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Nucleolar Proteins and Nuclear Ultrastructure in Preimplantation Bovine Embryos Produced In Vitro[1]

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ABSTRACT

The aim of the present investigation was to describe the basic cell biology of the postfertilization activation of rRNA genes using in vitro-produced bovine embryos as a model. We used immunofluorescence confocal laser scanning microscopy and transmission electron microscopy to study nucleolar development in the nuclei of embryos up to the fifth postfertilization cell cycle. During the first cell cycle (1-cell stage), fibrillarin, upstream binding factor (UBF), nucleolin (C23), and RNA polymerase I were localized to distinct foci in the pronuclei, and, ultrastructurally, compact spherical fibrillar masses were the most prominent pronuclear finding. During the second cell cycle (2-cell stage), the findings were similar except for a lack of nucleolin and RNA polymerase I labeling. During the third cell cycle (4-cell stage), fibrillarin, UBF, nucleophosmin, and nucleolin were localized to distinct foci. Ultrastructurally, spherical fibrillar masses that developed a central vacuole over the course of the cell cycle were observed. Early in the fourth cell cycle (8-cell stage), fibrillarin, nucleophosmin, and nucleolin were localized to small bodies that with time developed a central vacuole. UBF and topoisomerase I were localized to clusters of small foci. Ultrastructurally, spherical fibrillar masses with a large eccentric vacuole and later small peripheral vacuoles were seen. Late in the fourth cell cycle, nucleophosmin and nucleolin were localized to large shell-like bodies; and fibrillarin, UBF, topoisomerase I, and RNA polymerase I were localized to clusters of small foci. Ultrastructurally, a presumptive dense fibrillar component (DFC) and fibrillar centers (FCs) were observed peripherally in the vacuolated spherical fibrillar masses. Subsequently, the presumptive granular component (GC) gradually became embedded in the substance of this entity, resulting in the formation of a fibrillo-granular nucleolus. During the fifth cell cycle (16-cell stage), a spherical fibrillo-granular nucleolus developed from the start of the cell cycle. In conclusion, the nucleolar protein compartment in in vitro-produced preimplantation bovine embryos is assembled over several cell cycles. In particular, RNA polymerase I and topoisomerase I are detected for the first time late during the fourth embryonic cell cycle, which coincides with the first recognition of the DFC, FCs, and GC at the ultrastructural level.

INTRODUCTION

The initial period of mammalian preimplantation development is governed by gene transcripts and polypeptides produced by, and stored in, the oocyte during its development [1]. However, after one to three cleavage divisions, dependent on the species, control of development is taken over by expression of portions of the embryonic genome, and the maternally derived transcripts and proteins are gradually degraded [2,3]. This transition from maternal to embryonic control of development is referred to as the maternal-embryonic transition.

As the preimplantation embryo has a profound need for protein synthesis, a substantial number of ribosomes is crucial. Activation of the rRNA genes follows a specific program in the bovine embryo, and because of the characteristic ultrastructure of nucleolus formation associated with this process, these genes may serve as markers for embryonic gene activation as proposed by Kopecny and Niemann [4]. In general, it has been accepted that the rRNA genes are activated during the fourth embryonic cell cycle, i.e., at the 8-cell stage in cattle, when they participate in the establishment of functional nucleoli [5–7]. In the preceding cell cycles, different nuclear entities are observed, none of which possess the ultrastructural components that characterize the functional nucleolus [8–10]. Thus, it is believed that during the first three cell cycles, protein synthesis in the bovine embryo is based upon ribosomes inherited from the oocyte.

In the functionally active mammalian nucleolus, three main ultrastructural components can be identified: the fibrillar components consisting of the fibrillar centres (FCs) and the dense fibrillar component (DFC), and the granular component (GC) (for review, see [11]). These components of the so-called fibrillo-granular nucleolus reflect the steps in the biosynthesis of ribosomes according to the following simplified model: the FCs and the DFC house the enzymatic apparatus for transcription; the DFC carries the primary unprocessed transcripts, while the GC represents processed transcripts associated with proteins in the form of preribosomal particles that, in their final form, represent the ribosomal subunits.

It is, however, unknown how the protein components of the nucleolus necessary for transcription and further processing are assembled in the bovine embryo. Therefore, the localization of topoisomerase I, RNA-polymerase I, upstream binding factor (UBF), fibrillarin, nucleophosmin (B23), and nucleolin (C23) in 2- to 16-cell in vitro-produced bovine embryos was investigated by immunocytochemistry and confocal laser scanning microscopy and related to the nuclear ultrastructure at identical developmental stages.

MATERIALS AND METHODS

Oocyte Recovery and In Vitro Maturation

The protocol was mainly as described previously [12,13]. All the chemicals were purchased from Sigma (Bie

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& Berntsen, Roedovre, Denmark), unless otherwise indicated. Briefly, bovine abattoir ovaries, collected from herds of unknown origin (mainly dairy cows of Jersey, Holstein-Friesian, and Danish Red breeds), were kept at 35°C in 0.9% NaCl. After arrival at the laboratory 2–3 h after slaughter, the ovaries were rinsed in warm saline, and oocytes were aspirated with an 18-gauge needle from follicles > 2 mm by use of a pump (KNF Miniport, type N74KN18; Neuberger, Germany). Follicular aspirates were collected into 50-ml plastic tubes (Nunc, Roskilde, Denmark) containing 3 ml Hepes-buffered basic medium-3 (HBM-3; [14,15]) with 1 mg/ml polyvinyl alcohol (PVA), 50 mg/ml gentamicin sulphate, and 20 U/ml heparin to prevent clotting. Cumulus-enclosed oocytes were isolated from the pellet and washed 3 times in HBM-3 without heparin, and once in maturation medium. The maturation medium was tissue culture medium (TCM)-199 with Earle's salts (Gibco, Life Technologies, Taastrup, Denmark), containing 1 mg/ml PVA and 0.2 U/ml gonadotropins (eCG:hCG 2:1 mixture, Suigonan Vet; Intervet Scandinavia A/S, Skovlunde, Denmark). Groups of up to 75 oocytes were matured in 0.5 ml maturation medium in 4-well culture dishes without oil (Nunc) for 24 h at 38.8°C in 5% CO_2 in air with saturated humidity.

Sperm Preparation and In Vitro Insemination

After 24 h of culture in maturation medium, oocytes were washed once in in vitro fertilization (IVF)-Tyrode's albumin lactate pyruvate (TALP) [16–19], before transfer to 0.5 ml IVF-TALP in 4-well plates without oil. The IVF-TALP medium contained 6 mg/ml BSA (fraction V; Sigma A 4919), 0.25 mM sodium pyruvate, 30 mg/ml heparin (170 USP/mg), and PHE (20 mM D-penicillamine, 10 mM hypotaurine, 1 mM epinephrine). SPERM-TALP contained 6 mg/ml BSA, 1 mM sodium pyruvate, and 50 mg/ml gentamicin sulphate. Straws of frozen bovine semen from one bull of proven fertility with 25 million live spermatozoa per straw were quickly thawed for 1 min at 37°C, washed twice by centrifugation at $300 \times g$ for 5 min in SPERM-TALP medium, and adjusted to 25×10^6/ml. The final sperm concentration used for in vitro insemination was 2×10^6/ml. Spermatozoa and oocytes were coincubated at 38.8°C in 5% CO_2 in air with saturated humidity.

In Vitro Embryo Culture

After coincubation for 20–22 h, cumulus cells and excess spermatozoa were removed by vortexing for 1 min and washing 3 times in HBM-3 and 1 time in Menezo-B2 medium (INRA Laboratoire, France). The Menezo-B2 medium, which contains 10 mg/ml BSA, was supplemented with 10% estrous cow serum (not heat-inactivated) from a single standardized batch. Groups of 20 inseminated oocytes were cocultured with a suspension of bovine oviduct epithelial cells in 100-ml droplets of Menezo-B2 medium covered with paraffin oil (Uvasol, Merck, Struers Kebo-lab, Copenhagen, Denmark). The embryos were cultured at 38.8°C in 5% CO_2 in air with saturated humidity.

The zygotes and embryos used in this investigation were collected from 5 replicates in such a way that all groups defined in the following included samples from each replicate. Presumptive zygotes were harvested for fixation at 10, 19, and 26 h postinsemination. Later embryonic stages were assessed first at 28, 37, 43, and 80 h postinsemination, and re-evaluated 2 and 4 h after each of the timepoints. At each of these evaluations, newly cleaved embryos were isolated and cultured separately for different periods until they were harvested for microscopical processing. Only embryos in which the blastomeres cleaved synchronously were used. Embryos were harvested for fixation at 3, 6, and 9 h postcleavage (hpc) to the 2-cell stage; at 3 and 6 hpc to the 4-cell stage; at 3, 9, 15, 21, 27, 33, and 39 hpc to the 8-cell stage; and at 3, 9, and 15 hpc to the 16-cell stage.

Processing for Light and Transmission Electron Microscopy

The ova (a minimum of 2 for each time interval) were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). Subsequently, the specimens were washed in buffer, postfixed using the osmium-ferricyanide method [14], stained en bloc in uranyl acetate, dehydrated, and embedded in epoxy resin. Serial semithin sections (2 μm) were cut and stained by 1% basic toluidine blue. Specific semithin sections with nuclear bodies were selected by light microscopy for re-embedding and subsequent ultrathin sectioning, and the grids were contrasted with uranyl acetate and lead citrate for transmission electron microscopy. The sections were examined on a Philips CM 100 microscope (Eindhoven, The Netherlands).

Immunofluorescence Microscopy

The following primary antibodies against key nucleolar proteins were used: mouse monoclonal anti-nucleolin (C23; 1:1000; [20]), mouse monoclonal anti-nucleophosmin (B23; 1:1000; [20]), human anti-fibrillarin (1:1000; [21]), human anti-topoisomerase I (1:100; [22]), human anti-RNA polymerase I (1:500; [23]), and human anti-UBF (1:500; [24]).

For indirect immunofluorescence, embryos were fixed in a mixture of 4% paraformaldehyde and 0.1% Triton X-100 for 3 h at 4°C; at 10, 19, and 26 h postinsemination at the 1-cell stage ($N = 75$); at 3, 6, and 9 hpc to the 2-cell stage ($N = 135$); at 3 and 6 hpc to the 4-cell stage ($N = 90$); at 3, 9, 15, 21, 27, 33, and 39 hpc to the 8-cell stage ($N = 420$) and at 3, 9, and 15 hpc to the 16-cell stage ($N = 135$). Subsequently, the embryos were washed in 1% Triton X-100 in PBS and preincubated for 2 h with 5% rabbit serum (Dako, Glostrup, Denmark) in PBS at room temperature. After this procedure, the embryos were incubated with the primary antibodies diluted in PBS containing 5% rabbit serum for 2 h at room temperature. Excess primary antibodies were removed by extensive washing in PBS before a 4-h (at 4°C) and 1-h (at room temperature) incubation in rabbit anti-human/biotin (Dako; for anti-topoisomerase I, anti-RNA-polymerase I, anti-UBF, and anti-fibrillarin) or rabbit anti-mouse/biotin (Dako; for anti-nucleolin and anti-nucleophosmin), diluted in PBS containing 5% rabbit serum. The secondary antibodies were visualized by Streptavidin/fluorescein isothiocyanate (FITC; Dako) in PBS. Finally, the embryos were mounted on glass slides using Dako fluorescent mounting medium and examined on a Leitz (Wetzlar GBH, Wetzlar, Germany) confocal laser scanning microscope. Control immunostaining was performed by omitting the primary antibodies. All nuclear entities that exhibited immunocytochemical labeling were recorded; diffuse labeling of the nucleoplasm was not addressed in the present study.

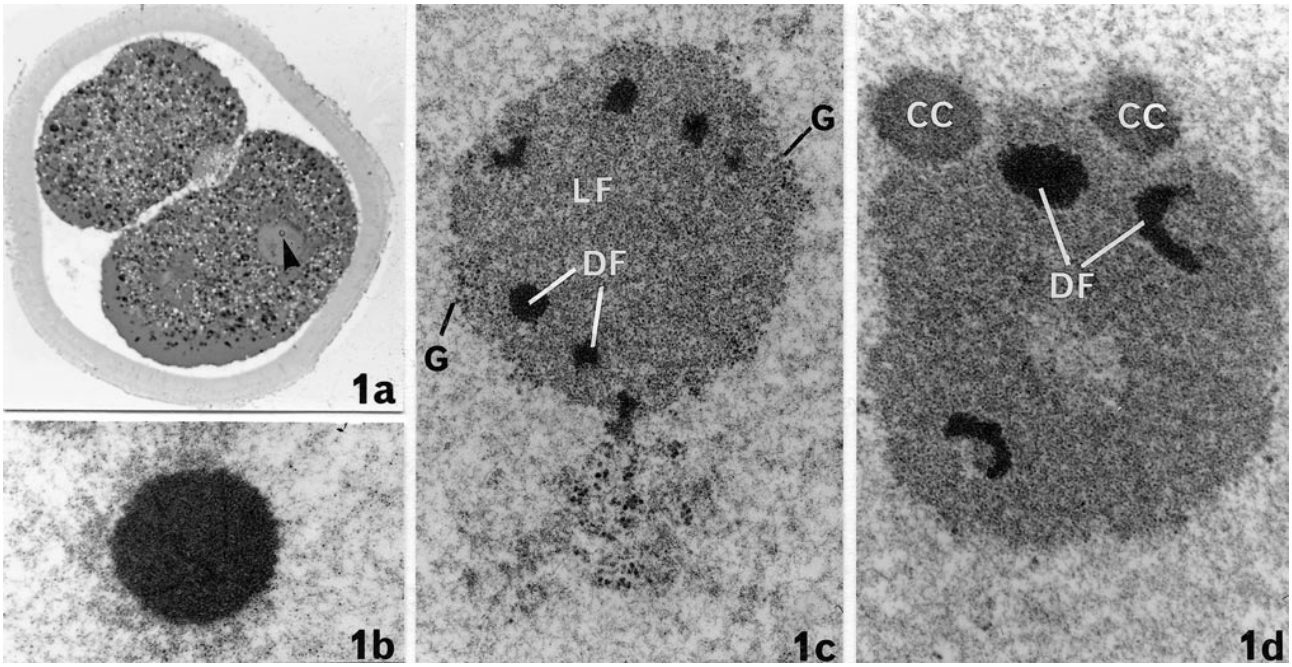

FIG. 1. The second cell cycle, i.e., the 2-cell embryo. **a**) An embryo fixed at 9 hpc. Note the nuclear entities (arrowheads). Magnification ×380. **b**) A compact spherical mass of electron-dense fibrillar material in an embryo fixed at 3 hpc. Note the chromatin halo extending from the mass. Magnification ×31 200. **c**) A fibrillo-granular complex consisting of clusters of electron-dense granules (G) closely attached to masses of loosely packed fibrillar material (LF) in which small foci of electron-dense fibrillar material (DF) was found. The embryo was fixed at 3 hpc. Magnification ×23 100. **d**) A fibrillo-granular complex presenting larger horseshoe-shaped bodies of electron-dense fibrillar material (DF). Note the chromatin-like caps (CC). The embryo was fixed at 6 hpc. Magnification ×27 300.

RESULTS

First Cell Cycle

The most prominent ultrastructural entities of the pronuclei were compact spherical masses of electron-dense fibrillar material often referred to as the nucleolus precursor bodies (identical to the 2-cell stage: Fig. 1, a and b). These bodies were associated with a halo of slightly condensed chromatin. On other locations in the pronuclei, fibrillo-granular complexes were observed. These complexes consisted of clusters of electron-dense granules closely attached to masses of loosely packed fibrillar material in which small foci of electron-dense fibrillar material were found (identical to the 2-cell stage: Fig. 1, c and d). In addition, the granule clusters were associated with caps of condensed chromatin-like material.

Fibrillarin—and also UBF, nucleolin, and RNA polymerase I in about half of the specimens—were localized by confocal microscopy to small spherical bodies in the pronuclei (see Fig. 5). None of the remaining antibodies resulted in labeling of nuclear entities.

Second Cell Cycle

At 3 hpc, ultrastructural observations similar to those described for the first cell cycle were made (Fig. 1). At 6 hpc, the electron-dense fibrillar material in the fibrillo-granular complexes had developed into larger horseshoe-shaped

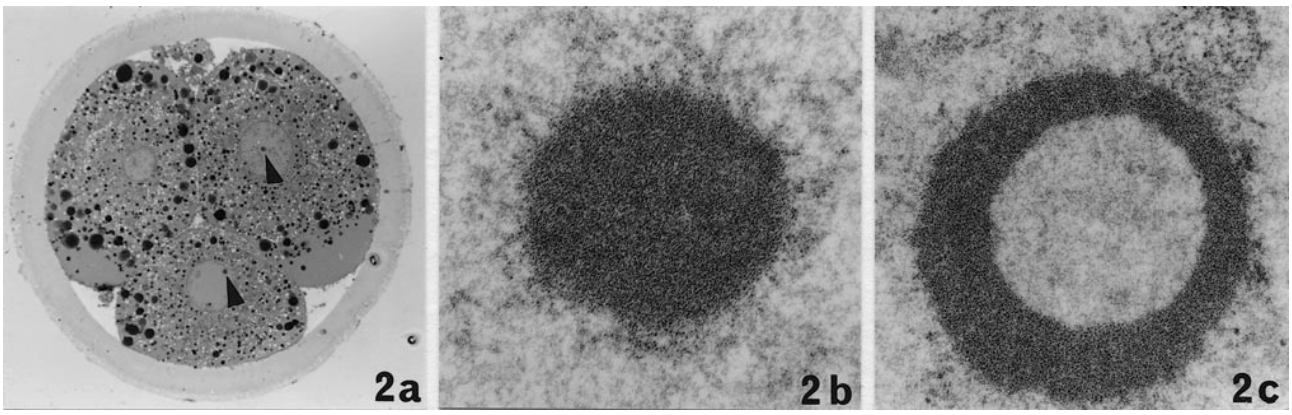

FIG. 2. The third cell cycle, i.e., the 4-cell embryo. **a**) An embryo fixed at 3 hpc. Note the nuclear entities (arrowheads). Magnification ×370. **b**) A compact spherical mass of electron-dense fibrillar material in an embryo fixed at 3 hpc. Note the chromatin halo extending from the mass. Magnification ×42 000. **c**) A vacuolated spherical mass of electron-dense fibrillar material in an embryo fixed at 6 hpc. Note the chromatin halo extending from the mass. Magnification ×29 400.

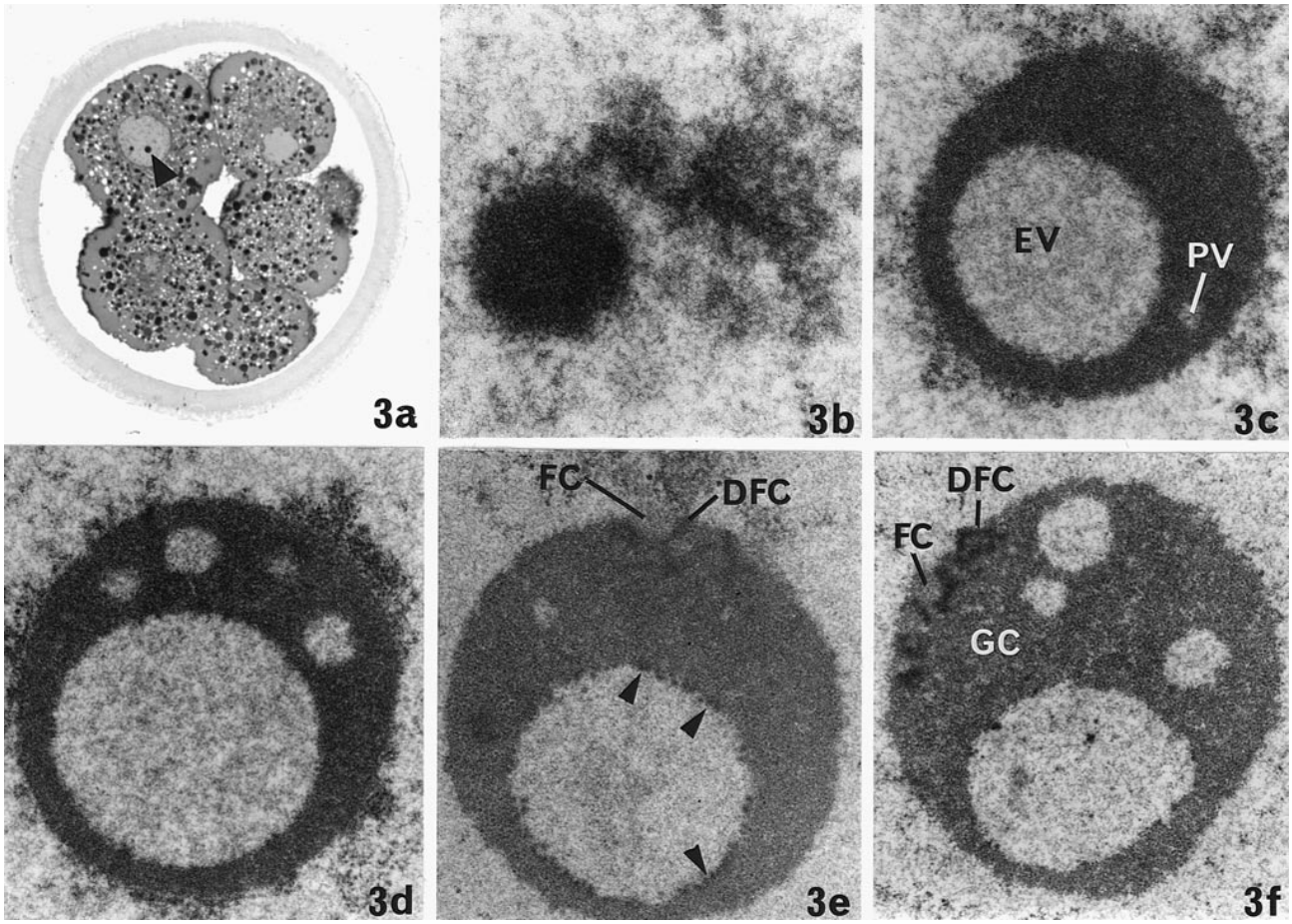

FIG. 3. The fourth cell cycle, i.e., the 8-cell embryo. **a**) An embryo fixed at 21 hpc. Note the nuclear entities (arrowhead). Magnification ×450. **b**) A compact spherical mass of electron-dense fibrillar material in an embryo fixed at 3 hpc. Note the chromatin halo extending from the mass. Magnification ×48 500. **c**) A spherical mass of electron-dense fibrillar material displaying a large eccentric vacuole (EV) and a tiny peripheral vacuole (PV) in an embryo fixed at 3 hpc. Note the chromatin halo extending from the mass. Magnification ×26 400. **d**) Same as **c** except that the peripheral vacuoles have developed and become more numerous. The embryo was fixed at 9 hpc. Magnification ×27 300. **e**) A vacuolated spherical mass of electron-dense fibrillar material presenting a presumptive DFC surrounding an FC. Note that the FC is connected to condensed chromatin and that small portions of a presumptive DFC are also found delineating the large eccentric vacuole (arrowheads). The embryo was fixed at 27 hpc. Magnification ×25 300. **f**) A spherical fibrillo-granular nucleolus presenting a presumptive DFC surrounding a presumptive FC and a GC. The embryo was fixed at 33 hpc. Magnification ×17 100.

structures. This morphological appearance was maintained at 9 hpc.

From 3 hpc to the 2-cell stage, fibrillarin—and UBF in about half of the specimens—were detected by confocal microscopy in the nucleus (see Fig. 5). Both proteins were localized to small spherical bodies. The same labeling pattern was maintained throughout the cell cycle (6 and 9 hpc). None of the remaining antibodies resulted in labeling of nuclear entities.

Third Cell Cycle

At 3 hpc, compact spherical masses of electron-dense fibrillar material associated with slightly condensed chromatin were again observed, together with fibrillo-granular complexes located at other sites in the nucleus (Fig. 2). At 6 hpc, vacuolization of the spherical masses of electron-dense fibrillar material to a shell-like structure, i.e., a ring-like profile in the sections, was observed. The development of abundant electron-dense fibrillar material in the fibrillo-granular complexes characteristic for the second cell cycle was not observed.

Throughout the cell cycle (3 and 6 hpc), fibrillarin, and in about half of the specimens UBF, displayed the same labeling pattern as during the previous cycle (see Fig. 5) as observed by confocal microscopy. In addition, nucleophosmin and nucleolin were detected in about half of the specimens from 3 hpc. Both proteins were localized to small spherical bodies, which in the case of nucleolin in many instances displayed a central vacuole. None of the remaining antibodies resulted in labeling of nuclear entities.

Fourth Cell Cycle

At 3 hpc, both compact and vacuolized spherical masses of electron-dense fibrillar material were observed (Fig. 3). On some occasions, small secondary vacuoles were observed peripheral to the primary eccentric vacuole. The fibrillo-granular complexes were no longer observed. At 9 hpc, the eccentric vacuole of the spherical masses of electron-dense fibrillar material had enlarged and several peripheral secondary vacuoles had developed. Identical observations were made at 15 and 21 hpc. At 27 hpc, the formation of some of the presumptive components of the nucleolus was seen. Spherical masses of electron-dense fibrillar material presenting primary and secondary vacuoles were observed again. However, in these shell-like bodies, peripheral areas of increased electron density, representing

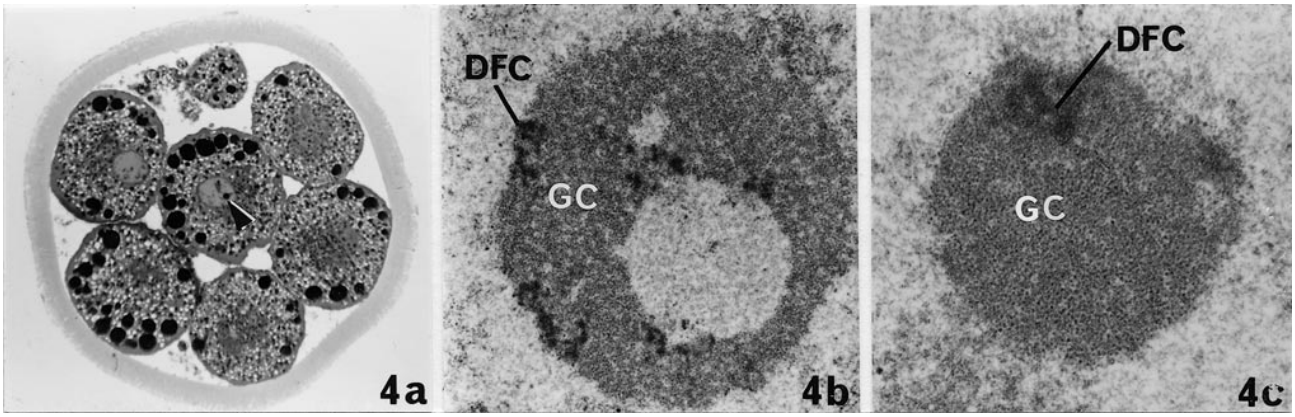

FIG. 4. The fifth cell cycle, i.e., the 16-cell embryo. **a**) An embryo fixed at 3 hpc. Note the nuclear entities (arrowhead). Magnification ×350. **b**) A spherical fibrillo-granular nucleolus presenting a DFC and a GC in an embryo fixed at 3 hpc. Magnification ×11 500. **c**) Same as **b** in an embryo fixed at 15 hpc. Magnification ×25 200.

the first signs of a presumptive DFC, appeared. On some occasions, the presumptive DFC delineated more electron-lucent fibrillar areas, representing the first signs of presumptive FCs. The FCs were associated with slightly condensed chromatin. A presumptive DFC was also noticed as small bodies lining the inside of the primary vacuole. At 33 hpc, similar observations were made. In addition, however, electron-dense granules, representing the first signs of the granular component (GC), had become embedded in the slightly loosened fibrillar substance of the electron-dense spherical masses. At 39 hpc, the major portion of these bodies had been transformed into presumptive GC.

By confocal microscopy, fibrillarin was localized to small compact spherical bodies up to 15 hpc in about half of the specimens. At 21 h, labeled nuclear entities were recorded in all embryos, and the labeled bodies displayed a central vacuole. At 33 and 39 hpc, fibrillarin was localized to more complex bodies showing several distinct intensely labeled foci embedded in a less intensively labeled matrix. UBF was localized to small discrete foci in about half of the specimens up to 21 hpc, when several foci appeared to cluster. At 27, 33, and 39 hpc, UBF was localized to complexes similar to those described for fibrillarin. The labeling was more discrete, however, than for fibrillarin. Nucleophosmin and nucleolin were localized to small discrete foci at 3 hpc, and at 15 hpc spherical bodies that typically displayed a central vacuole emerged. Up to 39 h, these shell-like bodies gradually enlarged. Topoisomerase I was detected for the first time at 15 hpc. It was localized to discrete foci that appeared to cluster at 33 and 39 hpc. RNA polymerase I was detected for the first time at 27 hpc in clusters of discrete foci. This labeling pattern was maintained throughout the cycle.

Fifth Cell Cycle

At 3 hpc, a spherical nucleolus composed of an abundant presumptive GC with strands of a peripherally located presumptive DFC was observed (Fig. 4). The nucleolus contained several large vacuoles. At 9 and 15 hpc, similar observations were made. However, the presumptive DFC was observed both peripherally and deep in the nucleolus, and, moreover, it delineated small FCs.

By confocal microscopy, fibrillarin was localized to complex bodies similar to those described for the previous cell cycle (Fig. 5). At 3 hpc, the structure of the bodies was more loose and irregular than at 9 and 15 hpc. UBF was localized to clusters of discrete foci throughout the cycle. Nucleophosmin and nucleolin were localized to spherical bodies, some of which displayed a central vacuole at 3 hpc. At 9 and 15 hpc, labeling was confined to large shell-like bodies. Occasionally, what appeared as fusion of shell-like bodies was noticed. Topoisomerase I and RNA polymerase I were localized to clusters of discrete foci throughout the cycle.

DISCUSSION

The preimplantation mammalian embryo represents an alternative model for understanding nucleolar formation. Thus, in the embryo, a protracted nucleologenesis lasting for several cell cycles occurs while the embryo is reprogrammed from meiotically imprinted to normal mitotic cell cycles. However, the information concerning rRNA transcription and the biosynthesis of ribosomes in the preimplantation bovine embryo is limited.

From the ultrastructural point of view, it is clear that functional fibrillo-granular nucleoli are established late during the fourth postfertilization cell cycle ([6,7], this study). During the initial three cell cycles, spherical masses of electron-dense fibrillar material and fibrillo-granular complexes are observed, none of which possess the structural characteristics of a functional nucleolus. The spherical masses of electron-dense fibrillar material have often been referred to as the nucleolus precursor bodies. In the investigation reported here, we demonstrated that these bodies during the first three cell cycles do not contain the full complement of proteins characterizing the nucleolus and that other entities as well may be involved in nucleolar formation in the preimplantation bovine embryo. We therefore avoid the use of the term nucleolus precursor body.

According to both the present investigation and the detailed study by Kopecny et al. [7], the transformation of the spherical masses of electron-dense fibrillar material into a fibrillo-granular nucleolus during the fourth cell cycle includes the formation of a primary eccentric and secondary peripheral vacuoles. We found that the next step in nucleologenesis was the establishment of a presumptive DFC surrounding tiny FCs peripherally in the vacuolated fibrillar mass at sites where condensed chromatin penetrated into it. Simultaneously, small portions of a presumptive DFC delineated the eccentric vacuole. Previously, Kopecny et al. [7] described the formation of a presumptive DFC delineating the vacuole, but the peripheral transformation has

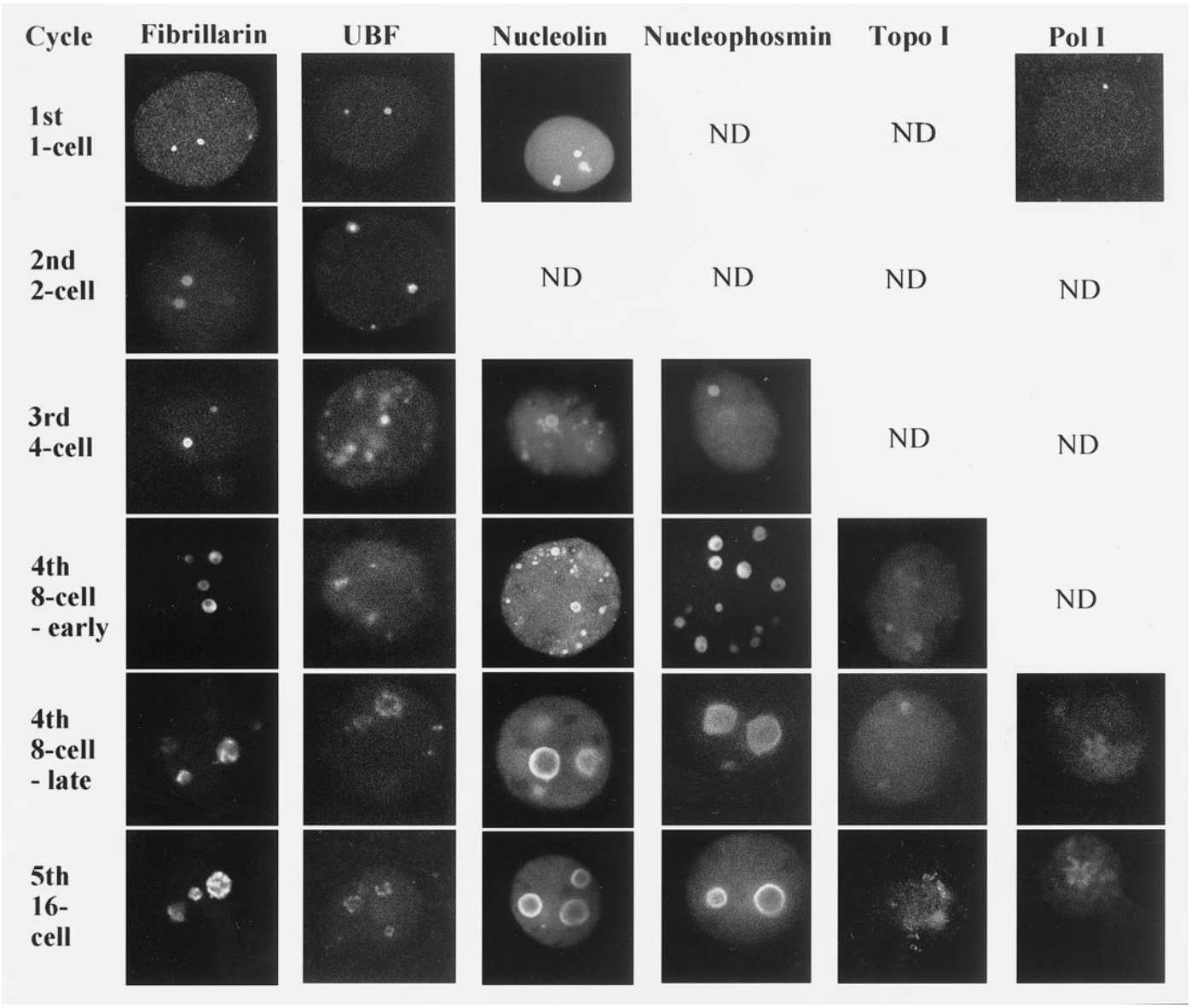

FIG. 5. Confocal laser scanning microscopy of single nuclei from 1-cell, 2-cell, 4-cell, early (3–21 hpc) and late (27 to 39 hpc) 8-cell, and 16-cell bovine embryos labeled with antibodies against the nucleolar proteins fibrillarin, UBF, nucleolin, nucleophosmin, topoisomerase I (Topo I), and RNA polymerase I (Pol I). ND, No immunocytochemical labeling detected.

never been addressed. Two models of transformation of the spherical fibrillar mass into a nucleolus during mammalian preimplantation nucleologenesis have been proposed: one in which the initial formation of the presumptive DFC occurs in the center, i.e., in the vacuole, and propagates in a centrifugal pattern, and one in which the presumptive DFC is formed in the periphery and propagates in a centripetal pattern. The bovine embryo has been considered a representative of the former model [7] whereas, e.g., the porcine embryo [25] and the mouse embryo have been considered representatives of the latter. The present investigation questions this concept in that the initial formation of the presumptive DFC and FCs was clearly demonstrated to occur peripherally in the vacuolized electron-dense fibrillar spheres in the bovine embryo as well.

During the initial two cell cycles in the preimplantation bovine embryo, the ultrastructural findings indicate that the spherical masses of electron-dense fibrillar material became dislocated into the fibrillo-granular complexes, a phenomenon described earlier [10]. The cell biological background for this observation is unknown but may signal that the fibrillo-granular complexes participate in nucleolar building or that the spherical masses of electron-dense fibrillar material may serve non-nucleolar functions during the initial cell cycles.

The nucleolar proteins investigated in the present study may be divided into two categories: proteins such as RNA polymerase I, UBF, and topoisomerase I that are characterized by a well-defined action during transcription; and others such as fibrillarin, nucleolin, and nucleophosmin that are characterized by their spatial localization within the nucleolus. The functions of the latter category of proteins are less defined.

Transcription of the rRNA genes is dependent on the activity of RNA polymerase I. Except for labeling in about half of the zygotes, the first immunocytochemical labeling for RNA polymerase I was detected during the fourth cell cycle at 27 hpc. Remarkably, this finding coincided with

the first ultrastructural detection of the presumptive DFC and FCs, which is in accordance with the fact that RNA polymerase I is localized mainly to the FCs and to some degree to the DFC in other cell types [26]. Moreover, the observed labeling of clusters of discrete foci corresponds well with the spatial distribution of the presumptive DFC and FCs observed in the electron microscope. In early mouse embryos, this enzyme is absent from the prenucleolar bodies in 1-and 2-cell embryos and is first detected in the 4-cell embryo, in which rRNA transcription is resumed in this species [27]. The labeling of entities in the zygotes noted in the present study probably represents targeting of maternally derived proteins to the pronuclei.

UBF is one of several transcription factors required for the binding of RNA polymerase I to DNA [28]. UBF is thought to bind to the promoter and to recruit another transcription factor, the promotor selectivity factor (SL1; [29]), thus forming a preinitiation complex to which RNA polymerase I can bind and initiate transcription [30]. UBF was immunocytochemically detected from the first cell cycle, in which it was found in small spherical bodies. Late during the fourth cycle, however, the labeling shifted to clusters of discrete foci compatible with the localization to the presumptive DFC and FCs as reported earlier for other cell types (for review see [11]). The detection of UBF before that of RNA polymerase I is in accordance with the sequential function of the two proteins in the transcription process as alluded to above.

A prerequisite for transcription of the rRNA genes is that the supercoiled DNA must be uncoiled. This process is mediated by topoisomerase I [31]. In the bovine embryo, this protein was immunocytochemically detected for the first time during the fourth cell cycle at 15 hpc, when it was localized to discrete foci that clustered at 33 hpc. Again, this spatial distribution is compatible with a localization to the presumptive DFC and FCs as reported earlier in other cell types [11].

Fibrillarin was first identified by human autoimmune sera from patients with scleroderma and was localized to the DFC and the FCs [21,26]. Fibrillarin is a small nucleolar ribonucleoprotein (snoRNP) associated with U3 small nucleolar RNA (snoRNA) [32,33] and with U8 and U13 snoRNAs [34,35]. SnoRNAs are believed to be involved in the processing of the primary rRNA transcript [36–39]. Fibrillarin was immunocytochemically detected already from the first cell cycle, and the protein was typically localized to small spherical bodies that vacuolized over the initial cell cycles. During the fourth cell cycle at 33 hpc, the labeling shifted to more complex bodies compatible with a localization to the presumptive DFC and FCs. In mouse embryos, immunofluorescent staining of fibrillarin was associated with the cortical region of the prenucleolar bodies in 2-cell embryos [40], i.e., one cell cycle before the expected onset of rRNA transcription in this species [27]. The fact that fibrillarin is detectable well before a functional nucleolus is established may signal that this protein serves a structural role during the assembly of the nucleolar compartment before it becomes involved in rRNA processing, in conjunction with the onset of rRNA transcription. Indeed, Ochs and Smetana [41] postulated that there is more than one population of fibrillarin: one with a structural role and another with a role in rRNA processing.

Nucleolin (C23) is a phosphorylated protein present in large amounts in nucleoli with active ribosomal biogenesis [42]. It has RNA-binding properties [43] and, furthermore, it is identical to the human DNA helicase IV, which unwinds RNA-RNA, DNA-DNA, and DNA-RNA duplexes [44]. The protein is associated with the primary rRNA transcripts, in particular with the 18S and 28S sequences. Nucleolin may act in promoting the functional secondary structures of 18S and 28S RNA that are necessary for the assembly of the preribosomal particles [45]. However, it is not part of the final product [46]. Nucleolin has been localized to the DFC and the GC [47]. Nucleophosmin (B23) may be involved in shuttling other proteins, such as nucleolin [48] and the nucleolar protein p120 [49], into the nucleolus. However, nucleophosmin also has DNA- and RNA-binding properties [50] and ribonuclease activity [51], and associates with the most mature nucleolar preribosomal RNP [52]. Therefore, it has been proposed that nucleophosmin, together with nucleolin, functions in assembly of preribosomal particles. Nucleophosmin has been localized to the DFC and the GC [47]. Nucleolin was immunocytochemically detected as early as the first cell cycle; it was not detected during the second cycle, but reappeared during the third. Over both the third, and especially the fourth, cell cycle, the bodies labeling for nucleolin and nucleophosmin became vacuolized and shell-like. The large shell-like structures established towards the end of the fourth cell cycle are compatible with a localization to the GC. Previous immunocytochemical studies of bovine embryos at the ultrastructural level during the fourth cell cycle indicate that nucleolin and nucleophosmin are not present in the compact fibrillar masses, i.e., the nucleolus precursor bodies [53]. Nucleolin becomes detectable in this entity with the formation of the primary vacuole, and nucleophosmin becomes detectable with the formation of the secondary vacuoles. The discrepancy between these data and ours may be due to the lower sensitivity of the immunocytochemical approach at the ultrastructural level. The lack of immunocytochemical detection of nucleolin during the second cell cycle can be explained in at least two ways. It may be due to a lack of relocalization of maternal proteins to the nucleus in sufficient concentrations for immunocytochemical detection during this cell cycle. On the other hand, it cannot be excluded that the protein detected during the first cell cycle is of maternal origin, that it is degraded during the second cell cycle, and that the protein detected during the third cycle is derived from transcription of the embryonic genome.

Several of the nucleolar proteins investigated in the present study were detected in one or more cell cycles before the establishment of a functional fibrillo-granular nucleolus during the fourth cell cycle. At present, it is unknown to which structural entities these proteins are localized. It is tempting to suggest that fibrillarin, nucleolin, and nucleophosmin, all of which during periods of embryonic development display shell-like labeling patterns, are localized to the spherical masses of electron-dense fibrillar material that also undergo shell-like transformation during the same periods. This aspect, however, awaits further elucidation by immunocytochemistry at the electron microscopic level.

Another matter of controversy is whether the proteins are synthesized de novo during initial embryonic development or whether they simply become detectable because of coalescence resulting in higher local concentrations. Furthermore, if the proteins are synthesized de novo, they may result from transcripts inherited from the oocyte or from de novo transcription of the embryonic genome. Thus, it is clear that although major transcription is not activated in the bovine embryo until the fourth cell cycle, a low level of transcription is seen from at least the second cycle [54].

It should be stressed that the present investigation was performed on in vitro-produced embryos. Previous reports have substantiated that the expression of certain genes is different in in vitro-produced bovine embryos as compared with their in vivo-developed counterparts [55–57]. Whether the same situation applies for the rRNA genes is unknown but presently under investigation.

In conclusion, the nucleolar protein compartment in in vitro-produced preimplantation bovine embryos is assembled over several cell cycles. In particular, RNA polymerase I and topoisomerase I are detected for the first time late during the fourth embryonic cell cycle, which coincides with the first recognition of the DFC, FCs, and GC at the ultrastructural level. It is our belief that the protracted nucleologenesis described in the preimplantation bovine embryo may prove useful as an alternative model for understanding nucleolar biology.

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