

Ultrastructural study of nucleolar changes in rat embryos during diapause and reactivation

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Summary — Changes in nucleolar ultrastructure were studied in preimplantation rat embryos before diapause (d 5), during diapause (d 6, 7 and 8) and after reactivation brought about either by *in vitro* culture for 24 h (d 7 and 8) or by estradiol-17 β treatment of the mothers (d 11). Before diapause, fully developed nucleoli contained several low-density fibrillar centers surrounded by a dense fibrillar component and an abundant granular component. This type of nucleolus indicates a high activity of ribosomal RNA synthesis. During diapause, nucleoli revealed a disorganization of the fibrillar and granular elements typical of a diminution in transcriptional activity. During reactivation, nucleoli progressively returned to a reticulo-fibrillar configuration characteristic of the onset of intense transcriptional activity. It is concluded that the structure of the nucleolus in rat preimplantation embryos corresponds to the level of transcriptional activity and is a reliable model for studying structure-function relationships during early development.

rat embryo / nucleolus / RNA synthesis / ultrastructure / genome reactivation

Résumé — Étude ultrastructurale des changements dans le nucléole d'embryon de rat pendant la diapause et la réactivation. Les changements d'ultrastructure des nucléoles ont été étudiés chez des embryons de rat au stade de préimplantation avant la diapause (j 5), pendant la diapause (j 6, 7 et 8) et après une réactivation déclenchée par la culture *in vitro* pendant 24 h (j 7 et 8) ou par une injection d'estradiol-17 β aux femelles gravides (j 11). Avant la diapause, les nucléoles pleinement développés renferment de nombreux centres fibrillaires de faible densité aux électrons entourés d'une couche de composant fibrillaire dense, baignant dans un composant granulaire abondant. Il s'agit donc de nucléoles de type réticulé indicatifs d'une importante activité de synthèse de l'acide ribonucléique ribosomique. Pendant la diapause, les nucléoles présentent une désorganisation des éléments fibrillaires et granulaires typiques d'une diminution de l'activité de transcription. Pendant la réactivation, les nucléoles reprennent progressivement une configuration réticulo-fibrillaire caractéristique du début d'une intense activité de transcription. La structure du nucléole d'embryon de rat traduirait donc le niveau d'activité de transcription et serait un modèle fiable pour l'étude des relations entre la structure et la fonction pendant les premiers stades du développement embryonnaire.

embryon de rat / nucléole / synthèse de l'ARN / ultrastructure / réactivation du génome

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INTRODUCTION

In rats, the embryo enters the uterus at the morula stage on the 4th d of pregnancy and normally attaches to the endometrium 24 h later at the blastocyst stage. In a number of species, blastocysts can enter a quiescent period called diapause which expresses itself by a delay in implantation or attachment (Renfree and Calaby, 1981). In rodents such as rats and mice, diapause occurs after *post-partum* mating in response to suckling stimuli of the newborn (McLaren, 1968) or can be provoked by experimental ovariectomy before the afternoon of d 4 (Canivenc *et al*, 1956; McLaren, 1971; Bergström, 1978). This resting period is characterized by a gradual arrest in mitotic activity (Baevisky, 1963) and DNA synthesis (Clark and Poole, 1967; McLaren, 1968; Van Blerkom *et al*, 1979; Given, 1988), a significant reduction in protein synthesis (Prasad *et al*, 1968; Weitlauf, 1973; Holmes and Dickson, 1975; Van Blerkom *et al*, 1979), RNA (Mohla and Prasad, 1971; Weitlauf and Kiessling, 1980) and a reduction of carbon dioxide production (Menke and McLaren, 1970a,b). Resumption of development can be triggered artificially by estrogen treatment of the mother or by *in vitro* culture of the embryos (McLaren, 1973; Weitlauf and Kiessling, 1981) and is marked by an increase in RNA (McLaren, 1968; Holmes and Dickson, 1975) and protein synthesis (Weitlauf, 1974; Holmes and Dickson, 1975) replication of DNA (Given and Weitlauf, 1981) and embryo implantation in the uterine wall within 24 h (Aitken, 1977).

The nucleolus, a prominent substructure of the nucleus, reflects morphologically the changes in cellular metabolism (Goessens, 1984). As a marker, it has provided valuable information on the activity of the embryonic genome of a number of species (King *et al*, 1989; Kopečný, 1989; Tománek *et al*, 1989). The purpose of this

study was to characterize the ultrastructure of the nucleoli of rat embryos during different periods of metabolic activity associated with diapause and reactivation.

MATERIAL AND METHODS

Animals

Female Sprague-Dawley rats (Charles River Canada Inc, St Constant, Quebec) were used in this study. Males were kept in individual breeding cages. Animals (weighing between 45–50 g, age 21–25 d) were maintained in animal quarters at 20–23 °C, with lighting from 06:00 to 20:00 h. Superovulation of the females was initiated 4 d after arrival. Each rat received a subcutaneous injection of 16 IU pregnant mare's serum gonadotropin (Equinex, Laboratory Ayerst, Montreal, Quebec) between 07:00 and 08:00 h at d -2 (d 0 was the day of mating). Fifty-six h later, at 16:00 h on d 0, females were caged individually with male breeders. Opening of the vagina had previously been performed with a plastic tip. Mating was confirmed the next morning by the presence of a vaginal plug of spermatozoa in the vagina. The morning of vaginal plug formation was designated as d 1 of pregnancy.

Embryo collection

To obtain embryos before diapause, donor rats were sham-operated on d 3 of pregnancy. Rats were killed by cervical dislocation on d 4 and 5 of pregnancy. Embryos were recovered by flushing uterine horns with warm (37 °C) Dulbecco's phosphate-buffered saline (PBS; Gibco Laboratories, Chagrin Falls, OH), with 2% fetal calf serum (FCS; Gibco Laboratories, Grand Island Biological Co, Grand Island) and antibiotics (100 IU penicillin, 100 µg streptomycin/ml). To obtain diapausing and reactivated embryos, donor rats were bilaterally ovariectomized in the evening of d 3 of pregnancy and received a daily subcutaneous injection of 2 mg progesterone in 0.2 ml 10% ethanol and 90% sesame oil to maintain the embryos in diapause. Diapausing blasto-

cysts were recovered on d 6, 7 and 8 of pregnancy. Days 7 and 8 diapausing (inactive) embryos were reactivated by *in vitro* culture in Ham's F-10 (Flow Laboratories Inc, McLean, VA) enriched with 20% FCS and antibiotics for 24 h at 37 °C in a humid atmosphere. The embryos were washed in fresh PBS flushing medium immediately after collection and then transferred to culture dishes. *In vitro* reactivated embryos were therefore called d 7+1 and d 8+1. *In vivo* reactivated blastocysts were recovered 6 h after subcutaneous injection of 1 µg estradiol-17β in 0.1 ml saline given in addition to the daily progesterone on d 11 of pregnancy in diapausing rats. Except for d 4 embryos which were morulae, all recovered embryos were at the blastocyst stage.

Electron microscopy

All embryos were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.2 at 4 °C for 1 h, then washed 3 times with 0.1 M PB for 5 min, embedded in 4% agar to facilitate manipulation (Hyttel and Madsen, 1987) and post-fixed in 2% osmium tetroxide, pH 7.2, in 0.1 M PB for 1 h at 4 °C. After 2 rinses with 0.1 M PB (pH 7.2) and 2 others with deionized distilled water of 5 min each, the embryos were stained *en bloc* in 0.5% uranyl acetate for 1 h at room temperature (24 °C). Embryos were then rinsed twice with distilled water and dehydrated in ascending ethanol concentrations and embedded in Spurr (JBEM Services Inc, Pointe-Claire, Quebec, Canada). Thin sections were cut on Reichert-Jung (Ultracut 41, Vienna, Austria) ultramicrotome and stained with lead citrate (Reynolds, 1963). Grids were examined in a Philips EM 201 electron microscope at 60 kV. Semi-thin sections were stained with toluidine blue. The classification of nucleoli and descriptive terminology used was according to Jordan (1984).

RESULTS

Embryos

A total of 103 embryos were examined in this study. They were separated into the

following groups: active embryos, d 4 ($n = 11$), d 5 ($n = 16$); diapausing embryos, d 6 ($n = 12$), d 7 ($n = 18$), d 8 ($n = 10$); embryos reactivated *in vitro*, d 7 ($n = 15$), d 8 ($n = 11$); and embryos reactivated *in vivo*, d 11 ($n = 10$). The embryos in each group came from at least 3 different donors.

Active embryos

The nucleoli of d 4 morula nuclei contained a compact mass of tightly packed electron-dense filaments which occupied at least half of the area of the nucleolus in most of the sections examined (*eg*, see fig 1). It was surrounded by a dense fibrillar component organized in a 3-dimensional network with very little granular component in its interstices. In some nuclei, the nucleolus was completely devoid of granules and consisted exclusively of the mass of dense fibrils with or without peripheral condensed nucleolus-associated chromatin. These nucleoli were found in cells from morulae that were not yet compacted and there was no difference in nucleoli from either the internal or external cells. In the nucleolus of d 5 active blastocysts, the 3 components described by Goessens (1984) were found in addition to the chromatin (fig 2). These functional reticulated nucleoli were consistently observed in the inner cell mass cells of active blastocysts. However, in the trophoblastic cells, nucleoli similar to those observed in morulae, *ie* devoid of fibrillar centers with only a compact fibrillar mass and some peripheral fibrillar component, were also present. Both types of nucleoli were found in equal proportions in trophoblastic cells. Occasionally, there was asynchrony in the nucleologenesis as both types of nucleoli were observed in the same nucleus (fig 3). Sometimes, fusion between 2 nucleoli was noted in cells from both trophoblast and inner cell mass (fig 4).

Diapausing embryos

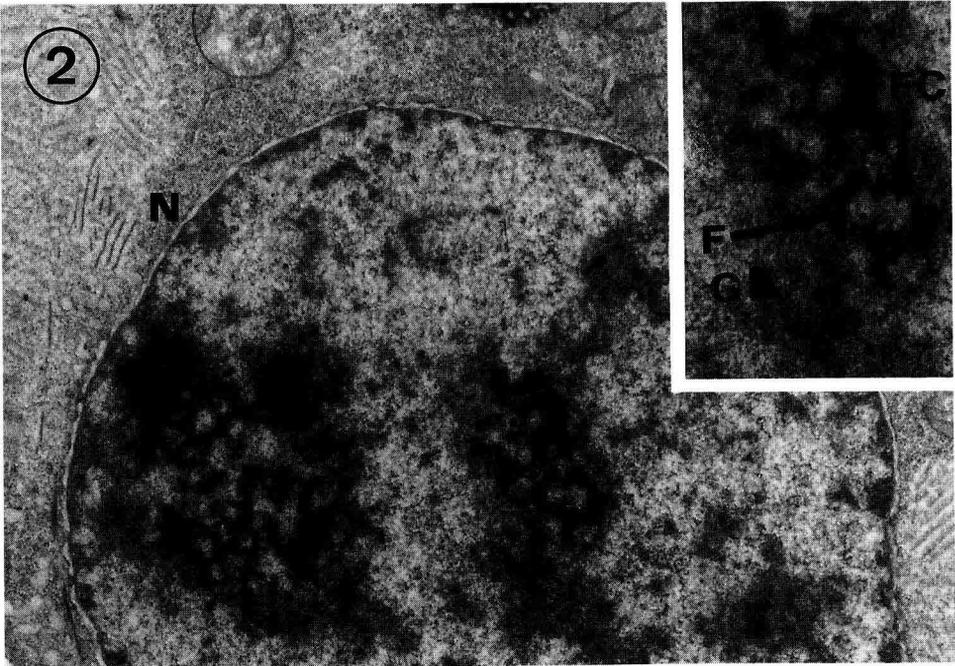
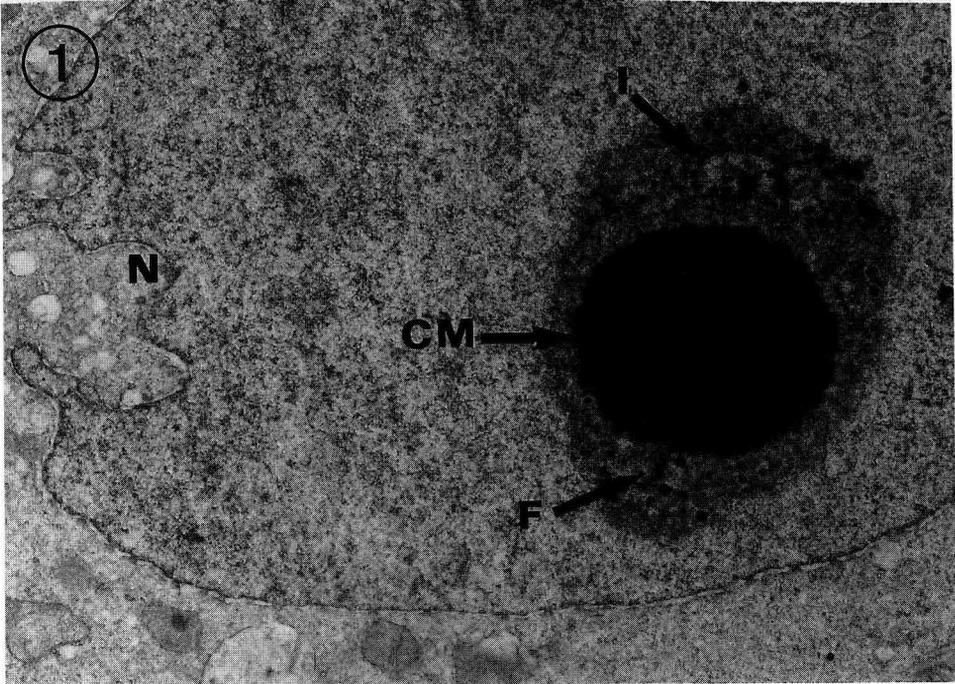
The nucleoli of diapausing embryos at the beginning of the quiescent period (d 6) presented a clear disorganization of the 3 nucleolar components, indicating a diminution in cellular activity. Fibrillar centers were much larger than those observed in the nucleoli of active blastocysts, and always closely associated with the fibrillar component. There was no difference between the nucleoli of cells from the inner cell mass and the trophoblast during diapause.

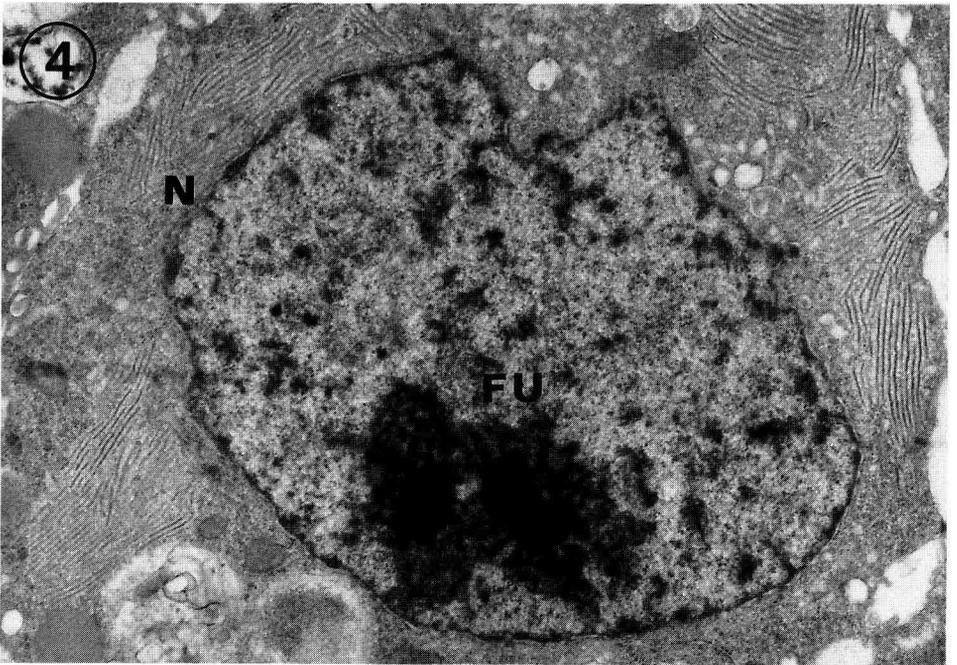
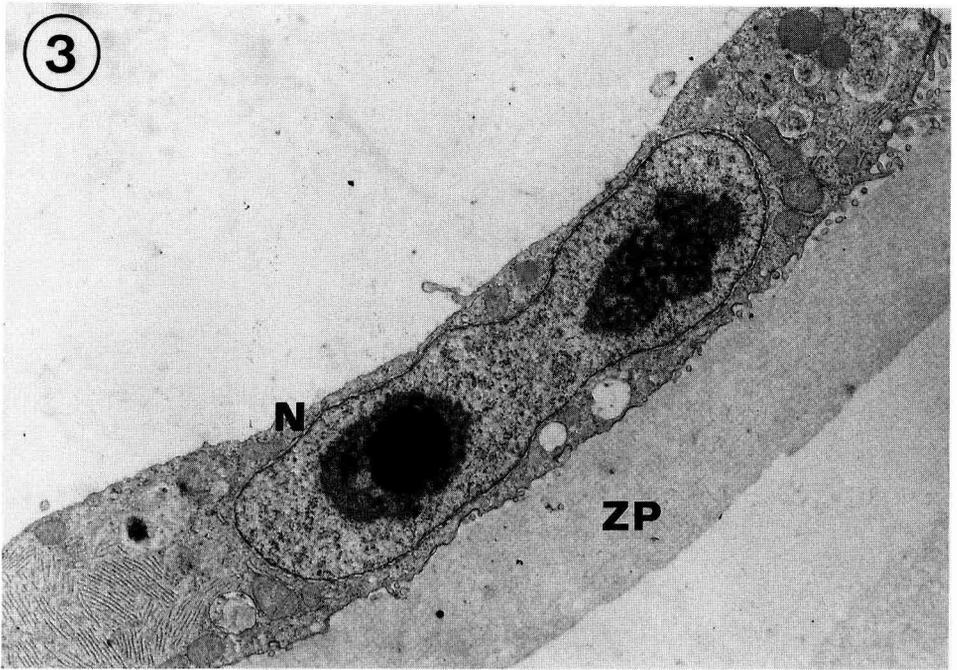
In the nucleoli of d 7 diapausing blastocysts, fibrillar centers were scarce and often absent, and the other nucleolar components were disorganized (fig 5). The

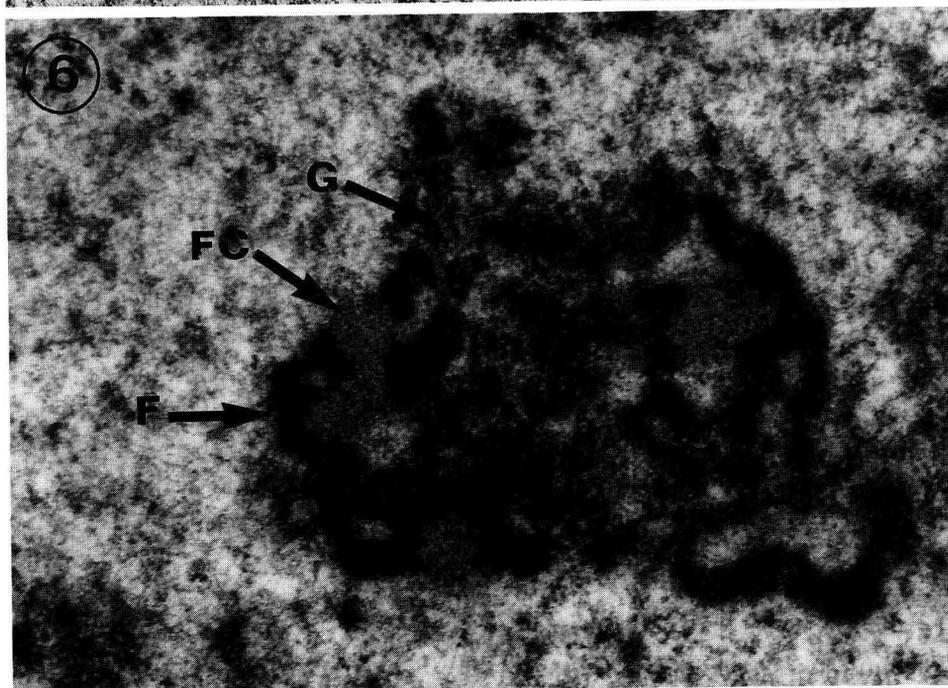
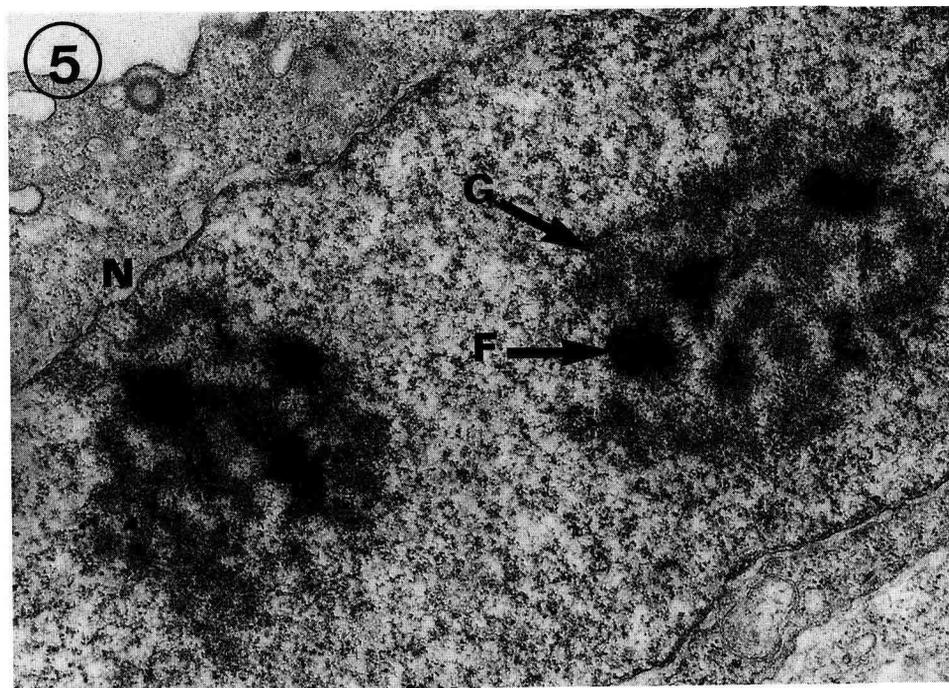
most prominent part of such nucleoli was the granular component with a greatly reduced fibrillar component (fig 5). Nucleoli of these inactive embryos were smaller in size and more numerous than those of d 5 active blastocysts.

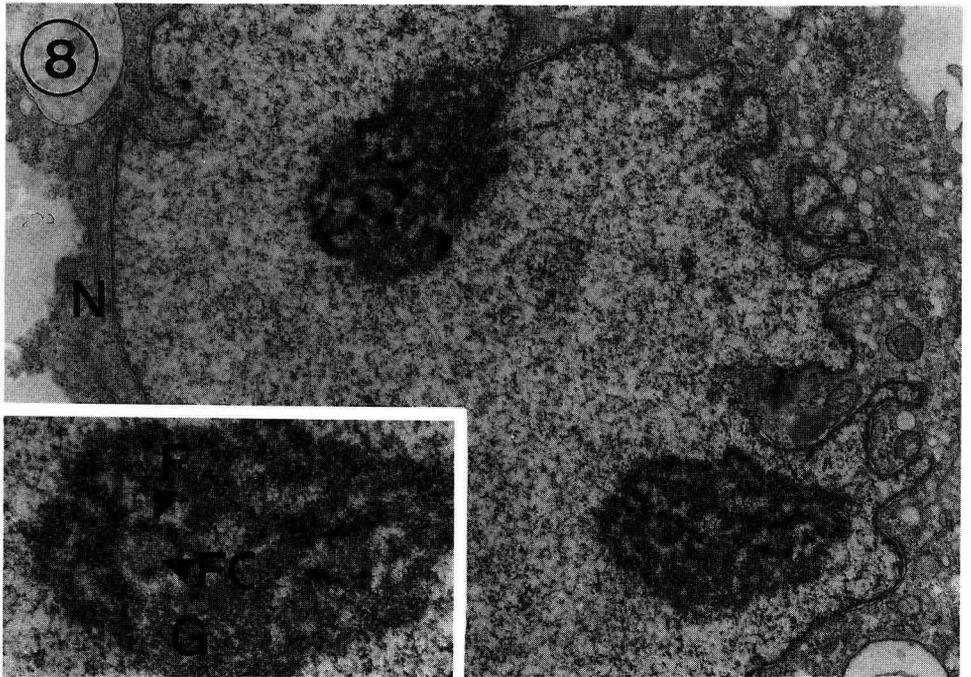
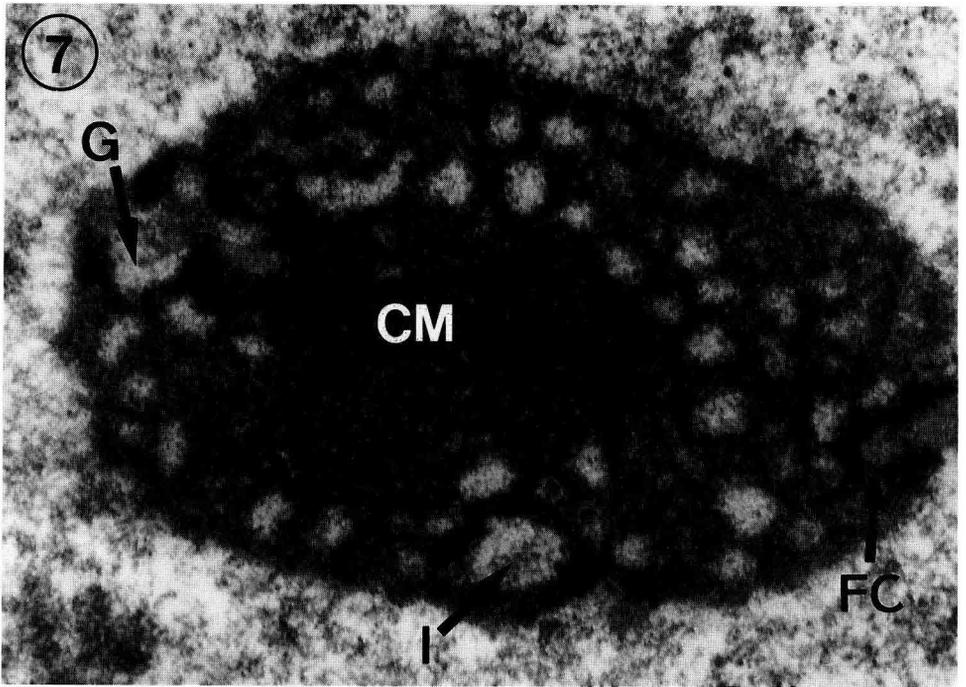
Day 8 diapausing blastocysts had nucleoli that were similar to those of d 7 diapausing blastocysts; however, they possessed other nucleoli with large fibrillar centers almost entirely surrounded by a thick layer of dense fibrillar component (fig 6). Sometimes an extended dense fibrillar zone formed a network in which zones of granular component were interspersed. Disorganization of the nucleolar zones was observed and nucleolar volume was greatly reduced compared to active blastocysts.

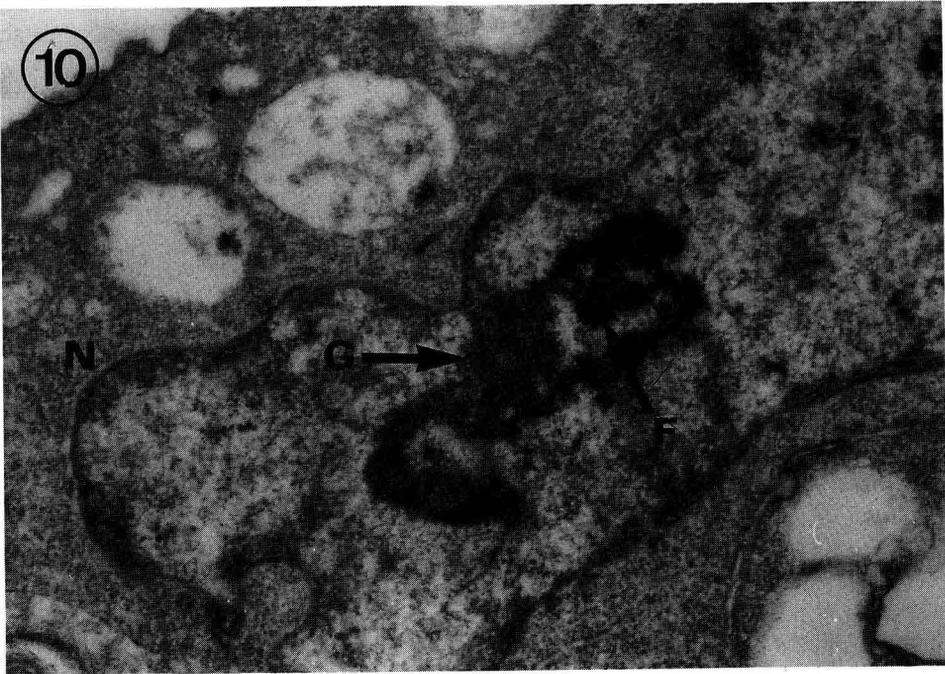
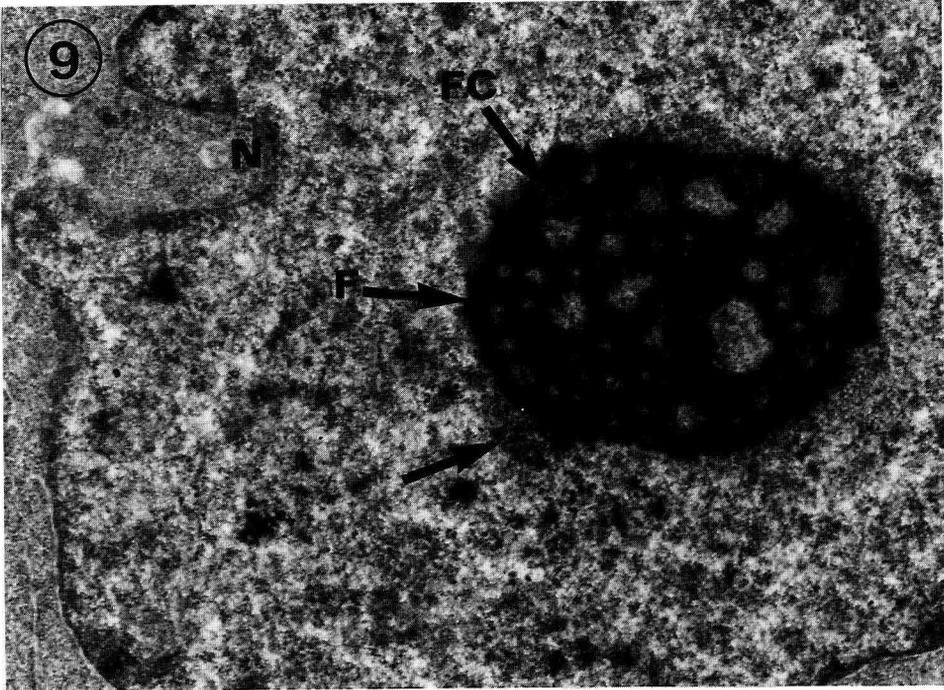
Fig 1–4. **1.** Electron micrograph of a d 4 morula nucleolus formed of a compact mass (CM) of tightly packed filaments surrounded by a 3-dimensional network of dense fibrillar component (F) comprising interstices (I). Nuclear envelope (N); x 12 400. **2.** Electron micrograph of an inner cell mass blastomere from a d 5 active blastocyst. Functional reticulated nucleoli (inset, x 33 600) include an abundant granular component (G) with numerous small fibrillar centers (FC) (with electron-density different to that of the nucleolar matrix and nucleoplasm), each surrounded by a layer of dense fibrillar component (F). Nuclear envelope (N); x 18 500. **3.** Electron micrograph of 2 different nucleoli in a trophoblastic cell of a d 5 blastocyst. The upper nucleolus is fully developed and active, whereas the lower nucleolus is constituted of a compact mass with a peripheral dense fibrillar component network and corresponds to a less advanced developmental stage of nucleologenesis. Nuclear envelope (N); zona pellucida (ZP); x 7 500. **4.** Electron micrograph of a possible fusion (FU) occurring between 2 reticulated nucleoli in an inner cell mass cell. Nuclear envelope (N); x 12 700. **Figs 5–6.** Electron micrographs of the nucleoli of diapausing embryos. **5.** Day 7: disorganized nucleoli from a trophoblastic cell mostly containing a granular component (G) and a few bundles of dense fibrillar component (F). Nuclear envelope (N); x 29 000. **6.** Day 8: nucleolus from an inner cell mass cell showing clear disorganization of its components significative of a lower cellular activity. Fibrillar centers (FC); dense fibrillar component (F); granular component (G) much reduced; x 36 400. **Figs 7–8.** Electron micrographs of *in vitro* reactivated blastocysts. **7.** Reticulated inner cell mass nucleolus composed of a compact mass (CM) surrounded by a fibrillar network containing many interstices (I) and small amounts of granular component (G) between filaments of the network. Some fibrillar centers are observed (FC); x 33 600. **8.** These nucleoli from a trophoblastic cell are beginning to reticulate and are composed of a poorly defined network of granular (G) and dense fibrillar component (F); inset x 25 000. Some fibrillar centers are present (FC). Nuclear envelope (N); x 13 750. **Figs 9–10.** Electron micrographs of *in vivo* (estradiol-17 β) reactivated embryos recovered on d 11 of gestation. **9.** Inner cell mass nucleolus formed of dense fibrillar component (F) arranged in a 3-dimensional network (nucleolonema) with many interstices (I) and some granular component (G). Some fibrillar centers can be observed (FC). Nuclear envelope (N); x 20 000. **10.** Nucleolus in a trophoblastic cell showing segregation of the dense fibrillar component (F) and granular component (G) which suggests a reduction in cellular activity. Nuclear envelope (N); x 23 800.











Reactivated embryos

Day 7+1 and d 8+1 *in vitro* reactivated blastocysts were similar. Nucleoli from inner cell mass cells were reticulated, composed of a dense fibrillar component organized in a 3-dimensional network with some dispersed small regions of granular component. These nucleolonema-like nucleoli were large and sometimes contained a central mass of dense fibrillar component and many interstices (fig 7). Granular component was present between meshes of the network and some fibrillar centers were observed (fig 7). Nucleoli were not counted but appeared to be more numerous and smaller in the trophoblast than in the inner cell mass. They were less advanced in the formation of the nucleolonema (fig 8). Small fibrillar centers were occasionally observed in nucleoli from the trophoblast.

In the estrogen-reactivated embryos, the nucleoli of the trophoblastic cells were less advanced in the reticulation process than those of the inner cell mass blastomeres. No fibrillar centers were seen in these nucleoli. In the inner cell mass cells, however, nucleolonema-like nucleoli with a 3-dimensional network of dense fibrillar component with numerous interstices and some fibrillar centers were observed (fig 9). Granular component was scarce (fig 9). In the trophoblastic cells, nucleoli consisted either of an undefined reticular structure residing in the granular component or presented disorganization of granular and dense fibrillar components (fig 10). It was also noticed in both types of cells that the number of nucleoli per nucleus seemed to diminish as the size of nucleoli increased.

DISCUSSION AND CONCLUSION

Changes in the nucleolus ultrastructure observed during periods of different cellu-

lar activity are related to RNA metabolism of the cell (Goessens, 1984). The compact fibrillar mass surrounded by a peripheral reticulo-granular network of the d 4 morula nucleoli observed in the present study corresponds to the morphology of the nucleoli found in rat embryos at the 4-cell stage (Szöllösi, 1971). Formation of the peripheral network is related to the first detection of rRNA synthesis in the mouse (Hillman and Tasca, 1969), pig (Tománek *et al*, 1989), man (Tesarík *et al*, 1987) and cow (Kopečný *et al*, 1989). Some nucleoli exclusively formed of a compact mass without the peripheral network in embryos at the morula stage may still be inactive, as described in other species (Kopečný, 1989).

The nucleoli from inner cell mass cells of d 5 blastocysts were similar to those observed in cells with a high rate of RNA synthesis (Wachtler *et al*, 1980) indicating an intense transcriptional activity in the embryos at this developmental stage. In addition to this, the large number of fibrillar centers encountered in the nucleoli of active blastocysts agrees with the suggestion that the number of fibrillar centers increases proportionally with cellular activity (Jordan and McGovern, 1981). The nucleoli of the trophoblastic cells of control blastocysts were morphologically similar to the nucleoli of morulae which have been shown to have a relatively low rate of ³H-uridine incorporation (Daentl and Epstein, 1971). Since this kind of nucleolus was not found in the inner cell mass cells, and trophoblastic cells seemed to contain nucleoli which were in morphological transition toward a higher level of activity, it is possible that the nucleolar transition occurs in advance in the inner cell mass. In the mouse, the inner cell mass is vital to normal embryonic development and influences the proliferation of the adjacent trophoblast to enable the production of the ectoplacental cone and the extraembryonic ectoderm (Gardner *et al*, 1973). It is also

interesting to note that in cattle, blastocysts show a higher [^3H]-uridine labelling in the nucleus of the inner cell mass compared to that of the trophoblast (Pivko *et al.*, 1986).

Although there was variation in nucleolar morphology among diapausing embryos on different days after ovariectomy, they were generally characterized by disorganization of the nucleolar components, particularly of fibrillar centers and fibrillar components. These changes are also characteristic features of cells, embryos and oocytes exposed to inhibitors of transcription such as α -amanitin (Brasch, 1990; Plante *et al.*, 1991) or actinomycin D (Jordan and McGovern, 1981) and inhibitors of protein synthesis such as cycloheximide (Crozet, 1983). Together these observations suggest that there is a sharp reduction or an absence of rDNA transcription associated with diapause.

While it has been demonstrated that *de novo* RNA synthesis starts within 30 min of estradiol-17 β stimulation in diapausing mice embryos (Holmes and Dickson, 1975), reactivation of diapausing blastocysts 6 h post-estradiol-17 β in the present study was characterized by reticulation of the inner cell mass nucleoli. This type of nucleolus is also observed in cells at the beginning of intense transcriptional activity (Wachtler *et al.*, 1980; Raska *et al.*, 1983). The nucleoli of trophoblastic cells of estradiol-17 β reactivated embryos were clearly less advanced in the reticulation process. Granular and fibrillar components did not always form a distinct network and these nucleoli resembled those observed in cells after actinomycin D inhibition of rRNA synthesis (Recher *et al.*, 1976). These asynchronous modifications of the nucleoli of inner cell mass cells and trophoblastic cells were also noticed in 24-h *in vitro* reactivated blastocysts. Interstices have been observed in the nucleolus of inner cell mass cells of both *in vitro* and *in vivo* reactivated embryos. The appearance

of interstices is the result of a rapid loss of the granular component in the nucleolus and correlates with the beginning of incorporation of labelled uridine in rhizoma cells of *Zea mays* (Goessens, 1984).

Nucleoli of embryos reactivated *in vivo* and *in vitro* had one feature in common; they were smaller and more numerous in the trophoblastic cells than in the inner cell mass cells.

In summary, during diapause the nucleoli went through a progressive disorganization of the nucleolar components, and during reactivation returned to a reticulated configuration. Both of these transformations are consistent with the diminution and the resumption of transcriptional activity, respectively. Small nucleoli in reactivated trophoblastic cells seem to correspond to nucleoli which are less active in rDNA transcription (Goessens, 1984), which indicates that the nucleolar transition occurs earlier in the inner cell mass cells than in the trophoblast cells. The present study confirms that, as in embryos of species that do not experience diapause, the nucleolus of the diapausing rat embryos can be used as a structure-function model.

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