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Cytochemical and immunocytochemical ultrastructural study of nucleoproteins during nucleogenesis in 8-cell bovine embryos

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Summary — The aim of this study was to characterize embryonic nucleogenesis by determining the appearance and localization of acid argyrophilic, basic lysine-rich and histone proteins in 8-cell bovine embryos. Two silver staining techniques, ethanolic phosphotungstic acid (PTA) and immunocytochemical methods using specific antibodies, were applied at the ultrastructural level. The silver-stained proteins were detected at the onset of nucleogenesis on the periphery of the dense nucleolus precursor bodies (NPBs). The amounts of these proteins increased during the transformation of the NPBs into the fibrillogranular nucleolus. At this stage the well-developed dense fibrillar components encircling fibrillar centres showed intense staining. PTA-positive (basic lysine-rich) proteins were present within most nucleolar structures during nucleogenesis as well as in the chromatin. Histones H2B, H3 and H4 were concentrated throughout the chromatin including the nucleolus-associated chromatin. At the onset of nucleogenesis, histones were absent in the NPBs. The first weak histone labelling was detected in the multivacuolated NPBs, both in the fibrous mass as well as inside the vacuoles. Nucleolar histones appeared with the massive penetration of DNA into the NPBs. We suggest that nucleogenesis may serve as a criterion of normal early embryonic development and that the proteins involved in the process of nucleogenesis and transcription could be used as chemical markers of nucleolar function.

nucleolus / nucleoprotein / cattle / embryo

Résumé — Étude cytochimique et immunocytochimique ultrastructurale de nucléoprotéines pendant la nucléogénèse dans l'embryon bovin de 8 cellules. L'objet de cette étude est d'analyser la nucléogénèse en déterminant l'apparition et la localisation de protéines acides argyrophiles, basiques, riches en lysine et d'histones dans les embryons bovins de 8 cellules. Des techniques à l'argent, à l'acide phosphotungstique (PTA) éthanolique et 2 techniques immunocytochimiques ont été appliquées en microscopie électronique. Les protéines argyrophiles ont été détectées au début de la nucléogénèse à la périphérie des corps précurseurs de nucléole (NPB). La quantité de ces protéines augmente pendant la transformation du NPB en nucléole fibrogranulaire. À ce stade le composant fibrillaire dense bien développé entourant les centres fibrillaires montre une coloration intense. Des protéines basiques riches en lysine (PTA+) sont présentes dans la plupart des composants nucléolaires pendant la nucléogénèse et dans la chromatine. Les his-

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tones H2B, H3 et H4 sont concentrées dans la chromatine, y compris la chromatine associée au nucléole. Au début de la nucléogénèse, les histones sont absentes des NPBs. Le premier marquage faible est détectable dans les NPBs en cours de vacuolisation, dans la masse compacte et dans les valvoles (agrégats). Les histones nucléolaires accompagnent la pénétration massive d'ADN dans le NPB. Nous suggérons que la morphogénèse nucléolaire soit utilisée comme un critère du développement embryonnaire normal et que les protéines impliquées dans la nucléogénèse et la transcription soient utilisées comme marqueurs de la fonction nucléolaire.

nucléole / nucléoprotéine / espèce bovine / embryon

INTRODUCTION

Nucleoli are nuclear structures reflecting the state of activity and differentiation of each cell. They are the morphological expression of a complex machinery in which repetitive genes of rDNA are transcribed into rRNA, the latter being processed and packed into the ribosomal subunit.

During early mammalian embryonic development, functional nucleoli are absent. They develop gradually *via* the process of nucleogenesis linking morphological events with the onset of functional activity. This process of embryonic nucleogenesis has been described in a few domestic ruminants (Chartrain *et al*, 1987; King *et al*, 1988, 1989).

In cow embryos, the whole nucleogenesis process takes place at the 8-cell stage (Camous *et al*, 1986; Kopečný *et al*, 1989a). The authors have divided this process into 4 clear-cut stages: stage 1 (S1) — after fertilization, nucleolar precursor bodies (NPBs) are present as small, round-shaped structures, composed of a compact homogeneous mass of fibrils; stage 2 (S2) — a large central nucleoplasm-like vacuole develops in the NPBs; stage 3 (S3) — nucleolar differentiation proceeds with the formation of numerous small secondary vacuoles. Dense NPBs as well as vacuolated NPBs are in close association with perinucleolar chromatin; stage 4 (S4) — typical fibrillogranular nu-

cleoli contain fibrillar centers surrounded by dense fibrillar components and increasing amounts of granular component which becomes apparent.

It is now accepted that the 8-cell stage of early embryonic development in the cow is an important period during which nucleolar transformation takes place and the onset of transcriptional activity is detected for the first time (Kopečný *et al*, 1989a; Telford *et al*, 1990). We therefore concentrated our attention on this developmental stage.

The aim of the present study was to analyze the localization of: 1) acidic argyrophilic proteins; 2) basic lysine-rich proteins; and 3) histones, which are in direct relation to the nucleolar structures and are thought to play an important regulatory function in the process of transcription. By correlating the distribution and quantity of 3 types of nucleoproteins in nucleoli undergoing differentiation we hope to improve our knowledge of the relationship between these proteins and of their physiological role in nucleolar activity.

MATERIAL AND METHODS

The 8-cell bovine embryos ($n = 17$) originated from superovulated heifers. The animal were killed 3–4 d after the onset of oestrus. The embryos were flushed from the oviducts and carefully evaluated. Only those embryos showing perfect morphology were used for further experi-

ments. They were briefly precultured for 1–2 h in 0.1 ml culture medium (TC 199) as droplets under paraffin oil at 37.5°C in an atmosphere of 5% CO₂ in air (Fulka Jr *et al*, 1986). After this time interval, embryos were re-evaluated, washed briefly in PBS without protein and used for the following procedures.

Silver staining applied in toto before embedding in Epon 812

After brief glutaraldehyde fixation (2.5% for 30 min at 4°C), embryos were postfixed in Carnoy's solution (methanol / glacial acetic acid, 3:1, for 20 min at room temperature) and carefully washed in distilled water. Silver staining procedure (Likovsky and Smetana, 1981), using a mixture 100% AgNO₃ and formaldehyde developer 1:1 for 6–8 min at 60°C was applied. After repeated brief washing in distilled water, the samples were dehydrated in ethanol and embedded in Epon 812. One set of sections were stained with uranyl acetate and lead citrate, while the second set served as unstained controls.

Staining with ethanolic solution of phosphotungstic acid (PTA) before embedding in Epon 812

After glutaraldehyde fixation (2.5%, 2 h, 4°C), the embryos were dehydrated with an ethanol series and incubated for 16–18 h at 4°C in 3% PTA in absolute ethanol according to Sheridan and Barnett (1969). Samples were then washed in absolute ethanol and embedded in Epon 812. Grids with ultrathin sections were washed, dried and observed without further contrast.

Embedding in the hydrophilic resin Lowicryl K4M

The procedure was performed according to the method described by Altman *et al* (1984). After glutaraldehyde fixation and brief washing, samples were dehydrated in a series of ethanol, infiltrated in Lowicryl and polymerized by indirect ul-

traviolet irradiation. Ultrathin sections were mounted directly on gold grids and processed for the following step.

Silver staining with a 1-step method according to Howell and Black (1980)

Grids with sections were floated for 10 min at room temperature in Carnoy's solution and after washing, a mixture containing 2 vol 50% AgNO₃ and 1 vol 1% gelatin in 1% formic acid was applied for 10 min at room temperature. After washing and drying, the sections were not post-stained.

Immunocytochemical demonstration of histones

Reagents

Rabbit specific anti-histone sera which had been previously tested, H2B (Müller *et al*, 1985), H3 (Müller *et al*, 1982) and H4 (Müller *et al*, 1987), were used. The anti-rabbit IgG antibodies were: goat anti-rabbit IgG conjugated peroxidase; donkey biotinylated anti-rabbit IgG; and 15 nm colloidal gold streptavidin-coated particles (Amersham). Buffer A was composed of PBS pH 7.2, 0.01% Tween, and 10% decomplexed goat serum. Buffer B for washing was composed of PBS pH 7.2 and 0.01% Tween.

Peroxidase method

Ultrathin Lowicryl sections preincubated in buffer A for 20 min at 20°C were treated for 16 h at 4°C with specific antisera diluted 1:500 in the same buffer. Following extensive washing in buffer B, they were incubated for 1 h at 20°C in 1:50 goat anti-rabbit IgG coupled to horseradish peroxidase in buffer A. After several washes in PBS, the peroxidase was revealed by 3 min incubation in 22 mg diaminobenzidine + 175 ml PBS + 3 µl 5% H₂O₂ followed by extensive washing in PBS and distilled water and drying of the sections. The final peroxidase-diaminobenzidine product was osmicated for 45 min at 60°C in OsO₄ vapor.

Colloidal gold method

The pre-incubation, washing and incubation with specific antisera were similar to the peroxidase

method. The second antibody was anti-rabbit IgG coupled to biotin diluted 1:50 in buffer A. After 1 h at 20°C, it was washed carefully in buffer B. The sections were finally incubated for 30 min at 20°C in 1:50 colloidal gold-streptavidin in PBS, and washed extensively in PBS and distilled water.

For both methods, the negative controls were carried out. The possible nonspecific absorption of either the antibody or the contrasting agents were tested by replacement of the first antiserum by normal rabbit serum, and or by replacement of one of the steps of the technique by PBS incubation.

Ultrathin sections were prepared on an Ultratome III LKB Nova ultramicrotome and examined under a JEOL 100 CX II electron microscope at 80 kV.

RESULTS

Four main stages of nucleologenesis in the early cow embryo were observed according to the ultrastructural morphology described by Kopečný *et al* (1989a).

S1: this is characterized by the presence of rounded NPBs, which persist up to the beginning of the 8-cell stage.

S2: a large central lucid area develops in the NPB. According to the recent nucleolar terminology, this entity will be termed the NPB-vacuole.

S3: the primary vacuole loses its sharply delineated boundary, and a clump of chromatin appears within it. A continuity with perinucleolar chromatin is visible. Around the secondary vacuoles, the dense fibrillar component can be distinguished as well as the first granular component.

S4: typical nucleolonemal structure appears as well as small fibrillar centers.

Silver staining

Localization of acidic argyrophilic proteins was detected in dense NPB of stage 1 within a convex-shaped area (lenticle) at the NPB periphery (fig 1).

During stage 2 silver grains were present in the peripheral filamentous area around the central vacuole (fig 2). The inside of the central vacuole was devoid of silver.

Stage 3 involved the formation of small secondary vacuoles which remained free

Fig 1. Silver staining; pre-embedding silver staining, sections were contrasted; NPB with the lenticle containing silver positive material (arrow); x 80 000.

Fig 2. Silver staining; post-embedding silver staining on Lowicryl section without any further contrast; NPB with central vacuole (CV): the peripheral part of the fibrous material contains the fine silver grains; x 42 000.

Fig 3. Silver staining; Pre-embedding silver staining, sections were contrasted; NPB with secondary vacuoles (SV); dense fibrillar component surrounding vacuoles is impregnated with silver; x 76 000.

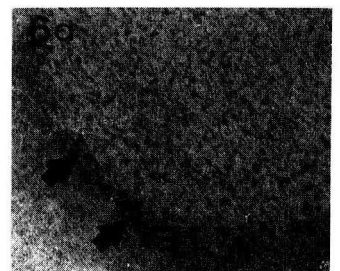
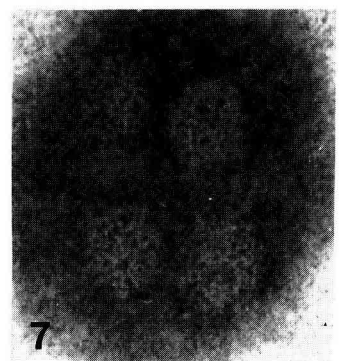
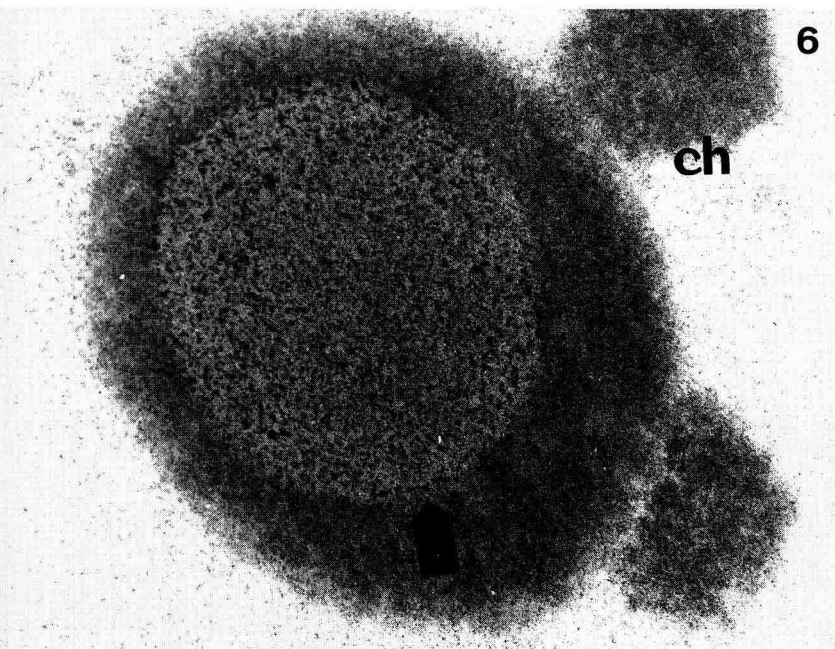
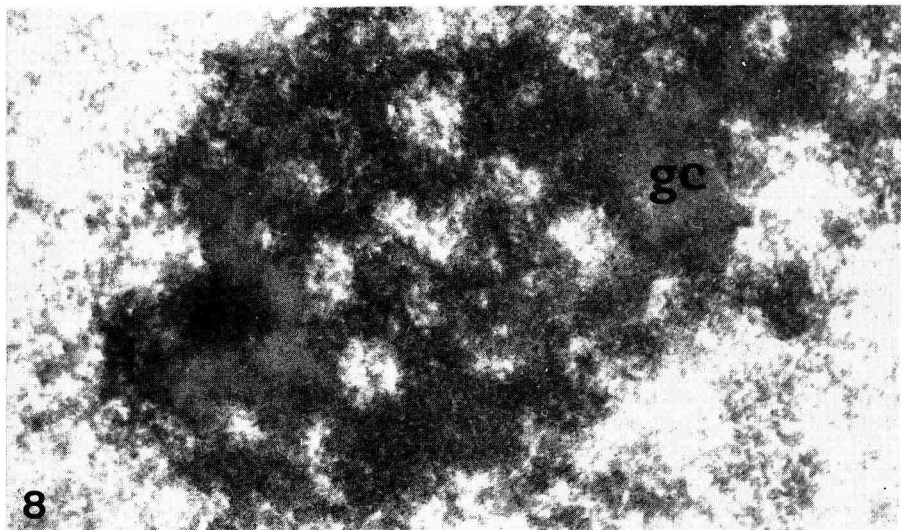
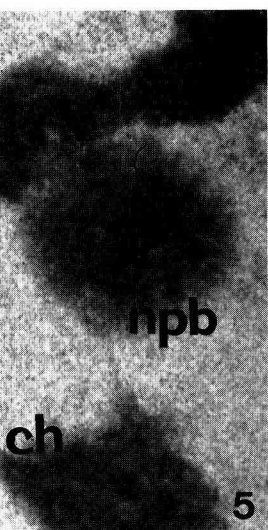
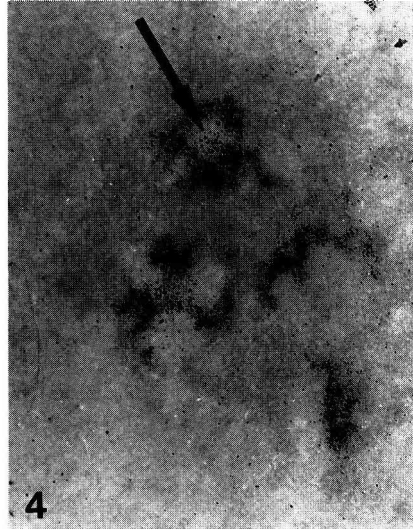
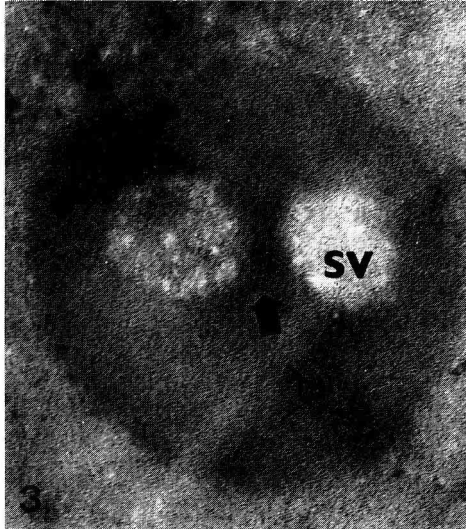
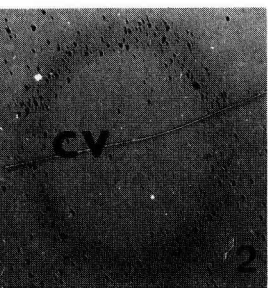
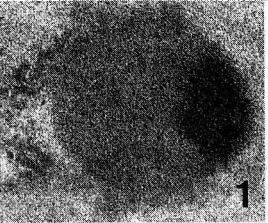
Fig 4. Silver staining; post-embedding silver staining on Lowicryl section without any further contrast; part of fibrillogranular nucleolus; silver granules are accumulated within the dense fibrillar component and in a small fibrillar center (arrow); x 44 000.

Fig 5. PTA staining; dense nucleolus precursor body (npb) and the masses of chromatin (ch) are intensely stained; x 39 000.

Fig 6. PTA staining; NPB with a central vacuole which is filled with many clumps of PTA-positive material (arrow); NPB wall and nucleolus-associated chromatin (ch) are also stained; x 65 000; PTA-positive clusters including the inner vacuolar surface; x 65 000.

Fig 7. PTA staining; multivacuolated NPB with PTA-positive material inside secondary vacuoles; x 62 000.

Fig 8. PTA staining; fibrillogranular nucleolus; strongly stained dense fibrillar component and fibrillar centers; gc: granular component; x 60 000.



of silver. Irregular silver deposits were seen in the dense fibrillar component surrounding the vacuoles (fig 3).

At the end of the 8-cell stage (stage 4), numerous silver grains were deposited on the dense fibrillar component while fibrillar centers appeared to be less stained (fig 4). The granular component was devoid of silver staining.

PTA staining

Basic lysine-rich proteins identified using ethanolic PTA were present in high quantities within the S1 compact rounded NPB (fig 5). Clumps of condensed chromatin closely associated with NPB (nucleolus-associated chromatin) also displayed strong PTA staining (fig 5).

At the following stage of nucleologenesis (S2), NPBs were characterized by a large central vacuole containing many PTA-staining patches. Similar patches were arranged on the inner vacuolar surface (fig 6, 6a).

Multivacuolated NPBs (S3) showed secondary vacuoles filled with clumps of PTA-positive material (fig 7). In several areas increased staining was visible, especially around and amongst the vacuoles.

During the last step (S4) small fibrillar centers could also be recognized. All nucleolar components were PTA-positive with a different intensity of staining. The

dense fibrillar component surrounding the fibrillar centers as well as the fibrillar centers themselves showed a very strong reaction, while the granular component was stained to a lesser degree (fig 8).

Demonstration of histones

The staining patterns of histones H2B, H3 and H4 obtained in the present study largely coincide with chromatin distribution within the nucleus. The labelling was located over the nucleolus-associated chromatin (figs 9–12). At the beginning of nucleologenesis (S1), the compact NPB was without any labelling as well as those NPBs containing a large central vacuole (fig 2). A weak labelling of histones was first detected within the multivacuolated nucleoli (S3) in various localization: 1) in transition areas between nucleolus-associated chromatin and the nucleolus itself (fig 9); 2) within NPB = filamentous material (fig 11); and 3) inside the secondary vacuoles (fig 12).

DISCUSSION

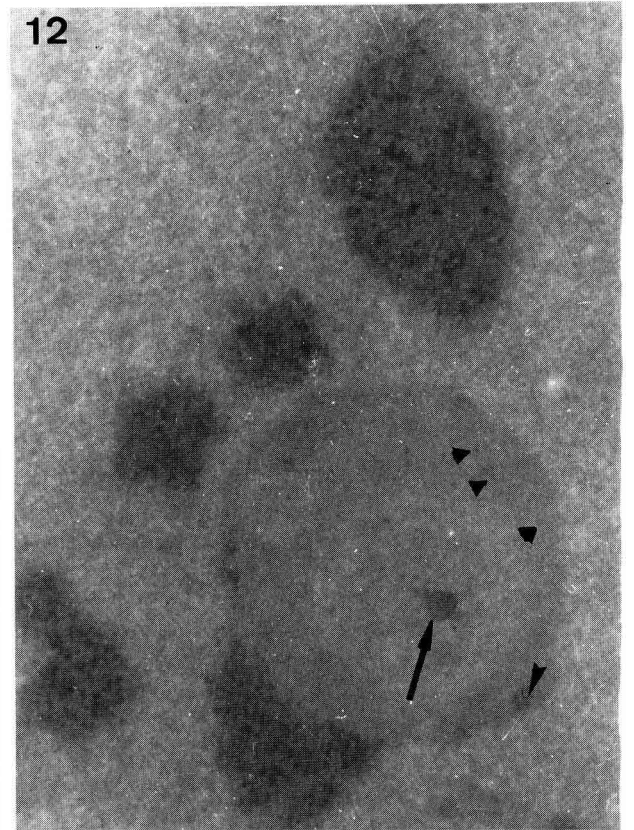
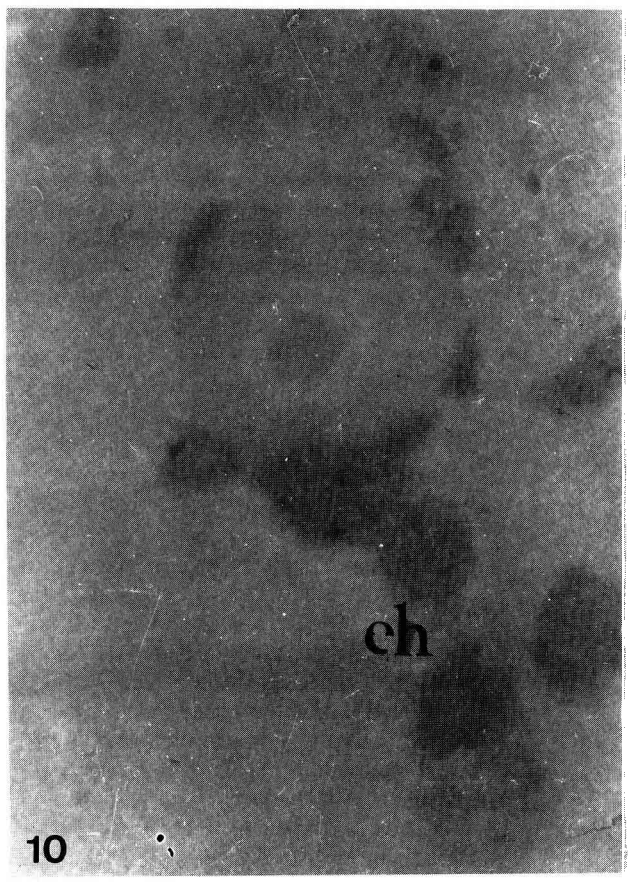
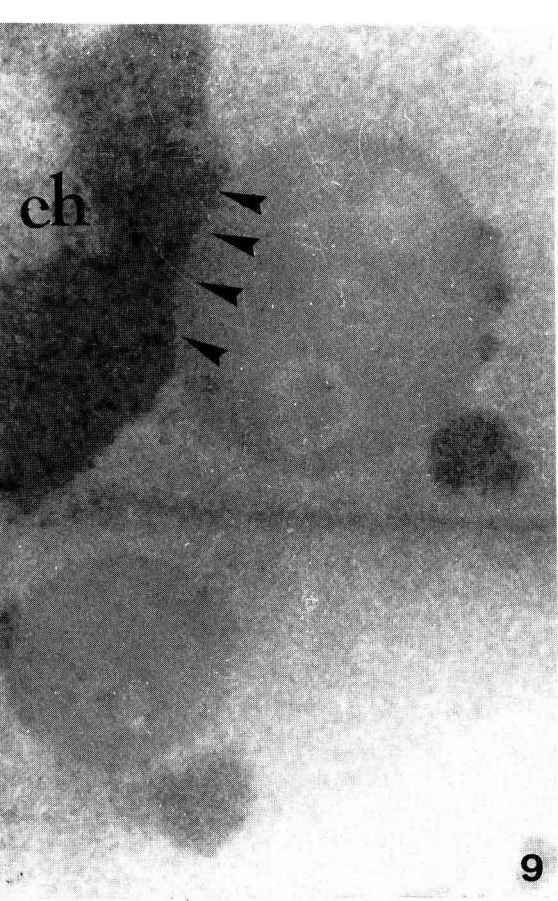
In this report we extend our previous observations which described silver-stained nuclear proteins in preimplantation cow embryos (Antalíková and Fulka Jr, 1990). Silver staining techniques adapted for electron microscopy have enabled us to local-

Fig 9. Immunocytochemical demonstration of histone; histone H3, transitional area between labelled nucleolus-associated chromatin (ch) and multivacuolated NPB; weak labelling at the periphery of NPB (small arrowheads); x 30 000.

Fig 10. Immunocytochemical demonstration of histone, histone H4; weak labelling of nucleolus-associated chromatin (ch); x 30 000.

Fig 11. Immunocytochemical demonstration of histone; histone H2B; weak labelling within the chromatin and in the fibrous wall of vacuolated NPB (arrowheads); x 30 000.

Fig 12. Immunocytochemical demonstration of histone; histone H3; labelling of histones in chromatin and in the peripheral part of NPB (arrow heads) as well as inside the secondary vacuole (arrow); x 40 000.



ize metal deposits associated mainly with the dense fibrillar components. These deposits were therefore thought to indicate the acquisition of rDNA transcriptional activity (Fakan and Hernandez-Verdun, 1986). Based on autoradiographic data (Kopečný, 1989), it is known that the onset of transcription in bovine embryos takes place during stage 3 of nucleogenesis, *ie*, within the multivacuolated NPB. Our observations indicate that the presence of acidic argyrophilic proteins precedes the onset of transcription, since they are already clearly detected in stages 1 and 2 of nucleogenesis. Similarly, Takeuchi and Takeuchi (1986) also detected silver-stained proteins in mouse embryos before the onset of the transcriptional activity. King *et al* (1989) explained these findings by 3 possible interpretations: 1) pre-ribosomal particles present in the ooplasm associate with the as yet non-functioning nucleolus; 2) the silver deposits are associated with a very low level of rRNA gene activity; or 3) there is lack of silver-staining specificity.

Since silver staining also takes place in nucleoli without transcriptional activity, it cannot be considered as a general marker of nucleolar activity. Nevertheless, the increasing amount and density of silver deposits (aggregates) within the dense fibrillar component which are involved in ribosomal RNA synthesis and processing suggests some involvement of acidic argyrophilic proteins in the building of the pre-ribosomal RNP machinery.

The specificity of PTA staining has been validated by several authors. Bloom and Aghajanian (1968) demonstrated that the treatment of nervous tissues with a 100% ethanol PTA solution resulted in intense staining of proteins rich in lysine, arginine and histidine. Courtens and Loir (1981) have reported that the PTA method may be specific for lateral lysine residues of proteins in mammalian sperm nuclei.

Takeuchi (1986) showed that in the growing oocytes engaging in active rRNA synthesis, the nucleolar dense fibrillar component and also the fibrillar centers were intensely stained with PTA; the granular component was only slightly stained. In the course of embryonic nucleogenesis we found relatively strong PTA staining in the dense NPB, in the fibrous mass of univacuolated NPB and inside the primary and secondary vacuoles. In the fibrillogranular functional nucleolus, the dense fibrillar component is stained most intensely. Results obtained with PTA staining are very similar to those obtained with silver staining. Both types of proteins are localized in the dense fibrillar component. It is possible that basic PTA-positive proteins which colocalize with the acidic argyrophilic proteins may have a cooperative regulatory function in transcription and ribosome synthesis.

Our study demonstrates that all types of histones tested are present, especially in chromatin structures. However, the initial phases of nucleogenesis (stages 1 and 2) seem to be devoid of histones. In multivacuolated NPBs histones may be present in the nucleolar periphery and in the vacuoles. The presence of histones within the NPBs is probably associated with massive penetration of DNA into the NPBs in this time. Penetration of embryonic DNA into the NPB is the key event of nucleogenesis in early embryo development (Tesařík *et al*, 1987; Kopečný *et al*, 1989b).

The low labelling within the NPB in our material is probably a result of the small quantity of histones which may well be below the sensitivity of the peroxidase assay.

Taken together, all these methods provide a clear cytochemical picture of the differentiating nucleolus (fig 13). In brief, acidic argyrophilic proteins accumulate preferentially in the dense fibrillar component, whereas basic proteins and more specifically histones are mostly associated













	Ag+ proteins	PTA+ proteins	histones H2B, H3, H4
S1 nucleolar precursor body (NPB)			
S2 NPB with central vacuole			
S3 nucleolus with secondary vacuoles			
S4 fibrillogranular nucleolus			

Fig 13. Summary of results obtained on the localization of Ag+ proteins, PTA+ proteins and histones in stages S1 to S4. Ag+ proteins are found in a lenticle (S1), within the material of the wall encircling the central vacuole (S2) and in the forming dense fibrillar component (S3 and S4). PTA+ proteins are localized in associated chromatin at all stages and in NPB (S1), in the wall of the vacuoles (S2 and S3), in the vacuoles themselves (patches) and mostly in the dense fibrillar component and the fibrillar centers (S4). Histones are almost entirely restricted to the chromatin at all stages.

with nucleolar chromatin. Our analysis of nucleogenesis will probably be very useful for the evaluation and understanding of changes that occur during nuclear reprogramming, especially following micromanipulation procedures such as nuclear transplantation and the production of cloned embryos.

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