Protein requirements for germinal vesicle breakdown in ovine oocytes

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SUMMARY

The regulation of the cell cycle during the transition from prophase to metaphase I was studied by analysing protein changes and introducing protein blocks during the transition phase. The results show that the progression to metaphase in ovine oocytes is totally dependent on new protein synthesis. By delaying the addition of the inhibitor, cycloheximide, for progressively longer periods after the resumption of meiosis it was established that the required synthesis occurs in the 1–2 h immediately preceding germinal vesicle breakdown (GVBD). The action of cycloheximide was fully reversible: removal of the drug resulted in GVBD between 3 and 4 h later.

The synthesis and modification of proteins during these first few hours of maturation were studied by short-term radiolabelling of oocytes with [^{35}S]methionine and [^{32}P]phosphate followed by rapid assessment of their precise nuclear configuration. Changes in phosphorylation of two polypeptides were detected 4–5 h after the beginning of culture, but these changes were not dependent upon protein synthesis. The earliest change in synthesis was the appearance of a new polypeptide 6–8 h after explantation, immediately before GVBD. This polypeptide (M_r 47×10^3, pI 5·8) was not significantly phosphorylated and was relatively stable. Oocytes released from cycloheximide treatment began to synthesize this molecule 3–4 h later, again coinciding with GVBD. Synthesis of the polypeptide was suppressed by inhibition of transcription with α-amanitin.

INTRODUCTION

The competence to undergo fertilization and early embryonic development is not acquired by mammalian oocytes until the completion of a final phase of differentiation immediately before ovulation. During this phase, referred to as the maturation period, many intracellular changes occur, including a reprogramming of protein synthesis and the progression of the cell cycle from the late prophase of the first meiotic division to the metaphase of the second meiotic division. In order to investigate the relationship between these two events a number of investigators have used various inhibitors to block transcription or protein synthesis at selected stages during maturation. The experiments carried out on mouse oocytes have consistently shown that neither new transcription nor new protein synthesis is required for the condensation of chromosomes and the breakdown of the germinal vesicle (Jagiello, 1969; Stern, Rayyis & Kennedy, 1972; Golbus & Stein, 1976; Crozet & Szollosi, 1980). Protein synthesis is, however, required for the continued

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progression of meiosis after germinal vesicle breakdown (GVBD) since continuous protein inhibition during maturation blocks the chromosomes in mouse oocytes at the circular bivalent stage (Wassarman, Josefowicz & Letourneau, 1976). On the other hand, a delay in the inhibition of protein synthesis until after the metaphase I stage of maturation is followed by the emission of a polar body and the premature formation of a nucleus containing decondensed chromosomes (Clarke & Masui, 1983).

The relationship between the nuclear and cytoplasmic events in mouse oocytes appears, however, to differ in several important respects from that in the other mammals that have been studied. Thus, Ekholm & Magnusson (1979) report that rat oocytes, unlike those of the mouse, depend on protein synthesis at or before the resumption of meiosis for subsequent germinal vesicle breakdown. These workers conclude further that the proteins required for germinal vesicle breakdown in rats are characterized by a high rate of turnover. The sheep oocyte also differs from that of the mouse, primarily because of its requirement for new RNA synthesis within the first 1–2 h after the onset of maturation (Osborn & Moor, 1983). Inhibition of transcription during this period blocks germinal vesicle breakdown and the accompanying reprogramming of protein synthesis.

In this paper we show that the ovine oocyte is dependent upon new protein synthesis for the breakdown of the germinal vesicle. Moreover, a protein has been identified whose synthesis is dependent on early transcription and whose appearance coincides closely with nuclear membrane change.

MATERIALS AND METHODS

Collection and culture of oocytes

Ovaries were obtained from Welsh Mountain ewes previously injected with 3×1.5 mg equine FSH-CB (Moor & Crosby, 1985), 48, 40 and 24 h before slaughter on day 10–12 of the oestrous cycle. Follicles >3 mm in diameter were dissected from the ovaries at room temperature as previously described (Moor & Trounson, 1977). Cumulus-enclosed oocytes were removed from the follicles and immediately placed into culture in Medium 199+10 % FCS using the gentle agitation system described by Staigmiller & Moor (1984). Gonadotrophins added to the culture medium were: $5 \mu g$ NIH-LH-S22 ml^{-1}; $2.5 \mu g$ NIH-FSH-S13 ml^{-1}. In all cases, freshly prepared supplementary granulosa cells, approximately 10^6 ml^{-1}, were added to the culture dishes.

Inhibition of protein synthesis

Protein synthesis was inhibited by the addition of $10 \mu g$ ml^{-1} cycloheximide (BDH Chemicals Ltd) to the culture medium.

Two regimes of administration were chosen. In the first, cycloheximide was added 0–15 h after the start of culture, in order to study the dependence of the oocyte on continuing protein synthesis throughout the meiotic process. In the second experiment, protein synthesis was inhibited from the beginning of culture, the oocytes were subsequently washed thoroughly in plain medium and culture was continued in the absence of cycloheximide in order to study the effects of the resumption of protein synthesis on the progress of meiosis.

Radiolabelling of oocyte proteins

In order to study protein synthesis, cumulus-enclosed oocytes were incubated for 30 min in [^{35}S]methionine (>1000 Ci mmol^{-1}, Amersham) at a radioactive concentration of 500 μCi ml^{-1},

using phosphate-buffered saline as described previously (Moor, Osborn, Cran & Walters, 1981). Labelled oocytes were denuded of cumulus cells before collection for electrophoresis.

Phosphorylation of proteins was studied by labelling oocytes for 60 min in [^{32}P]phosphate (carrier-free, Amersham) at a concentration of 200 μCi ml^{-1}, using phosphate-free EMEM (Flow Laboratories).

The subsequent fate of labelled proteins was studied in chase experiments. Oocytes were labelled in methionine as above for 6–7 h, washed five times to remove all free label and cultured with added unlabelled methionine (up to 1 mM) for 1–3 h before collection for electrophoresis.

Assessment of meiotic progress

The effects of cycloheximide on nuclear maturation were determined by fixing of whole oocytes in ethanol:acetic acid (3:1) and staining with lacmoid.

Oocytes which had been labelled with [^{35}S]methionine were incubated for 2 min in the fluorescent, DNA-binding dye, 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co. Ltd), at a concentration of 40 μg ml^{-1} and then examined under fluorescence before freeze-drying for electrophoresis. In this way it was possible to relate the protein synthetic pattern to the exact meiotic state of each oocyte.

Gel electrophoresis

Samples were freeze-dried, dissolved in sample buffer and 10 % removed for measurement of incorporation of label by TCA precipitation as described by Moor *et al.* (1981).

One-dimensional (1-D) electrophoresis was performed on 8–15 % linear gradient SDS polyacrylamide gels (Moor *et al.* 1981).

For two-dimensional (2-D) analysis, samples were first separated by isoelectric focusing, essentially according to the method of O'Farrell (1975) except that multiple samples were run simultaneously on a single horizontal gel on a plastic backing (GelBond PAG film, FMC Corporation). The pH gradient was formed using 4 % Pharmalyte 3–10 and 1 % Pharmalyte 4·5–6·5 giving a wide range on a single gel. Individual tracks were cut out and run on second dimension SDS gels identical to those used for one-dimensional analysis.

Fluorography was carried out on [^{35}S]methionine-labelled gels according to the methods of Bonner & Laskey (1974) and dried gels were exposed to preflashed Kodak X-Omat S X-ray film (Laskey & Mills, 1975). Intensifying screens (Philips Ultrascreens) were used to enhance image formation by ^{32}P-labelled gels.

One-dimensional gels were scanned using a Helena QuickScan R&D densitometer and bands were quantified using an Apple II microcomputer.

RESULTS

Oocytes removed from ovine follicles and cultured *in vitro* undergo germinal vesicle breakdown at about 8 h after explantation. The metaphase I stage is extended and persists for about 10 h. This is followed by a short anaphase and telophase and the formation of the second metaphase plate at about 24 h after the initiation of maturation. The timing of the meiotic cycle in individual oocytes may, however, vary by about 2 h from the general outline presented above.

Effect of cycloheximide and α-amanitin on protein synthesis by oocytes

Oocytes incubated in cycloheximide (10 μg ml^{-1}) for 30 min incorporated only 252 cts min^{-1} per oocyte during a subsequent 60 min labelling period. When the addition of labelled methionine was further delayed, until 1 h after cycloheximide, only 352 cts min^{-1} per oocyte were incorporated in the following 4 h. Control
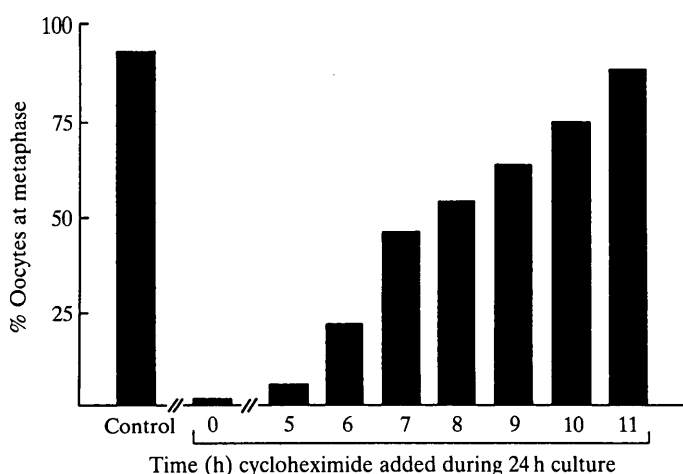
Fig. 1. Proportion of oocytes undergoing the prophase to metaphase transition after 24 h in culture in the absence (control) or presence of $10\,\mu g$ cycloheximide ml^{-1}. Oocytes were first exposed to the protein inhibitor at the initiation of maturation or at the time shown thereafter and then continuously maintained with cycloheximide in the medium until fixation at 24 h after explantation. The number of oocytes in each group varied from 17 to 42.

oocytes incorporated $6512\,cts\,min^{-1}$ in 3 h immediately after removal from the follicle. Oocytes removed from cycloheximide after 14 h of exposure incorporated a mean of $4400\,cts\,min^{-1}$ within 1 h.

α-Amanitin had no significant effect on the amount of methionine incorporation by oocytes during labelling periods between 6 and 9·5 h after exposure to the drug.

Requirements for protein synthesis during the meiotic cycle

Fig. 1 illustrates the effect on germinal vesicle breakdown of inhibiting protein synthesis at various times after the initiation of maturation. The addition of cycloheximide to extrafollicular oocytes during the first 4 h after initiation invariably blocks nuclear breakdown (percentage of metaphase oocytes <5 % in all groups from 0–4 h). On the other hand over 50 % of oocytes undergo GVBD when the addition of the inhibitor is delayed until 7 h after the initiation of maturation. The addition of cycloheximide between 10 and 15 h after initiation is without effect on GVBD but blocks meiosis at the transition from metaphase I to metaphase II (data not shown).

Oocytes removed from follicles and cultured thereafter in the presence of cycloheximide remain continuously arrested in the germinal vesicle stage of meiosis. Fig. 2 shows the effect on the nucleus of removing the protein inhibitor from oocytes after a 24 h period of inhibition. All oocytes showed signs of resuming meiosis and 65 % reached metaphase I within 4 h. The results show, firstly, that the effects of cycloheximide on the meiotic cycle are fully reversible. Secondly, it has been found that the nuclear membrane consistently breaks down between 3 and 4 h after the removal of the inhibitor. Use has been made both of

this model and that of the extrafollicular oocyte maturing in culture to identify the synthesis or phosphorylation of proteins in the period immediately preceding germinal vesicle breakdown.

Protein changes preceding germinal vesicle breakdown

Protein changes potentially associated with nuclear breakdown were sought by radiolabelling different groups of oocytes for successive 30 min periods throughout the first 10 h of maturation. The nuclear status of each oocyte was determined at the end of labelling to enable protein patterns to be related directly to meiotic events in individual oocytes. The results of these studies show that two distinct changes in protein patterns occur in oocytes before the breakdown of the nuclear membrane.

The earliest of these events occur between 4 and 5 h after the initiation of maturation and involve changes in protein phosphorylation. Fig. 3A compares the phosphorylation patterns in oocytes during the period of protein modification. No consistent changes were observed during the first 4 h after the initiation of maturation. However, in the ensuing 30–60 min a protein of approximately 38×10^3 M_r became dephosphorylated (marked 1 on Fig. 3A) and two others (designated 2a and 2b) became more heavily labelled. New protein synthesis is not required for the observed early phosphorylation modifications. The possible association of these phosphorylation changes with germinal vesicle breakdown is difficult to ascertain, especially since they occur in cycloheximide-treated oocytes in which meiotic change is totally inhibited (data not shown).

A further modification to the protein pattern occurs between 6 and 8 h after the induction of a maturation. Within individual oocytes this protein change is closely associated, in a temporal manner, with the first stages of germinal vesicle breakdown and is characterized by the appearance of a new protein band with a
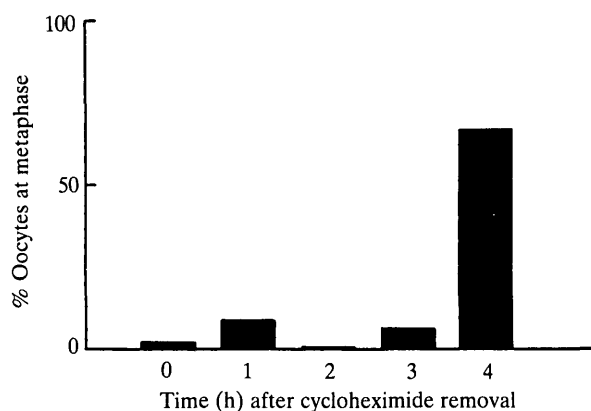

Fig. 2. Time required to undergo the transition from prophase to metaphase in oocytes maintained for 24 h in medium containing cycloheximide ($10\,\mu g\,ml^{-1}$) before being washed and cultured in inhibitor-free medium. Between 12 and 20 oocytes were included in each group.
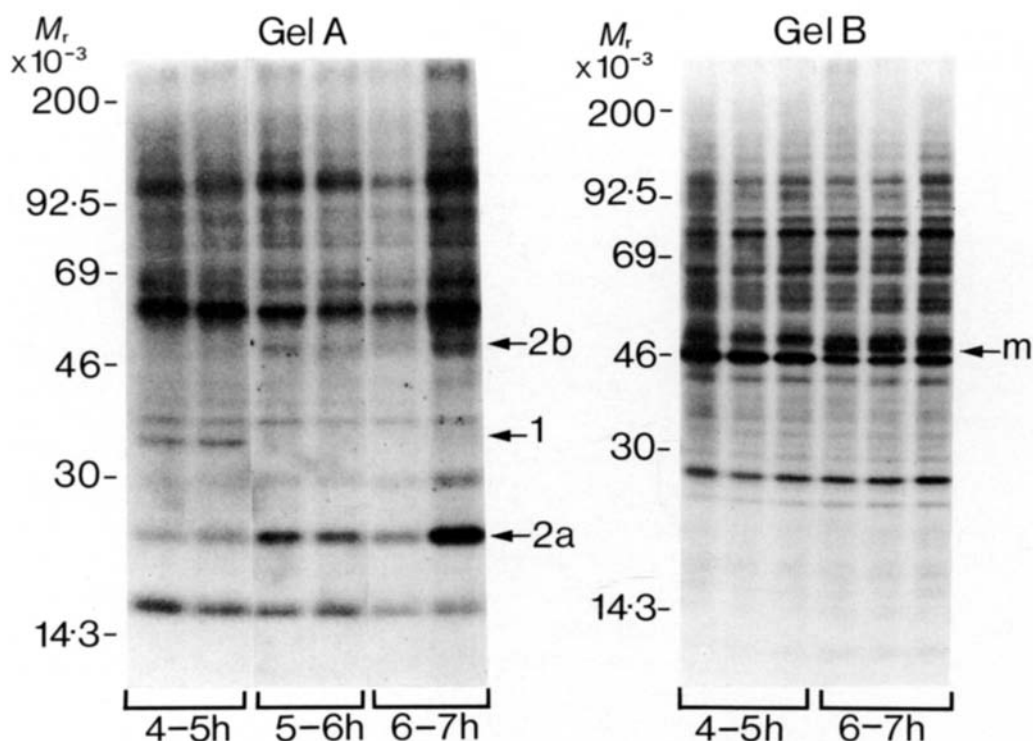
Fig. 3. Changes in protein patterns in oocytes immediately before germinal vesicle breakdown. Polypeptides were separated on 8–15 % linear-gradient SDS poly-acrylamide gels. Gel A shows phosphoprotein changes in oocytes incubated with [^{32}P]phosphate before (4–5 h) or during (5–7 h) the early stages in the transition from prophase to metaphase I. Phosphoproteins labelled 1, 2a and 2b undergo consistent changes during this early phase. Gel B shows the synthesis of new polypeptides in oocytes incubated with [^{35}S]methionine before (4–5 h) or during (6–7 h) the early stages in the transition from prophase to metaphase I. The appearance of a new protein (arrowed and designated protein 'm') coincides with the earliest morphological signs of germinal vesicle breakdown.

molecular weight of 46 to 48×10^3. The new band, designated as protein 'm' in Fig. 3B, is located between two of the most intensively labelled proteins in the oocyte and is therefore often obscured on 1-D gels. Separation of proteins on 2-D gels illustrates more clearly the appearance of protein 'm' before GVBD (Fig. 4). It is clear from these gels that the new protein is slightly more basic (pI approximately 5·8) than β-actin. Moreover, protein 'm' is not phosphorylated to any measurable extent, and from pulse-chase experiments, appears to be relatively stable. Densitometric measurements show that protein 'm' accounts for 4·9 % of the total radiolabelled protein in prometaphase oocytes incubated with [^{35}S]methionine for 30 min. The protein was still present in a similar proportion (4·5 % of total labelled protein) in oocytes that had been extensively washed after labelling and cultured thereafter for 3 h in the presence of up to 1 mM unlabelled L-methionine.

Protein synthesis and meiosis in oocytes removed from cycloheximide inhibition

Fig. 5 shows the 2-D pattern of proteins obtained from oocytes that were subjected to cycloheximide-induced meiotic arrest for 24 h and then released from inhibition for 2, 3 or 4 h. The synthesis of protein 'm' is low at 3 h and is slightly
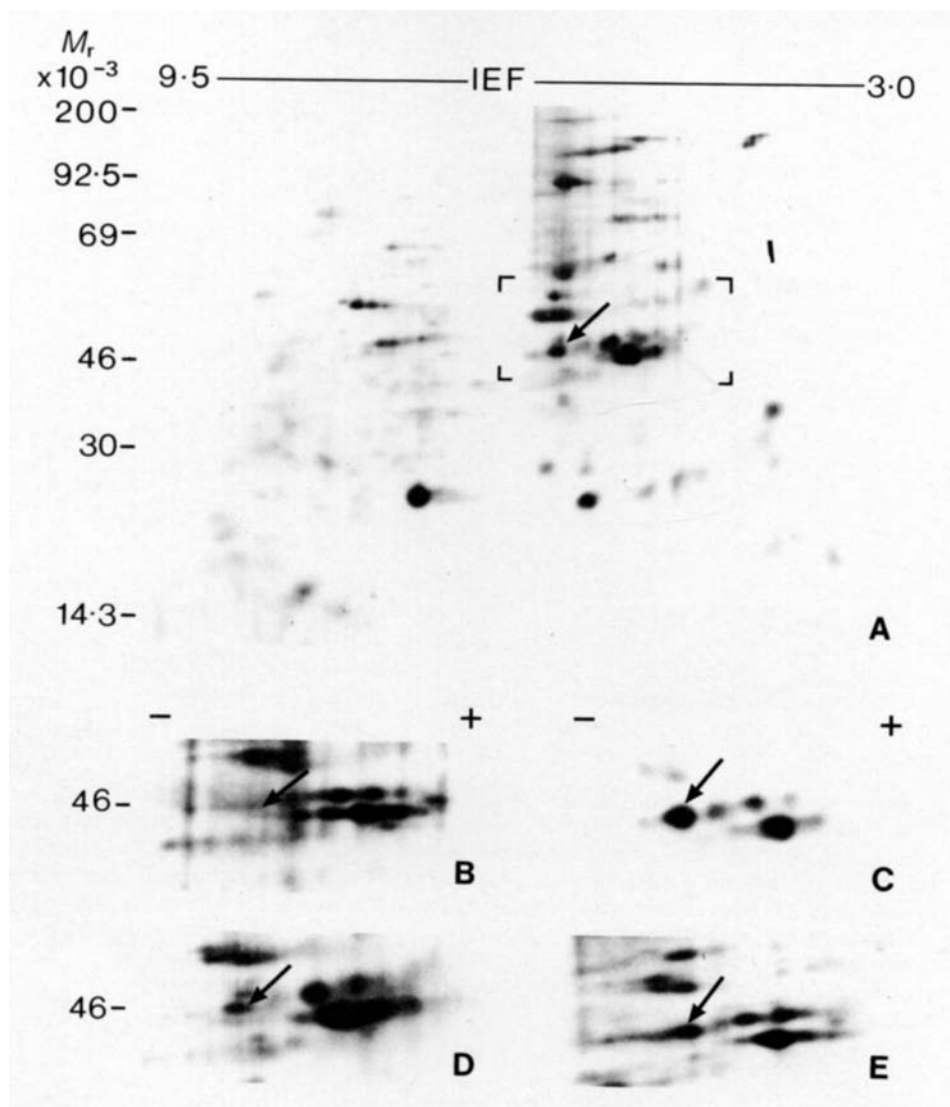

Fig. 4. Fluorograms of two-dimensional gel separations of [^{35}S]methionine polypeptides from oocytes at different stages during the prophase to metaphase transition. The polypeptides were separated by flat-bed IEF followed by electrophoresis on 8–15 % linear gradient SDS polyacrylamide gels (see Materials and Methods). The area marked by corners in the fluorogram (Fig. 4A) encompasses the region containing protein 'm' (arrowed). This protein which is barely detectable in germinal vesicle oocytes at 4–5 h after the initiation of maturation (Fig. 4B), becomes heavily labelled in prometaphase oocytes at 6–7 h (Fig. 4C) or 7–8 h (Fig. 4D) and is still synthesized by early metaphase I oocytes at 8–9 h after initiation of maturation (Fig. 4E).
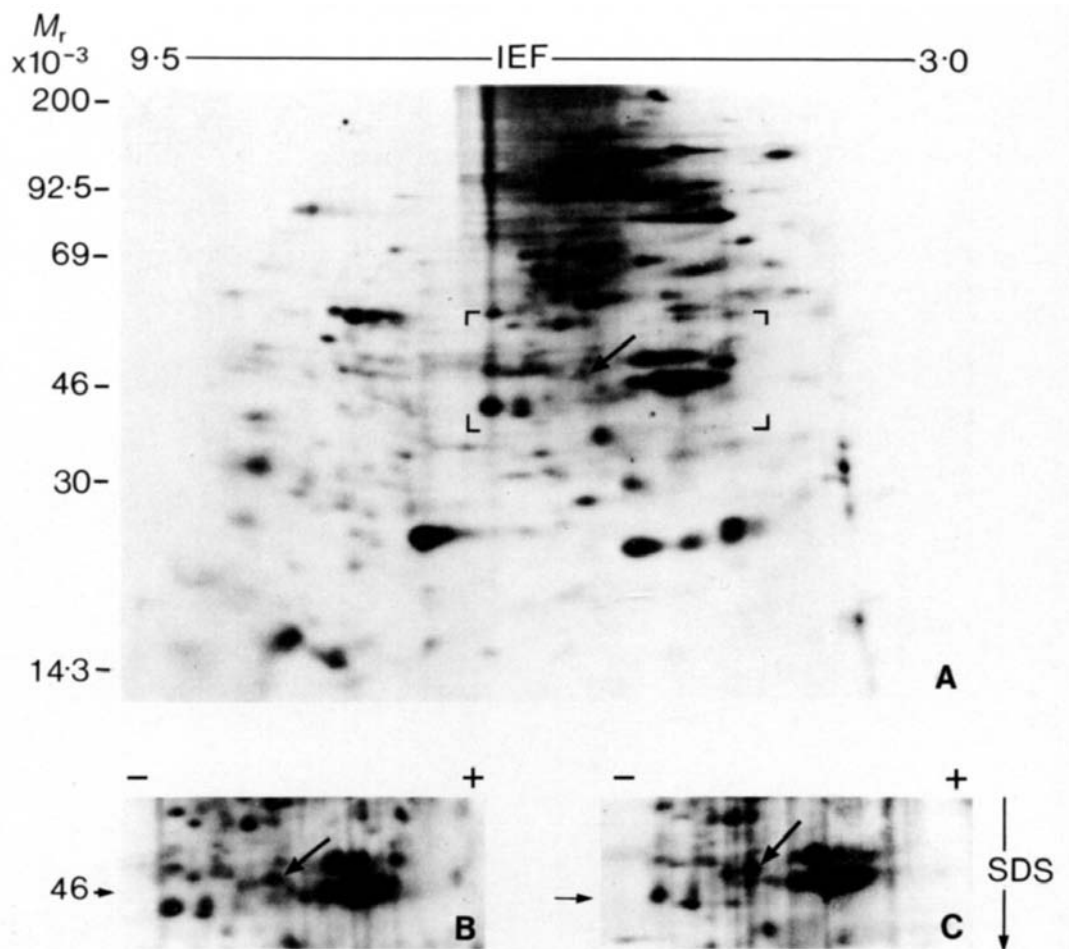
Fig. 5. Fluorograms of two-dimensional gels of oocytes labelled with [^{35}S]methionine from 1–2 h (Fig. 5A), 2–3 h (Fig. 5B) or 3–4 h (Fig. 5C) after release from 24 h of cycloheximide inhibition. The polypeptides were separated by flat-bed IEF followed by electrophoresis on 8–15 % linear gradient SDS polyacrylamide gels (see Materials and Methods). The area marked by corners in Fig. 4A is selected for use in Fig. 4B and C since it encompasses the area containing protein 'm' (arrowed). Overexposure of the fluorograms has been necessary to demonstrate protein 'm' which appears to be synthesized at a lower level in oocytes released from cycloheximide inhibition than in untreated oocytes at prometaphase.

more intense at 4 h when the nuclear membrane is in the process of breaking down. However, the synthesis of this protein is less marked in oocytes released from cycloheximide arrest than in normal prometaphase oocytes.

Relationship between transcription, protein synthesis and nuclear membrane breakdown

We have previously reported that transcription, restricted to the first 2 h of maturation, is required in ovine oocytes for the subsequent breakdown of the

germinal vesicle (Osborn & Moor, 1983). It has become relevant to determine whether the mRNA synthesized during this obligatory early transcriptional phase codes for protein 'm'. Accordingly, oocytes were cultured in the presence or absence of α-amanitin and their protein profiles were determined at intervals between 4 and 8h after the initiation of culture. As expected, protein 'm' was synthesized by untreated oocytes undergoing GVBD. Fig. 6 shows that the protein was not, however, synthesized in oocytes cultured in the presence of α-amanitin. Moreover, an analysis of the 1-D gels by densitometry suggested that protein 'm' was being selectively suppressed by the transcriptional inhibitor. A more detailed 2-D analysis of the protein patterns from control and α-amanitin-treated oocytes was carried out to verify this conclusion. The fluorograms shown in Fig. 7 confirm
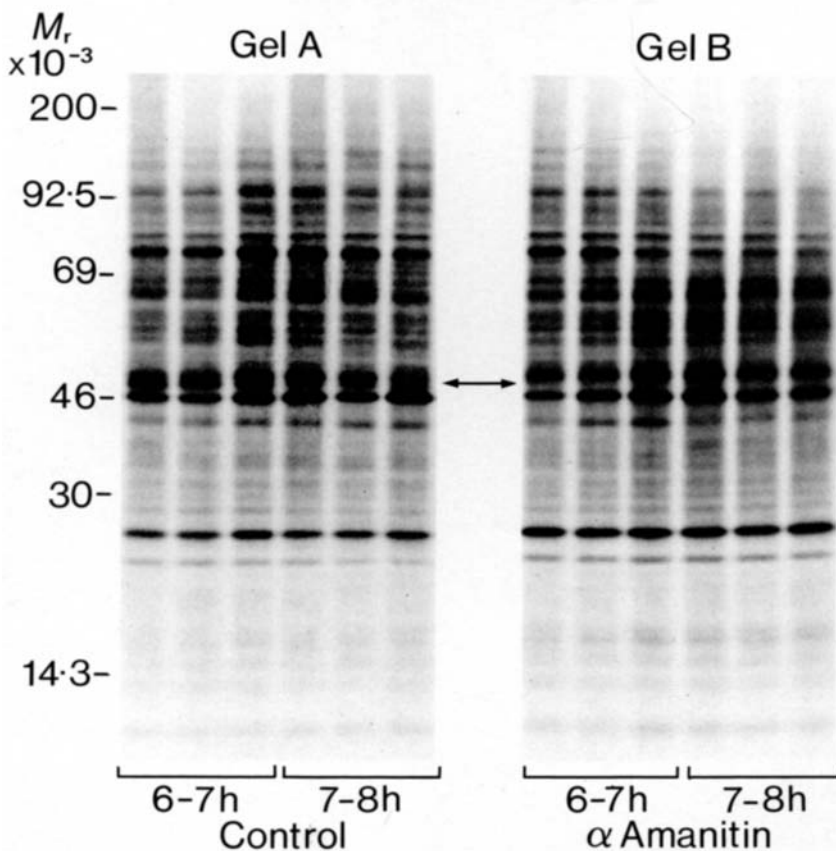

Fig. 6. The effect of inhibiting transcription on the synthesis of protein during the prophase-to-metaphase transition. Oocytes were incubated with [^{35}S]methionine for 1h, examined for nuclear change and then prepared for electrophoresis. Polypeptides were separated on 8–15 % linear gradient SDS polyacrylamide gels. Gel A shows the profile of proteins in control oocytes during the prometaphase stage. The synthesis of protein 'm' (arrowed) accounts for approximately 5 % of the total protein synthesis at this stage. Gel B shows the pattern of proteins synthesized by oocytes cultures for the same period as the controls but in the presence of α-amanitin (10 μg ml^{-1}).
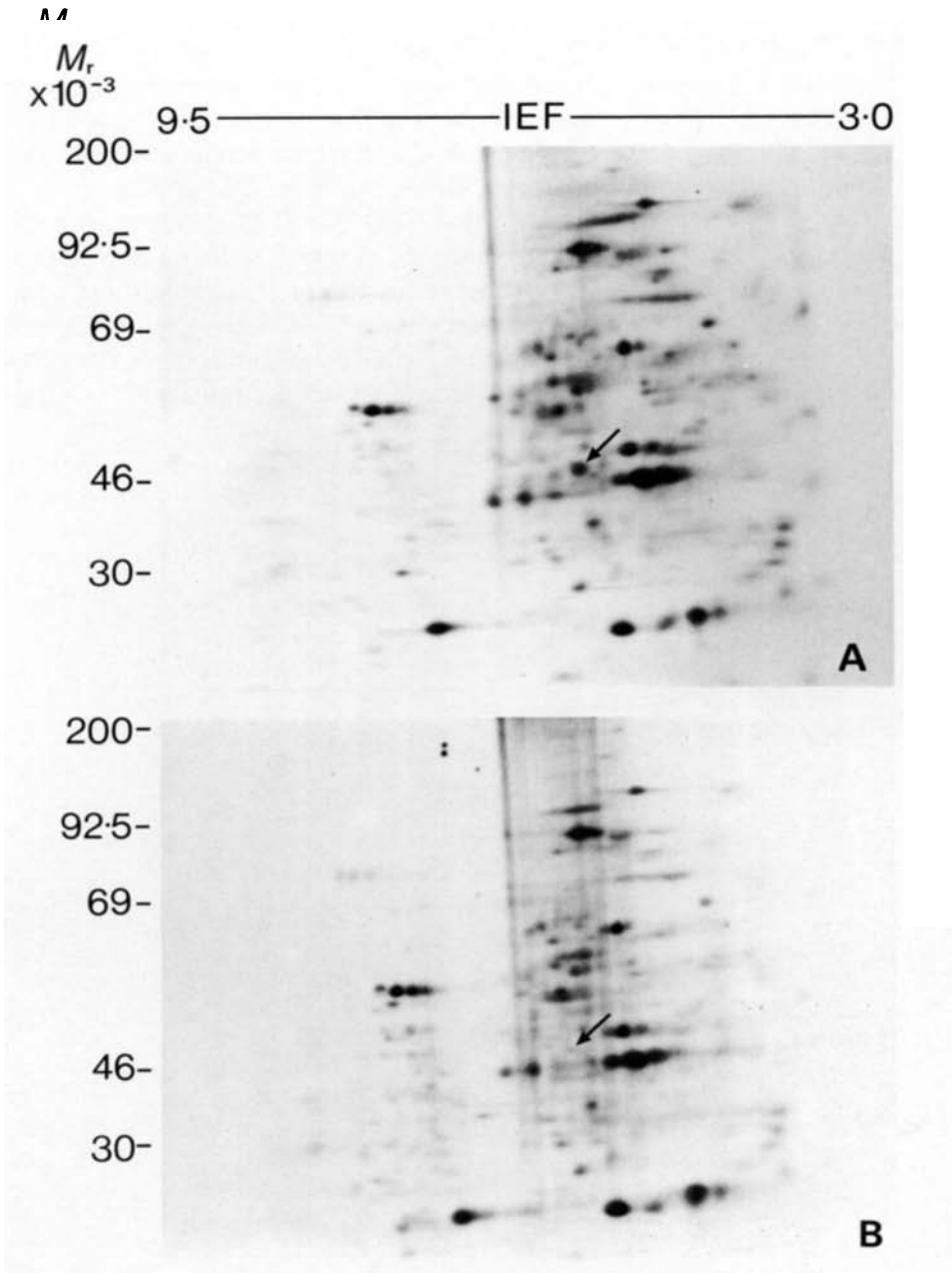
Fig. 7. Fluorogram of two-dimensional gels of oocytes labelled with [^{35}S]methionine from 7–8 h after the initiation of maturation. Fig. 5A shows the pattern of synthesis in control oocytes undergoing the prophase-to-metaphase transition. Protein 'm' (arrowed) is being synthesized at a high level. By contrast no synthesis of this protein is occurring in oocytes cultured in the presence of α-amanitin (10 μg ml^{-1}). The polypeptides were separated by flat-bed IEF followed by electrophoresis on 8–15 % linear gradient SDS polyacrylamide gels as described in the Materials and Methods section.

that the inhibition of transcription during maturation inhibits synthesis of protein 'm' but has little effect on the numerous other proteins synthesized at this time.

DISCUSSION

We report on the intracellular events in ovine oocytes during progression from the G_2-phase (prophase) to the M-phase (metaphase I) of the meiotic cycle. The results show that new protein synthesis acts as a trigger for this transition phase and that the required proteins are synthesized within 1 h or so preceding germinal vesicle breakdown. Inhibition of protein synthesis immediately before GVBD arrests the meiotic cycle in prophase, whilst removal of the protein synthesis inhibitor even after prolonged inhibition results in nuclear envelope breakdown within 4 h. Furthermore, an analysis of protein changes before GVBD show that the patterns are affected both by post-translational modifications and by the synthesis of at least one new protein. This newly synthesized protein (referred to as protein 'm') has a relative molecular mass of about 47×10^3 and is probably coded for by mRNA transcribed during the first 2 h after the initiation of maturation. The addition of α-amanitin during the early stages of maturation inhibits transcription, prevents the synthesis of protein 'm' and blocks germinal vesicle breakdown.

Although some of these conclusions rely upon the use of inhibitors and must, therefore, be treated with caution, there were few signs of undesirable side effects. Cycloheximide blocked oocyte protein synthesis quickly and completely and oocytes resumed synthesis in an apparently normal manner when the drug was removed. Similarly, cycloheximide acted extremely rapidly on meiosis, blocking GVBD when added only 1 h beforehand, and all oocytes showed signs of resuming meiosis after treatment ended, 65 % reaching MI within 4 h. α-Amanitin had no measurable effect on the rate of incorporation and little effect on the pattern of synthesis except of protein 'm'.

Changes in the pattern of protein phosphorylation were the earliest events observed in oocytes undergoing meiotic maturation. The use of short labelling periods enabled us to identify changes at earlier stages than had previously been reported (Crosby, Osborn & Moor, 1984) and revealed phosphorylation and dephosphorylation events. The crucial role of protein phosphorylation in governing GVBD in amphibian oocytes has repeatedly been stressed (for review see Maller & Smith, 1985). For example, the burst of protein phosphorylation before nuclear breakdown has been associated both with increased MPF activity and the coincident phosphorylation of the 40S ribosomal subunit known as S6 (Maller, Wu & Gerhart, 1977; Wu & Gerhart, 1980; Hanocq-Quertier & Baltus, 1981). In addition, phosphorylation changes are essential for the disassembly of the nuclear lamins and the consequent breakdown of the nuclear membrane (Gerace & Blobel, 1980). We have so far failed to establish any direct association between phosphorylation changes and the specific events associated with the transition

from prophase to metaphase I in ovine oocytes. The elution of specific phosphoproteins from gels and their subsequent microinjection into oocytes might assist in elucidating the role of these proteins during the metaphase transition.

Considerable progress has recently been made in identifying changes in the synthesis and degradation of proteins which appear to be associated with specific phases of the cell cycle in newly fertilized eggs. Most attention has been focused on the three proteins (M_r 40, 51 and 58×10^3) found in the eggs of the sea urchins and clams (Evans *et al.* 1983). Synthesis of these proteins, whilst barely detectable before fertilization, becomes marked during cleavage and is programmed by stored maternal mRNA. One of these proteins (M_r 40×10^3) has been identified as a subunit of ribonucleotide reductase (Standart *et al.* 1985). The others, called cyclins, are destroyed during each cell cycle and are thought to play a role in catalysing the entry of the egg into the M-phase (Evans *et al.* 1983). Likewise, in mouse eggs two proteins (M_r 46×10^3 and 30×10^3) show cyclic behaviour associated with the interphase-to-M-phase transition in the second meiotic and first mitotic division (Howlett & Bolton, 1985; Howlett, 1986). A third protein complex (M_r 35×10^3) undergoes one set of changes during the M-phase and another specifically during interphase. We have not found protein changes during the metaphase I transition that correspond either to the cyclins or to the 30 and 35×10^3 changes seen in the M-phase transitions in mouse eggs after fertilization. However, the appearance of the 46×10^3 protein immediately before nuclear breakdown in the first mitotic cycle of the mouse could possibly be analogous to protein 'm' in our system. Both proteins appear at similar stages of the cell cycle and are of a comparable molecular weight and charge. However, the 46×10^3 mouse protein is a heavily phosphorylated variant of a smaller protein which is continuously synthesized (Howlett, 1986). The protein which appears immediately before the breakdown of the germinal vesicle in ovine oocytes is, by contrast, not heavily phosphorylated and requires new mRNA synthesis for its appearance. Clearly, much further work on the characterization of both of these proteins is necessary before further comparison can be made.

It is clear that protein synthesis is not required for the transition from the prophase to metaphase I in many species ranging from invertebrates to amphibia and mice (reviewed by Masui & Clarke, 1979). In these species the proteins required for the M-phase transition are already present in the prophase oocyte in an inactive form. The key regulatory complex in these, as indeed in all oocytes and somatic cells, is the so-called maturation promoting factor (MPF), originally described by Masui & Markert (1971). Detailed investigations on *Xenopus* oocytes have indicated that the MPF complex is maintained in an inactive state by a cyclic AMP-dependent phosphoprotein. Activation of MPF necessitates the dephosphorylation of the regulator protein, a process induced by progesterone acting to depress intracellular cyclic AMP levels (Maller & Krebs, 1977). It is probable that somewhat similar protein regulators operate to control the M-phase in mouse oocytes. On the other hand, mechanisms that regulate the transition from prophase to metaphase I in ovine oocytes differ in many respects from those described

above. Indeed, the regulatory mechanisms in ovine oocytes have important similarities with those regulating the G_2-to-M-phase transition in the first mitotic cycles in eggs. Thus, ovine ooyctes, in common both with porcine and bovine oocytes (R. M. Moor, A. Hunter & F. Gandolfi, unpublished observations) and with cleaving embryos of all animals so far studied require new protein synthesis for the breakdown of the nuclear membrane. In addition, the reduction in cyclic AMP, considered to be essential for MPF activation in *Xenopus* and mouse oocytes, does not occur in ovine oocytes (Moor & Heslop, 1981) or in most mitotic cells.

Despite intensive study, particularly on marine invertebrates and amphibia, there is still no detailed understanding of the sequence of events controlling the cell cycle during development. Important advances have been made in the purification of MPF (Wu & Gerhart, 1980) and in the identification of a number of proteins whose synthesis or degradation is linked in a temporal manner to events in the meiotic cycle (Evans *et al.* 1983; Howlett, 1986). We have added to this list by describing a protein whose synthesis occurs immediately before germinal vesicle breakdown. Unfortunately, in no instance has an unequivocal physiological role yet been ascribed to any of the cycle-dependent proteins. It is improbable that the protein described by us is MPF but work is progressing on the assumption that protein 'm' forms part of the MPF activation system outlined by Kirschner and colleagues (Gerhart, Wu & Kirschner, 1984; Newport & Kirschner, 1984).

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