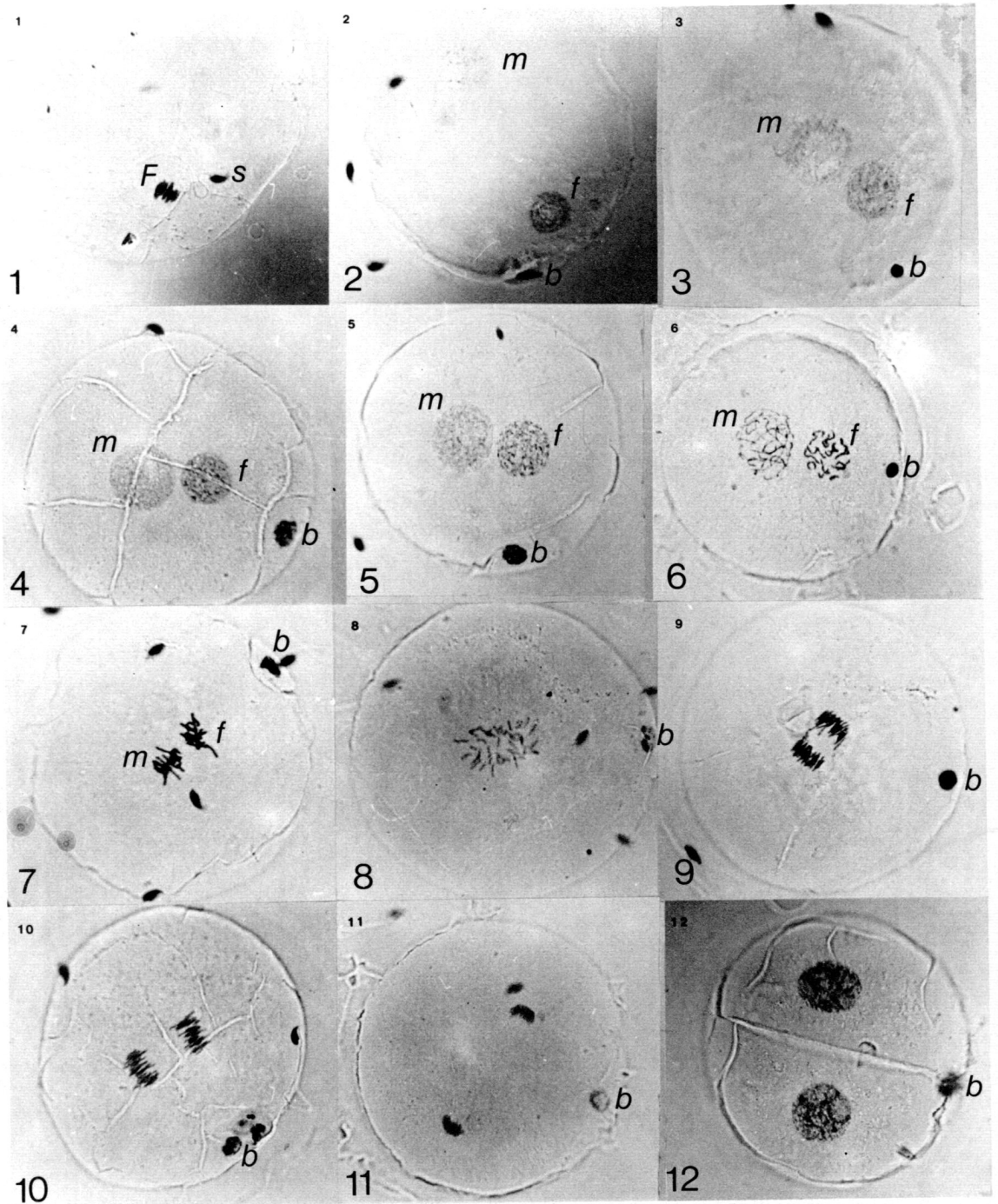


Fig. 5

(19.5 post hCG) synthesis of 35 kD lower band resumes (track 6, Fig. 6), increasing gradually until by 20 hpi (33.5 h post hCG) it is the only band of the 35 kD complex synthesized (track 20, Fig. 6). Synthesis of 35 kD lower band declines throughout the second cell cycle, until by the late 2-cell stage (34 hpi; 47.5 h post hCG) it is no longer detectable (Bolton *et al.* 1984).

In contrast, in ageing oocytes, synthesis of 35 kD upper band persists throughout the period of study and into the 2-cell stage (Fig. 7). Synthesis of 35 kD middle and lower bands does increase, although to a lesser extent, and later, than the corresponding increases detected in fertilized eggs. Thus, increased synthesis of 35 kD middle band is detectable in oocytes at 12 hpi (25.5 h post hCG; track 12, Fig. 7). Similarly, the ageing oocyte resumes synthesis of 35 kD lower band by 24 h post ovulation (43.5 h post hCG; track 24, Fig. 7), and this continues for a further 40 h (data not shown). Thus, the cessation of synthesis of 35 kD upper band represents a *fertilization-dependent* polypeptide synthetic change, while the transition to synthesis of 35 kD middle and lower band polypeptides represents a *fertilization-accelerated* polypeptide synthetic change.

These patterns of polypeptide synthesis were resolved more clearly in two-dimensional separations of labelled polypeptides synthesized during 3 h labelling periods at selected time points by oocytes and by developing embryos derived from fertilization *in vivo*. The polypeptides synthesized by early 1-cell embryos and oocytes labelled 16–19 h post hCG (which corresponds to approximately 3–6 hpi; panels a & b, Fig. 9) and by 2-cell embryos and ageing oocytes labelled 37–40 h post

Fig. 5. Appearance of 1- and 2-cell embryos following Feulgen staining.

1. Unfertilized egg, showing the highly condensed diploid (2C) maternal chromosomes lying on the metaphase plate (*F*) and the highly condensed haploid (1C) paternal chromatin within a supernumerary sperm head (*s*).

2. Fertilized egg at 8 hpi. The female pronucleus (*f*) is smaller, more densely stained, and nearer to the densely stained nucleus of the second polar body (*b*), than the male pronucleus (*m*). Three supernumerary sperm heads can be seen.

3. Fertilized egg at 12 hpi. The male and female pronuclei are positioned centrally. The female pronucleus (*f*) is still closer to the densely stained second polar body nucleus. Two supernumerary sperm heads visible.

4. Fertilized egg at 14 hpi. The male and female pronuclei are more highly stained than previously, indicating that DNA replication is underway.

5. Fertilized egg at 16 hpi. The chromatin in each pronucleus is still in interphase. Panels 6–11 show fertilized eggs in various stages of mitosis 17–20 hpi.

6. Chromosomes beginning to condense (early prophase), DNA replication having been completed.

7. Fully condensed chromosomes (late prophase). Several supernumerary sperm heads visible.

8. Chromosomes spread on the metaphase plate.

9. Homologous chromosomes begin to separate (early anaphase).

10. Mid anaphase (note second polar body positioned in the plane of the developing cleavage furrow).

11. Late anaphase.

12. Mid 2-cell, each nucleus having undergone the second round of DNA replication (29 hpi).

hCG (approximately 24–27 hpi; panels c & d, Fig. 9) are shown. The overall similarity between polypeptide synthetic profiles of fertilized eggs and ageing oocytes at different time points seen in one-dimensional analysis is confirmed by resolution in two dimensions (e.g. see reference polypeptides A,C,D,G,J, Fig. 9). The 35 kD complex polypeptides (arrowheads and arrows, Fig. 9), and other polypeptides whose pattern of synthesis changes with time and/or that differs consistently between fertilized eggs and ageing oocytes (numbers, Fig. 9; Table 1) have been marked.

The three bands of the 35 kD complex can be resolved into at least 15 components in two dimensions (Fig. 9). Thus, at 16–19 h post hCG, early 1-cell embryos

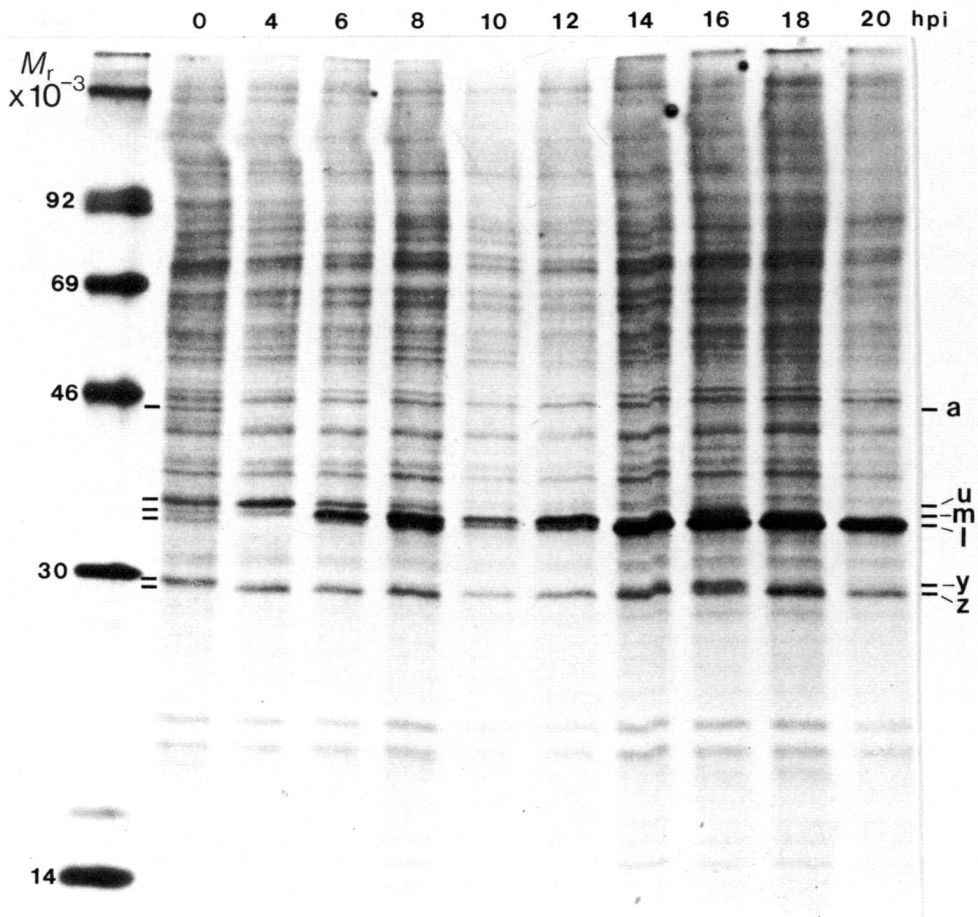


Fig. 6. One-dimensional SDS PAGE separation of [^{35}S]methionine-labelled polypeptides synthesized during the first cell cycle of fertilized eggs. Relative molecular mass markers (M_r): 92, 69, 46, 30 and 14×10^3 . Embryos cultured in M16 + BSA containing [^{35}S]methionine for 1 h at 0, 4, 6, 8, 10, 12, 14, 16, 18 and 20 hpi. Bars indicate positions of polypeptides whose pattern of synthesis changes throughout the culture period: relative molecular masses 45×10^3 (a), 35×10^3 (u, m, 1) and 30×10^3 (y, z).

synthesize one set of polypeptides (small arrowheads; panel a, Fig. 9) corresponding to 35 kD upper band, a major set (large arrowheads; panel a, Fig. 9) corresponding to 35 kD middle band, and a minor set (large arrows; panel a, Fig. 9) corresponding to lower band (*cf* track 6, Fig. 6).

By the early to mid 2-cell stage (37–40 h post hCG; panel c, Fig. 9) synthesis of

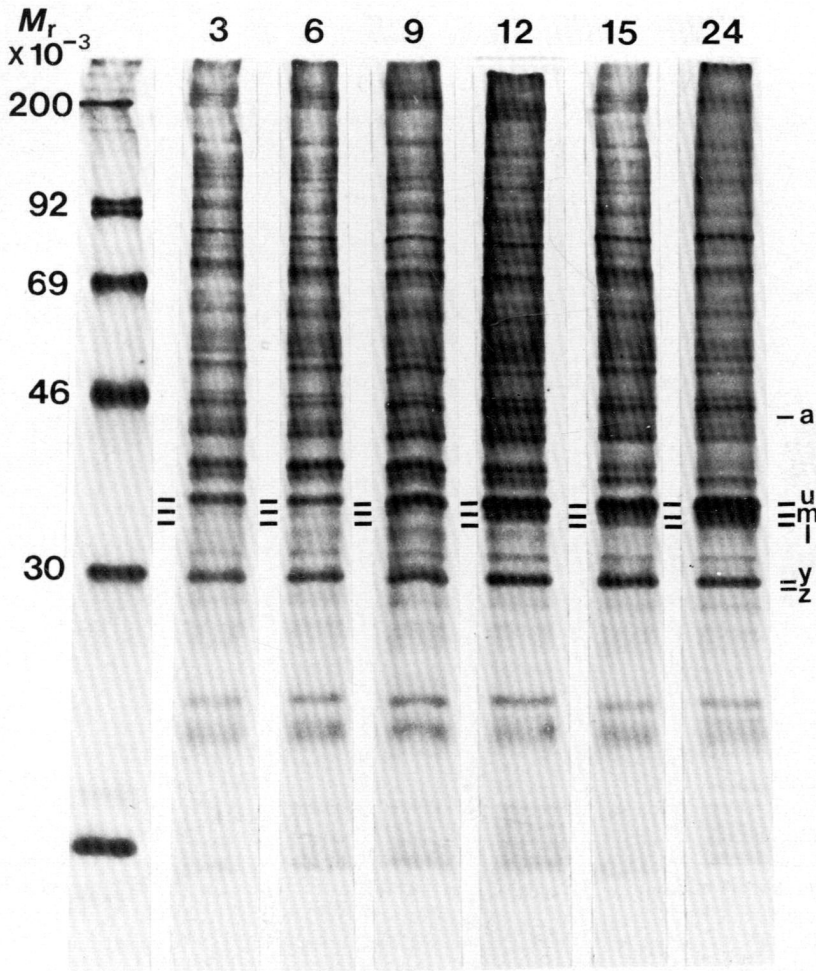


Fig. 7. One-dimensional SDS PAGE separation of [³⁵S]methionine-labelled polypeptides synthesized by ageing unfertilized oocytes. Relative molecular mass markers (M_r) and annotation as for Fig. 6. Eggs were cultured from 12.5 h post hCG in M16 + BSA and labelled: (3) 16.5–19.5 h post hCG (3–6 ‘hpi’); (6) 19.5–22.5 h post hCG (6–9 ‘hpi’); (9) 22.5–25.5 h post hCG (9–12 ‘hpi’); (12) 25.5–28.5 h post hCG (12–15 ‘hpi’); (15) 28.5–31.5 h post hCG (15–18 ‘hpi’); (24) 37.5–41.5 h post hCG (24–27 ‘hpi’).

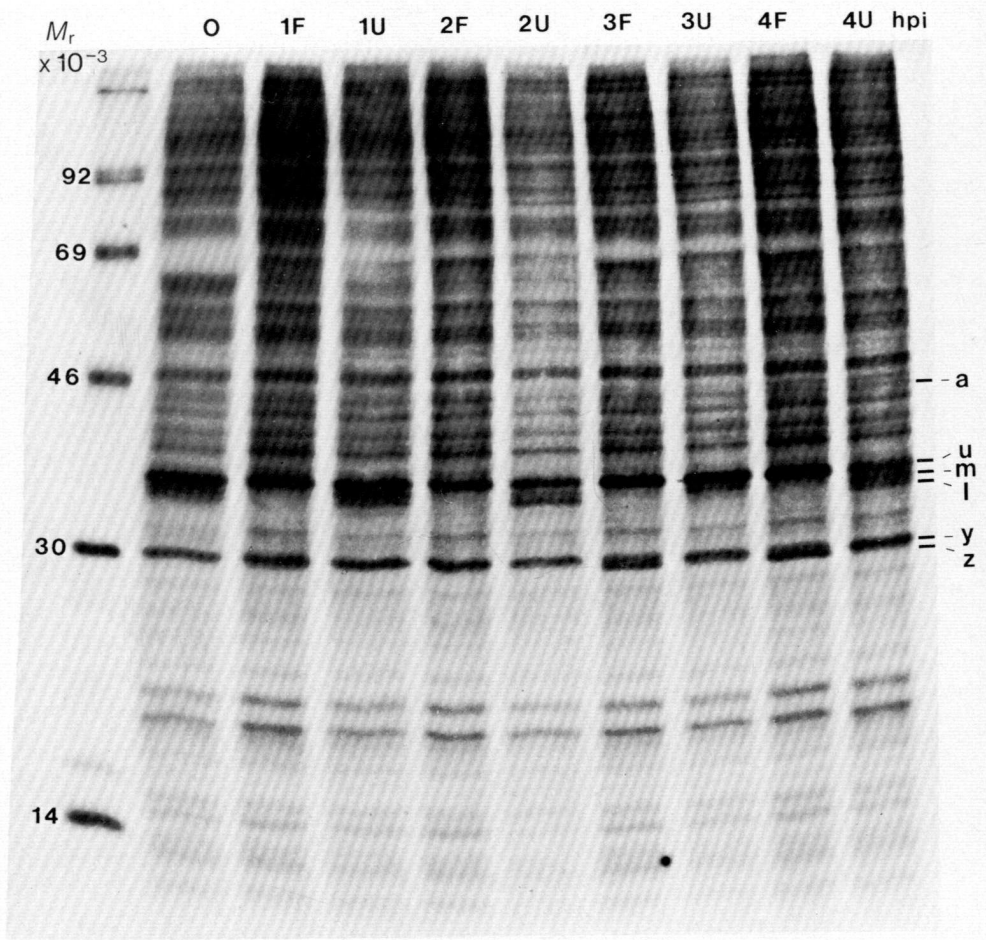


Fig. 8. One-dimensional SDS PAGE separation of [^{35}S]-methionine-labelled polypeptides synthesized by fertilized eggs (F) and unfertilized oocytes (U) of the same chronological age (h post hCG). Relative molecular mass (M_r) as for Fig. 6. Embryos and unfertilized oocytes cultured in M16 + BSA containing [^{35}S]-methionine for 1 h, beginning at 0, 1, 2, 3 and 4 hpi (13.5, 14.5, 15.5 and 16.5 h post h CG).

Fig. 9. Two-dimensional SDS PAGE separation of [^{35}S]-methionine-labelled polypeptides synthesized by (a) fertilized eggs cultured in M16 + BSA after fertilization *in vivo*, labelled 16–19 h post hCG; (b) unfertilized oocytes cultured in M16 + BSA, labelled 16–19 h post hCG; (c) early to mid 2-cell embryos cultured in M16 + BSA after fertilization *in vivo*, labelled 37–40 h post hCG; (d) unfertilized oocytes cultured in M16 + BSA, labelled 37–40 h post hCG. Polypeptides A, C, D, G, J represent reference polypeptides used for localization (annotation modified from Pratt *et al.*, 1983).

Polypeptides of the 35×10^3 complex are annotated according to their mobility in the second dimension: upper band = small arrowheads; middle band = large arrowheads; lower band = arrows.

Numbers indicate additional polypeptides whose pattern of synthesis changes with time and/or between fertilized eggs and unfertilized oocytes (see text for details). Isoelectric focusing is from left (approx. pH 7.0) to right (approx. pH 4.5).

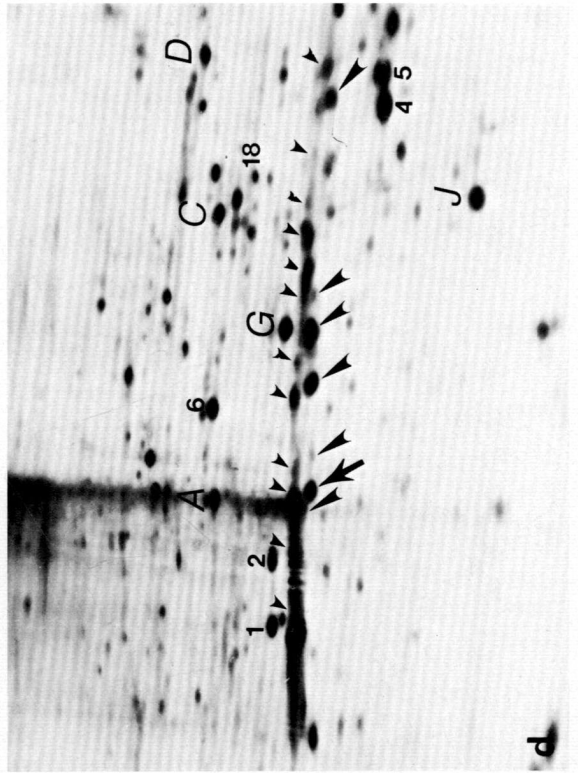
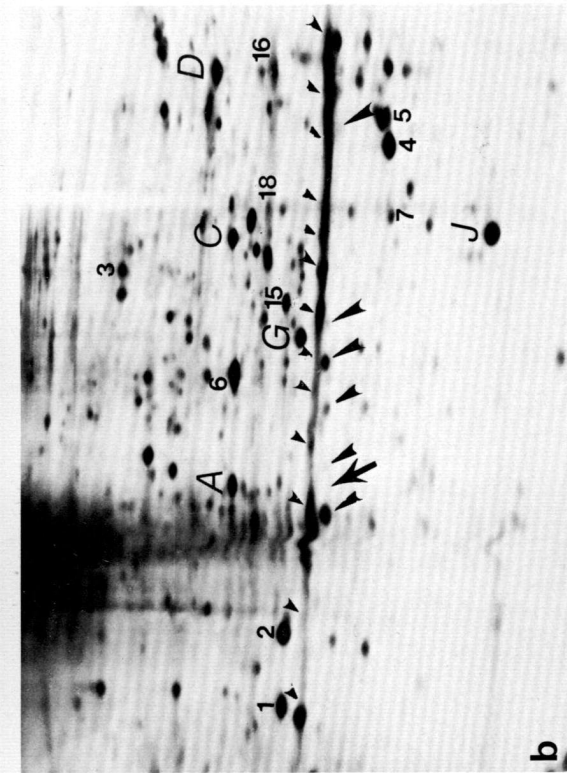


Table 1. Summary of polypeptide synthetic changes observed in fertilized and unfertilized eggs during the 24 h following ovulation

Polypeptide	Oocyte programme	Fertilization programme	Fertilization- dependent changes	Fertilization- accelerated changes	Fertilization- independent changes	Comment
1	C	O	+			} Synthesis ceases soon after fertilization.
2	C	O	+			
3	C	▼		+		-
4 (y)	C	▼	+			mRNA 'inactivated' by 3 h after fertilization.
5 (x)	C	▼	+			formed by slow modification of 4.
6	C	▼		+		-
7 (z)	O	C	+			mRNA activated selectively by 3 h after fertilization.
8	O	C	+			-
9	O	▲	+			Heavily labelled 'streak'. Possibly lamins.
11	O	O	+			} Embryonic gene products - heat-shock proteins. ¹
12	O	O	+			
13	O	▲	+			-
15	▼	C	+			-
16	▼	▼		+		-
17	O	▼	+			-
18 (a)	▼	▼		+		Short half-life
U	C	▼		+		} Conversion of U into M over chase period in fertilized eggs. Cessation of synthesis is fertilization-dependent.
M	▲	▲		+		
L	▲	▲		+		

C synthesis remains constant; ▲ synthesis increases; ▼ synthesis decreases; O synthesis undetectable ¹ Flach *et al.* 1982; Bensaude *et al.* (1984).

upper band polypeptides (small arrowheads) is negligible, synthesis of middle band polypeptides (large arrowheads) is reduced substantially, while that of lower band polypeptides (large arrows) predominates.

In contrast, oocytes at 16–19 h post hCG (panel b, Fig. 9) synthesize predominantly a linear array of 35 kD polypeptides (small arrowheads) corresponding to upper band on one-dimensional gels; these polypeptides are concentrated on the right (lower pH region) of two-dimensional gels. Some synthesis of putative middle band polypeptides (large arrowheads) is also detectable, but no polypeptides corresponding to 35 kD lower band on one-dimensional gels (large arrow) can be detected. At 37–40 h post hCG (panel d, Fig. 9), there is a marked shift of upper band polypeptides (small arrowheads) to the left of two-dimensional gels (higher pH region). The synthesis of several polypeptides apparently corresponding to one-dimensional middle band (large arrowheads) increases, while synthesis of only one putative lower band polypeptide (large arrow) can be detected.

In order to examine the contribution that post-translational modifications make to the changing patterns of synthesis of the 35 kD complex polypeptides, a series of pulse-chase experiments was undertaken. When fertilized eggs were pulsed with [³⁵S]methionine at 5 hpi, label was incorporated into 35 kD upper band polypeptides (track 5 F, Fig. 10). During a chase period of 4 h, label was 'chased' into 35 kD middle band, but not into 35 kD lower band (track 5–9 F, Fig. 10). Thus, it appears that 35 kD upper band polypeptides undergo post-translational modification to 35 kD middle band polypeptides, as inferred from the relative loss and gain of label in these two bands respectively, and that this modification occurs during a period of development when *in situ* synthesis of 35 kD middle band polypeptides takes place (track 8 F, Fig. 10). In contrast, when ageing oocytes of the same chronological ages (h post hCG) were pulsed with [³⁵S]methionine, they showed synthesis of 35 kD upper band at both 5 h and 8 h time points (tracks 5 U & 8 U, Fig. 10) and, as expected from the above analysis, label incorporated at 5 h could not be 'chased' to 35 kD middle band (track 5–9 U, Fig. 10).

Synthesis of 35 kD lower band polypeptides, in contrast to that of 35 kD middle band polypeptides, does not appear to be due to post-translational modification of pre-synthesized polypeptides, since label incorporated by developing embryos at 5 hpi (track 5 F, Fig. 10) cannot be 'chased' into 35 kD lower band (track 5–9 F, Fig. 10). This suggests that lower band polypeptides are unrelated to other polypeptides of the 35 kD complex, and are synthesized after approximately 6 hpi.

Thus, while some changes in polypeptide synthesis during this period of development can be explained by post-translational modification, others cannot. To determine whether these latter changes are the result of selective mRNA utilization, total RNA was extracted from oocytes and developing embryos, and after translation *in vitro* in the presence of [³⁵S]methionine, the labelled polypeptides were resolved in two dimensions (Fig. 11).

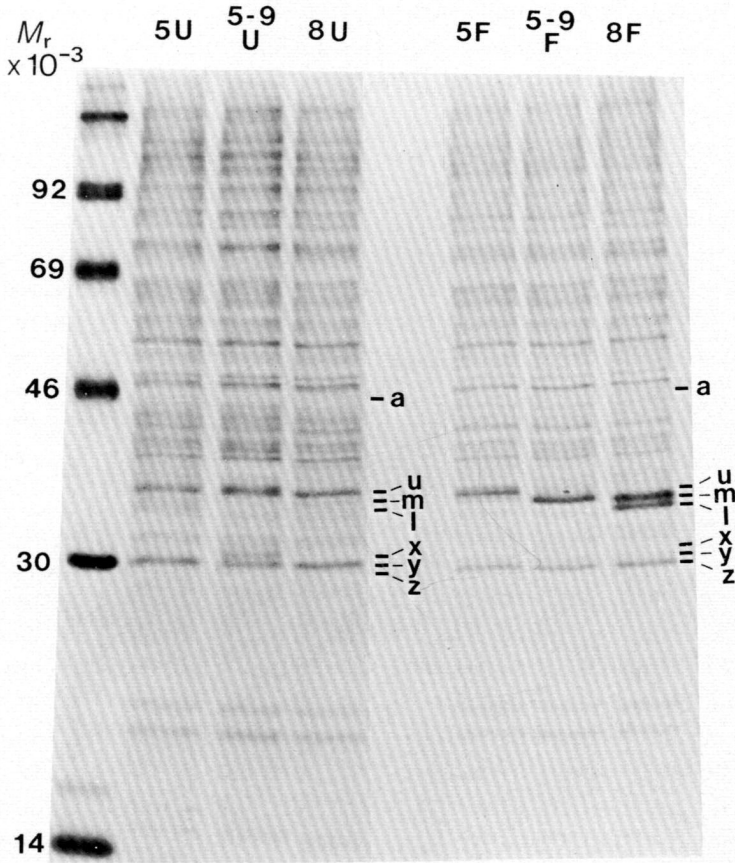


Fig. 10. One-dimensional SDS PAGE separation of [^{35}S]methionine-labelled polypeptides synthesized by fertilized eggs (F) and unfertilized (U) oocytes placed for 1 h in M16 + BSA + $0.01\ \mu\text{M}$ unlabelled methionine containing diluted, lyophilized [^{35}S]methionine. Relative molecular mass markers (M_r) as in Fig. 6. (For unfertilized oocytes 'hpi' refers to age with reference to 13.5 h post-hCG, the time of insemination for fertilized eggs). (5) eggs or oocytes labelled for 1 h at 5 hpi and harvested immediately; (5-9) eggs labelled as for 5, washed and cultured in M16 + BSA containing $100\ \mu\text{M}$ unlabelled methionine, phenylalanine and leucine and harvested at 9 hpi; (8) eggs or oocytes labelled for 1 h starting at 8 hpi and harvested at 9 hpi. Bars indicate positions of the 45×10^3 (a), 35×10^3 (u, m, l) and 30×10^3 (x, y, z) polypeptide complexes.

Despite the many changes with time that occur among the 35 kD complex polypeptides that are translated *in situ*, there are no corresponding changes among the many fewer 35 kD complex polypeptides translated *in vitro* (arrowheads and arrows, Fig. 11). Furthermore, of the 35 kD complex polypeptides, only two in the middle band, and three in the lower band are readily detectable in the *in vitro* translate pattern, confirming the notion that the polypeptides in each of these bands are unrelated. In contrast, only a trace of one upper band polypeptide can be detected, and this probably arises from a limited capacity for post-translational

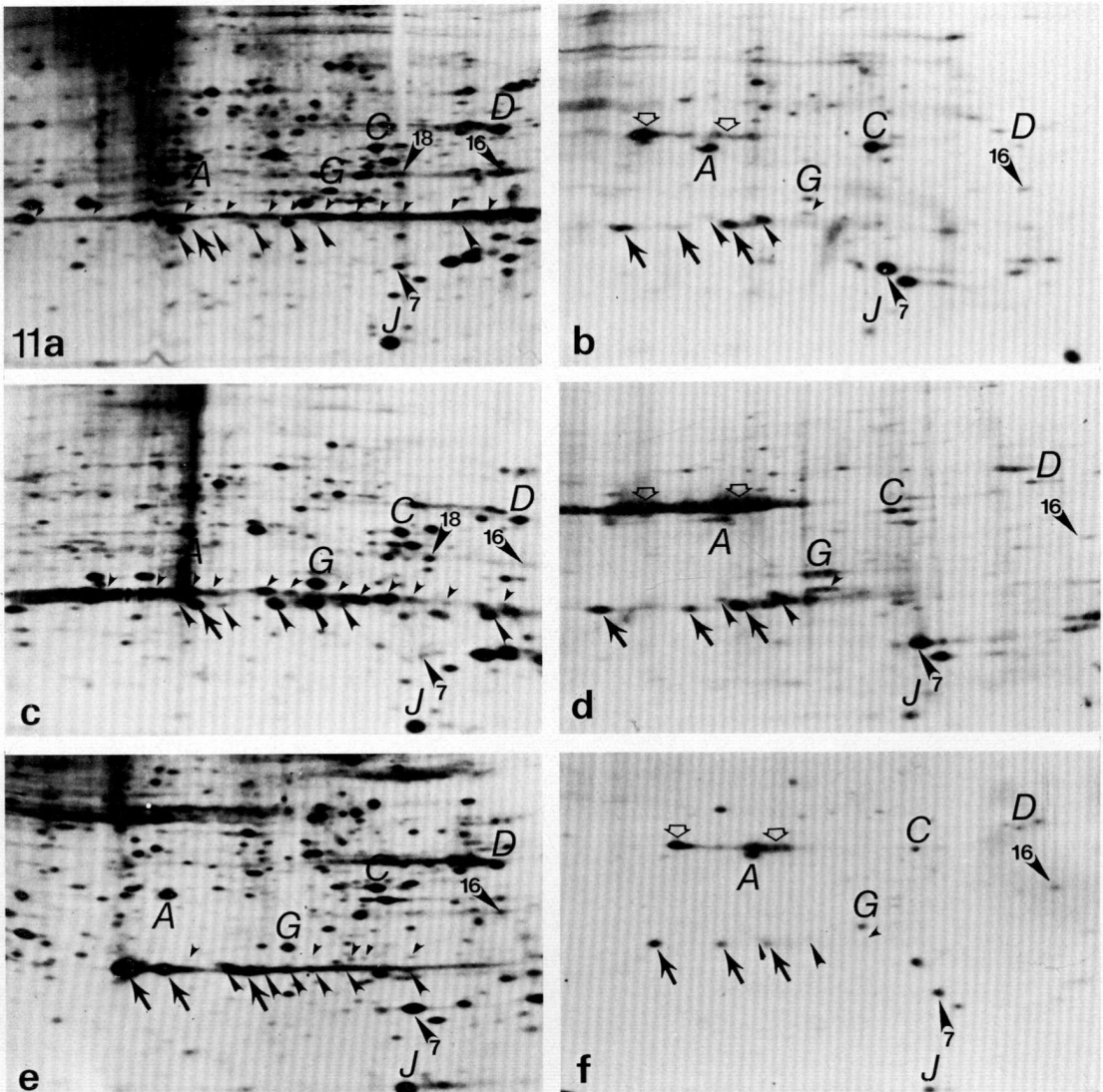


Fig. 11. Two-dimensional separation of [^{35}S]methionine-labelled (a) unfertilized oocytes cultured in M16 + BSA and labelled *in situ* 16–19 h post hCG; (b) polypeptides translated *in vitro* on mRNA extracted from unfertilized oocytes at 19 h post hCG; (c) unfertilized oocytes cultured in M16 + BSA and labelled *in situ* 37–40 h post hCG; (d) polypeptides translated *in vitro* on mRNA extracted from unfertilized oocytes at 37 h post hCG; (e) early to mid 2-cell embryos cultured in M16 + BSA and labelled *in situ* 37–40 h post hCG; (f) polypeptides translated *in vitro* on mRNA extracted from early 2-cell embryos at 32 h post hCG.

Open arrows in panels (b), (d), (f) indicate mRNA-independent incorporation of [^{35}S]methionine (Braude & Pelham, 1979); annotation as for Fig. 9.

modification *in vitro*. These results therefore suggest that both selective mRNA utilization and post-translational modification are important processes in the regulation of protein synthesis. This is consistent with previous work (Van Blerkom, 1981; Cascio & Wassarman, 1982; Pratt *et al.* 1983) and supports the idea that middle and lower band 35 kD polypeptides are the primary translation products of maternal mRNAs, and are synthesized *in situ* as a result of their selective, sequential activation; in contrast, synthesis of 35 kD upper band polypeptides results from post-translational modification(s) to precursor polypeptide(s). This observation, together with the results of pulse-chase experiments, suggests that upper band polypeptide synthesis results from the rapid post-translational modification of middle band polypeptides, and that this post-translational modification is slowly reversed. It is also clear from Fig. 11 that the horizontal shifts of upper, middle and lower band primary polypeptides observed *in situ* must also arise through post-translational modifications that alter the charges on the proteins.

(ii) *The 30 kD complex*

One-dimensional analysis of polypeptides synthesized during 1 h labelling periods reveals that in oocytes, a prominent 30 kD polypeptide band is synthesized throughout the period of study (band y; Fig. 7). Over the period 3–5 h post fertilization, synthesis of a slightly lower relative molecular mass (higher mobility) polypeptide (band z) first supplements (tracks 3 F & 4 F, Fig. 8), and then replaces band y (track 4, Fig. 6). This lower relative molecular mass species is then synthesized continuously, and at the same apparent rate, throughout the remainder of the first cell cycle (Fig. 6). No such shift from synthesis of band y to band z occurs in ageing oocytes (Fig. 7). Therefore, this represents a *fertilization-dependent* polypeptide synthetic change.

The possibility that these changes among the 30 kD complex polypeptides might involve post-translational modifications was examined by pulse-chase experiments. Oocytes were labelled with [³⁵S]methionine for 1 h at 12.5 h post hCG, and then cultured for a chase period of 7 h, either after fertilization *in vitro*, or as ageing oocytes (Fig. 12). Label incorporated into band y cannot be chased into the fertilization-specific band z (track 0–8 F, Fig. 12). This result suggests that in fertilized eggs (a) loss of band y represents either loss of its mRNA activity or increased turnover of the protein itself, and (b) synthesis of band z represents the translation of a new subset of maternal mRNA, rather than the post-translational modification of presynthesized polypeptide(s). Once synthesized, band z polypeptide appears to be stable, remaining unmodified during a chase period of 4 h (track 5–9 F, Fig. 10).

In the absence of fertilization, band y undergoes a slow post-translational modification to produce an additional, higher relative molecular mass band; this can be seen after a chase period of either 7 h (band x; track 0–8 U, Fig. 12) or 4 h (band x; track 5–9 U, Fig. 10). No such post-translational modification can be detected in fertilized eggs (track 0–8 F, Fig. 12).

The individual polypeptides involved in this inter-relationship of the 30 kD complex were identified by two-dimensional analysis both of *in situ* translation products, and after pulse-chase experiments. When the labelled polypeptides synthesized during a 3 h period by developing embryos and ageing oocytes were separated in two dimensions, three 30 kD polypeptides, whose patterns of synthesis changes during the culture period, were resolved (polypeptides 4, 5 & 7. Fig. 9). These polypeptides may correspond to bands y, x and z respectively on one-dimensional gels. Thus, synthesis of polypeptides 4 (equivalent to band y) and 5

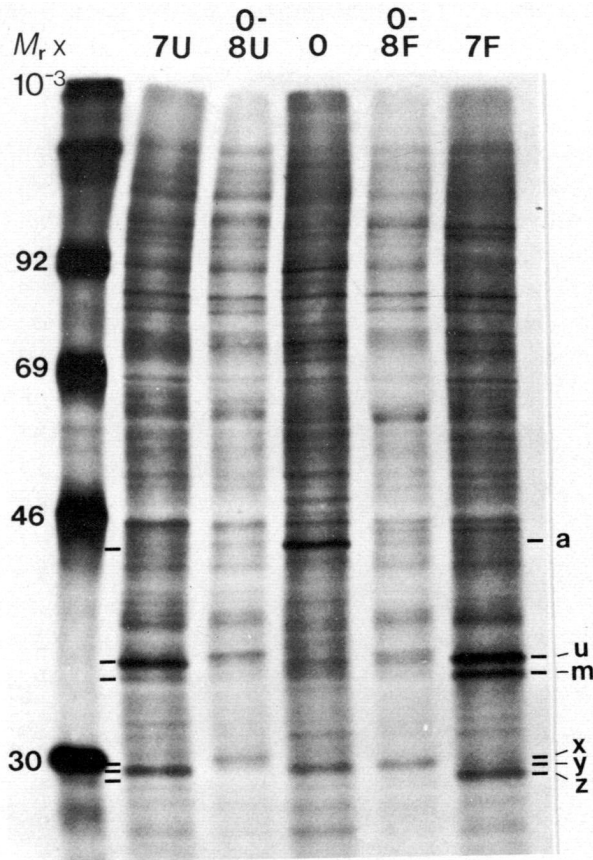


Fig. 12. One-dimensional SDS PAGE separation of [³⁵S]methionine-labelled polypeptides synthesized by fertilized eggs (F) and unfertilized oocytes (U). Relative molecular mass markers (M_r) and annotation as for Fig. 10.

All eggs were labelled in Whittingham's medium + 0.01 μ M unlabelled methionine containing diluted, lyophilized [³⁵S]methionine: (0) oocytes labelled from 12.5–13.5 h post hCG and harvested immediately; (0–8 U & 0–8 F) oocytes labelled from 12.5–13.5 h post hCG, washed and cultured in Whittingham's medium containing 100 μ M unlabelled methionine, phenylalanine and leucine, one group was cultured as ageing unfertilized oocytes until 8 'hpi' (21.5 h post hCG; 0–8 U), a second was fertilized by addition of sperm and harvested at 8 hpi (0–8 F); (7 U & 7 F) unfertilized oocytes or fertilized eggs, respectively, labelled 7–8 hpi (20.5–21.5 h post hCG) and harvested immediately.

(equivalent to band x) is marked in both fertilized eggs and oocytes at 16–19 h post hCG (panels a & b, Fig. 9), is reduced by 37–40 h post hCG in 2-cell embryos (panel c, Fig. 9), but persists in oocytes of the same chronological age (panel d, Fig. 9) thus representing a *fertilization-dependent* change. Polypeptide 7 (equivalent to band z) is synthesized substantially only by fertilized eggs at both times examined (*fertilization-dependent* synthesis). An increase in synthesis of polypeptide 7 and a decrease in synthesis of polypeptides 4/5 may be the same transition from synthesis of the one-dimensional 30 kD band y to that of band z in fertilized eggs. In these experiments, relatively long (3 h) labelling periods were used compared with the 1 h labelling period used for pulse-chase experiments, and therefore both unmodified polypeptide 4, and the product of its slow post-translational modification (polypeptide 5), show incorporation of label (panels a, b & d, Fig. 9). Two-dimensional separation of polypeptides synthesized by oocytes during 1 h labelling periods, and after chase periods of 5 h are shown in Fig. 13, and confirms the suspected equivalence of polypeptide 4 & 5 to bands y & x. Thus, label incorporated into polypeptide 4 (panel a, Fig. 13) is reduced during the 5 h chase period, while there is an increase, over the same period, in the label present in polypeptide 5 (panel b, Fig. 13).

Examination of *in vitro* translation products of total mRNA extracted from oocytes and developing embryos reveals that of the 30 kD complex polypeptides, only polypeptide 7 (band z) is encoded by an abundant maternal mRNA (Fig. 11). This observation supports the notion that synthesis of polypeptide 7 is the result of selective utilization of a specific maternal message. It is possible that the message for polypeptide 4 is present, but is either less abundant or less efficiently translated *in vitro* than message for polypeptide 7 (Fig. 11). Alternatively, polypeptides 4 and 5 might both be post-translationally modified products of some third primary polypeptide product. If this latter explanation is correct, then their post-translational conversion *in situ* must be extremely rapid and was not detected here.

(iii) *The 45 kD complex*

One-dimensional separation of polypeptides synthesized during 1 h labelling periods indicates that the newly ovulated oocyte synthesizes a 45 kD polypeptide band (band a; track 0, Fig. 6) which appears to show a *fertilization-accelerated*, though variable, reduction in synthesis. This band is not always well resolved on one-dimensional gels (for example see Fig. 7), but results from several experiments suggest that synthesis of band a decreases earlier, and is reduced to a lower level, in fertilized eggs (around 4 hpi; track 4, Fig. 6) than in oocytes (around 8 hpi; 21.5 h post hCG; data not shown). Furthermore, from pulse-chase analysis, this polypeptide shows a higher rate of turnover than the majority of polypeptides synthesized. Thus, label that is incorporated into band a in the newly ovulated oocyte (track 0, Fig. 12) is lost after a chase period of 8 h in both fertilized eggs and ageing oocytes (tracks 0–8F & 0–8U, Fig. 12). It is possible, therefore, that its loss post-fertilization results simply from an increased turnover rate.

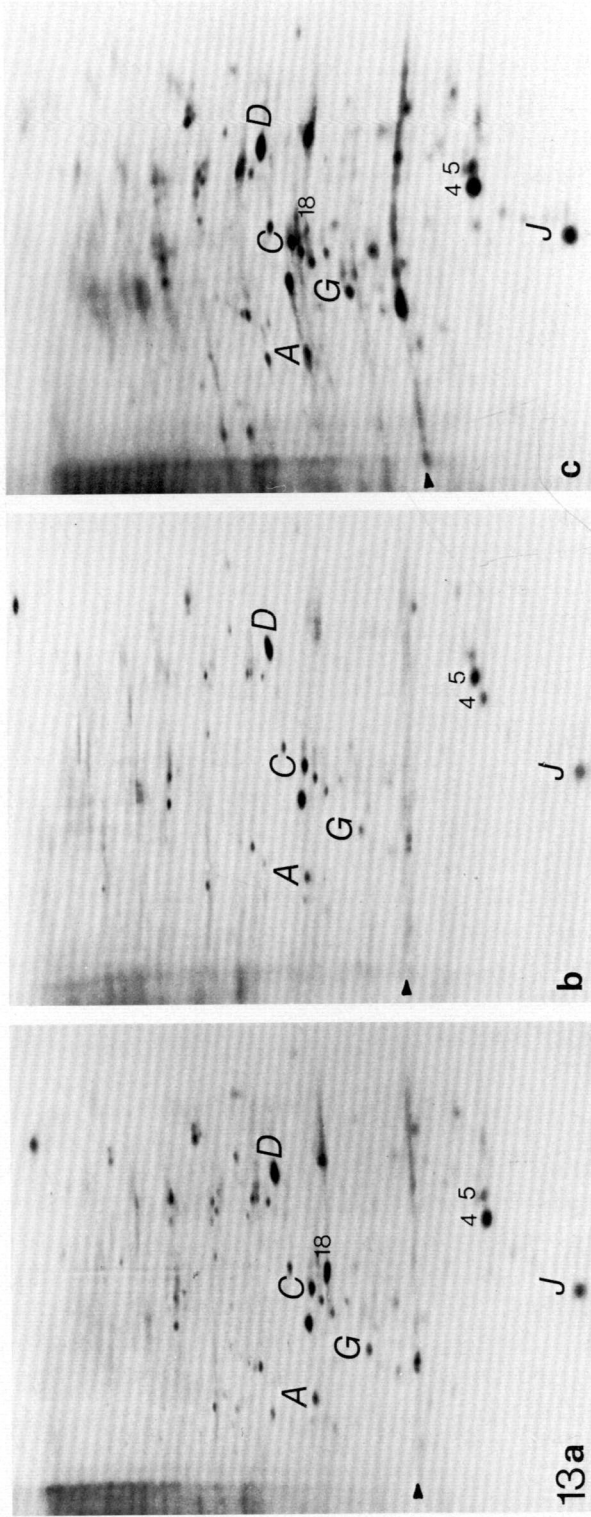


Fig. 13. Two-dimensional separation of [³⁵S]methionine-labelled polypeptides synthesized by unfertilized oocytes. Oocytes were labelled in M16 + BSA + 0.01 μ M unlabelled methionine containing diluted, lyophilized [³⁵S]methionine: (a) oocytes labelled 15.5–16.5 h post hCG (2–3 'hpi') and harvested immediately; (b) oocytes labelled 15.5–16.5 h post hCG, washed and cultured in M16 + BSA containing 100 μ M unlabelled methionine, phenylalanine and leucine and harvested at 20.5 h post hCG (7 'hpi'); (c) oocytes labelled 19.5–20.5 h post hCG (6–7 'hpi') and harvested immediately. Letters and numbers indicate reference and additional polypeptides, respectively, as for Fig. 9. Small arrowheads indicate the position of upper band 35×10^3 polypeptides.

Of the polypeptides with relative molecular masses of approximately 45 kD that are resolved by two-dimensional separation, polypeptide 18 (see Figs 9 & 13) appears to show a pattern of synthesis that corresponds to band a (Fig. 6). It is not possible to resolve polypeptide 18 among the *in vitro* translation products of maternal mRNA which suggests that it is either encoded by a rare or poorly translated message, or represents the post-translationally modified product of an unidentified primary transcript.

(iv) *Additional polypeptides*

Two-dimensional separation reveals a number of polypeptides, in addition to those in the 35, 30 and 45 kD regions of the gels, whose pattern of synthesis changes during the period of study, and that are not resolved in one dimension. Details of these are recorded in Fig. 9 & Table 2. The cessation of synthesis of polypeptides 1, 2 and 6 appears to be fertilization-dependent, while that of polypeptide 3 appears to be fertilization-accelerated. The commencement of synthesis of polypeptides 8, 9, 11, 12 and 13, the transitional synthesis of polypeptide 17, and the persisting synthesis of polypeptide 15 appear to be fertilization-dependent. Polypeptides 11 and 12 are the first two detectable translation products from expression of the embryonic genome (Flach *et al.* 1982; Bensaude, Babinet, Morange & Jacob, 1983).

Of these polypeptides, only one can be identified unambiguously among the *in vitro* translation products (number 16, Fig. 11) and is therefore presumably the primary translation product of a maternal mRNA. Polypeptide 16 shows a time-dependent decrease in synthesis *in situ* that is independent of fertilization, and is not reflected in any obvious loss of mRNA. Thus, despite the temporal and developmental changes in the pattern of its synthesis, the mRNA coding for this polypeptide remains present in a stable, translatable form, to at least 32 h post hCG in developing embryos, and to 37 h post hCG in ageing oocytes. The remaining polypeptides are presumably synthesized as a result of rapid post-translational modification, and furthermore may be stable proteins as no changes are detectable in pulse-chase analysis (Fig. 13).

DISCUSSION

In this paper, the timing and sequence of some of the morphological and molecular events that take place during the first cell cycle of mouse embryogenesis have been measured more precisely than previously, the general nature of some of the molecular changes defined, and the nature of the developmental trigger(s) that initiates this cycle investigated. The sequence of morphological events, the time course of DNA replication, and the major changes that are observed in one-dimensional patterns of polypeptide synthesis during this period of development are summarized diagrammatically in Fig. 14.

The time of onset, and the duration, of DNA replication described here are

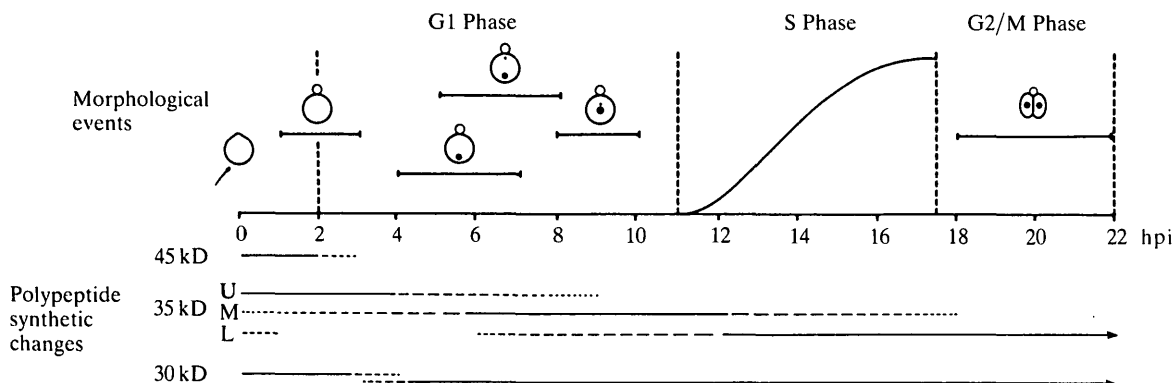


Fig. 14. Outline of events occurring during the first cell cycle of mouse development. The morphological events are summarized, and the timing of extrusion of the second polar body (1–3 hpi), of formation of the male (4–7 hpi) and female (5–8 hpi) pronucleus, of migration of the two pronuclei to the centre of the embryo (8–10 hpi), and of the first cleavage division (18–22 hpi) are shown. The timing of DNA replication within the 1-cell egg is indicated. *In situ* polypeptide synthesis is illustrated for the three sets of polypeptides (relative molecular masses $45, 35, 30 \times 10^3$) which show marked changes during the first cell cycle. Solid line represents maximal/near maximal synthesis, dotted line represents lower levels of synthesis, no line represents no detectable synthesis (see also Figs. 6 & 8).

similar to the times quoted by Luthardt & Donahue (1973) and Siracusa, Coletta & Monesi (1975), though rather later and longer than the values given by Abramczuk & Sawicki (1975). This difference may be a reflection of the developmental asynchrony of embryos used in previous studies, which were derived from fertilization *in vivo*, or it may be a manifestation of interstrain variation. These earlier studies report that the male pronucleus consistently enters S phase before the female pronucleus; in this study, earlier initiation of DNA replication appeared to occur at random in either pronucleus. We have no explanation for this difference.

Previous studies have shown that the genotype of the male and female gametes can alter the time of the first cleavage division by as much as 4 h and 6 h respectively (McLaren & Bowman, 1973; Shire & Whitten, 1980 *a, b*). However, since in these studies embryos were derived by fertilization *in vivo*, it is difficult to establish whether or not this is due to differences in the timing of sperm transport to, and entry into, the oocyte (Krzanowska, 1964; Nicol & McLaren, 1974). We have compared the relationship between the time of first cleavage and the times of both hCG injection and insemination, in embryos derived by fertilization *in vitro*, in order to determine which of these two events triggers the cell cycle 'clock' (Figs. 3 & 4). The results suggest that the clock is associated closely with, and may be initiated directly by, sperm entry. Furthermore, oocytes retain the capacity to respond to this trigger (i.e. are fertilizable) for at least 30 h following ovulation (Marston & Chang, 1964).

Fertilization in some species, or the resumption of meiosis in others, triggers certain metabolic changes that may include an increase in the rate of protein

synthesis and/or a change in the pattern of proteins synthesized. In all animals that have been studied, these changes appear to proceed for an initial period in the absence of transcription (reviewed by Davidson, 1976) and therefore are presumed to be controlled at the post-transcriptional level. In the sea urchin, for example, the rate of protein synthesis increases dramatically (30–50 fold) on fertilization (Grainger, Winkler, Shen & Steinhardt, 1979), and the pattern of protein synthesis changes markedly due both to selective activation of mRNAs and to cell cycle variations in polypeptide stability (Evans *et al.* 1983). The surf clam, *Spisula solidissima*, is an example of a species in which there is a relatively modest increase in the rate of total protein synthesis (2–4 fold) on fertilization, together with a clear switch in the classes of proteins that are synthesized (Rosenthal *et al.* 1980). Indeed, examples of specific mRNAs have been identified, the translation of which is elevated or depressed at fertilization (Tansey & Ruderman, 1983). The mouse embryo appears to employ a different balance of these two changes at fertilization. There is a slight decrease in the rate of protein synthesis during mouse oocyte maturation (Schultz & Wassarman, 1977). At fertilization only a modest increase in the rate of protein synthesis occurs, and while the biosynthetic profile of polypeptides is broadly similar, many changes do occur. These differences may be simply because the mouse embryo, with a first cell cycle lasting up to 20 h, has so much longer to synthesize and accumulate the material required for cleavage and development than do more rapidly dividing embryos such as *Arbacia* or *Spisula*, in which the first cell cycles last only 60 and 70 minutes respectively. It is also possible that cell cycle variations in mouse biosynthetic activity are more readily resolved, than with the shorter cell cycles in other species.

At the time of fertilization the *Spisula* oocyte, like that of the starfish *Asterias*, is arrested at first meiotic metaphase; the mouse oocyte is arrested at second meiotic metaphase, whereas the sea urchin oocyte has completed meiosis. Many of the qualitative changes in protein synthetic patterns at activation in *Asterias*, *Spisula* and the mouse may in fact relate to terminal events of meiotic maturation. Evidence consistent with this view comes from an analysis of the complexity of protein synthetic changes in mammalian oocytes undergoing only meiotic transition from the germinal vesicle phase to second metaphase (Schultz & Wassarman, 1977; Van Blerkom & McGaughey, 1978; Moor & Osborn, 1983).

Following ovulation and/or fertilization of the mouse oocyte, the broad patterns of polypeptides synthesized by ageing oocytes and by developing embryos change remarkably little over a period of at least 24 h. Although the most striking polypeptide synthetic changes during this time are those that occur among the 35 kD complex of polypeptides (Levinson, Goodfellow, Vadeboncoeur & McDevitt, 1978; Braude *et al.* 1979; Cullen *et al.* 1980; Van Blerkom, 1981; Cascio & Wassarman, 1982; Pratt *et al.* 1983), several other changes have now also been identified and better characterized. The timings of certain of these changes have been described in detail (summarized in Fig. 14), and the underlying molecular mechanisms have been examined by use of *in vitro* translation of extracted mRNA and in

pulse-chase experiments. These approaches have shown that during the 24 h period following ovulation, the changing polypeptide synthetic patterns observed may be explained by temporally programmed changes in the availability of mRNA for translation, in the differential turnover of polypeptides and, unlike the situation in other types of embryo (Rosenthal *et al.* 1980; Rosenthal, Brandhorst & Ruderman 1982), in the nature of post-translational modifications to the primary translation products. Finally, a comparison of the polypeptides synthesized by developing embryos and by ageing oocytes indicates that while some of these processes take place irrespective of fertilization, others are accelerated following fertilization, and some may be fertilization-dependent. Thus, the data presented here (summarized in Table 1) support the notion that the polypeptide synthetic changes observed during the first cell cycle of mouse embryogenesis may be controlled at two levels, namely at the levels of the oocyte and fertilization programmes (Van Blerkom, 1981; Pratt *et al.* 1983).

The oocyte programme is defined as proceeding independently of fertilization. It may be set in train by the terminal events of oogenesis, and may serve to confer on the oocyte the potential to initiate development. Since mammalian oocytes must undergo full cytoplasmic and nuclear maturation to at least metaphase II before they are competent to yield viable embryos (Braden & Austin, 1954; Chang, 1955; Thibault, 1971; Moor & Trounson, 1970), this suggests that the oocyte programme controls certain processes (presumably the accumulation of proteins and various other cellular components) that must be completed before fertilization takes place. Indeed, many of the reference polypeptides (A, C, D, G, J; see Fig. 9) are already present in the ovulated oocyte, as they can be detected by silver staining (data not shown).

The fertilization programme, which is initiated by sperm penetration, is superimposed on the oocyte programme, so that while many polypeptides are common to both fertilized eggs and ageing oocytes (presumably those which are regulated by the oocyte programme, and which are required for the maintenance of "housekeeping" functions during the early post-ovulation period), other polypeptides undergo fertilization-accelerated or fertilization-dependent changes in their pattern of synthesis. It is envisaged that this latter group of polypeptides are more directly involved in the initiation of embryogenesis, perhaps by restarting the cell cycle "clock", and by reorganizing the chromatin in preparation for DNA replication and activation of embryonic transcription (Bolton *et al.* 1984). In addition, fertilization may catalyse the termination of the oocyte programme, once it has performed the function of producing a fertilizable oocyte. Thus, at the early and mid 2-cell stages, the fertilization programme induces the degradation of maternal messages, and allows their replacement by messages newly transcribed from the embryonic genome (Bachvarova & DeLeon, 1980; Brinster, Chen, Trumbauer & Avarbock, 1980; Flach *et al.* 1982; Piko & Clegg, 1982; Giebelhaus, Heikkila & Schultz, 1983; Giebelhaus, 1984; Bolton *et al.* 1984; reviewed by Johnson, McConnell & Van Blerkom, 1984).

An examination of the changes that take place among the 35 kD complex of polypeptides illustrates the processes of selective, sequential mRNA activation and post-translational modification, as well as the influence of the fertilization programme on polypeptide synthetic changes already regulated by the oocyte programme. The *in vitro* translation studies described here show that maternal mRNA encodes two middle and three lower band, but no upper band 35 kD polypeptides, suggesting that upper band polypeptides are post-translationally modified products. This conclusion is confirmed by pulse-chase experiments, which indicate that some or all upper and middle band polypeptides are related directly by post-translational modification(s). In contrast, lower band polypeptides appear to represent a distinct set, unrelated to upper and middle band polypeptides. These findings are consistent with peptide mapping analysis (Van Blerkom, 1981; Cascio & Wassarman, 1982), and *in vitro* translation studies (Cascio & Wassarman, 1982; Pratt *et al.*, 1983). Thus, the temporal transition that occurs among the 35 kD complex polypeptides can be explained by the following sequence of events.

Synthesis of the linear array of upper band polypeptides, which occurs during the early post-ovulation period irrespective of fertilization, may be due to the immediate post-translational modification of the (two) middle band polypeptides which are the primary translation products of maternal mRNA. This conversion of middle to upper band polypeptides probably involves phosphorylation, as ^{32}P is incorporated into upper, but not into middle band 35 kD polypeptides (Van Blerkom, 1981; Pratt *et al.* 1983). The conversion ceases in fertilized eggs at 4–6 hpi, and the primary middle band translation products remain unmodified throughout the rest of the first cell cycle (until around 16–18 hpi). In addition, existing upper band polypeptides undergo slow conversion back to middle band polypeptides, presumably by dephosphorylation. Although unmodified middle band polypeptides are synthesized after 25.5 h post hCG by ageing oocytes, elements of the middle to upper band conversion persist at least until 40.5 h post hCG (though they may proceed in a less ordered manner, producing the heavily labelled streak of upper band polypeptides in the high pH region of two-dimensional gels, probably by multiple glycosylations; Van Blerkom, 1981). By the time of first cleavage, developing embryos no longer synthesize middle band polypeptides, but middle and upper band polypeptides are synthesized by ageing oocytes of the same chronological age.

In the case of 35 kD lower band, mRNA coding for these polypeptides appears to be transiently accessible for translation around the time of ovulation. However, at fertilization it is rapidly rendered untranslatable (either directly, by modification of the mRNA itself, or indirectly, by competition from other mRNA species that become available for translation) and remains so for about 5 h, when synthesis of lower band polypeptides resumes; this then persists to the mid 2-cell stage (Bolton *et al.* 1984). In the unfertilized oocyte the inactivation of mRNA coding for lower band polypeptides takes 3–4 h longer than in the 1-cell embryo, and the subsequent reactivation is delayed by approximately 24 h.

Regarding the 30 kD complex polypeptides, there is a fertilization-dependent switch from synthesis of band y (polypeptide 4) to that of band z (polypeptide 7). This switch occurs within 2–3 h of fertilization, and may involve the alternate utilization of separate species of maternal mRNA, since pulse-chase experiments indicate that y is not converted to z, suggesting that y and z are encoded by two distinct mRNAs. However, *in vitro* translation of total mRNA shows that while message encoding polypeptide 7 (band z) is relatively abundant in oocytes and embryos, that encoding polypeptide 4 (band x) is either absent, rare, or inefficiently translated *in vitro*. In the oocyte, a slow post-translational modification of polypeptide 4 to produce polypeptide 5 occurs. This conversion of polypeptide 4 into the higher relative molecular mass polypeptide 5 may involve phosphorylation (Pratt *et al.* 1983).

The 45 kD complex may represent a single polypeptide with a short half-life, whose synthesis ceases or turnover increases earlier in fertilized eggs than in ageing oocytes. It is difficult to identify an *in vitro* translation product corresponding to this polypeptide which therefore may be a secondary product of maternal mRNA.

This description of the temporal sequence of the morphological and molecular events that take place during the first cell cycle of mouse embryogenesis, and of the nature of some of the molecular controls, provides the basis for an investigation of their developmental significance and regulation. For example, both Ca^{2+} and pH play regulatory roles in the protein synthetic changes that occur after fertilization and/or meiotic maturation (Winkler, Steinhardt, Grainger & Minning, 1980); in some species this may be achieved by effecting phosphorylation of the ribosomal protein S6, which may control the translational capacity of the oocyte (Ballinger & Hunt, 1980; Ballinger, Bray & Hunt, 1984; Wasserman & Housel, 1984). However, it is also clear that there are other, as yet uncharacterized, intracellular changes, such as in levels of NADPH or phosphoinositides (Turner, Sheetz & Jaffe, 1984), involved in the translationally mediated control mechanisms operating around the time of meiotic maturation and fertilization. By analogy, it is possible that the changing pattern of translation, and of post-translational modification and stability, that occurs in the mouse oocyte may be induced in part by changes in pH and in intracellular Ca^{2+} levels occurring at fertilization. In this context, it is important to emphasize that the polypeptide analysis undertaken in this paper has concentrated on *synthetic* patterns which are not the same as the pattern of proteins present, as assessed for example by silver staining. However, the evident importance of modifications to the structure and stability of polypeptides over this period will and does apply to polypeptides regardless of their time of synthesis. Indeed, it is possible that the developmentally regulated selective mRNA utilization may also result from modification of regulatory proteins. If this is so, then many of the events of early development may be independent of *de novo* protein synthesis. The results reported in this paper shift emphasis towards an analysis of the role of protein modification in the first cell cycle, and provide a base line of descriptive and analytical information

that is essential if the control of events in this and the ensuing cell cycle are to be understood.

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REFERENCES

- ABRAMCZUK, J. & SAWICKI, W. (1975). Pronuclear synthesis of DNA in fertilised and parthenogenetically activated mouse eggs. *Expl Cell Res.* **92**, 361–372.
- BACHVAROVA, R. (1974). Incorporation of tritiated adenosine into mouse ovum RNA. *Devl Biol.* **40**, 52–58.
- BACHVAROVA, R. & DELEON, V. (1980). Polyadenylated RNA of mouse ova and loss of maternal RNA in early development. *Devl Biol.* **74**, 1–8.
- BALLINGER, D. G. & HUNT, T. (1981). Fertilisation of sea urchin eggs is accompanied by 40S ribosomal subunit phosphorylation. *Devl Biol.* **87**, 277–285.
- BALLINGER, D. G., BRAY, S. J. & HUNT, T. (1984). Studies of the kinetics and ionic requirements for the phosphorylation of ribosomal protein S6 after fertilisation of *Arbacia punctulata* eggs. *Devl Biol.* **101**, 192–200.
- BENSAUDE, O., BABINET, C., MORANGE, M. & JACOB, F. (1983). Heat shock proteins, first major products of zygotic gene activity. *Nature* **305**, 331–333.
- BOLTON, V. N., OADES, P. J. & JOHNSON, M. H. (1984). The relationship between cleavage, DNA replication and activation of transcription in the mouse 2-cell embryo. *J. Embryol. exp. Morph.* **79**, 139–163.
- BONNER, W. M. & LASKEY, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83–88.
- BRADEN, A. W. H. & AUSTIN, C. R. (1954). Fertilisation of the mouse egg and the effect of delayed coitus and of heat-shock treatment. *Aust. J. biol. Sci.* **7**, 552.
- BRAUDE, P. R., PELHAM, H. R. B., FLACH, G. & LOBATO, R. (1979). Post-transcriptional control in the early mouse embryo. *Nature* **282**, 102–105.
- BRINSTER, R. L., CHEN, H. Y., TRUMBAUER, M. E. & AVARBOCK, M. R. (1980). Translation of globin messenger RNA by the mouse ovum. *Nature* **283**, 499–501.
- CASCIO, S. M. & WASSARMAN, P. M. (1982). Program of early development in the mammal: post-transcriptional control of a class of proteins synthesised by mouse oocytes and early embryos. *Devl Biol.* **89**, 397–408.
- CHANG, M. C. (1955). Fertilisation and normal development of follicular oocytes in the rabbit. *Science* **121**, 867.
- CULLEN, B., EMIGHOLZ, K. & MONAHAN, J. (1980). The transient appearance of specific proteins in 1-cell mouse embryos. *Devl Biol.* **76**, 215–221.
- DAVIDSON, E. H. (1976). *Gene Activity in Early Development*, 2nd edn. New York & London: Academic Press.
- DIETCH, A. D., WAGNER, D. & RICHARDT, R. M. (1967). Conditions influencing the intensity of the Feulgen reaction. *J. Histochem. Cytochem.* **16**, 371–379.
- EVANS, T., ROSENTHAL, E. T., YOUNGBLOM, J., DISTEL, D. & HUNT, T. (1983). Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* **33**, 389–396.
- FLACH, G., JOHNSON, M. H., BRAUDE, P. R., TAYLOR, R. S. & BOLTON, V. N. (1982). The transition from maternal to embryonic control in the 2-cell mouse embryo. *The EMBO J.* **1**, 681–686.

- FRASER, L. R. (1983). Mouse sperm capacitation assessed by kinetics and morphology of fertilization *in vitro*. *J. Reprod. Fert.* **69**, 419–428.
- FRASER, L. R. & DRURY, L. (1975). The relationship between sperm concentration and fertilisation *in vitro* of mouse eggs. *Biol. Reprod.* **13**, 513–518.
- FULTON, B. P. & WHITTINGHAM, D. G. (1978). Activation of mammalian oocytes by intracellular injection of calcium. *Nature* **273**, 149–151.
- GIEBELHAUS, D. H. (1984). mRNA in early mouse embryos. PhD Thesis. University of Calgary, Alberta, Canada.
- GIEBELHAUS, D. H., HEIKKILA, J. J. & SCHULTZ, G. A. (1983). Changes in the quantity of histone and actin messenger RNA during the development of preimplantation mouse embryos. *Devl Biol.* **98**, 148–154.
- GRAINGER, J. L., WINKLER, M. M., SHEN, S. S. & STEINHARDT, R. A. (1979). Intracellular pH controls protein synthesis rate in the sea urchin egg and early embryo. *Devl Biol.* **68**, 396–406.
- HOUK, M. S. & EPEL, D. (1974). Protein synthesis during hormonally induced meiotic maturation and fertilisation of starfish oocytes. *Devl Biol.* **40**, 298–309.
- JOHNSON, M. H. (1981). The molecular and cellular basis of preimplantation mouse development. *Biol. Rev.* **56**, 463–498.
- JOHNSON, M. H., MCCONNELL, J. & VAN BLERKOM, J. (1984). Programmed development in the mouse embryo. *J. Embryol. exp. Morph.* **83**, suppl. 197–231.
- JOHNSON, M. H. & ROSSANT, J. (1981). Molecular studies on cells of the trophectodermal lineage of the postimplantation mouse embryo. *J. Embryol. exp. Morph.* **61**, 103–116.
- KRZANOWSKA, H. (1964). Time interval between copulation and fertilization in inbred lines of mice and their crosses. *Folia biol. Krakow* **12**, 231–244.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- LAMARCA, M. J. & WASSARMAN, P. M. (1979). Program of early development in the mammal: changes in absolute rate of synthesis of ribosomal proteins during oogenesis and early embryogenesis in the mouse. *Devl Biol.* **73**, 103–119.
- LASKEY, R. A. & MILLS, A. D. (1975). Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**, 335–341.
- LEVEY, I. L. & BRINSTER, R. L. (1978). Effects of α -amanitin on RNA synthesis by mouse embryos in culture. *J. exp. Zool.* **203**, 351–360.
- LEVEY, I. L., STULL, G. B. & BRINSTER, R. L. (1978). Poly (A) and synthesis of polyadenylated RNA in the preimplantation mouse embryo. *Devl Biol.* **64**, 140–148.
- LEVINSON, J., GOODFELLOW, P., VADEBONCOEUR, M. & MCDEVITT, H. (1978). Identification of stage-specific polypeptides synthesised during murine preimplantation development. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3332–3336.
- LUTHARDT, F. W. & DONAHUE, R. P. (1973). Pronuclear DNA synthesis in mouse eggs. *Expl Cell Res.* **82**, 143–151.
- MARO, B., JOHNSON, M. H., PICKERING, S. J. & FLACH, G. (1984). Changes in actin distribution during fertilisation of the mouse egg. *J. Embryol. exp. Morph.* **81**, 211–237.
- MARSTON, J. H. & CHANG, M. C. (1964). The fertilisable life of ova and their morphology following delayed insemination in mature and immature mice. *J. exp. Zool.* **155**, 237–252.
- MCLAREN, A. & BOWMAN, P. (1973). Genetic effects on the timing of early development in the mouse. *J. Embryol. exp. Morph.* **30**, 491–498.
- MOOR, R. M. & OSBORN, J. C. (1983). Somatic control of protein synthesis in mammalian oocytes during maturation. In *Molecular Biology of Egg Maturation*, pp. 178–196, Ciba Foundation Symposium **98**.
- MOOR, R. M. & TROUNSON, A. O. (1977). Hormonal and follicular factors affecting maturation of sheep oocytes *in vitro* and their subsequent developmental capacity. *J. Reprod. Fert.* **49**, 101–109.
- NICOL, A. & MCLAREN, A. (1974). An effect of the female genotype on sperm transport in mice. *J. Reprod. Fert.* **39**, 421–424.
- O'FARRELL, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. biol. Chem.* **250**, 4007–4021.
- PETZOLDT, V., HOPPE, P. C. & ILLMENSEE, K. (1980). Protein synthesis in enucleated fertilised and unfertilised mouse eggs. *Wilhelm Roux' Arch. devl Biol.* **189**, 215–219.

- PIKO, L. & CLEGG, K.B. (1982). Quantitative changes in total RNA, total Poly A and ribosomes in early mouse embryos. *Devl Biol.* **89**, 362–378.
- PRATT, H. P. M., BOLTON, V. N. & GUDGEON, K. A. (1983). The Legacy from the oocyte and its role in controlling early development of the mouse embryo. In *Molecular Biology of Egg Maturation*, pp. 197–227, CIBA Foundation Symposium 98.
- RICHTER, J. D. & MCGAUGHEY, R. W. (1981). Patterns of polypeptide synthesis in mouse oocytes during germinal vesicle breakdown and during maintenance of the germinal vesicle stage by dibutyl cAMP. *Devl Biol.* **83**, 188–192.
- ROSENTHAL, E. T., HUNT, T. & RUDERMAN, J. V. (1980). Selective translation of mRNA controls the pattern of protein synthesis during early development of the surf clam, *Spisula solidissima*. *Cell* **20**, 487–494.
- ROSENTHAL, E. T., BRANDHORST, B. P. & RUDERMAN, J. V. (1982). Translationally mediated changes in patterns of protein synthesis during maturation of starfish oocytes. *Devl Biol.* **91**, 215–220.
- SATO, K. & BLANDAU, R. J. (1979). Second meiotic division and polar body formation in mouse eggs fertilised *in vitro*. *Gam. Res.* **2**, 283–293.
- SCHULTZ, G. A., CLOUGH, J. R., BRAUDE, P. R., PELHAM, H. R. B. & JOHNSON, M. H. (1981). A reexamination of messenger RNA populations in the preimplantation mouse embryo. In *Cellular and Molecular Aspects of Implantation* (ed. Glasser, S. R. & Bullock, D. W.) pp. 137–154, New York & London: Plenum Press.
- SCHULTZ, R. M. & WASSARMAN, P. M. (1977). Biochemical studies of mammalian oogenesis: Protein synthesis during oocyte growth and meiotic maturation in the mouse. *J. Cell Sci.* **24**, 167–194.
- SHIRE, J. G. M. & WHITTEN, W. K. (1980a). Genetic variation in the timing of the first cleavage in mice: effect of paternal genotype. *Biol. Reprod.* **23**, 363–368.
- SHIRE, J. G. M. & WHITTEN, W. K. (1980b). Genetic variation in the timing of the first cleavage in mice: effect of maternal genotype. *Biol. Reprod.* **23**, 369–376.
- SIRACUSA, G., COLETTA, M. & MONESI, V. (1975). Duplication of DNA during the first cell cycle in the mouse embryo. *J. Reprod. Fert.* **42**, 395–398.
- TANSEY, T. R. & RUDERMAN, J. V. (1983). Differential mRNA accumulation and translation during *Spisula* development. *Devl Biol.* **99**, 338–351.
- THIBAUT, C. (1971). *In vitro* maturation and fertilisation of rabbit and cattle oocytes. In *Regulation of Mammalian Reproduction* (ed. S. J. Segal, R. Crozier & P. A. Corfman), Springfield, Illinois: Charles C. Thomas.
- TURNER, P. R., SHEETZ, M. P. & JAFFE, L. A. (1984). Fertilisation increases the polyphosphoinositides content of sea urchin eggs. *Nature* **310**, 414–415.
- VAN BLERKOM, J. (1981). Structural relationship and post-translational modification of stage-specific proteins synthesised during early preimplantation development in the mouse. *Proc. natn. Acad. Sci., U.S.A.* **78**, 7629–7633.
- VAN BLERKOM, J. & MCGAUGHEY, R. W. (1978). Molecular differentiation in the rabbit ovum. I. During oocyte maturation *in vivo* and *in vitro*. *Devl Biol.* **63**, 139–150.
- WASSERMAN, W. J. & HOUSEL, J. G. (1984). The regulation of ribosomal protein S6 phosphorylation in *Xenopus* oocytes: A potential role for intracellular pH. *Devl Biol.* **101**, 436–445.
- WINKLER, M. M., STEINHARDT, R. A., GRAINGER, J. L. & MINNING, L. (1980). Dual controls for the activation of protein synthesis at fertilisation. *Nature* **287**, 558–560.
- WHITTINGHAM, D. G. (1971). Culture of mouse ova. *J. Reprod. Fert. Suppl.* **14**, 7–21.
- WHITTINGHAM, D. G. & WALES, R. G. (1969). Storage of two-cell mouse embryos *in vitro*. *Austr. J. biol. Sci.* **22**, 1065–1068.
- WOODLAND, H. R., FLYNN, J. M. & WYLIE, A. J. (1979). Utilisation of stored mRNA in *Xenopus* embryos and its replacement by newly synthesised transcripts: histone H1 species using interspecies hybrids. *Cell* **18**, 165–171.
- YOUNG, R. J., SWEENEY, K. & BEDFORD, J. M. (1978). Uridine and guanosine incorporation by the mouse one-cell embryo. *J. Embryol. exp. Morph.* **44**, 133–148.

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