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Original article

Somatic cells and the G2 to M-phase transition in sheep oocytes

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Summary — New protein synthesis is essential in sheep oocytes for the progression of the meiotic cycle to metaphase. Inhibition of transcription by α-amanitin, a specific inhibitor of RNA polymerase II, arrests meiosis in the G2 phase in cumulus enclosed oocytes but denuded oocytes progress to M-phase. Denuded oocytes injected with α-amanitin also undergo maturation. Cumulus-enclosed and denuded oocytes were also cultured on transcriptionally inactive follicle shells to examine the influence of transcription in the granulosa cell compartment on the progression of meiosis. No effect of α-amanitin was detected on the maturation rate of both cumulus enclosed and denuded oocytes. We conclude that transcription is required in the cumulus cells to allow translation of stored messages within the oocytes coding for cell-cycle proteins.

oocyte maturation / α-amanitin / granulosa cell / sheep

Résumé — Cellules somatiques et la transition G2-M dans les ovocytes ovins. *Il est nécessaire que des protéines soient synthétisées dans les ovocytes pour que la méïose atteigne le stade métaphase. Lorsque la transcription est inhibée par l'α-amanitine, inhibiteur spécifique de l'ARN polymérase II, la meïose est bloquée à la phase G2 dans les ovocytes entourés de leur cumulus oophorus, mais les ovocytes dénudés atteignent la phase M. Les ovocytes dénudés injectés avec de l'α-amanitine achèvent aussi leur maturation. Des ovocytes dénudés ou non ont été cultivés sur la granulosa de parois folliculaires pour tester l'influence de la transcription dans la granulosa sur la progression de la méïose. Aucun effet de l'α-amanitine n'a été détecté sur la maturation des ovocytes des 2 groupes. Nous concluons que la transcription dans le cumulus est essentielle pour permettre la traduction des messages des protéines du cycle cellulaire stockés dans les ovocytes.*

maturation ovocytaire / α-amanitine / granulosa / mouton

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INTRODUCTION

The meiotic cycle in mammalian oocytes is arrested during primordial development in prophase of the first meiotic division (G2-cell cycle stage). This period of arrest persists until oocyte growth is completed and enters its final preovulatory phase of differentiation (maturation phase) at which time meiosis is resumed and the oocyte becomes programmed for fertilization. It is now clearly established that the transition from the G2- to the M-phase (metaphase) of the cell cycle is driven by the protein complex, maturation promoting factor or MPF (Masui and Markert, 1971; Lee and Nurse, 1987). It is further clear that the core molecule in MPF is a 34 kDa phosphoprotein with a close homology to the yeast cdc2+ mitotic regulator (Lee and Nurse, 1987). Whilst the abundance of this protein does not appear to be altered during the cell cycle, its activation involves changes firstly in its state of phosphorylation (Gould and Nurse, 1989) and secondly in the formation of complexes between it and a family of oscillatory proteins called cyclins (Dunphy *et al*, 1988). The highly conserved nature of the proteins involved in cell cycle control contrasts sharply with the apparent diversity of underlying mechanisms that influence the intracellular function of MPF. Thus, in some species such as the mouse, neither new RNA nor protein synthesis is required for the resumption of meiosis (Masui and Clarke, 1979). In these animals activation of presynthesised latent polypeptides by changes in protein phosphorylation probably initiates the MPF cascade (Rime *et al*, 1989). By contrast, new protein synthesis is essential in the oocytes of sheep, pigs and cattle for the progression of the meiotic cycle to metaphase I (Fulka *et al*, 1986; Moor and Crosby, 1986; Hunter and Moor, 1987). Likewise, inhibition of the transcription by α-amanitin, a specific inhibitor of RNA polymerase II, arrests meiosis in the G2-phase of the cycle in these animals (Osborn and Moor, 1983; Hunter and Moor, 1987). However, the effectiveness of α-amatin in inhibiting meiosis depends entirely on two factors. The first is that the block must be inserted within the first 1-2 h after the initiation of maturation and the second is that the oocyte must be in junctional communication with follicle cells (Osborn and Moor, 1983). It was however, uncertain whether this crucial early transcriptional event occurs within the oocyte or in the associated follicle cells.

Two series of experiments have, consequently, been undertaken to identify the intrafollicular compartments in which new mRNA synthesis is required for the progression of the meiotic cycle from the G2- to M-phase. The first study was designed to determine the need for transcription within the oocyte itself while the second examined the influence of transcription in the granulosa compartment on the progression of meiosis. Since this work was undertaken, recent findings of Motlik *et al* suggest that the resumption of meiosis in rabbit cumulus-complex oocytes is dependent upon early transcriptional and translational events in the cumulus cells (Motlik *et al*, 1989).

MATERIALS AND METHODS

Tissue preparation and culture methods

Ovaries were obtained from slaughtered sheep and intact non-atretic 3.0 to 5.0 mm diameter follicles were isolated by the method of Moor and Trounson (1977). The intact follicles were thereafter utilised differently in the 2 separate series of experiment.

Series 1: Intrafollicular site of α-amanitin action

Culture experiments

Cumulus enclosed oocytes (oocyte complexes) were removed from non-atretic follicles and allocated to one of the following groups: i) control complexes cultured for 24 h without the addition of α-amanitin (10 μg/ml; Boeringher, Mannheim); ii) complexes treated with α-amanitin for 24 h; iii) oocytes devoid of cumulus cells (denuded oocytes cultured with α-amanitin for 24 h or iv) complexes treated with α-amanintin for 4 h followed by the removal of the cumulus and the subsequent culture of the denuded oocytes in α-amanitin for a further 20 h. The methods of tissue preparation, culture procedures and inhibitor additions were all as described previously (Osborn and Moor, 1983).

Microinjection of α-amanitin

Oocytes devoid of cumulus cells were microinjected with 10 pl each of a solution of α-amanitin (10 μg/ml) using the procedure of cytoplasmic injection described previously (Moor and Powell, 1989). Oocytes were thereafter cultured for 24 h in medium containing 10 μg/ml of α-amanitin (Osborn and Moor, 1983). At the end of the culture period oocytes were mounted on slides, fixed and stained with 1% lacmoid for chromosomal analysis.

Series 2: Follicle cell transcription and meiotic regulation

Non-atretic follicles were carefully opened, washed to remove follicular fluid and spread out on stainless steel grids with the theca adjacent to the grid and the granulosa layer directly exposed to the culture medium. After culture for 4 h in culture medium (control) or in medium containing α-amanitin (10 μ g/ml) the follicle shells were washed thoroughly (5 x) to remove all traces of extracellular α-amanitin before cumulus-enclosed or denuded oocytes were placed by micropipette onto the membrane granulosa. Co-culture was for 24 h in complete culture medium (Osborn and Moor, 1983) supplemented with 5 μg/ml FSH (NIH-FSH- S12) and 3 μg/ml LH (NIH-LH S18). At the end of the culture period, oocytes were either mounted, fixed and stained with 1% lacmoid for nuclear examination or radiolabelled for the analysis of protein synthesis.

Radiolabelling and electrophoretic analysis of oocyte proteins

Oocyte complexes were removed from coculture, washed 3 times in incubation medium consisting of Dulbecco's phosphate-buffered saline supplemented with bovine serum albumin (4 mg/ml^{-1}) and energy source (pyruvate 0.36 mM, lactate 23.9 mM and glucose 5.5 mM (Moor *et al,* 1980) and thereafter labelled at 38° for 3 h in 50 μl of incubation medium containing 500 μCi/ml (^{35}S) methionine (specific activity > 1 000 Ci/mmol, Amersham International, UK). After radiolabelling, oocytes were washed, denuded of follicle cells and collected singly in 3 μl 10 mM Tris-HCL, ph 7.4 buffer before being lyophilised and frozen at -70°C until required for electrophoresis. Labelled oocyte polypeptides were separated on 8-15% SDS-gradient polyacrylamide gels (PAGE) as described previously (Osborn and Moor, 1983). Briefly, individual oocytes were lysed in 30 μl SDS-sample buffer and 3 μl was used for determining incorporation of radioactivity into TCA-precipitable material. Equal numbers of TCA-precipitable counts from individual oocytes were loaded onto separate lanes of the linear-gradient PAGE gels and polypeptide separations were made at constant current. Labelled polypeptides were visualised by fluorography (Bonner and Laskey, 1974) using preflashed Kodak X-Omat film at –70°C to ensure linearity and reduce background (Laskey and Mills, 1975). Densitometric scans were made using a Chromoscan III (Joyce-Loebl, UK).

RESULTS

Series 1: Intrafollicular site of α-amanitinaction

Previous results showed that the presence of α-amanitin from the initiation of matura-

tion inhibited the transition from the G2- to M-phase of the cycle in cumulus-enclosed but not in denuded ovine oocytes (Osborn and Moor, 1983). The present results (table I) confirm these observations and extend them by showing that exit from GV arrest occurs in oocytes that are exposed to α-amanitin for the crucial first 4 h of maturation and are thereafter denuded. That all the expected protein changes associated with maturation occur in such oocytes is shown in figure 1. The major maturational changes can be summarised as follows: The synthesis of some polypeptides (labelled a to h) is sharply reduced as the oocyte progresses from the GV to the metaphase I stage of the cell cycle. By contrast, sharply increased levels of sythesis occur in other polypeptides (labelled i to l). Since oocytes denuded 4 h after exposure to α-amanitin ("Cumulus 4 h" group in the figure) show the metaphase-type polypeptide pattern, we conclude that the action of the inhibitor is on transcription in the follicle cells rather than directly on the oocyte. This conclusion was further strengthened by our finding that α-amanitin injected directly into the denuded oocyte at the initiation of maturation did not prevent the resumption of meiosis. Thus, each of 15 oocytes were injected with approximately 100 pg of α-amanitin and cultured thereafter for 24 h. After culture, 3 oocytes showed clear mechanical damage and were discarded; 8 of the remaining 12 oocytes had progressed to metaphase despite the high intracellular concentration of α-amanitin.

Series 2: Follicle cell transcription and meiotic regulation

The results from the first series of experiments showed that the inhibition of transcription in the follicle cells at the initiation of the maturation was sufficient to prevent entry into metaphase I. The second series of experiments was designed to test whether inhibitory molecules existed in follicle shells whose transcriptional activity had previously been arrested by α-amanitin. The presence of inhibitory factors was investigated by culturing corona–oocyte complexes or denuded oocytes on such shells and studying the subsequent progression of the meiotic cycle. The results of the experiments do not appear to support the hypothesis that factors capable

Table I. Action of α-amanitin (10 μg/ml) on the resumption of meiosis in sheep oocytes cultured for 24 h with or without associated follicle cells.

Status of the oocyte during culture	*α -Amanitin in media*		*Total No oocytes*	*Percent oocytes after culture*	
	Presence	*Duration (h)*		*GV*	*MI-II*
Cumulus enclosed	–	–	43	12	88
Denuded	–	–	47	21	79
Cumulus enclosed	+	24	40	80	20
Denuded	+	24	45	24	76
Cumulus enclosed* for 4 h	+	first 4	38	17	83
Cumulus enclosed	+	first 4	13	69	31

* Oocytes denuded of cumulus cells 4 h after the initiation of maturation.

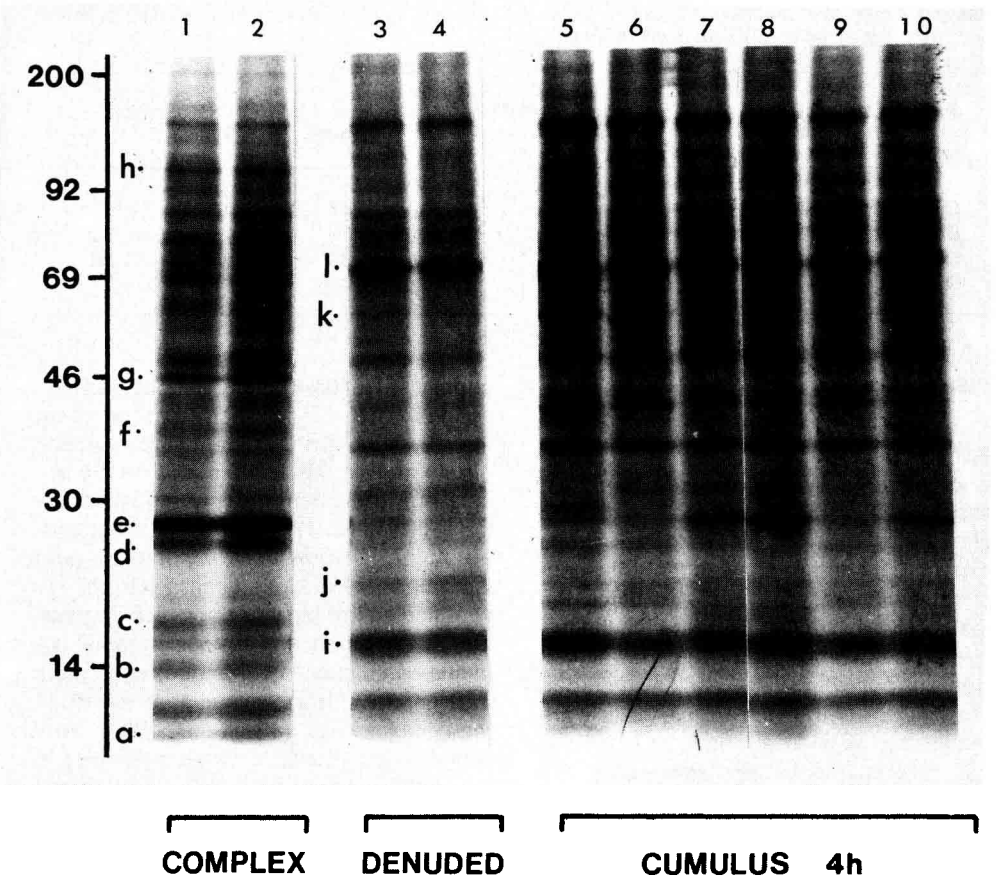

Fig 1. Inhibition of transcription on protein synthesis during maturation. Cumulus-complex oocytes show GV pattern (tracks 1 and 2) while denuded oocytes (tracks 3 and 4) and oocytes denuded 4 h after exposure to α-amanitin (tracks 5-10) undergo maturational changes.

of inhibiting the G2 to M-phase transition accumulated in follicle cells after α-amanitin treatment. Thus, it is clear from table II that the proportion of cumulus-enclosed oocytes that reached metaphase after 24 h culture on transcriptionally inactivated shells (50.4%) was similar to that on untreated shells (53.2%). However, the possibility that the 6 to 8 layers of transcriptionally active corona cells around each oocyte acted as a buffer between the follicle shell and oocyte cannot be discounted. That denuded oocytes also progressed to metaphase was uninformative since our previous findings have shown that junctional integrity is essential for the imposition of an α-amanitin block to meiosis. We are thus unable at present to determine whether the co-culture system chosen by us provides an acceptable means of detecting meiotic inhibitors after α-amanitin treatment.

Table II. Action of α-amanitin (10 μg/ml) treated follicle shells on the resumption of meiosis in sheep oocytes cultured with or without cumulus cells.

Status of the oocytes during culture	*α-Amanitin on follicle shell for 4 h*	*Total No oocytes*	*% Oocytes GV*	*MI-II*
Cumulus enclosed	–	47	47	53
Denuded	–	31	41	59
Cumulus enclosed	+	38	50	50
Denuded	+	21	43	57

DISCUSSION

Previous studies indicate that transcription in cumulus–oocyte complexes is necessary for the resumption of meiotic cycle in sheep (Osborn and Moor, 1983). Those experiments further showed that transcriptional inhibitors are unable to block the meiotic cycle in oocytes from which the follicle cells have been removed. However, it was not possible in the earlier studies to determine whether the follicle cells were required to provide a means of junctional entry and α-amanitin and cordysepin into the oocyte or whether the inhibitors acted directly on the follicle cells themselves. The present experiments make use of the intercellular communication system in the follicle, the highly restricted period of α-amanitin sensitivity displayed by the oocyte complex and the irreversible effect of α-amanitin to determine its site of action in the follicle. It is clear both from the present and past results (Osborn and Moor, 1983) that cumulus complexes treated with α-amanitin remained blocked at the GV stage of meiosis; however, this only occurs when the inhibition is present during the crucial first 4 h after induction of maturation. Any entry and binding of α-amanitin to the oocyte DNA must therefore occur during this period and the subsequent removal of the follicle cells should consequently not influence amanitin-oocyte interactions. That this is clearly not the case, strongly suggests that the inhibitory action of this drug is not a direct one on the oocyte but is almost certainly mediated *via* the follicle cells. This conclusion is supported by the microinjection experiments and is furthermore in close accord with the recent findings of Motlik *et al* which suggest that the resumption of meiosis in rabbit cumulus-complexes is dependent upon early transcriptional and translational events in the cumulus cells (Motlik *et al*, 1989). Very similar experiments to those described in the present paper have been carried out by Meinecke *et al* on pig oocytes (Meinecke, personal communication). Their results agree closely with those in the sheep and underline the conclusion that the inhibitory action of α-amanitin during maturation is mediated *via* the follicle cells.

The precise nature of the transcription-dependent signal from the follicle cells is still uncertain. However, many interesting possibilities exist. On the one hand, the argument for meiotic cycle regulation by cycle nucleotides is frequently rehearsed (Eppig, 1989); it is possible that transcription could be required for the synthesis of molecules involved in the production, degradation or putative transport of cyclic AMP through gap junctions. Our results on the levels of cyclic AMP in ovine oocytes dur-

ing maturation and on the effects of this cyclic nucleotide on the transition from the germinal vesicle to metaphase 1 stage of meiosis do not, however, persuade us of its key role in the initial regulation of meiosis in sheep (Crosby *et al*, 1985). On the other hand, factors which initiate translation of specific stored mRNA in the ovine oocyte appear to be of much greater interest in the breakdown of the germinal vesicle. Thus, new protein synthesis is an early obligatory step in the process and the inhibition of transcription in the cumulus complex (follicle cells) prevents this translational process and blocks entry into metaphase I (Moor and Crosby, 1986).

The search for regulatory molecules within the follicle shells after α-amanitin treatment has not provided convincing answers. The observation that the presence of α-amanitin treated shells did not block the G2-M transition in cumulus enclosed oocytes suggests that the requirement for transcription could be limited to the cumulus; this emphasising differences in the biological function of the mural granulosa and cumulus cells. If this putative cell difference were to exist then denuded oocytes placed on granulosa cells would not be expected to remain in GV. However, our experimental design is unable to differentiate between this explanation and others related to the possible requirement for a high concentration of inhibitory molecules or for transmission through intercellular junctions. Attempts to overcome the dilution problem were made by introducing denuded oocytes into α-amanitin treated shells rebuilt into artificial follicles. Although these oocytes did not resume meiosis in the reconstituted follicles, evidence from amino acid incorporation studies suggested that these oocytes were probably suffering from a nutrient deficiency due to the artificial microenvironment. New approaches using intact follicles on the one hand and the analysis of peptides and proteins secreted by follicle cells on the other are presently being undertaken in further attempts to identify transcription-dependent signals that affect the G2 to M phase transition in sheep oocytes.

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