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Original article

Onset of RNA synthesis and poly (A) content of early rabbit embryos. Comparison with sheep

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Summary — RNA synthesis in 2–32 cell embryos, as assessed by α-amanitin-sensitive ^{3}H-uridine incorporation, was first detectable in 4-cell stage rabbit and 8-cell stage sheep embryos. In the rabbit, uridine incorporation was detectable at the 2-cell stage but was unaltered by α-amanitin, indicating synthesis of non-polymerase II-dependent RNA species. Initiation of mRNA synthesis as determined by *in situ* hybridization with ^{3}H-poly (U) probe was first detectable in late 2-cell stage rabbit and 4-cell stage sheep embryos. In the rabbit embryos, nuclear labelling increased from the late 2-cell stage to the 16-cell stage, following a pattern similar to that of ^{3}H-uridine incorporation. In contrast, the intensity of cytoplasmic labelling decreased from the 1- to the 8-cell stage and then increased up to the 32-cell stage. In sheep embryos, nuclear labelling by the poly (U) probe increased from the 4- to the 16-cell stage. It is concluded that initiation of transcription of the embryonic genome (mRNA) can be detected *via* the current methods used at the 4-cell stage in the rabbit and the 8-cell stage in the sheep.

embryo / RNA synthesis / autoradiography / poly(A) tail / *in situ* hybridization

Résumé — Début de la synthèse d'ARN et teneur en poly(A) des embryons préimplantatoires de lapin (comparaison avec le mouton). *La synthèse d'ARN dans les embryons aux stades 2 à 32 cellules, mise en évidence par l'incorporation d'uridine-^{3}H sensible à l'α-amanitine, a été détectée dans les embryons de lapin au stade 4 cellules et de mouton au stade 8 cellules. Chez le lapin, l'incorporation d'uridine a été détectée au stade 2 cellules mais elle n'a pas été altérée par l'α-amanitine indiquant une synthèse d'ARN non dépendante de la polymérase II. L'initiation de la synthèse d'ARNm déterminée par hybridation* in situ *à l'aide de poly(U)-^{3}H a été détectée chez le lapin au stade 2 cellules tardif et chez le mouton au stade 4 cellules. Dans l'embryon de lapin le marquage du noyau augmente à partir du stade 2 cellules jusqu'au stade 16 cellules suivant un profil semblable à l'incorporation d'uridine-^{3}H. Au contraire, l'intensité du marquage dans le cytoplasme diminue à partir du stade 1 cellule jusqu'au stade 8 cellules et ensuite remonte au stade 32 cellules. Dans l'embryon de mouton le marquage du noyau avec la sonde poly(U) augmente à partir du stade 4–16 cellules. Les résultats montrent que l'initiation de la transcription à partir du génome embryonnaire est détectée, avec les méthodes utilisées, à partir du stade 4 cellules chez le lapin et du stade 8 cellules chez le mouton.*

***embryon / synthèse d'ARN / autoradiographie / polyadénylation / hybridization* in situ**

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INTRODUCTION

The onset of transcription of the embryonic genome marks an important event in early embryonic development. The period when the zygote begins producing its own RNA while still translating RNA that was stored during oogenesis has been termed the maternal-to-zygotic transition and appears to occur at a species-specific cell stage (Kopečný, 1989; Telford *et al,* 1990).

Two principal cytochemical methods exist for determining the onset of zygote transcripts, *ie*: i) uridine incorporation into total RNA or in mRNA after eventual inhibition of heterogeneous nuclear (hnRNA) synthesis with α-amanitin, a specific inhibitor of RNA polymerase II (Wieland, 1968); and ii) detection of mRNAs by *in situ* hybridization.

In the mouse, uridine is incorporated by 2-cell stage embryos (Mintz, 1964), in agreement with production of α-amanitin-sensitive heat-shock proteins at G2 (Bensaude *et al,* 1983; Bolton *et al,* 1984).

In the cow embryo, uridine incorporation was first detected at the 8-cell stage (Camous *et al,* 1986; Kopečný *et al,* 1989), while the initial α-amanitin-sensitive proteins were produced at the late 4-cell stage (Barnes, 1988). However, the major change in protein profiles occurs at the 8- to 16-cell stage (Barnes, 1988).

In the pig embryo, uridine is incorporated from the 4-cell stage onwards, this incorporation being α-amanitin-sensitive (Tománek *et al,* 1989).

Manes (1969, 1973) first reported significant incorporation of ^{3}H-uridine into the acid-insoluble fraction 12 h post-fertilization in the rabbit zygote. However, Manes (1977) later found that this incorporation could be ascribed to contamination of the embryonic samples by follicle cells, and was unable to detect any embryonic RNA synthesis prior to the 16-cell stage. On the basis of his experiments, he termed the period between fertilization and fourth cleavage the "dark period", thus indicating the difficulties of characterizing RNA synthesis in rabbit embryos by radiolabelling during this period. Direct examination of rabbit embryos by electron microscopic analysis showed significant amounts of RNA synthesis occurring in 2- to 4-cell stage embryos (Cotton *et al,* 1980). However, the initial production of α-amanitin-sensitive protein occurred only at the 8- to 16-cell stage (Johnson, 1981).

In the sheep embryo, changes in α-amanitin-sensitive proteins have been detected at the 8- to 16-cell stage (Crosby *et al,* 1988); however, no direct results on uridine incorporation have yet been published.

In the present study we have examined the time of initiation of hnRNA synthesis in the rabbit and sheep embryo using autoradiography after ^{3}H-uridine incorporation and *in situ* hybridization with polyuridylic (^{3}H-poly (U)) probe (Pikó and Clegg, 1982).

MATERIAL AND METHODS

Embryo culture and radiolabelling

Rabbit and ovine embryos (table I) were cultured in TCM 199 (Pavlok and McLaren, 1988). For radiolabelling embryos were transferred to a medium enriched with 5-[^{3}H]-uridine (UVVVR, Prague, Czechoslovakia, spec act 765 GBq/mol) at a final concentration of 3.7 MBq/ml for a 30-min period. Alpha-amanitin (10 μg/ml, Sigma, St Louis, MO, USA) was added 2 h before radiolabelling in some experiments with rabbit embryos.

Table I. Developmental stages and numbers of rabbit and sheep embryos used for autoradiography and *in situ* hybridization.

Developmental stage	*ARG*			In situ *hybridization*	
	Rabbit	*Rabbit + α-amanitin*	*Sheep*	*Rabbit*	*Sheep*
1	0	0	1	3	0
2	10	10	4	5	3
4	10	10	5	5	3
8	10	10	5	5	3
16	5	5	0	5	3
Morula	5	5	0	5	0

Embryo fixation, histology and autoradiography

At the end of the labelling period, the embryos were washed 3 times in cold medium to remove unincorporated precursor and fixed for 60 min in 2.5% glutaraldehyde and 0.75% paraformaldehyde in 0.06 M cacodylate buffer, pH 7.4. After washing in this buffer at 4°C, the embryos were postfixed in 1% OsO_4 overnight, dehydrated in ethanol and propylene oxide and embedded in Epon 812 (Crozet *et al,* 1986). Semi-thin sections (1 μm) were prepared for autoradiography (Baserga and Malamud, 1969). The labelling intensity was estimated by counting the number of silver grains over background on equatorial sections of nuclei. The intensity of labelling in the whole nuclear volume is given by the formula valid for tritium: $N = (2D/3l)n$, where N = total number of grains, D = diameter of the nucleus, l= section thickness (1 μm) and n = number of grains on the equatorial section (Neyfakh *et al,* 1972). The assimilation of the nuclei into spheres introduces a systematic error which is, however, the same for all early stages and thus the latter remain comparable.

In situ *hybridization*

The embryos were fixed in fresh 4% paraformaldehyde in PBS at 4°C for 30 min, dehydrated in ethanol and embedded in paraffin (Haffner and Willison, 1987). One-μm sections were obtained with an ultramicrotome; control preparations were incubated in 1 mM EDTA in 10 mM Tris buffer containing 50 μg/ml RNase A and 25 U/ml RNase T1 (Sigma, St Louis, MO, USA). The pretreatment and hybridization protocol was carried out as described (Hogan *et al,* 1986). As a hybridization probe we used ^{3}H-polyuridylic acid (MW 12 500–45 000, spec act 740 GBq/mM, final concentration 370 kBq/10 μl, Amersham International, Amersham, UK). Liquid nuclear emulsion K5 (Ilford, Mobberley, UK) was used for autoradiography. The labelling intensity was estimated by counting the silver grains per unit surface, over the background, on the nucleus and the cytoplasm of blastomeres. The intensity per nuclear volume was estimated as above.

RESULTS

Uridine incorporation during early rabbit embryonal development

Evidence of tritiated uridine labelling was first found in the 2-cell stage rabbit embryo, but this incorporation was relatively weak (grain density 20–25% above background) and was not significantly affected by α-amanitin (figs 1,2A). Incorporation of ^{3}H-uridine increased gradually from the 4-cell stage onward and abruptly at the 16-

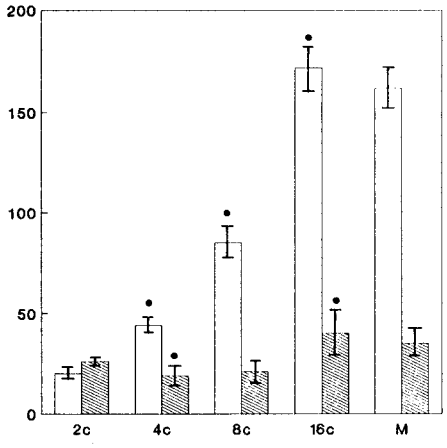

Fig 1. Incorporation of ^{3}H-uridine into nuclei during rabbit embryonal development. The number of silver grains over the largest section through the nucleus was evaluated; the background value was subtracted and the average number of silver grains per nuclear volume calculated. Each column represents the mean of 10 different nuclei and SE. *T*-test: $P < 0.05$ *vs* previous development stage (•). White columns: number of silver grains per nuclear volume; hatched columns: id, embryos cultured in the presence of α-amanitin (10 μg/ml) for 2 h before labelling with ^{3}H-uridine. On abscissa: number of cells; M: morula.

cell stage (fig 1) when nucleoli started to be labelled. It remained not significantly different at the 32-cell stage. From the 4-cell stage onward, incorporation of ^{3}H-uridine was significantly decreased by α-amanitin, even at low doses (10 μg/ml).

Uridine incorporation during early sheep embryonal development

No ^{3}H-uridine incorporation was found in 1-, 2- or 4-cell sheep embryos, the density of silver grains over the nuclei being indistinguishable from the background level. At the 8-cell stage, clear evidence of uridine incorporation was obtained in nuclei with a grain density that was significantly higher than the background (fig 2D). Insufficient numbers of sheep embryos were available to perform experiments with α-amanitin.

In situ *hybridization experiment in rabbit embryos*

The contents of poly (A) mRNA were determined by monitoring the number of silver grains over the nuclei and cytoplasm after *in situ* hybridization with the ^{3}H-poly (U) probe. Significant nuclear labelling appeared at the late 2-cell stage (fig 3, 4A). In 60% of late 2-cell embryos one labelled and one unlabelled nucleus was found, possibly reflecting nuclear asynchrony in early development. The number of silver grains per nuclear volume was increased from the late 2-cell to the 16-cell stage, but at the 32-cell stage a substantial decrease in silver grain density was found.

In the cytoplasm, intense labelling was observed in the 1-cell (zygote) rabbit embryos. Subsequently, labelling decreased till the 8-cell stage and then increased till the 32-cell stage where it again reached levels found at the 1-cell stage (fig 3).

In situ *hybridization experiment in sheep embryos*

In ovine embryos, nuclear labelling was not significant before the 4-cell stage with the signal increasing at the 8- and 16-cell stages (fig 5A, B). We were unable to observe a significant change in the intensity of cytoplasmic labelling, as our material was too limited (table I). However, the onset of nuclear labelling starting one cell cycle later than in rabbit embryos could be clearly shown.

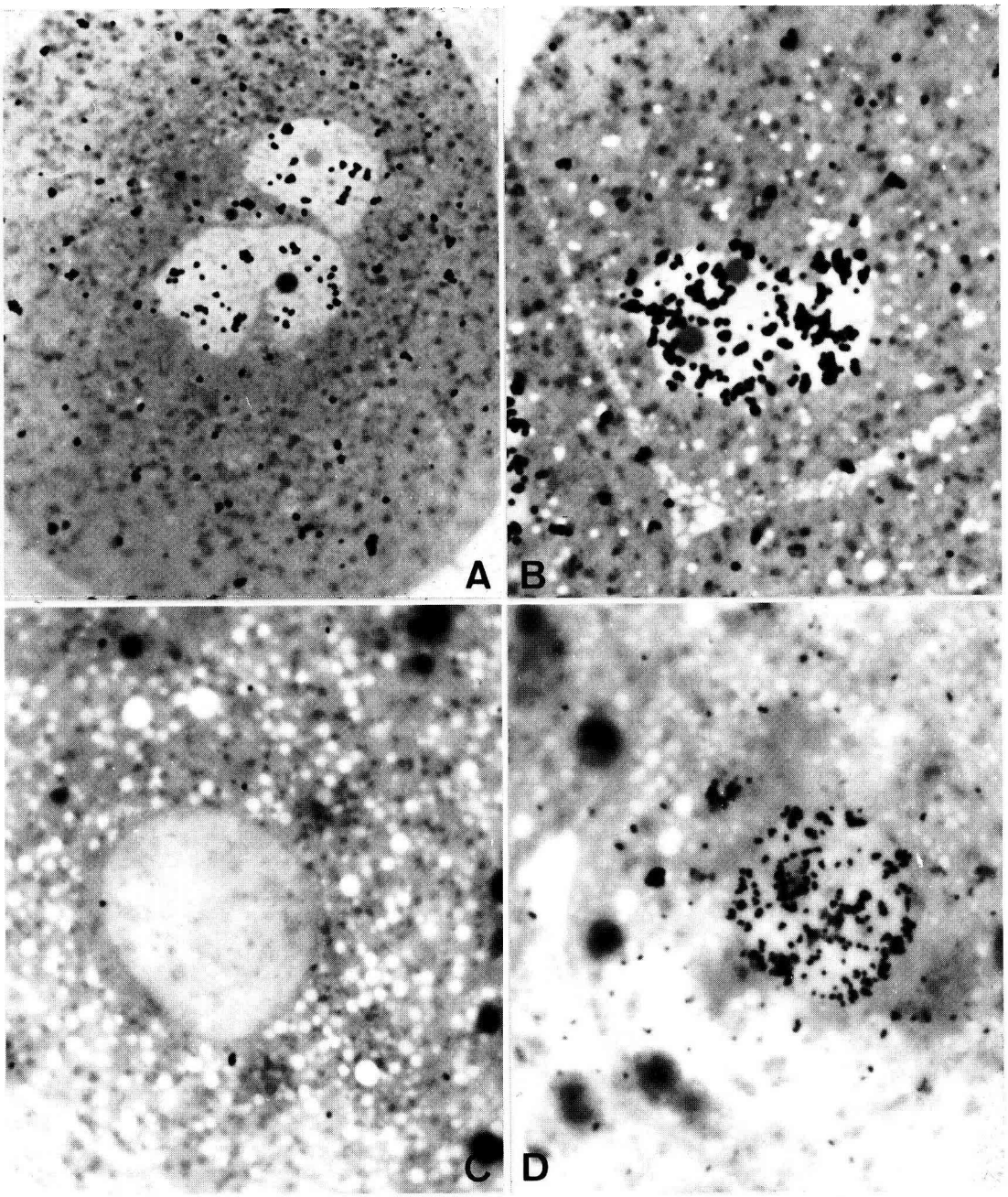

Fig 2. Incorporation of ^{3}H-uridine into rabbit and sheep embryos. **A, B**: 2- and 8-cell stage rabbit embryos. The labelled nuclei of early blastomeres are very irregular (lobes); x 1 200. **C, D**: 4- and 8-cell stage sheep embryos; x 1 500. Clearly negative labelling is observed at the 4-cell stage in sheep *versus* slight labelling at the 2-cell stage in the rabbit. The same labelling occurs at the 8-cell stage in both species.

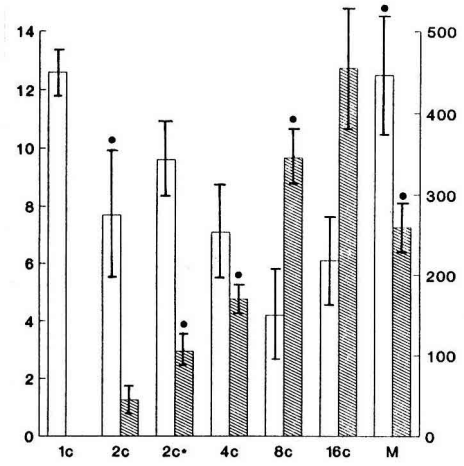

Fig 3. *In situ* hybridization using a poly (U) probe during early rabbit embryonal development. White columns: ^{3}H-poly (U) probe in the cytoplasm. The number of silver grains per 0.25 μm^2 over the cytoplasm was evaluated, the background was subtracted and the average number of silver grains determined. Each column represents the mean of 5 different cells ± SE. *T*-test: $P < 0.05$ *vs* previous developmental stage (•). Hatched columns: ^{3}H-poly (U) probe in nuclei. The number of silver grains over the largest section through the nucleus was evaluated, the background was subtracted and the average number of silver grains per nuclear volume calculated. Each column represents the mean of 5 different nuclei. Abscissa: see figure 1; asterisk: late 2-cell stage.

DISCUSSION

The initiation of transcription during early embryonal mouse development has been relatively well described (Johnson, 1981). On the other hand, precise data are still lacking for the rabbit and several other domestic species (Kopečný, 1989; Telford *et al*, 1990).

Our experiments indicate the low incorporation of ^{3}H-uridine in the 2-cell stage rabbit embryo. This low level of incorporation is not sensitive to α-amanitin, and may reflect RNA polymerase II-independent incorporation into transfer or small nuclear RNAs. Clear evidence of inhibitor-sensitive incorporation of ^{3}H-uridine was obtained in 4-cell stage embryos, marking the detected start of synthesis of new embryonal hnRNA. It remains to be determined: i) whether this RNA is transported from the nucleus to the cytoplasm (after 30-min incorporation without chase, only nuclear incorporation can be detected and no data exist on chase experiments); and ii) whether it serves as a matrix for new protein synthesis or for the regulation of transcription or stabilization of other mRNAs. Rabbit embryos exposed continuously to α-amanitin from the 1-cell stage onward show arrested cleavage at the 8-cell stage (Manes, 1973). If accurate, these data show that maternally inherited transcripts and/or stored proteins are sufficient to achieve initial cleavages.

Most eucaryotic mRNAs have a sequence of polyadenylic acid (poly (A)) at their 3' termini (Munroe and Jacobson, 1990). These poly (A) tails are added post-transcriptionally in the nucleus with an initial length of ≈ 200–250 adenylate residues. Candidate functions of this tail are the following: mRNA transport from the nucleus to the cytoplasm; control of mRNA stability; a role in the actual mechanism; or control of translation (Jackson and Standart, 1990).

In our experiments, *in situ* hybridization was used with a ^{3}H-poly (U) probe to detect RNA poly(A) tails during early embryonal rabbit and sheep development. Significant labelling over the nuclei was first detectable at the late 2-cell stage and 4-cell stage respectively, which coincides with ^{3}H-uridine incorporation. In the rabbit, the high density of labelling present in the cytoplasm of 1-cell stage embryos decreases up to the 8-cell stage and subse-

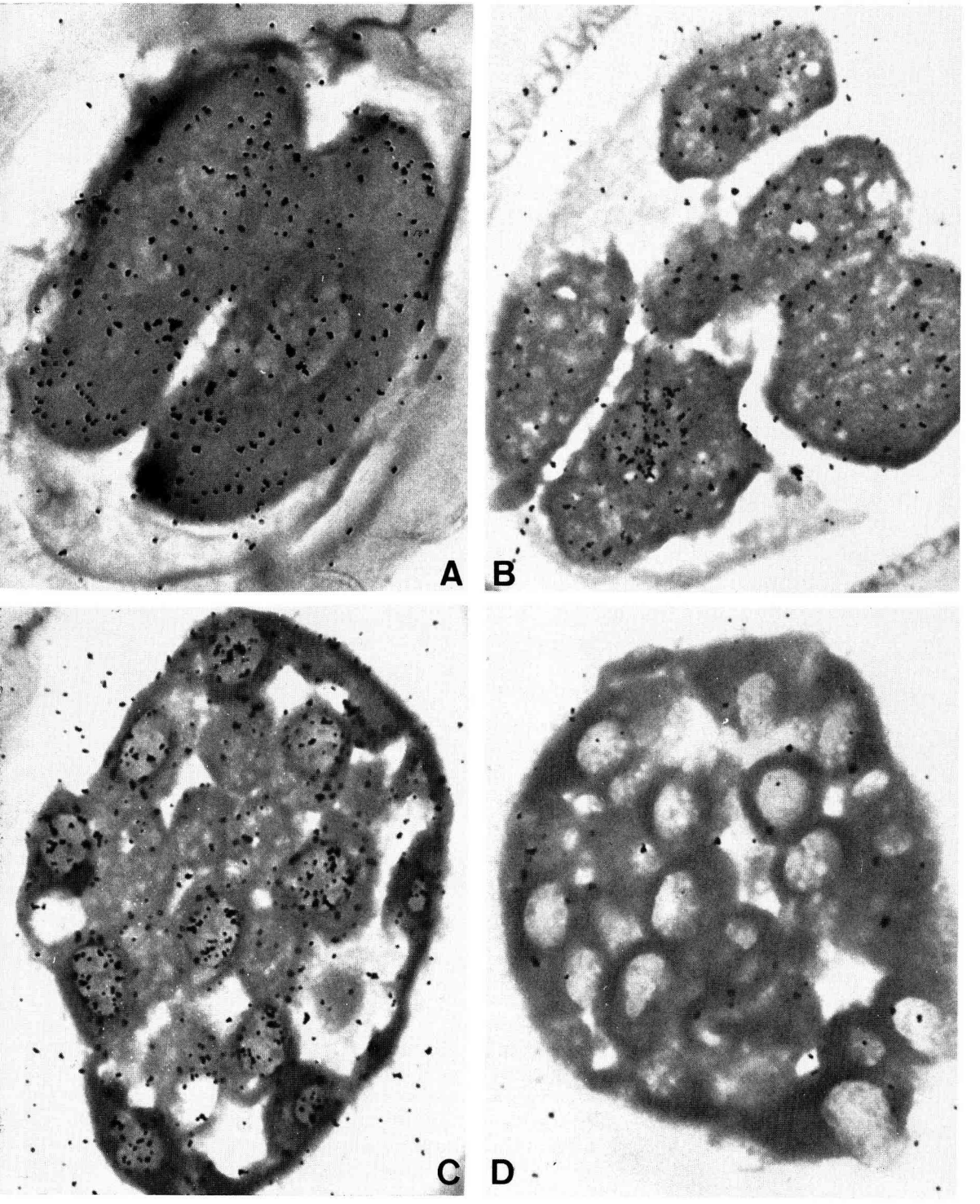

Fig 4. *In situ* hybridization using a poly (U) probe during rabbit early embryonal development on 1-μm thin sections in paraffin. The nuclear labelling starts at the 2-cell stage (visible asynchrony). **A**: 2-cell stage; **B**: 16-cell stage; **C**: morula stage; **D**: morula stage, control with RNase A and RNase T1. No labelling above background; x 900.

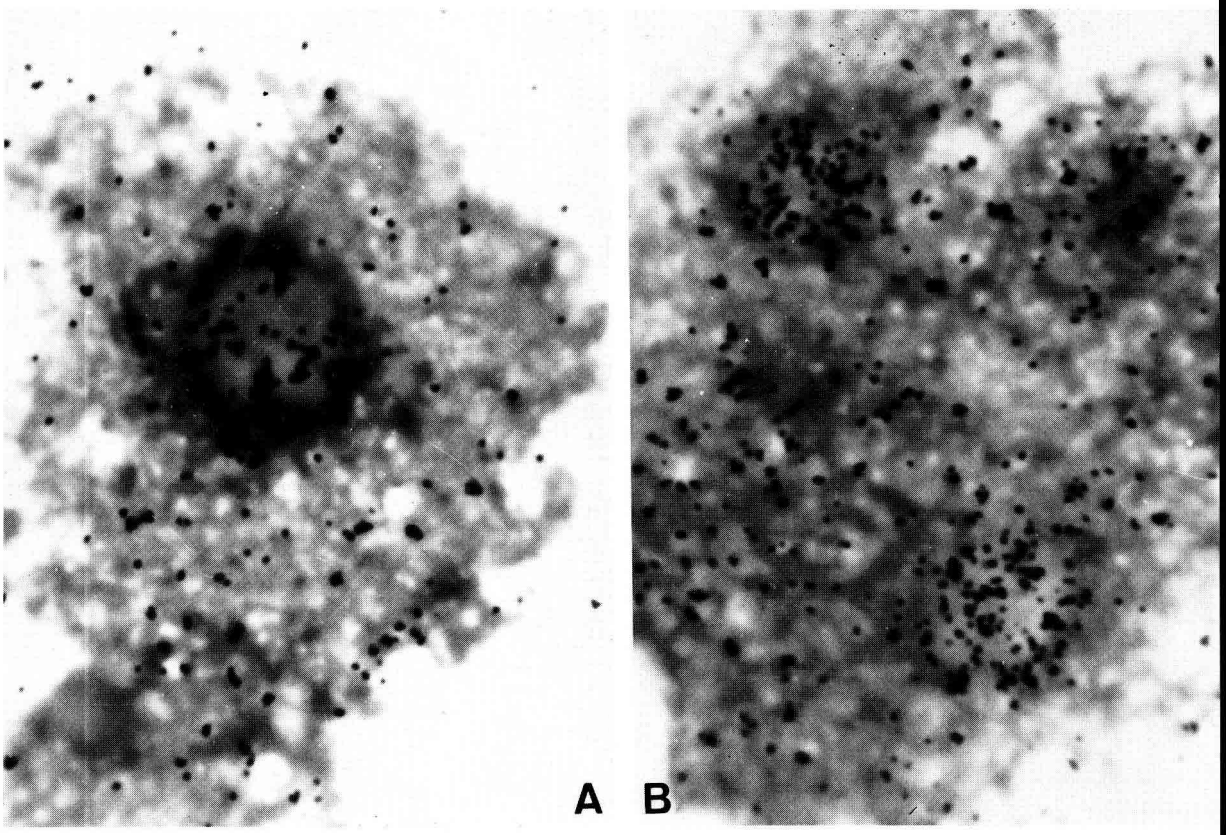

Fig 5. *In situ* hybridization with poly (U) probe during sheep early embryonal development (same technique). No labelling was found before the 4-cell stage; x 1 000. **A**: 4-cell stage; **B**: 16-cell stage.

quently increases. Cytoplasmic labelling at the 1-cell stage represents maternal transcripts stored in the cytoplasm during oogenesis. The 4- and 8-cell stage messenger population in the cytoplasm may consist of both maternal and newly synthesized mRNA. From these data we can conclude that the maternal-to-zygotic transition occurs at ≈ the 8-cell stage.

New poly (A) transcripts were first found in the cell nucleus 1 cell cycle prior to autoradiographic detection of initial RNA synthesis. Two possible explanations exist: i) *in situ* hybridization is a more sensitive method than ^{3}H-uridine incorporation; and/or ii) polyadenylation occurs 1 cell cycle before hnRNA synthesis as may be the case in mouse zygote (Pikó and Clegg, 1982). Generally, it is difficult from autoradiographic data without a parallel biochemical approach to estimate the precise time of initiation of RNA synthesis (Clegg and

Pikó, 1982). Our results on the progressive pattern of gene expression are supported by the following recent studies. Christians (personal communication) showed that with firefly luciferase reporter gene injected in the nuclei of rabbit zygotes, transcriptional ability is detectable by this sensitive technique as early as 15 h post-fertilization (during the 1-cell stage). Delouis *et al* (1992) investigated gene expression in rabbit early development by microinjecting *LacZ* DNA in 1-cell and 2-cell embryos. The microinjected DNA started to be expressed, according to this stable labelling at the 8–16-cell stage only, indicating a negative control of gene expression at earlier stages. Taken together, all these results show that transcriptional ability appears early in rabbit development but that most newly synthesized hnRNAs are probably not used as a matrix for protein synthesis before the 8-cell stage as already shown by Johnson (1981).

In sheep embryos, it is possible to conclude that new hnRNA synthesis started less progressively, *ie* only at the 8-cell stage; this result correlates with experiments demonstrating that sheep embryos cultured continuously from the 1-cell stage with α-amanitin cleaved regularly up to the 8-cell stage, but failed to continue to develop up to the 16-cell stage (Crosby *et al,* 1988).

PCR experiments and hybridization *in situ* with specific probes will constitute a useful method to determine the regulation of early embryonic gene expression and the nature of the mRNAs produced.

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