Development 101, 777–791 (1987)
Printed in Great Britain © The Company of Biologists Limited 1987

High-resolution autoradiographic localization of DNA-containing sites and RNA synthesis in developing nucleoli of human preimplantation embryos: a new concept of embryonic nucleologenesis

JAN TESAŘÍK[1,3]*, VÁCLAV KOPEČNÝ[2], MICHELLE PLACHOT[3] and JACQUELINE MANDELBAUM[3]

[1]*Station Centrale de Physiologie Animale, Jouy-en-Josas, France*
[2]*Research Institute of Animal Production, Prague, Czechoslovakia*
[3]*INSERM, U 173, Hôpital Necker-Enfants Malades, Paris, France*

* Address for reprints: Laboratory of Human Reproduction, First Department of Obstetrics and Gynecology, Faculty of Medicine, J E. Purkyně University, nám. Sov. hrdinů 11, 66233 Brno, Czechoslovakia

Summary

Human embryos from the 2-cell to the morula stage, obtained by *in vitro* fertilization, were incubated with [^{3}H]thymidine or [^{3}H]uridine so as to achieve labelling of all replicating nuclear DNA and the newly synthesized RNA, respectively. The label was localized in different structural components of developing nucleoli using electron microscopic autoradiography. Careful study of the relationship between the structural pattern and nucleic acid distribution made it possible to define four stages of embryonic nucleologenesis. *Homogeneous nucleolar precursors* (i) consist of nucleolar matrix elements appearing as filaments of 3 nm thickness, (ii) do not contain recently replicated DNA and (iii) lack RNA synthetic activity. Penetration of DNA into these bodies is a key event leading to their transformation into *heterogeneous nucleolar precursors*. In addition to the 3 nm matrix filaments, two types of 5 nm fibrillar components can be recognized in them. The denser type contains DNA and is the site of nucleolar RNA synthesis, while the more loosely arranged 5 nm fibrils are not labelled with [^{3}H]thymidine and apparently represent the newly produced pre-rRNA detached from the transcribing rDNA filament. *Compact fibrillogranular nucleoli* are characterized by the first appearance of the granular component and reduction of the nontranscribing part of the fibrillar component, both indicating the activation of the machinery for rRNA processing. Finally, the granular component is most evident in *reticulated nucleoli*, occupying mostly the inner parts of their nucleolonema, while the transcription sites tend to be located at the nucleolar periphery. Our findings advocate a unique concept of embryonic nucleologenesis, different from any other nucleolar event during the cell cycle of differentiated cells. This developmental pattern is characterized by a gradual activation of rRNA synthesis and processing, mediated by progressive association of rDNA and, later on, the newly formed pre-rRNA with pre-existing nucleolar matrix elements that are originally topically separated from nucleolar organizer regions. This model may have a general validity in early animal embryos despite some interspecies variability in the timing of individual steps and resulting structural peculiarities.

Key words: nucleolar development, preimplantation embryo, DNA, RNA, human, high-resolution autoradiography.

Introduction

The very early embryonic development of virtually all animals is characterized by the absence of transcription of embryonic genome, including ribosomal genes and, consequently, by the absence of nucleoli which are known to be sites of ribosomal gene transcription. Nucleologenesis in the early embryo involves, first,

the establishment of the typical nucleolar structure (for a review, see Geuskens & Alexandre, 1984) and cytochemistry (Engel, Zenzes & Schmid, 1977; Hansmann, Gebauer, Bihl & Grimm, 1978) and, second, the beginning of nucleolar functional activity reflected by measurable rRNA synthesis and production of ribosomes (Knowland & Graham, 1972; Clegg & Pikó, 1982). Most of these events occur at the 2-cell stage of mouse embryonic development. We have shown recently that, in the human embryo, major nucleologenetic changes take place no earlier than the fourth cell cycle (Tesařík, 1987; Tesařík *et al.* 1986*a*) when the nucleolar functional activity is also assumed, as manifested by detectable production of new ribosomes (Tesařík, Kopečný, Plachot & Mandelbaum, 1986*b*). It has been demonstrated that nucleolar precursors, appearing as dense, round and homogeneous intranuclear bodies in human 2- and 4-cell embryos, contain no embryonic DNA and that the penetration of DNA into these bodies from adjacent chromatin is an early step in the nucleologenetic process (Tesařík *et al.* 1986*a*). In the latter study using a combination of electron microscopy and light microscopic autoradiography, correlations were made between DNA penetration together with the onset of RNA synthesis in the transforming nucleolar precursors and the ultrastructures of these entities; however, a precise ultrastructural localization of the penetrating DNA and newly synthesized RNA was not possible with this methodological approach.

In this study, we performed a high-resolution autoradiographic analysis of the developing nucleoli of human preimplantation embryos cleaving *in vitro* in order to find structural correlates for DNA and RNA synthesis in different phases of nucleologenesis. The ultimate aim of this investigation was to define the structure–function correlation of this still rather poorly understood developmental process which is a subject of some controversial speculations, not only in human embryos but in animal embryos in general.

Materials and methods

Source of embryos

Embryos used in this study were surplus concepti from a human *in vitro* fertilization programme. The criteria for allocation of an embryo to experimental work were those described by Tesařík *et al.* (1986*a*,*b*). The embryos were obtained by *in vitro* fertilization of preovulatory oocytes recovered in ovarian cycles stimulated with clomiphene citrate, human menopausal gonadotropin and human chorionic gonadotropin. Details of ovarian stimulation, *in vitro* fertilization and embryo culture were reported earlier (Plachot *et al.* 1985). 20 embryos were the subject of the present investigation; four of them were at the 2-cell stage, four at the 4-cell stage, two at the 6-cell stage, five at the 8-cell stage and five at the early-morula stage (12- to 16-cell stage). All of the embryos showed a regular cleavage (Plachot *et al.* 1985) and were morphologically normal.

Media and culture conditions

All incubations and washes were performed using complete Menezo B_2 medium (API System, Montalieu-Vercieu, France) equilibrated with 90 % N_2, 5 % O_2 and 5 % CO_2. For nucleic acid labelling embryos were incubated at 37°C in a culture system filled with the above gas mixture.

DNA labelling

The strategy of DNA labelling was, in general, that used in our previous study (Tesařík *et al.* 1986*a*). Briefly, embryos were incubated in medium supplemented with 1 μCi ml^{-1} of [^{3}H]thymidine (29 Ci mmol^{-1}; Commissariat à l'Energie Atomique, Gif-sur-Yvette, France) for 24 h. This incubation time is longer than the average length of a cell cycle in human preimplantation embryos, so ensuring labelling of all nuclear DNA capable of replication. In fact, all embryos allocated to DNA labelling in this study doubled their number of blastomeres during the labelling period. Embryos were then thoroughly washed to remove unincorporated radioactivity and processed for electron microscopy (see below).

RNA labelling

For RNA labelling embryos were incubated in medium supplemented with 200 μCi ml^{-1} of [5-^{3}H]uridine (27 Ci mmol^{-1}; Commissariat à l'Energie Atomique, Gif-sur-Yvette, France) for 30 min. An incorporation time of 1 h was used in a limited number of cases in order to visualize the newly produced RNA apart from the sites of synthesis. Embryos were then washed thoroughly in medium and processed for electron microscopy.

Electron microscopic autoradiography

Labelled embryos were fixed in a solution containing 2·5 % glutaraldehyde and 0·6 % paraformaldehyde in 0·06 M-cacodylate buffer (pH 7·2) for 1 h. Fixed specimens were washed in 0·125 M-cacodylate-buffered 0·2 M-sucrose for 30 min, followed by a second fixation using the osmium–ferricyanide method (McDonald, 1984), dehydration in a graded ethanol series, treatment with propylene oxide and embedding in Epon. In the case of RNA-labelled embryos, 0·1 % (w/v) cold uridine (Prolabo, Paris, France) was added both to the aldehyde fixative and washing solution, and the washing period between aldehyde fixative and osmication was extended to 24–48 h. This prolonged washing was carried out at 4°C.

After polymerization, Epon blocks were sectioned on an Ultrotome III LKB ultramicrotome to give alternate series of thick (1 μm) and thin (70–100 nm) sections throughout the embryo. Thick sections were stained with methylene blue and examined in a light microscope so as to choose appropriate section planes for autoradiography. Thin sections cut at section planes of interest and gathered on nickel–palladium 150-mesh grids were coated with Ilford L.4 nuclear liquid emulsion using the loop technique. Following a 6-month exposure, autoradiographs were developed with D19, stained with aqueous uranyl acetate and

lead citrate, and examined in a Philips 300 electron microscope.

Nomenclature

The standard nomenclature (Goessens, 1984; Jordan, 1984) was used to denote different structural components of developing nucleoli.

Results

Structural background and timing of major nucleologenetic events

The ultrastructural changes accompanying the transformation of nucleolar precursors into mature nucleoli during early human embryogenesis represent a continuous process in which, however, several landmarks can be fixed. When applying such an approach to the definition of major intermediate steps of the nucleologenetic process, the latter can be characterized as progressing from the stage of *homogeneous nucleolar precursors* through the stages of *heterogeneous nucleolar precursors* and *compact fibrillogranular nucleoli*, and ending by the formation of *reticulated nucleoli*.

Homogeneous nucleolar precursors are spherical bodies of uniform structure consisting of a dense network of 3 nm-thick filaments.

Heterogeneous nucleolar precursors are characterized by the presence of distinct regions of greater electron opacity containing densely packed fibrils of 5 nm thickness.

Compact fibrillogranular nucleoli differ from the preceding stage by the first appearance of the granular component.

Reticulated nucleoli are larger as compared to all preceding stages and consist of nucleolonema with fibrillar and granular components surrounded by nucleolar interstices. Fibrillar centres may be present.

During the first three cell cycles of the human preimplantation embryo only the homogeneous nucleolar precursors are present. Nucleologenesis starts no earlier than the fourth cell cycle and is complete, in most blastomeres, by the end of the following cell cycle. However, the occurrence of individual blastomeres showing retardation of the nucleolar development is rather frequent. Such blastomeres are not included in this study. Hence, the homogeneous nucleolar precursors described here belong to 4-cell embryos, the heterogeneous nucleolar precursors and compact fibrillogranular nucleoli to 8-cell embryos, and reticulated nucleoli to morulae.

Localization of labelled DNA

(1) *Homogeneous nucleolar precursors*

No labelled DNA could ever be detected in the interior of these bodies which represent the starting point for the nucleologenetic process (Fig. 1). The efficiency of [^{3}H]thymidine incorporation into nuclear DNA was proven by intense labelling of nuclear chromatin (Figs 1, 2) strongly contrasting with the unlabelled cytoplasm (Fig. 2). Labelled chromatin was often seen to be in close apposition to the homogeneous nucleolar precursors; there was, however, no apparent accumulation of chromatin around these bodies (Fig. 1).

(2) *Heterogeneous nucleolar precursors*

Accumulation of labelled chromatin around nucleolar precursors was an early sign of transformation of the homogeneous form of this structure into the heterogeneous one (Fig. 2). As is also evident in Fig. 2, the labelled DNA associated with heterogeneous nucleolar precursors was not only in close contact with their periphery, but penetrated into them. The depth of DNA penetration was variable; the label was sometimes confined to their peripheral regions (Fig. 2); while, at other times, it could be observed deeper within these bodies (Fig. 3). Irrespective of the progression of DNA penetration, however, the regions infiltrated by DNA were ultrastructurally very uniform. Both at low (Fig. 2) and medium magnifications (Fig. 3) these DNA-containing sites were always the most electron-dense regions of the heterogeneous nucleolar precursors. At high magnifications (Figs 4, 5) extremely densely packed fibrils, 5 nm thick, could be recognized in those regions where in autoradiographs the structural background was not completely obscured by silver grains; it was also evident that the heterogeneous nucleolar precursors contained rather vast fibrillar regions which were devoid of label. These regions had a little lower electron density than the labelled sites, but they also consisted of 5 nm thick fibrils (though somewhat less-densely packed) and could be easily distinguished from other regions made up exclusively of the 3 nm filaments (Figs 4, 5).

(3) *Compact fibrillogranular nucleoli*

Somewhat surprisingly, and in contrast to heterogeneous nucleolar precursors at the later stages of their development, labelled DNA became once again preferentially localized in peripheral regions of these newly formed nucleoli (Fig. 6). As in the previous stage, labelling of adjacent chromatin remained very strong. At higher magnifications (Fig. 7) the fine-

structural appearance of the DNA-containing nucleolar sites was the same as in the case of heterogeneous nucleolar precursors. The label was localized in the highly electron-dense regions containing extremely densely packed 5 nm fibrils. Somewhat less-densely arranged 5 nm fibrils filled the unlabelled part

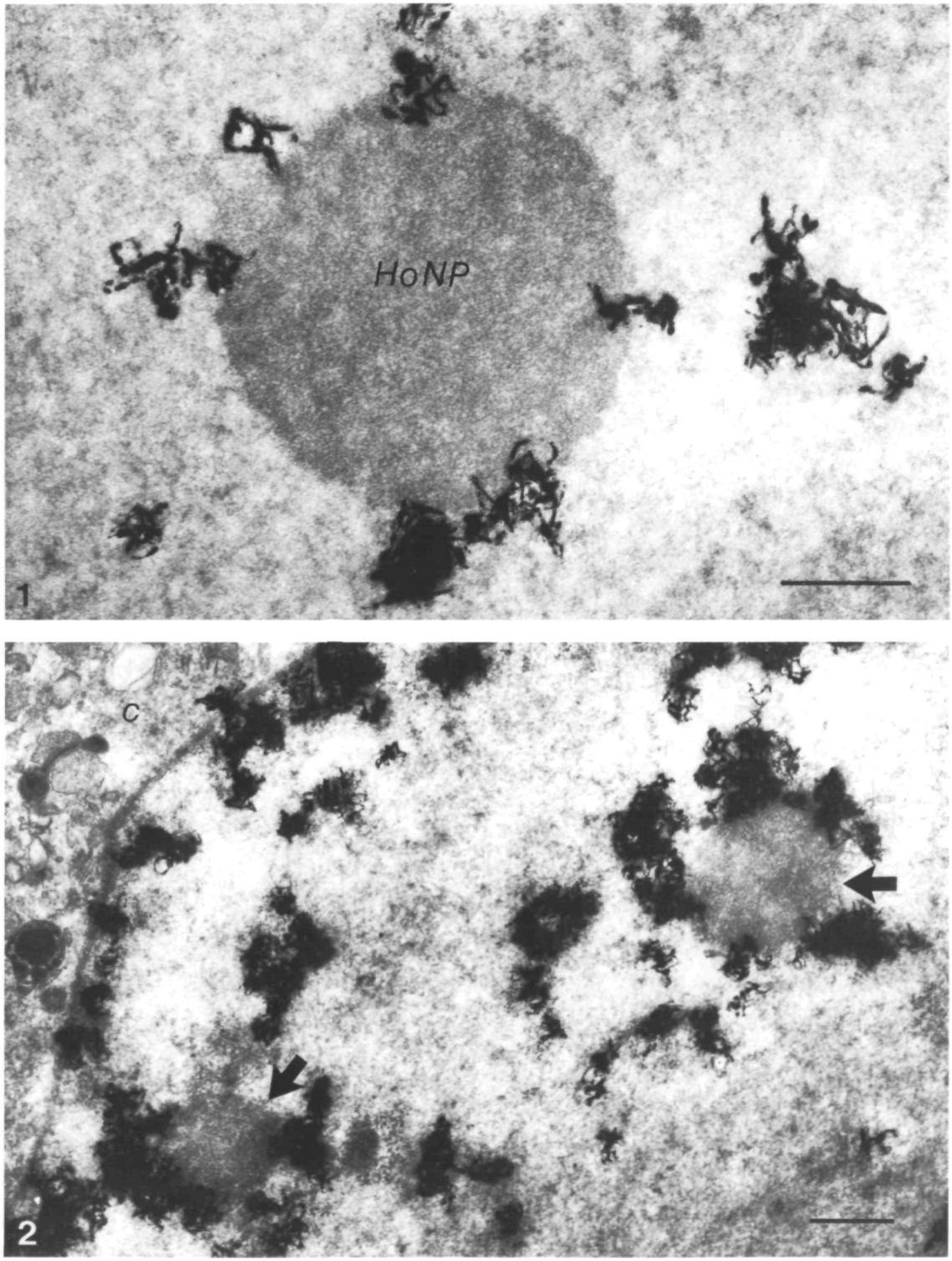


Fig. 1. Homogeneous nucleolar precursor (*HoNP*) of a 4-cell embryo labelled with [^{3}H]thymidine. Note the labelling of adjacent chromatin contrasting with the absence of label within the body. ×40 000; bar 0·5 μm.

Fig. 2. Part of the nucleus of an 8-cell embryo labelled with [^{3}H]thymidine showing two nucleolar precursors (arrows) at the beginning of DNA penetration. Note the accumulation of labelled DNA around the nucleolar precursors and the absence of label in the cytoplasm (*C*). ×15 000; bar 1 μm.

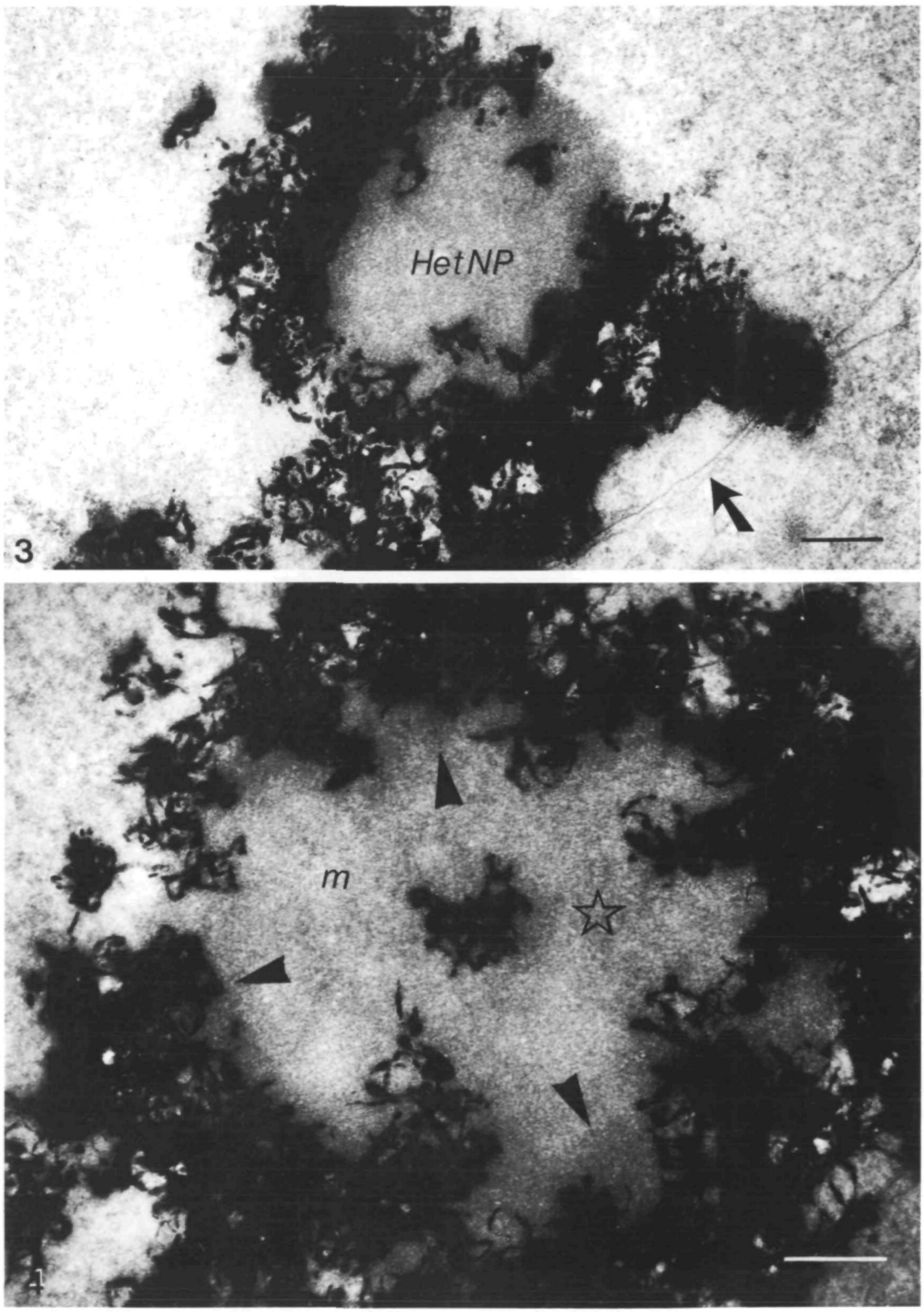


Fig. 3. Heterogeneous nucleolar precursor (*HetNP*) of an 8-cell embryo labelled with [^{3}H]thymidine showing penetration of labelled DNA into its interior. Arrow indicates the nuclear envelope. ×50 000; bar 0·25 μm.
Fig. 4. Heterogeneous nucleolar precursor of an 8-cell embryo labelled with [^{3}H]thymidine. Label is present in the denser parts of the fibrillar component (arrowheads), while its less-dense regions (star) as well as those formed by the 3 nm matrix filaments (*m*) are devoid of label. ×80 000; bar 0·2 μm.

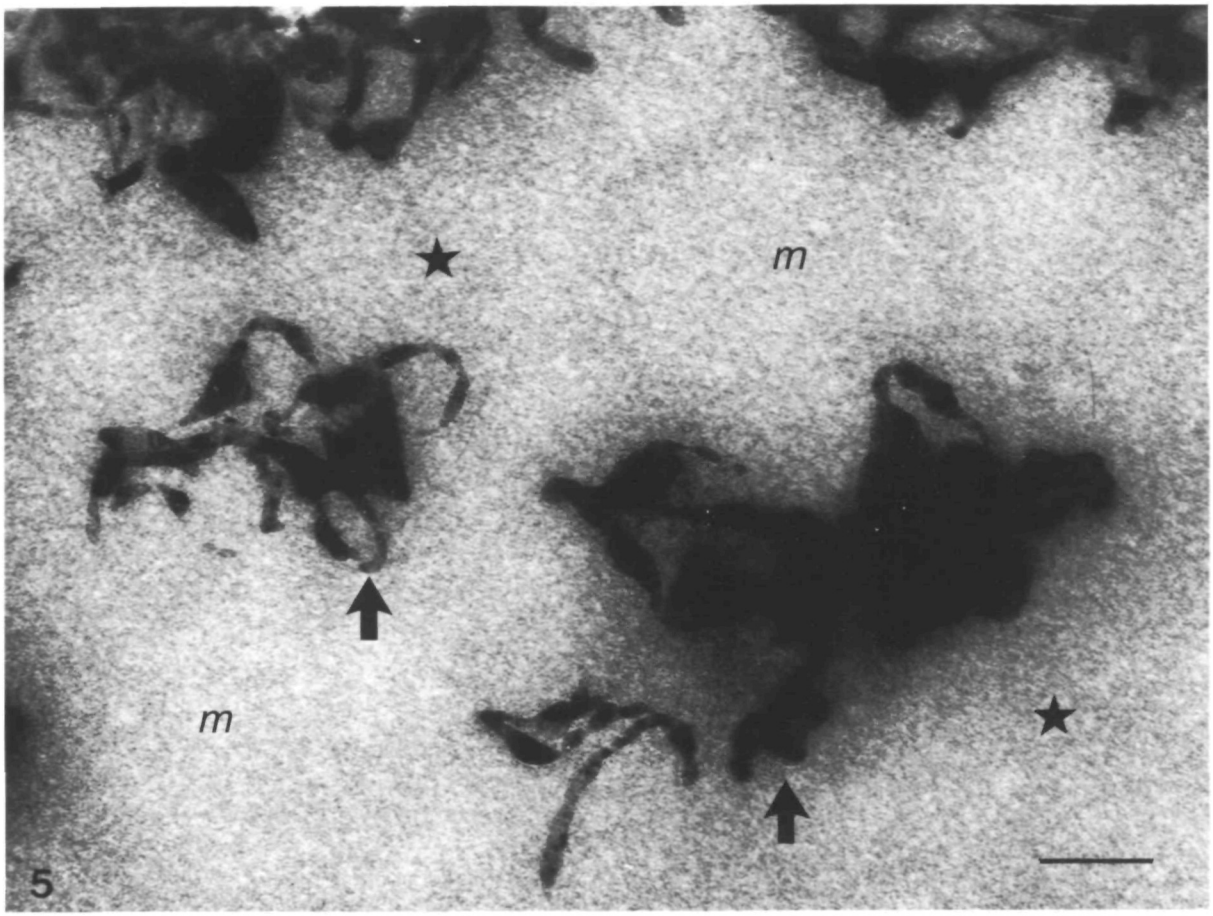


Fig. 5. Part of a heterogeneous nucleolar precursor of an 8-cell embryo labelled with [^{3}H]thymidine. Both the labelled denser part (arrows) and the unlabelled less-dense part (stars) of the fibrillar component consist of 5 nm fibrils, while 3 nm filaments can be recognized in the relatively light matrix (*m*). ×150,000; bar 0·1 μm.

of the nucleolar fibrillar component (Figs 6, 7), apparently less voluminous than in the heterogeneous nucleolar precursors. The rest of the nucleoli was formed by the 3 nm filaments intermingled to various extents with nucleolar granules. In typical cases, the DNA-containing sites localized at the nucleolar periphery were in contact with the unlabelled part of the fibrillar component which, in turn, contacted the granular component towards the centre of the nucleolus (Fig. 7).

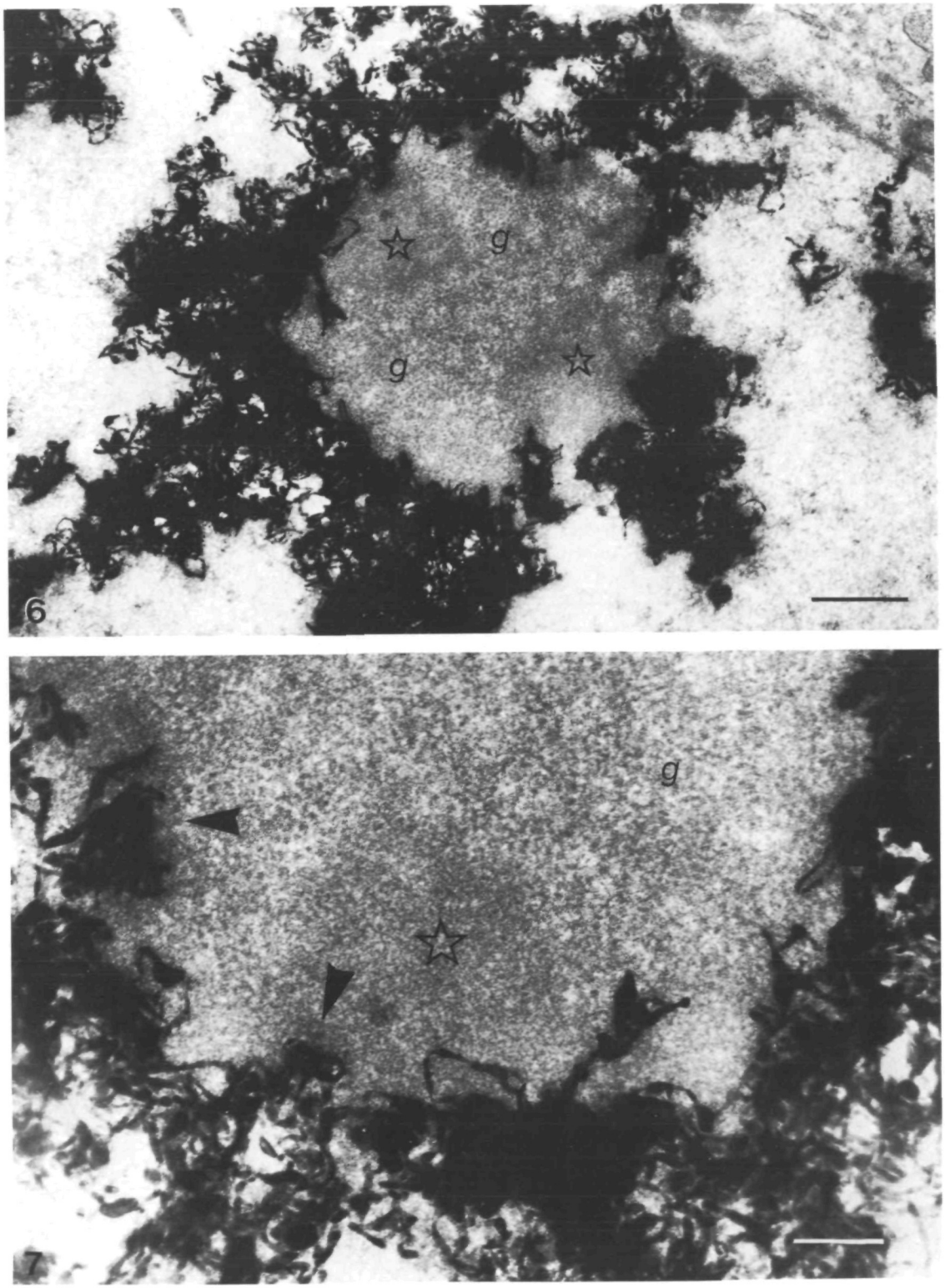
(4) *Reticulated nucleoli*

The preferential peripheral localization of labelled DNA, similar to that observed in the preceding stage of nucleologenesis, remained a characteristic feature of reticulated nucleoli (Fig. 8). Again the labelled DNA was found in the dense fibrillar component consisting of very densely packed 5 nm fibrils (Fig. 9). The unlabelled portion of the fibrillar component was apparently much less voluminous than in the compact fibrillogranular nucleoli. The 3 nm filaments could occasionally be distinguished in the granular component which occupied the greatest part of the nucleolonema. The label was always absent in the granular component as well as in nucleolar interstices (Figs 8, 9). When compared to the heterogeneous nucleolar precursors and compact fibrillogranular nucleoli, there was less nucleolus-associated chromatin. Heavily labelled chromatin did not completely surround the nucleoli, as was mostly the case in the two preceding stages, but was confined to areas adjacent to the strongly labelled regions of the nucleolar periphery (Fig. 8).

Localization of labelled RNA

(1) *Homogeneous nucleolar precursors*

Labelled RNA could never be detected in the interior of these bodies (Figs 10, 11). When the incorporation time was 30 min, extranucleolar RNA synthesis was found to occur at the border of condensed chromatin (Fig. 10), which is the typical site of RNA synthesis in eukaryotic cells (Fakan, 1986). In spite of the absence of labelled RNA within the homogeneous nucleolar precursors, newly synthesized RNA was sometimes observed to be associated with adjacent chromatin



Figs 6, 7. Compact fibrillogranular nucleolus of an 8-cell embryo labelled with [^{3}H]thymidine.

Fig. 6. Label is confined to a peripheral rim made up of the dense fibrillar component and neighbouring a less-dense part of the fibrillar component (stars) that is free of label in a similar way to as the granular component (*g*). ×30 000; bar 0·5 μm.

Fig. 7. Higher magnification view of a part of the nucleolus depicted in Fig. 6 showing the presence of 5 nm fibrils in both the labelled (arrowheads) and unlabelled (star) parts of the fibrillar component. The granular component (G) infiltrates to a varying extent the regions formed by the 3 nm matrix filaments. ×70 000; bar 0·2 μm.

(Fig. 11). In general, the labelling of nuclei containing this type of nucleolar precursors was low, in accordance with our previous findings using light microscopic autoradiography (Tesařík *et al.* 1986*b*).

(2) *Heterogeneous nucleolar precursors*

As the homogeneous nucleolar precursors transformed into heterogeneous ones, labelled RNA became localized within these bodies. When the

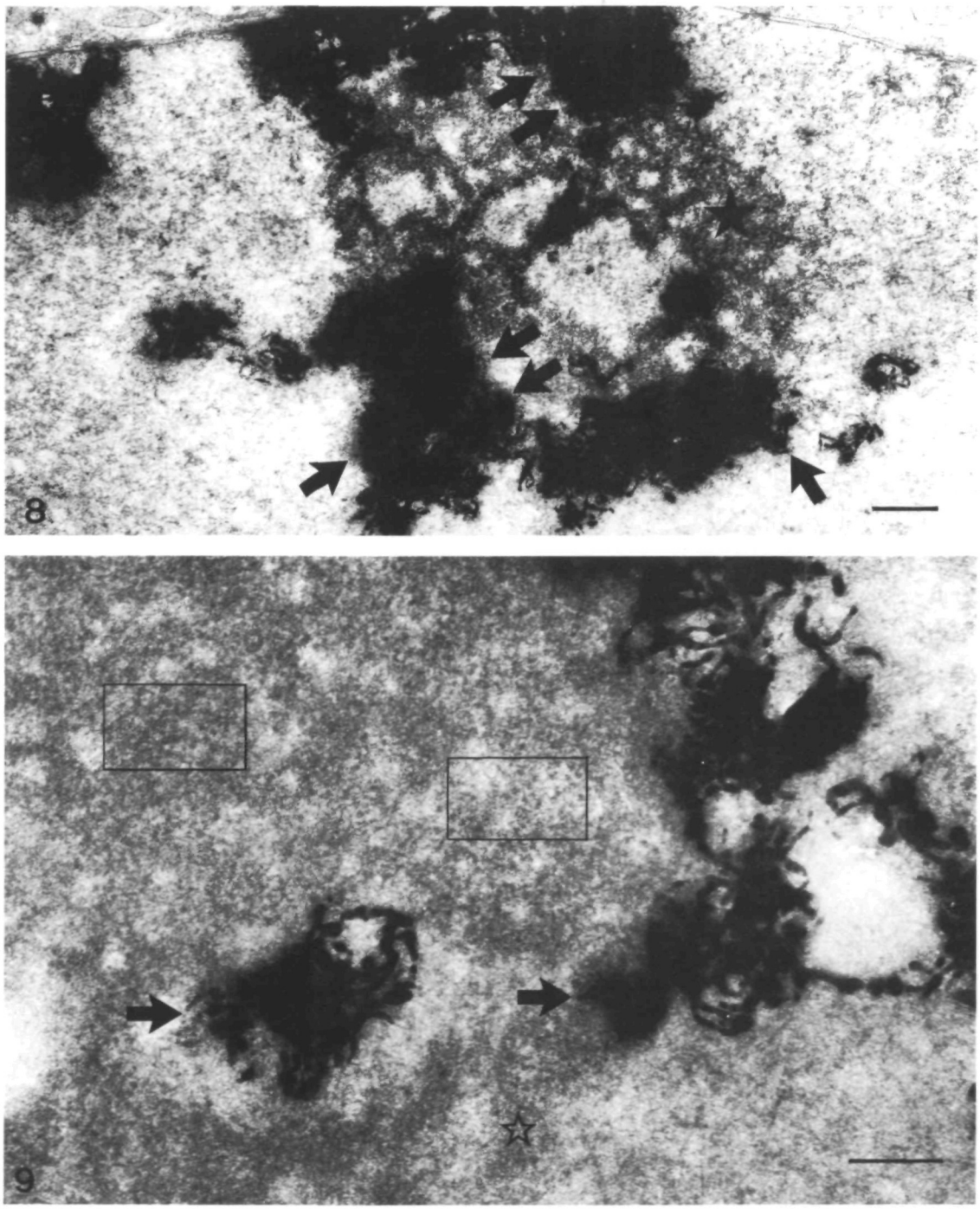


Fig. 8. Reticulated nucleolus of a morula labelled with [^{3}H]thymidine. Heavily labelled dense fibrillar component (double arrows) is preferentially located at the nucleolar periphery in contact with nucleolus-associated chromatin (single arrows). Label is absent in the granular component (star) and in nucleolar interstices. ×20 000; bar 0·5 μm.
Fig. 9. Part of a reticulated nucleolus of a morula labelled with [^{3}H]thymidine. Higher magnification permitted the resolution of 5 nm fibrils in both the labelled (arrows) and unlabelled (star) parts of the fibrillar component. Nucleolar granules are seen admixed to a varying extent both in the less-dense fibrillar regions (left-hand quadrangle) and amidst the 3 nm matrix filaments (right-hand quadrangle). ×70 000; bar 0·2 μm.

incorporation time was 30 min, it was always strictly limited to the extremely electron-dense regions formed by closely packed 5 nm fibrils (Fig. 12) where labelled DNA was also regularly found (cf. Figs 3, 4). However, when the incorporation time was prolonged to 1 h, labelled RNA could often be demonstrated in the less-densely arranged part of the fibrillar component, too (Figs 13, 14).

(3) *Compact fibrillogranular nucleoli*

Similarly, as in the case of the heterogeneous nucleolar precursors, labelled RNA occurred in the highly dense regions formed by 5 nm fibrils and representing the most-electron-opaque parts of the fibrillar component (Fig. 15). The granular component and regions formed exclusively by 3 nm filaments were free of label. The intranucleolar labelled sites were mostly in an intimate relation to heavily labelled nucleolus-associated chromatin.

(4) *Reticulated nucleoli*

Labelled RNA was typically localized in peripheral nucleolar regions (Fig. 16) showing, in general, the same intranucleolar distribution as labelled DNA (cf. Figs 8, 9). It should be noted, however, that all blastomeres containing reticulated nucleoli were from embryos after the shorter labelling period (30 min). At high magnifications (Fig. 17), it was evident that the labelled sites were consistent with the highly electron-dense fibrillar component. In contrast to the preceding stages, little labelling was found in the nucleolus-associated chromatin (Fig. 16).

Discussion

In this paper, the nucleolar development in human preimplantation embryos has been analysed by high-resolution autoradiography which has made possible, for the first time, an exact correlation between the ultrastructural changes during nucleologenesis in the early embryo and underlying molecular events. Using this approach, the proper position of individual intermediate stages of this process has been ascertained and the functional significance of structural changes occurring in the developing nucleoli elucidated.

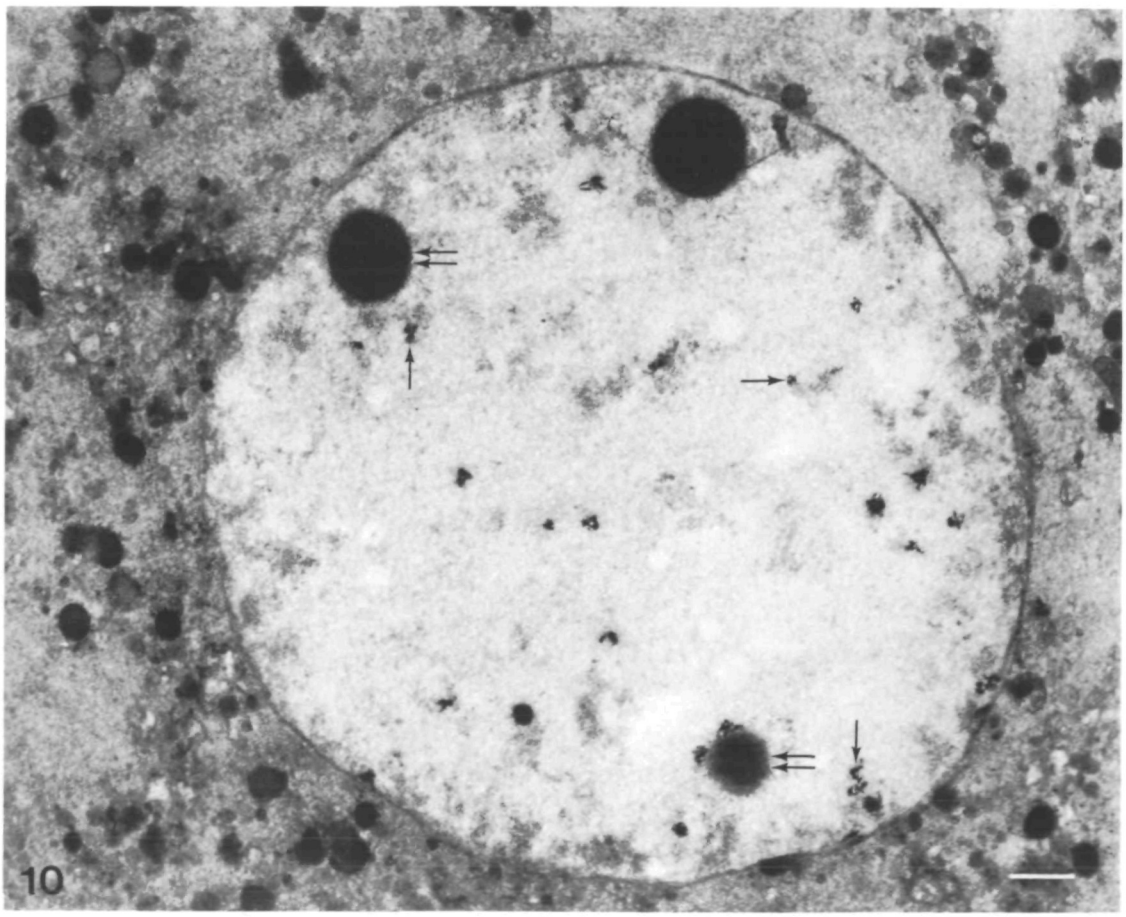
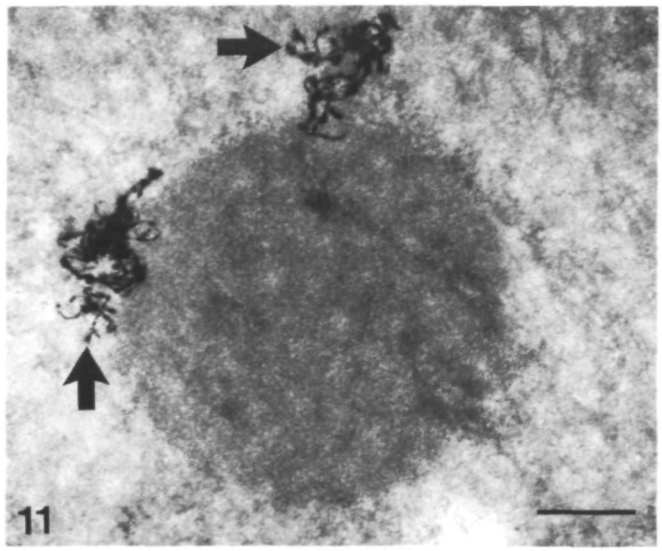
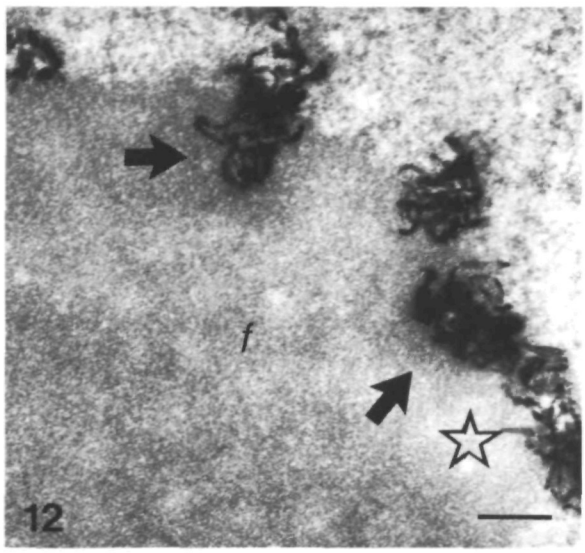


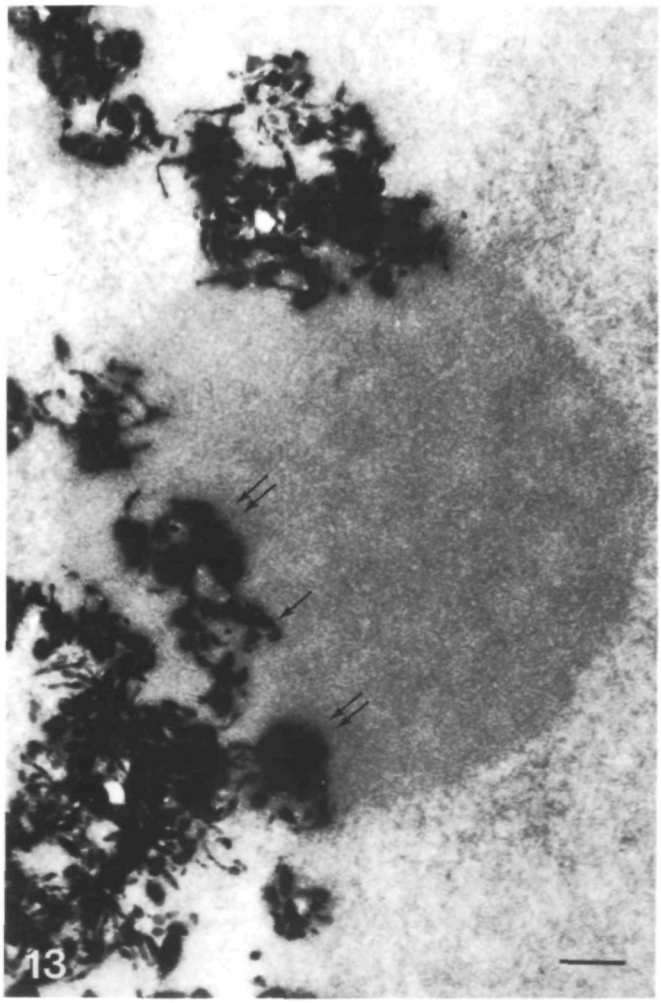
Fig. 10. Nucleus of a 4-cell embryo labelled with [^{3}H]uridine for 30 min. Note the labelling at the border of condensed chromatin (single arrows) and the absence of label in the homogeneous nucleolar precursors (double arrows). ×8000; bar 1 μm.

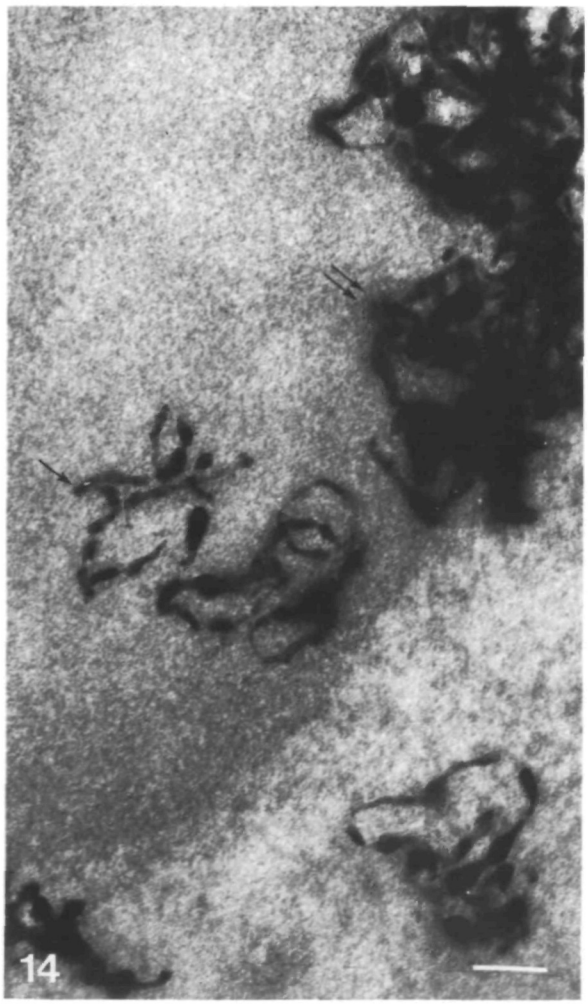
Homogeneous nucleolar precursors

This type of nucleolar precursor is characteristic of embryonic developmental stages before the onset of nucleolar activity and appears to have a consistent structure in all mammalian species studied so far (for a review, see Tesařík *et al.* 1986*a*). These entities have also been referred to as compact, agranular, round nucleoli (Fakan & Odartchenko, 1980) or primary nucleoli (Hillman & Tasca, 1969; Szollosi, 1971), but we consider the term 'nucleolar precursors' more suitable as these bodies lack all structural and functional attributes generally used for the definition of









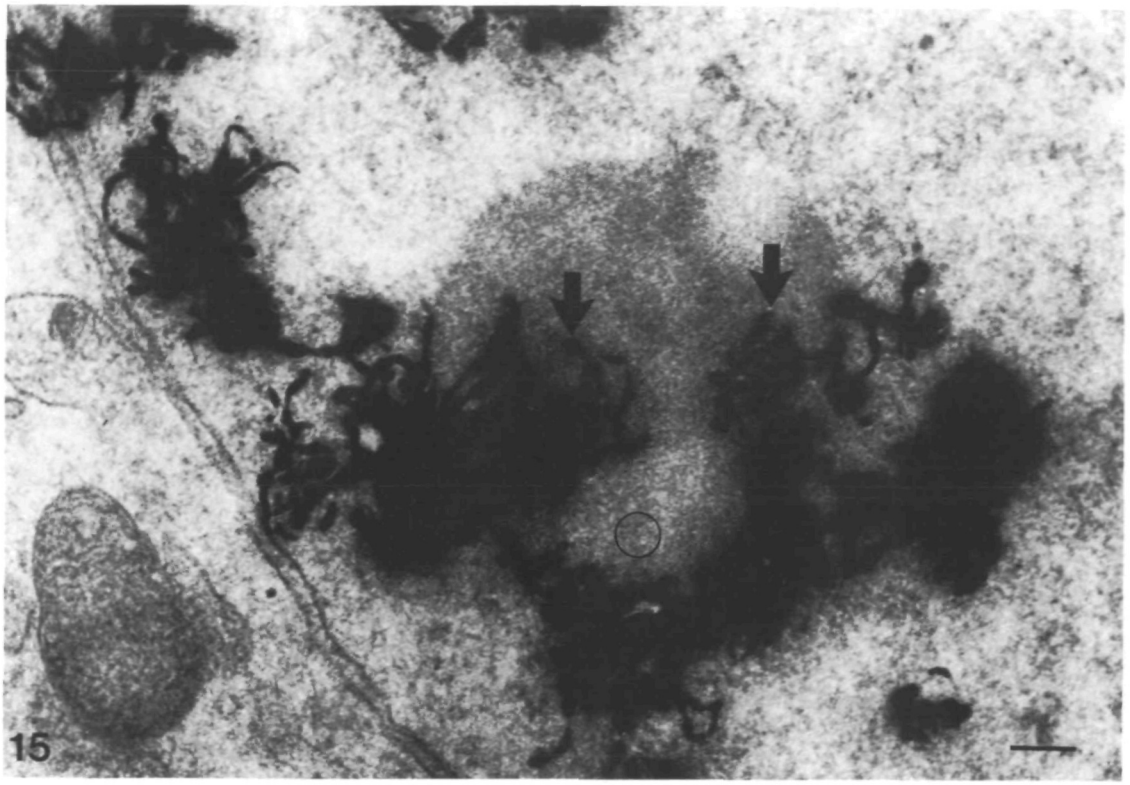


Fig. 15. Compact fibrillogranular nucleolus in an early phase of its development in an 8-cell embryo labelled with [^{3}H]uridine for 30 min. Label is confined to the dense fibrillar component (arrows). Only a few granules are visible (circled area). ×40 000; bar 0·2 μm.

nucleoli. In fact, the homogeneous nucleolar precursors of human preimplantation embryos display no RNA synthesis and, moreover, contain no recently replicated DNA. No argyrophilic nucleolar organizer region (NOR)-specific proteins have been demonstrated in these structures in mouse (Hansmann *et al.* 1978) or rat (Takeuchi & Takeuchi, 1987) preimplantation embryos. As to their fine structure, the homogeneous nucleolar precursors consisting of uniformly arranged osmiophilic 3 nm filaments clearly differ from the fibrillar component of nucleoli which is formed by elements of greater thickness, measuring about 4–5 nm in diameter (Goessens, 1984; Fakan, 1986). A question then arises about the molecular nature of the 3 nm filaments forming the homogeneous nucleolar precursors. Since neither DNA nor RNA can be accepted as potential candidates, these insoluble, and probably macromolecular, structures are most likely proteins. [^{3}H]arginine-labelled proteins have been reported to accumulate in homogeneous nucleolar precursors of pronuclei developing in zona-free hamster eggs inseminated *in vitro* with boar (Kopečný & Pavlok, 1984) and human spermatozoa (Tesařík, 1986). In view of the absence of argyrophilic NOR proteins (see above), the 3 nm filaments of the nucleolar precursors may represent nucleolar scaffold or skeletal matrix material of protein nature analogous to that described in rat liver cells (Todorov & Hadjiolov, 1979). Similar material formed by a meshwork of filaments and resistant to high-salt extraction of isolated *Xenopus laevis* nucleoli was referred to as nucleolar matrix and shown to contain only one predominant protein of 145 000 M_r (Franke *et al.* 1981).

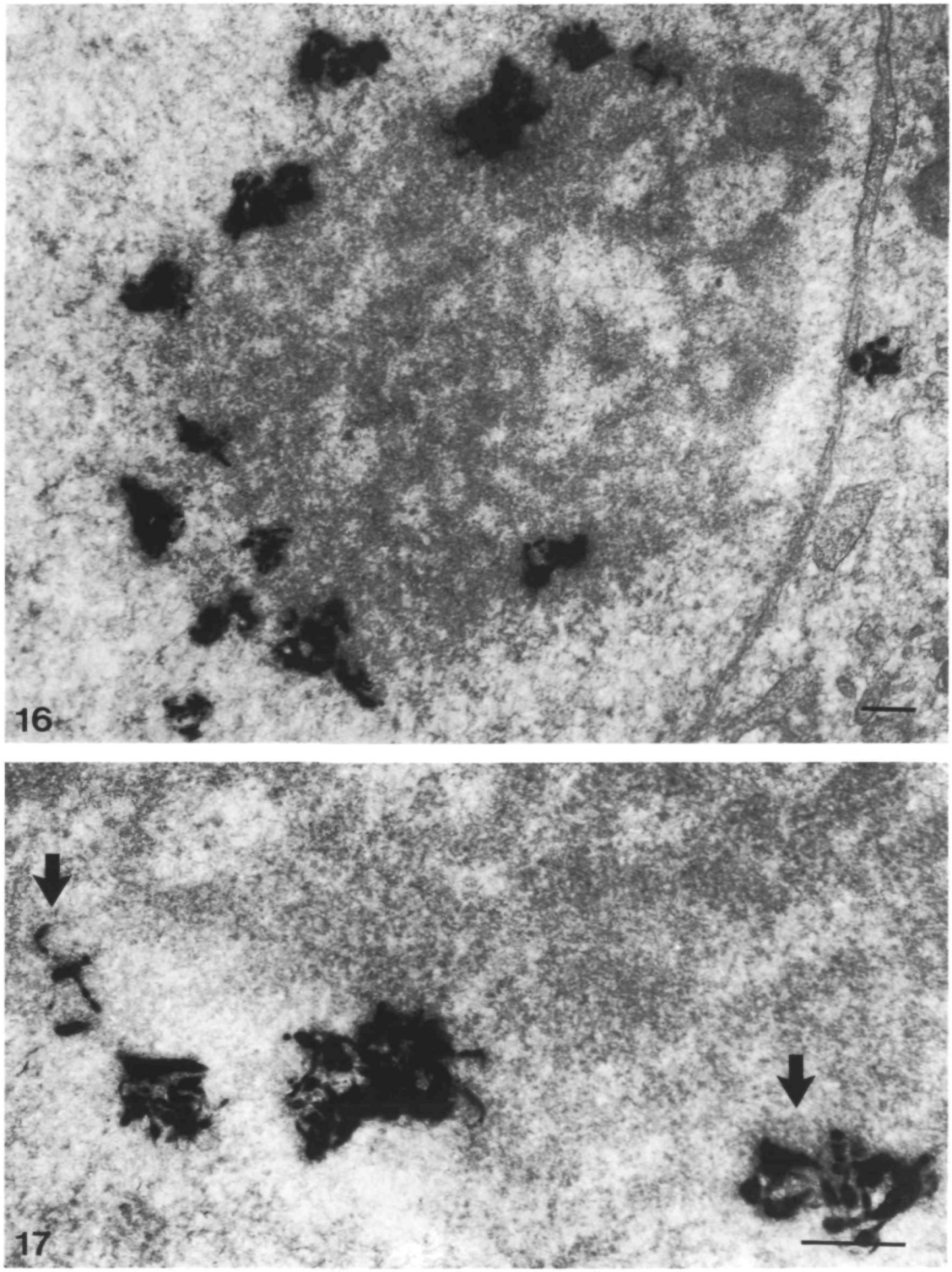
Fig. 11. Homogeneous nucleolar precursor of an 8-cell embryo labelled with [^{3}H]uridine for 1 h. Label is confined to adjacent chromatin (arrows). ×25 000; bar 0·5 μm.

Fig. 12. Part of a heterogeneous nucleolar precursor of an 8-cell embryo labelled with [^{3}H]uridine for 30 min. Label is confined to the dense fibrillar component (arrows) at the periphery of the body. Unlabelled less-dense part of the fibrillar component (*f*) and regions formed by 3 nm matrix filaments (star) can also be distinguished. ×50 000; bar 0·2 μm.

Figs 13, 14. Heterogeneous nucleolar precursors of an 8-cell embryo labelled with [^{3}H]uridine for 1 h. In addition to the denser part of the fibrillar component (double arrows), label is also present in some regions of its less-dense part (single arrows). Fig. 13, ×40 000; bar 0·2 μm. Fig. 14, ×100 000; bar 0·1 μm.

Heterogeneous nucleolar precursors

The heterogeneous structure of these bodies is the appearance of more-electron-opaque regions formed by densely packed 5 nm fibrils occurring together with other regions consisting of the 3 nm matrix filaments. As shown in our previous study (Tesařík *et al.* 1986*a*),



Figs 16, 17. Reticulated nucleolus of a morula labelled with [^{3}H]uridine for 30 min.

Fig. 16. Labelling is evident in the dense fibrillar component preferentially located at the nucleolar periphery. ×40 000; bar 0·2 μm.

Fig. 17. Higher magnification view of a part of the nucleolus shown in Fig. 16. The fibrillar structure of the labelled sites (arrows) is apparent, while the unlabelled parts are impregnated by numerous granules. ×80 000; bar 0·2 μm.

these fibrillar regions are typically located at the periphery of nucleolar precursors, in places where DNA penetrates into them from adjacent chromatin. In the present study, with the use of a high-resolution autoradiographic technique, we demonstrate that there are two structurally and functionally different types of this fibrillar material. The denser part is made up of extremely closely packed 5 nm fibrils, contains DNA and represents the site of nucleolar RNA synthesis. The other part, less electron dense, consists of more-loosely arranged 5 nm fibrils, is devoid of DNA and labelled RNA can only be identified in it using longer incorporation times.

This internal differentiation in the fibrillar component seems to be a unique feature associated with the embryonic nucleolar development. In fact, the fibrillar component of mature nucleoli is now generally accepted to be composed of both rDNA and newly synthesized RNP so that active rRNA genes are obscured by the products of transcription and maturation (Miller & Beatty, 1969; Franke *et al.* 1979), and the corresponding network of intranucleolar DNA filaments are only visible after the enzymic removal of RNP components (Smetana, Daskal & Busch, 1980). By contrast, the pre-rRNA processing is apparently still not very effective in the heterogeneous nucleolar precursors of human embryos, as also evidenced by the absence of the granular component and, under these conditions, the pre-rRNA detached from the transcribing rDNA filament can be visualized as the less-electron-dense portion of the fibrillar component.

Compact fibrillogranular nucleoli

In this stage, the term 'nucleolus' is obviously justified for the first time during human embryonic nucleologenesis in view of the first appearance of nucleolar granules. Correlated ultrastructural and biochemical studies of isolated nucleoli and their fractions obtained from various cell types (Koshiba, Thirumalachary, Daskal & Busch, 1971; Daskal, Prestayko & Busch, 1974; Royal & Simard, 1975) indicate that the dense fibrillar component is the site of 45 S pre-rRNA synthesis, while the granules contain processing intermediates as well as 28 S rRNA (for reviews, see also Hernandez-Verdun, 1986; Fakan, 1986). In accordance with the above concept, the compact fibrillogranular nucleoli of early human embryos seem capable of carrying on the entire process of rRNA synthesis and maturation, whereas the heterogeneous nucleolar precursors, in spite of their RNA synthetic activity, have not yet developed an efficient apparatus for pre-rRNA processing. Yet the two parts, one corresponding to the pre-rRNA transcription site, the other lacking DNA and presumably containing detached pre-rRNA in the course of processing, can be still distinguished in the fibrillar component, suggesting that the rRNA maturation process remains relatively inefficient as compared to mature nucleoli of differentiated cells.

Reticulated nucleoli

The reticulated nucleoli represent the final stage of human embryonic nucleologenesis. While all the above intermediate stages typically occur in the fourth cell cycle of human preimplantation development, the reticulated nucleoli retain the same structural organization throughout the subsequent morula and blastocyst stages. Intensive incorporation of [^{3}H]uridine, the presence of well-developed granular component and reduction of the DNA-free portion of the fibrillar component suggest high rRNA synthetic and processing activity.

Functional significance of structural changes during embryonic nucleologenesis

The results of this study, together with those reported earlier (Tesařík *et al.* 1986*a*), indicate that nucleologenesis in early human embryos is a unique biological process showing little analogy with any event occurring in nucleoli and NORs during the nucleolar cycle or differentiation of adult somatic cells. The probable sequence of events underlying the structural changes in developing nucleoli of preimplantation embryos is schematically summarized in Fig. 18. Unlike the periodical reconstitution of nucleoli in mitotic telophase of differentiated cells, when the dense fibrillar component followed by the granular component first appear around specific loci on chromosomes bearing rRNA genes, usually located at or close to a secondary constriction (for a review, see Goessens, 1984), rDNA of the mammalian zygote and early embryo appears to be incapable of starting transcription and so of triggering the nucleogenetic process unless associated with the nucleolar precursors. This association of rDNA with preformed nucleolar matrix elements contained in the nucleolar precursors is probably a pre-requisite for the beginning of pre-rRNA synthesis. The functionally relevant nucleolar matrix components, probably of protein nature, seem to have been lost from NORs during the relatively long period between the extinction of nucleolar activity in human gametogenesis (pachytene spermatocytes: Paniagua, Nistal, Amat & Rodríguez, 1986; germinal vesicle oocytes: Tesařík, Trávník, Kopečný & Kristek, 1983) and nucleolar reactivation after fertilization (fourth cell cycle: Tesařík *et al.* 1986*a*,*b*).

The first appearance of nucleolar granules that are believed to contain 28 S rRNA is indicative of the establishment of a functionally efficient apparatus for rRNA maturation. The expansion of the granular

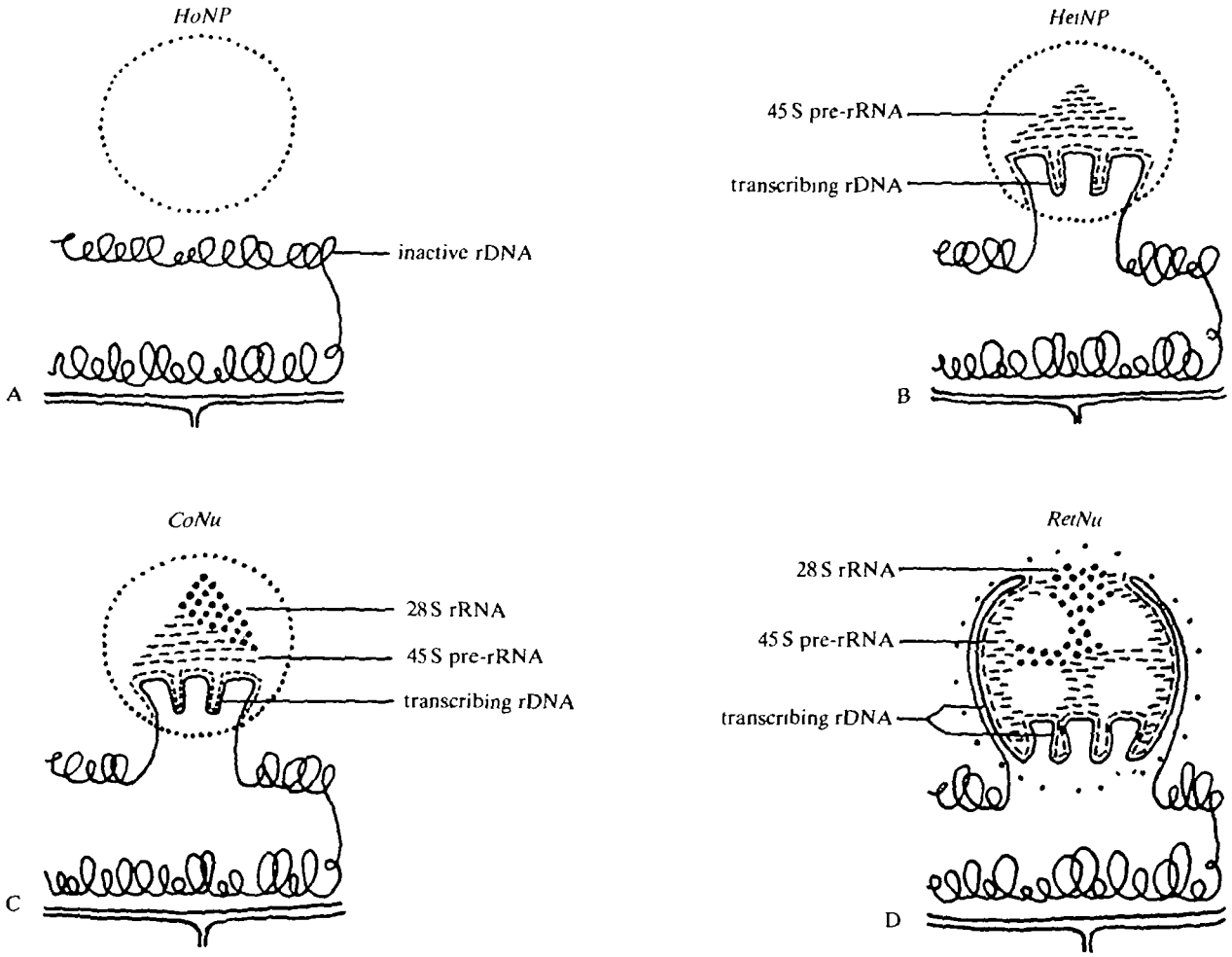


Fig. 18. Schematic representation of the putative molecular events underlying embryonic nucleologenesis. (A) Transcriptionally inactive rDNA has not yet penetrated into the homogeneous nucleolar precursor (*HoNP*). (B) rDNA, having penetrated into the heterogeneous nucleolar precursor (*HetNP*), starts the synthesis of pre-rRNA whose processing is still inactive. (C) Processing of pre-rRNA is progressively activated in compact fibrillogranular nucleoli (*CoNu*). (D) Active pre-rRNA synthesis and processing occur in reticulated nucleoli (*RetNu*).

component in reticulated nucleoli suggests an increase in the processing activity, while the newly formed nucleolar interstices probably serve to augment the interface at which the transport of nucleolar products to the nucleoplasm takes place.

In spite of the apparent differences in the ultrastructure of nucleologenesis amongst mammalian species (for a review, see Tesařík *et al.* 1986*a*), the underlying molecular events, as outlined in Fig. 18, are probably related. The structural variability seems to be caused by different timing of this process in relation to the progression of embryogenesis and to different rates of utilization of nucleolar precursors (i.e. extension through several cell cycles in mice *versus* virtual completion within a single cell cycle in humans). The preliminary results of a current analysis of the nucleologenetic process in farm animals (Kopečný *et al.* 1985; Camous, Kopečný & Fléchon, 1986; Tománek, Kopečný & Kaňka, 1986) increase confidence in the broader validity of the nucleologenetic mechanism suggested in this study. Experiments involving various mammalian species are now in progress in a collaborative multicentre study in order to re-evaluate this mechanism and distinguish the general from the special in animal embryonic nucleologenesis.

References

Camous, S., Kopečný, V. & Fléchon, J.-E. (1986). Autoradiographic detection of the earliest stage of [^{3}H]uridine incorporation into the cow embryo. *Biol. Cell* **58**, 195–200.

Clegg, K. B. & Piko, L. (1982). RNA synthesis and cytoplasmic polyadenylation in the one-cell mouse embryo. *Nature, Lond.* **295**, 342–345.

Daskal, Y., Prestayko, A. W. & Busch, H. (1974). Ultrastructural and biochemical studies of the isolated

fibrillar component of nucleoli from Novikoff hepatoma ascites cells. *Expl Cell Res.* **88**, 1–14.

ENGEL, W., ZENZES, M. T. & SCHMID, M. (1977). Activation of mouse ribosomal genes at the 2-cell stage. *Hum. Genet.* **38**, 57–63.

FAKAN, S. (1986). Structural support for RNA synthesis in the cell nucleus. In *Methods and Achievements in Experimental Pathology*, vol. 12 (ed. G. Jasmin & R. Simard), pp. 105–140. Basel: S. Karger.

FAKAN, S. & ODARTCHENKO, N. (1980). Ultrastructural organization of the cell nucleus in early mouse embryos. *Biol. Cell* **37**, 211–218.

FRANKE, W. W., KLEINSCHMIDT, J. A., SPRING, H., KROHNE, G., GRUND, C., TRENDELENBURG, M. F., STOEHR, M. & SCHEER, U. (1981). A nucleolar skeleton of protein filaments demonstrated in amplified nucleoli of *Xenopus laevis*. *J. Cell Biol.* **90**, 289–299.

FRANKE, W. W., SCHEER, U., SPRING, H., TRENDELENBURG, M. F. & ZENTGRAF, H. (1979). Organization of nucleolar chromatin. In *The Cell Nucleus*, vol. 7 (ed. H. Busch), pp. 49–95. New York: Academic Press.

GEUSKENS, M. & ALEXANDRE, H. (1984). Ultrastructural and autoradiographic studies of nucleolar development and rDNA transcription in preimplantation mouse embryos. *Cell Differ.* **14**, 125–134.

GOESSENS, G. (1984). Nucleolar structure. *Int. Rev. Cytol.* **87**, 107–158.

HANSMANN, I., GEBAUER, J., BIHL, L. & GRIMM, T. (1978). Onset of nucleolar organizer activity in early mouse embryogenesis and evidence of its regulation. *Expl Cell Res.* **114**, 263–268.

HERNANDEZ-VERDUN, D. (1986). Structural organization of the nucleolus in mammalian cells. In *Methods and Achievements in Experimental Pathology*, vol. 12 (ed. G. Jasmin & R. Simard), pp. 26–62. Basel: S. Karger.

HILLMAN, N. & TASCA, R. J. (1969). Ultrastructural and autoradiographic studies of mouse cleavage stages. *Am. J. Anat.* **126**, 151–174.

JORDAN, E. G. (1984). Nucleolar nomenclature. *J. Cell Sci.* **67**, 217–220.

KNOWLAND, J. & GRAHAM, C. (1972). RNA synthesis at the two-cell stage of mouse development. *J. Embryol. exp. Morph.* **27**, 167–176.

KOPEČNÝ, V., FLÉCHON, J.-E., TOMÁNEK, M., CAMOUS, S. & KAŇKA, J. (1985). Ultrastructural analysis of ^{3}H-uridine incorporation in early embryos of pig and cow (Abstr.). *ECBO 9th Nucleolar Workshop*, p. 31, Krakow.

KOPEČNÝ, V. & PAVLOK, A. (1984). Association of newly synthesized ^{3}H-arginine-labelled proteins with chromatin structures in fertilization. *Gamete Res.* **9**, 339–408.

KOSHIBA, K., THIRUMALACHARY, C., DASKAL, Y. & BUSCH, H. (1971). Ultrastructural and biochemical studies on ribonucleoprotein particles from isolated nucleoli of thioacetamide-treated rat liver. *Expl Cell Res.* **68**, 235–246.

MCDONALD, K. (1984). Osmium ferricyanide fixation improves microfilament preservation and membrane visualization in a variety of animal cell types. *J. Ultrastruct. Res.* **86**, 107–118.

MILLER, O. L. & BEATTY, B. R. (1969). Visualization of nucleolar genes. *Science* **164**, 955–957.

PANIAGUA, R., NISTAL, M., AMAT, P. & RODRÍGUEZ, M. C. (1986). Ultrastructural observations on nucleoli and related structures during human spermatogenesis. *Anat. Embryol.* **174**, 301–306.

PLACHOT, M., MANDELBAUM, J., COHEN, J. J., DEBACHE, C., PIGEAU, F. & JUNCA, A.-M. (1985). Sequential use of clomiphene citrate, human menopausal gonadotropin, and human chorionic gonadotropin in human in vitro fertilization. I. Follicular growth and oocyte suitability. *Fert. Steril.* **43**, 255–262.

ROYAL, A. & SIMARD, R. (1975). RNA synthesis in the ultrastructural and biochemical components of the nucleolus of Chinese hamster ovary cells. *J. Cell Biol.* **66**, 577–585.

SMETANA, K., DASKAL, I. & BUSCH, H. (1980). Cytochemistry of the microtrabecular network in compact nucleoli of hepatocytes treated with cycloheximide. *Histochemistry* **65**, 301–308.

SZOLLOSI, D. (1971). Nucleoli and ribonucleoprotein particles in the preimplantation conceptus of the rat and mouse. In *The Biology of the Blastocyst* (ed. R. J. Blandau), pp. 95–114. Chicago: University of Chicago Press.

TAKEUCHI, I. K. & TAKEUCHI, Y. K. (1987). Ultrastructural localization of Ag-NOR proteins in full-grown oocytes and preimplantation embryos of mice. *J. Electron Microsc.* **35**, 280–287.

TESAŘÍK, J. (1986). From the cellular to the molecular dimension: The actual challenge for human fertilization research. *Gamete Res.* **13**, 47–89.

TESAŘÍK, J. (1987). Gene activation in the human embryo developing in vitro. In *Future Aspects in Human in Vitro Fertilization* (ed. W. Feichtinger & P. Kemeter), pp. 251–261. Berlin, Heidelberg: Springer-Verlag.

TESAŘÍK, J., TRÁVNÍK, P., KOPEČNÝ, V. & KRISTEK, F. (1983). Nucleolar transformations in the human oocyte after completion of growth. *Gamete Res.* **8**, 267–277.

TESAŘÍK, J., KOPEČNÝ, V., PLACHOT, M., MANDELBAUM, J., DA LAGE, C. & FLÉCHON, J.-E. (1986*a*). Nucleologenesis in the human embryo developing *in vitro*: Ultrastructural and autoradiographic analysis. *Devl Biol.* **115**, 193–203.

TESAŘÍK, J., KOPEČNÝ, V., PLACHOT, M. & MANDELBAUM, J. (1986*b*). Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing *in vitro*. *J. Reprod. Fert.* **78**, 463–470.

TODOROV, I. T. & HADJIOLOV, A. A. (1979). A comparison of nuclear and nucleolar matrix proteins from rat liver. *Cell Biol. int. Rep.* **3**, 743–757.

TOMÁNEK, M., KOPEČNÝ, V. & KAŇKA, J. (1986). Studies on RNA synthesis in early pig embryos (Abstr.). *Histochem. J.* **18**, 138.

(*Accepted 29 July 1987*)