

## Review

# High-resolution autoradiographic studies of comparative nucleogenesis and genome reactivation during early embryogenesis in pig, man and cattle

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**Summary** — Using high-resolution autoradiography, simultaneous studies of ultrastructure and nucleic acid dynamics were performed during nucleogenesis in early porcine, human and bovine embryos. In contrast to the early genome activation known to occur during the second cell cycle in the mouse, the onset of rRNA synthesis detected by (5-<sup>3</sup>H) uridine incorporation in the nucleolar compartment is delayed by cleavage of one cell cycle (to the third cell cycle) in the early pig embryo and by two cell cycles (to the fourth cell cycle) in human and cattle embryos. Extranucleolar RNA synthesis, as detected by nucleoplasm labelling, generally started shortly before rRNA synthesis. The timing of nucleolar labelling was well correlated with the penetration of embryonic DNA into the nucleolus precursor body and with nucleolus structure differentiation. Ultrastructural and/or autoradiographic techniques are suggested for the study of the onset of embryonic transcription, e.g. in embryos "reconstructed" by micromanipulation.

**Early embryo — transcription — pig — man — cattle**

**Résumé** — *Etude comparative par autoradiographie ultrastructurale de la nucléogénèse et de la réactivation du génome au début du développement embryonnaire porcin, humain et bovin.* Cet article présente la synthèse des résultats obtenus par l'autoradiographie ultrastructurale sur la formation du nucléole et le début de l'expression du génome dans les embryons bovins, humains et porcins. Par rapport à l'embryon de souris, l'incorporation d'uridine (<sup>3</sup>H) dans le compartiment nucléolaire est retardée d'un cycle cellulaire dans l'embryon porcin, et de deux cycles cellulaires dans les embryons humain et bovin. Comme l'incorporation nucléolaire est concomitante de la pénétration d'ADN périnucléolaire dans le nucléole et de l'apparition du composant fibrillaire dense, l'ensemble de ces phénomènes correspond bien au début de la synthèse de l'ARNr. Le marquage du compartiment extranucléolaire précède de peu celui du nucléole. Comme cela commence à être confirmé par d'autres types d'expériences, l'activation du génome embryonnaire se produit à des stades différents dans les quelques espèces de mammifères étudiées.

*L'emploi simultané de la microscopie électronique et de l'autoradiographie est proposé comme une technique de choix pour l'analyse de l'activité nucléaire, notamment dans des embryons "reconstruits" par micromanipulation.*

**Embryon précoce — transcription — porc — homme — bovin**

"... peut-on envisager une définition plus restrictive qui ne reconnaîtrait l'existence d'un nouvel être qu'après l'expression chimique du message génétique composite..."

Jacques Testart, l'Œuf Transparent.

## REACTIVATION OF EMBRYONIC GENOME AFTER FERTILIZATION

Early embryonic animal development is characterized by an initial period during which the embryonic genome, composed of paternal and maternal contributions, remains inactive. Early development is directed by maternal RNAs of all classes already synthesized during oocyte growth and maturation and stored in the cytoplasm. The transitional period to the developmental control by the embryonic genome itself varies among species; rather than a sharp shift from maternal gene products to that of the embryo, there is a co-existence of gene products from these two genomic sources — maternal vs embryonic — for a certain period of time (Davidson, 1986).

In mammals, most of the published data concern mouse embryos (Johnson, 1981; Geuskens & Alexandre, 1984). Studies on embryos from other mammalian species are scarce (see King *et al.*, 1989; and Kopečný *et al.*, 1989a). For this reason we decided to study the process of the transition from the maternal to embryonic control in embryos of farm animals, as embryo transfer and genetic manipulation techniques are used at such early embryonic stages; and also in the human species in which *in vitro* fertilization and embryo transfer are primary concerns. In addition, all the species are phylogenetically distant from Rodentia.

The availability of early human and cattle embryos is evidently limited, so we concluded that the method of choice would be high-resolution autoradiography, a technique already used for the study of early embryonic development in mouse (see, Geuskens & Alexandre, 1984). In fact, the application of this methodology has allowed us to investigate simultaneously in early pig, human and cattle embryos : (i) the ultrastructural morphology of nucleologenesis; (ii) the localization of embryonic-DNA containing sites, especially in the differentiating nucleolus; and finally (iii) the onset of extranucleolar and nucleolar transcription. Based on the 3 parameters studied, these complex studies confirmed a different localization for transcription in early preimplantation embryos of pig, man and cattle, as far as the cleavage stage was concerned. Our findings suggest that the idea should be abandoned of equivalence in differentiation degree at a given division stage among different mammalian species, which leads to a somewhat mechanistic stage-to-stage comparison, and to unjustified extrapolations to other mammalian species of data pertinent to the mouse, *e.g.* the utilization of the number of cleavage divisions "*in vitro*" as a criterion of the normality of development. In addition, our findings outline the need to study the consequences of such diversity in onset of gene expression for the biology of the earliest phases of mammalian embryogenesis; especially in timing of relevant developmental changes at molecular and cellular levels which may be reflected, *e.g.* in the outcome of nuclear transplantation in the early embryo (Prather *et al.*, 1987).

Our autoradiographic observations were confirmed by other methods of investigation of genome activation in early embryos of some of the same species (see below).

## ULTRASTRUCTURAL MORPHOLOGY

The main target of morphological observations was the nucleolus since marked changes in the formation of this organelle coincide closely with the period of transition from the maternal to the embryonic control in all 3 mammalian species investigated.

Soon after fertilization, a nucleolus precursor body (NPB) appears (for review and terminology used, see Tesařík *et al.*, 1987b; Kopečný *et al.*, 1989a). Although of different sizes, NPBs showed a high degree of similarity in all embryos from the 3 species investigated. The NPB is a spherical intranuclear body of uniform structure consisting of a dense network of filaments; in human NPB these filaments are about 3 nm thick and probably of proteinaceous composition only (Tesařík *et al.*, 1987b). The NPB is most prominent in one- and two-cell pig embryos where it takes up to one-third of the nucleus diameter and is sharply delineated from the nucleoplasm (Tománek *et al.* 1989). NPB's are quite inconspicuous, however in cattle embryos until the early 8-cell stage (Kopečný *et al.*, 1989a).

The morphology of the NPB transformation into a functional nucleolus differs unexpectedly in the 3 species investigated. In comparison to the morphology of embryonic nucleogenesis in mouse (Geuskens & Alexandre, 1984) the smallest difference is seen in the pig. At the beginning of the four-cell stage, the first association of fibrillar centres with the periphery of NPB is seen (Tománek *et al.*, 1989). Later, a rim of a typical nucleolonemal structure is gradually formed at the periphery of the NPB, showing a prominent dense fibrillar component and granular component as well (Tománek *et al.*, 1989). In man, the ultrastructure of NPB is constant until the 8-cell stage (Tesařík *et al.*, 1986a). In con-

trast to pig embryos, condensed chromatin usually forms, during this period, a continuous rim around the NPB. During the fourth cell cycle, NPB sharp boundary with the nucleoplasm is lost and the perinucleolar chromatin seems to be internalized within the NPB (Tesařík *et al.*, 1986a). In contrast to mouse and pig embryos, the differentiation of the NPB into a functional nucleolus formed by intermingled typical nucleolar components and the disappearance/obscuration of the primitive fibrillar component is a relatively rapid process in human embryos (Tesařík *et al.*, 1986a, 1987b). A further difference to the pig embryo consists of the synchrony of nucleolar differentiation within a given nucleus (Tesařík *et al.*, 1986a). In pig embryos, until the morula stage, all developmental steps of nucleogenesis may be seen within the same nucleus (Tománek *et al.*, 1989) (Fig. 1). On the other hand, there is no synchrony between blastomeres constituting an early human embryo (Tesařík *et al.*, 1986a, b, 1988).

In the human embryo during the fifth cycle of cleavage, the nucleolus enlarges twice to the diameter of the NPB and the most of its volume is occupied by granular and fibrillar components and several interstices are observed (Tesařík *et al.*, 1986a).

The greatest differences in the morphological events of nucleogenesis in comparison to the mouse are encountered in the early cow embryo. In the course of the 8-cell stage, probably during the second half of the 4th cell cycle, a large central lucid area develops in the NPB. According to the recent nucleolar terminology we named this entity "NPB-vacuole" (Kopečný *et al.*, 1989a). At the beginning of the vacuole development, the appearance of its contents is similar to that of the nucleoplasm. A contemporary feature is the association of the NPB with prominent patches of condensed chromatin, attached to the NPB in relatively few and limited areas of

contact. During further differentiation towards the typical nucleolar structure, secondary vacuoles appear (Fig. 2a; short transparent arrows), the primary vacuole loses its sharply delineated boundary and a clump of chromatin appears within it (Fig. 2a). Internalization of perinucleolar chromatin is seen (Fig. 2b; long arrows) and the first granules of the granular nucleolar component appear (Fig. 2a; centre of the white circle). In a very rapid subsequent development within the eight-cell stage, fibrillogranular nucleoli appear with fibrillar centres showing a typical nucleolonemal structure. Shortly after, the nucleoli lose their rounded shape by rapid accumulation of granular nucleolar component and occupy a considerable proportion of the nucleus (Kopečný *et al.*, 1989a). This process takes place at least in some blastomeres of the 8-cell cattle embryo. During the final stages of nucleologensis it is not possible to discern the primary embryonic component in NPB (Kopečný *et al.*, 1989a).

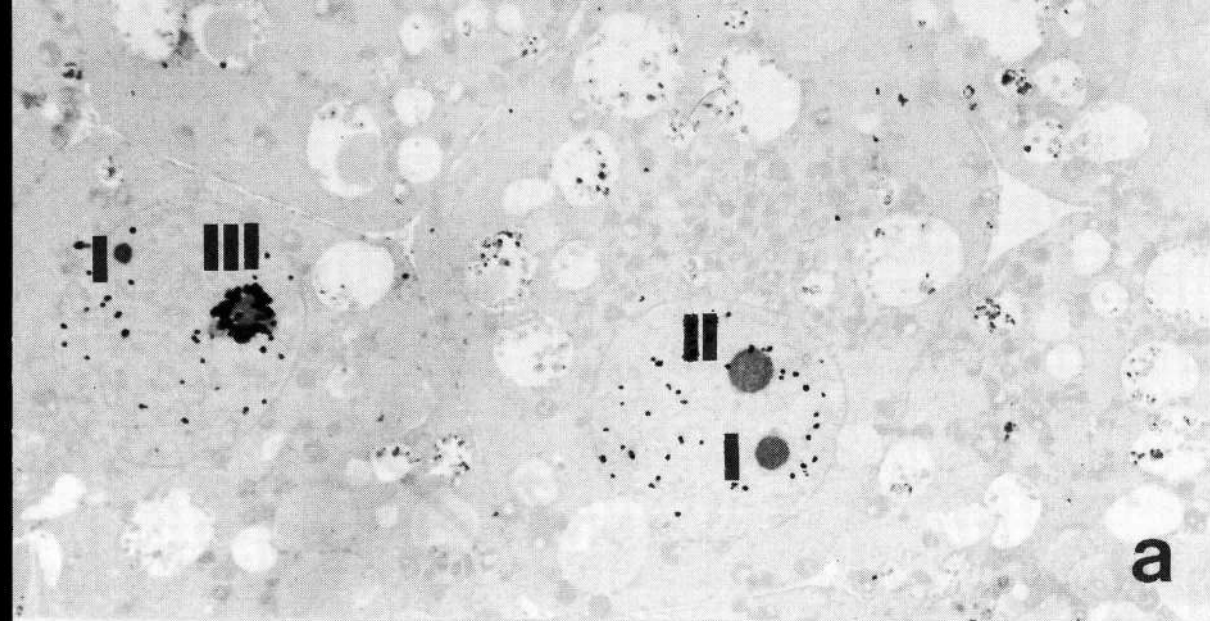
Taken as a whole, the development of nucleoli in early mammalian embryo, notwithstanding the identical biological significance of their differentiation, shows considerable differences among species, in the cleavage stage at which nucleologensis begins, in the number of cell cycles between the beginning and the completion of

this process, and in the remarkable diversity of the morphology of the intermediate stages.

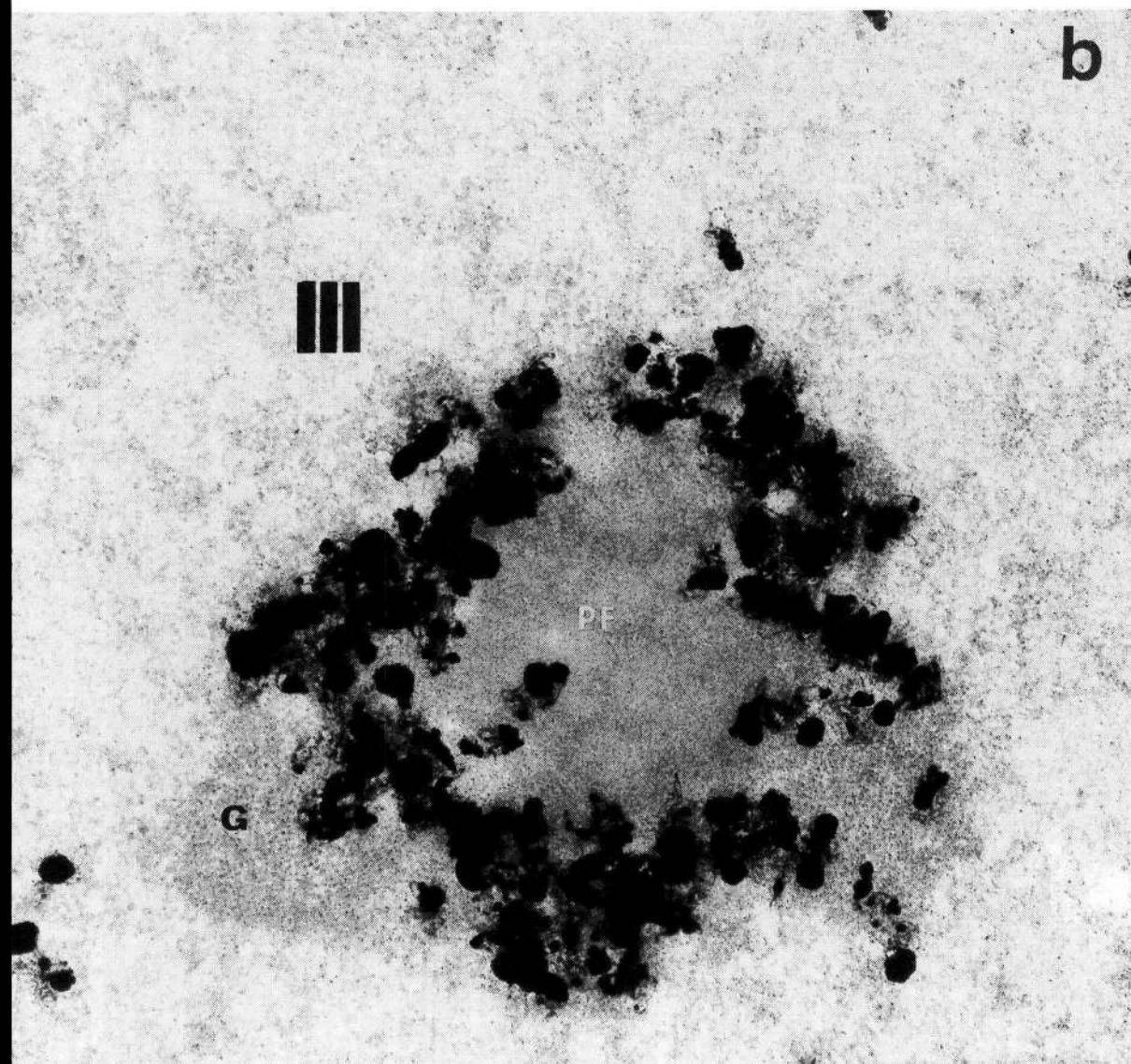
### FINE-STRUCTURE LOCALIZATION OF DNA-CONTAINING SITES

Nucleolus precursor bodies do not contain embryonic DNA and the penetration of DNA into these entities from perinucleolar chromatin is an early step in the nucleologenic process in man (Tesařík *et al.*, 1986a, 1987b) as well as in cattle (Kopečný *et al.*, 1989a, b). These conclusions were based on DNA labelling of cleaving embryos *in vitro*, for a time period which was more protracted than the expected length of the cell cycle by (methyl-<sup>3</sup>H) thymidine so that a general pattern of all replicated DNA distribution was obtained by autoradiographic analysis. In both species a close correlation was observed between the DNA-penetration process into NPB and morphological features of nucleologensis and the onset of transcription (Table I). In early human embryos, Tesařík *et al.* (1987b) suggested that the penetration of DNA into NPB, *i.e.* the gradual association of rDNA with the proteinaceous component

**Fig. 1.** Extranucleolar and nucleolar transcription in an 8-16-cell pig embryo. Fine-structure autoradiograms show labelling after a 20-minute pulse with (5-<sup>3</sup>H) uridine. **a.** The differentiation of the nucleolus from the nucleolus-precursor body is extended over several divisions in early pig embryos and is asynchronous for the nuclei within one blastomere nucleus. It is thus possible to observe the array of differentiation events from the nucleolus-precursor body (I) to a functional nucleolus (III) in two nuclei of two neighbouring blastomeres. The onset of nucleolar transcription, an "early nucleolar transcriptional pattern" is also seen (II), with only a few autoradiographic grains at the periphery. Note also the extranucleolar labelling in the nucleoplasm and lack of labelling in the cytoplasm of all blastomeres, since after a short pulse with (5-<sup>3</sup>H) uridine only the localization of newly synthesized RNAs is seen (x 2 600). **b.** A higher magnification of the functional nucleolus (III) shown in Figure 1a. This nucleolus is still composed of a prominent remnant of the primitive fibrillar material, probably only proteinaceous material (PF) not involved in transcription. On its periphery, however, a typical nucleolonemal structure composed of the dense fibrillar component is covered almost completely by heavy labelling and surrounded by the granular nucleolar component (G), not yet labelled after the short-duration pulse. (x 26 000).



**a**



**b**

of the nucleolar "anlage" is a key event of nucleologenesis, triggering an array of processes leading to activation of rDNA transcriptional capacity followed by pre-rRNA processing later on. In cattle embryos, the local penetration of labelled DNA into the NPB was found to coincide with the appearance of the large central vacuole (Fig. 2b). No labelling was observed in the vacuole. The inside of the differentiating nucleolus, however, was penetrated by embryonic DNA only in the next step of nucleolus development when secondary vacuoles were formed. Thereafter all vacuoles were a frequent site of labelling, in addition to other areas of the nucleolus (Kopečný *et al.*, 1989a, b).

Another interesting feature connected with the onset of transcription is the intranuclear distribution of DNA in cattle embryos. Before transcription started, the labelled DNA was seen only at the periphery of nuclei. As soon as transcription started, DNA distribution became homogeneous throughout the nucleus (Kopečný *et al.*, 1989b). This phenomenon might be the result of a change in the compactness of the DNA molecule, organized in a loop anchored to the nuclear periphery which may be differently extended in relation to different functional states of the DNA, *e.g.* in relation to transcription.

**Table 1.** Localization of fibrillar centres appearance, of DNA penetration into NPB, and of extranucleolar and nucleolar transcription during the first cleavage divisions of embryos of mouse, pig, man and cow as detected by fine-structure autoradiography.

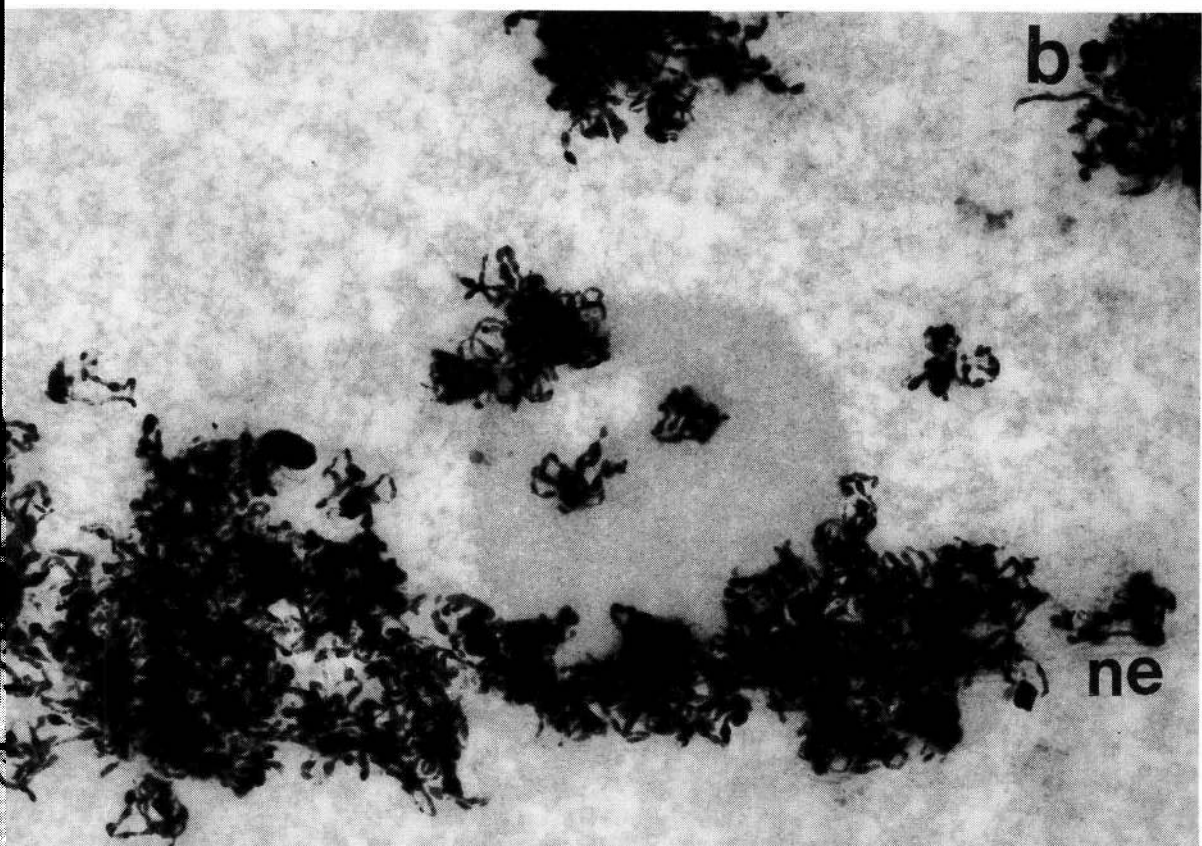
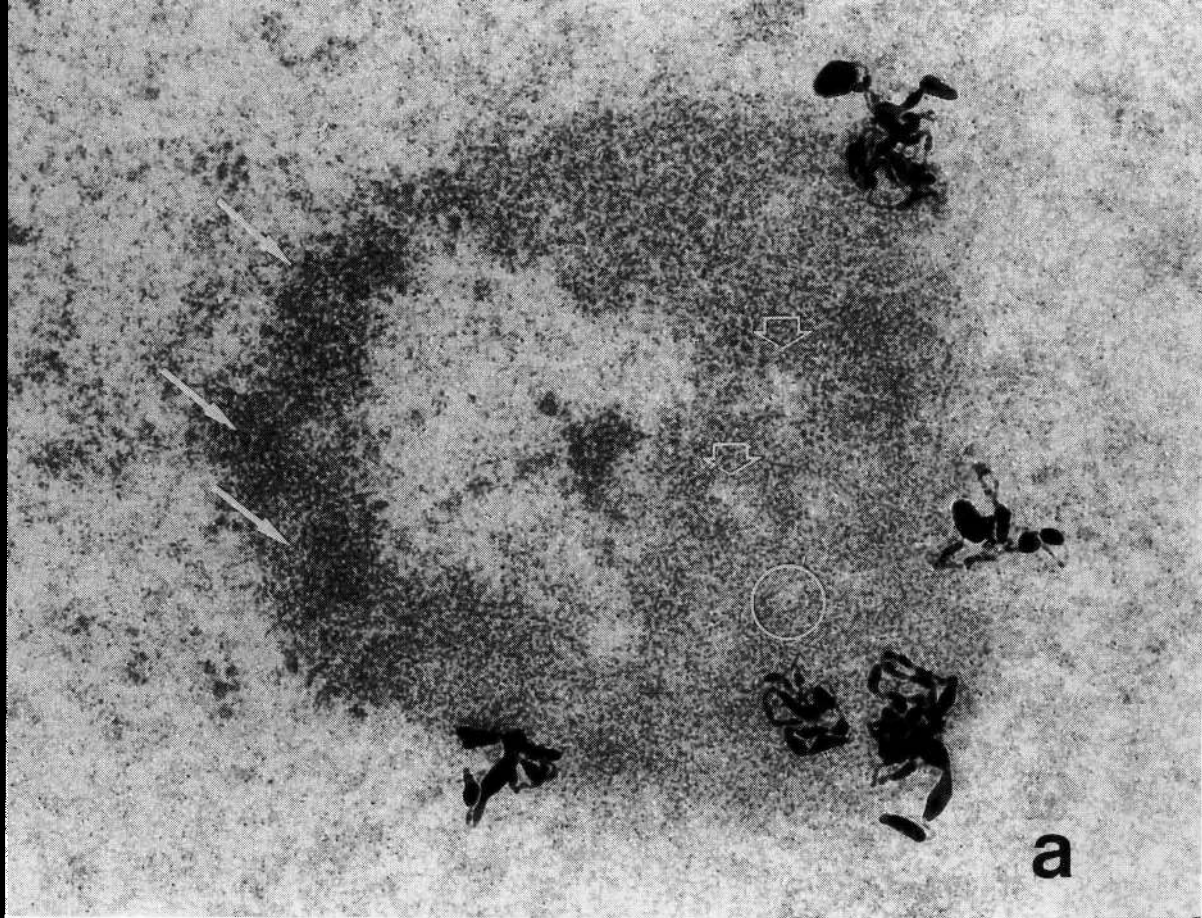
Mitoses	M1		M2		M3		M4	
Cell cycle *	1	2	3	4	5	6	7	8
Number of blastomeres	1	2	4	8	8	16	16	16
MOUSE <sup>1</sup>				FC				
PIG <sup>2</sup>				FC				
MAN <sup>3</sup>						••••	••••	
COW <sup>4</sup>						••••	••••	

<sup>1</sup> Geuskens & Alexandre, 1984. <sup>2</sup> Kopečný *et al.*, 1985, Tomanek *et al.*, 1986, 1989. <sup>3</sup> Tesařík *et al.*, 1986a, 1987b. <sup>4</sup> Kopečný *et al.*, 1985; Camous *et al.*, 1986; Kopečný *et al.*, 1989a.

FC — first appearance of fibrillar centres; •••• detection of DNA penetration into NPB; - - - - detection of extranucleolar transcription; ——— detection of nucleolar transcription.

\* In many cases the observations were made on asynchronously dividing embryos. For this reason the description and localization of particular events necessitates reference to the cell cycle and not to the number of blastomeres.

**Fig. 2.** Nucleolus of a late 8-cell cow embryo. Fine-structure autoradiographic demonstration of a) transcription sites; b) DNA-containing sites. **a.** Late 8-cell cow embryos were pulse-labelled with (5-<sup>3</sup>H) uridine so that the sites of newly synthesized RNA were visualized. The nucleolus at this stage was formed of intermingled nucleolar components. The autoradiographic grains indicate the localization of the appearing dense fibrillar component inside the otherwise less dense fibrillar structure, probably corresponding to the proteinaceous nucleolar "anlage" of the nucleolus-precursor body. In addition, first granules of the granular nucleolar component are already formed (centre of the white circle). In the big primary vacuole a clump of dense chromatin is seen. Secondary vacuoles (short transparent arrows) appear. Other dense-fibrillar nucleolar compartment (long arrows) not showing labelling may represent the penetrating nucleolus-associated chromatin (x 60 000). **b.** Four-cell cow embryos were cultured in the presence of (methyl-<sup>3</sup>H) thymidine so that after division to 8-cell stage all replicated embryonic DNA was labelled. In addition to an intensive extranucleolar labelling of all condensed chromatin, labelled DNA was detected inside the nucleolus. The intra-nucleolar labelling is interpreted as (perinucleolar) chromatin internalization leading to nucleolus differentiation from the NPB (x 38 000; ne : nuclear envelope).



## NUCLEOLAR AND EXTRANUCLEOLAR TRANSCRIPTION

The main aim of our ultrastructural autoradiographic studies was to detect the onset of transcription in early mammalian embryos by the allocation of labelling after a short (20 min) pulse of (5-<sup>3</sup>H) uridine *in vitro* to that stage of cleavage division at which the reactivation of embryonic genome occurs (Figs. 1, 2a). The validity of the results on the timing of the transition to the embryonic control of development in pig, man and cattle (see Table I) was confirmed by the simultaneous morphological changes already described and by observations of DNA interaction with the NPB. The extranucleolar labelling generally precedes nucleolar labelling in all investigated species (Table I). In human 8-cell embryos, a marked enhancement of both extranucleolar as well as nucleolar transcriptional activity was noted, but only in those embryos which continue their development (Tesařík *et al.*, 1988; review Tesařík, 1988). This increase in labelling intensity has been named "progressed-cleavage" transcriptional pattern in contrast to a preceding phase with low or absent labelling in the nucleolus which is named "early cleavage" transcriptional pattern. The proportion of blastomeres showing the progressed-cleavage transcriptional pattern was only about three-quarters at both 8-cell and morula stages (Tesařík *et al.*, 1986b). Production of ribosomes was detected only in blastomeres possessing the progressed-cleavage pattern of transcription (Tesařík *et al.*, 1986b). Application of that observation to different developmental situations in early human embryos allowed us to detect failures in differentiation of some blastomeres (Tesařík *et al.*, 1986b); it was indicative of a transcriptional defect in multinucleated blastomeres (Tesařík *et al.*, 1987a) and finally led to the detection

of the effect of early embryonic genome activity on phenotypic changes at the sub-cellular level (Tesařík *et al.*, 1988). However, whether a similar approach could be used in other mammalian species remains in question. In pig, in contrast to the human embryo, an asynchrony in nucleolar development within one nucleus is reflected in the simultaneous presence of "early" and "late" transcriptional patterns (Tománek *et al.*, 1989) (Fig. 1a, II and III). In reconstructed bovine embryos the arrest of nucleolus structure differentiation has been observed for three consecutive cell cycles after blastomere fusion with an enucleated oocyte. An "early" transcriptional pattern emerged only in the fourth cell cycle (Kaňka *et al.*, 1989), probably mimicking the usual situation in cattle embryos (Table I).

## DISCUSSION AND CONCLUSIONS

The main advantage of the autoradiographic technique in the study of the onset of transcription during early mammalian embryonic nucleogenesis is the possibility of showing the structure function relationship even at the level of particular nucleolar components in a single blastomere nucleus. This is a unique approach to the heterogeneous early mammalian embryo. Furthermore, it was possible to reveal two different types of transcriptional pattern in an apparently uniform population of blastomeres forming either 8-cell or morula stage human embryos. Autoradiography thus offered a tool for many new insights into the biology of early preimplantation human embryo, particularly into the impact of the activity of the embryonic genome on developmental events (Tesařík, 1988), which would hardly be detectable by other experimental approaches.



Correlation of structure and function, *i.e.* the coincidence of autoradiographic detection of the beginning embryonic of RNA synthesis or of the penetration of replicated embryonic DNA into the nucleolus-precursor body with the morphological features of nucleolus differentiation (Table I) was also a major criterion of our comparative analysis in different species.

Based on the present knowledge of the morphological support of nucleolar transcription in a variety of cells (Goessens, 1984) there was full agreement in the early embryos of all three investigated species between the first appearance of nucleolar labelling by ( $^3\text{H}$ ) uridine and the formation of the dense fibrillar component of nucleoli (the first morphological sign of nucleolar transcription). On the other hand, in human and bovine embryos, the appearance of fibrillar centres was delayed in regard to the onset of nucleolar transcription (Table I).

Our studies on penetration of replicated embryonic DNA, labelled by ( $^3\text{H}$ ) thymidine into the NBP (Tesařík *et al.*, 1987b; Kopečný *et al.*, 1989b) allowed us to identify clearly as DNA-containing structures the fibril originating in the perinucleolar chromatin and entering the NPB, a suggestion previously based on morphological analysis (Tesařík *et al.*, 1986a). Extranucleolar transcription generally appeared to start in the embryos of all investigated species a little earlier than nucleolar transcription, and its localization was found on the borderline of the condensed chromatin as in other cell nuclei. A new interesting feature emerged from our studies of labelled DNA distribution at the onset of transcription in nuclei of 8-cell cattle embryos (Kopečný *et al.*, 1989b), in that embryonic (labelled) DNA resettled stepwise to the central part of the blastomere nucleus after being localized, before nuclear transcription started, on the nuclear periphery.

The ontogenic localization of extranucleolar transcription in pig, human and cattle embryos (Table I) has also been tested in all experiments by light-microscope autoradiography whose sensitivity is known to be at least equal if not superior to the sensitivity of radiobiochemical measurements (Rodgers, 1987). The reliability of our autoradiographic data has been confirmed by various means of detection of the first embryonic genomic read-out. In human, sheep and cattle embryos using RNA-synthesis inhibitors, the sensitive period when embryonic RNA is essential for further development was localized to the 2-cell stage in mice (Bolton *et al.*, 1984; Kidder and McLachlin, 1985), but at the 4-cell stage in cattle embryo (Barnes, unpublished observation; cited by King *et al.*, 1988), in between the 4- and 8-cell stage in human embryo (Braude *et al.*, 1988) and at the 8-cell stage in sheep embryo (Crosby *et al.*, 1988). The first transcription of embryonic genes revealed by the electrophoretic profiles of newly synthesized proteins has again been detected at the 2-cell stage of the mouse embryonic development (Flach *et al.*, 1982) but only at the transition from the 4-cell to the 8-cell stage in the human embryo (Braude *et al.*, 1988), or even at the progressed 8-cell stage in cattle (Barnes *et al.*, 1987) and sheep embryos (Crosby *et al.*, 1988). A sharp increase in heat shock protein 70S synthesis was observed at the 8-cell stage in cow embryo (Renard *et al.*, 1988) and mRNA coding for hCG was first detected in 8-cell human embryo (Bonduelle *et al.*, 1987). Last but not least, a specific localization, during ontogenesis, of a vulnerable "block stage", representing a difficult period for culture *in vitro* interestingly corresponds with the expected shift from maternal to embryonic control of development (see Camous *et al.*, 1986; Tománek *et al.* 1989).

Based on these different lines of evidence, it can be concluded, that the activation of the embryonic genome really occurs in the studied mammalian species at that cell cycle when the incorporation of ( $^3\text{H}$ ) uridine was detected in nucleus and that the culmination of this process is the reactivation of nucleolar (= ribosomal RNA) genes, detectable both by morphological evidence of the nucleologenesis and by nucleolar ( $^3\text{H}$ ) uridine incorporation. The possibility of a minute RNA synthesis below the sensitivity threshold of ( $^3\text{H}$ ) uridine autoradiography cannot be excluded (Clegg & Pikó, 1982), but it is suggested that such possible gene action in human zygote or 2-cell embryo should not be responsible for the control of major cellular events (Tesařík, 1988). The maternal control in early human and cattle embryos is probably prolonged and at the same time more complex, as suggested in the case of early human embryos (Tesařík, 1988).

It is expected that the biochemical evidence for genome reactivation in different mammalian species will accumulate to an extent similar to that in the mouse, when sufficient experimental material is provided by *e.g. in vitro* fertilization of ovarian oocytes (Barnes *et al.*, 1987). On the contrary, the morphological and autoradiographic analyses remain the methods of choice in those experimental situations where single embryos or isolated blastomeres should be analyzed. This is the situation encountered at present in efforts of reconstructing cattle embryos by nuclear transplantation of single blastomere nuclei into enucleated oocytes. Since up to now the degree of success has differed considerably, an objective method for the assessment of the outcome of a micromanipulative procedure is necessary. In this situation the cytologic analysis based on fine-structure nucleolar morphology and

autoradiography proved to be extremely helpful (Kaňka *et al.*, 1989). Briefly, these authors showed that the reconstructed cattle embryo was not able, in some cases, to establish its own synthesis of ribosomal RNA since the process of nucleolus differentiation was arrested for several divisions of the reconstructed embryo at the vacuolated NPB (Stage II; Kopečný *et al.*, 1989a). Extranucleolar RNA synthesis and nucleoli with secondary vacuoles appeared in the fourth cell cycle in some embryos (Kaňka *et al.*, 1989). Therefore the nucleolar morphology of the cattle embryo might inform researcher immediately of the result of the experimental procedure.

In conclusion, in mammalian embryos, as well as in more thoroughly studied somatic cells, the nuclear and nucleolar morphology and chromatin topochemistry reflect the state of their differentiation and function (King *et al.*, 1989). The minute differences observed and discussed in this review may have been considered as of as purely academic interest just a short time ago. At present, they are, in the context of the efforts to understand the mechanisms of micromanipulative reconstruction of early mammalian embryos, among the information needed for further investigation in this promising field (Prather *et al.*, 1987; Betteridge and Fléchon, 1988).

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