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Localization and structure of nucleolar organizers in the oocyte during meiotic prophase I.

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Summary. The localization, structure and activity of nucleolar organizers were studied in quail and mouse oocytes during prophase I of meiosis using electron microscopy and *in situ* hybridization with tritium labelled 18S and 28S RNA.

The newly synthesized nucleolus is constituted of a fibrillar centre surrounded by a layer of electron-opaque fibrils. DNP fibres with a diameter of about 50 Å extend from the nucleolar organizer to the fibrillar centre and penetrate into the latter. At the onset of nucleolar synthesis, the position of the fibrillar centre coincides with that of the ribosomal cistrons revealed by *in situ* hybridization.

Following brief incorporation of tritiated uridine, labelling grains are localized over the electron-opaque fibrillar layer adjacent to the fibrillar centre.

These observations indicate that rDNA is present in both the fibrillar centre and the dense fibrillar layer which are always in association, and that transcription of the ribosomal cistrons essentially occurs in the dense fibrillar layer.

In a previous study we showed that the oocytes of birds and mammals represent particularly interesting material for studying ribosomal cistrons (Stahl *et al.*, 1975a). In various species, *de novo* synthesis of nucleolar material occurs during one of the phases of meiotic prophase I. Accordingly, newly synthesized nucleoli are formed during pachytene in the quail and the mouse (Mirre and Stahl, 1976, 1978 ; Stahl *et al.*, 1977). Ultrastructural studies of oocytes at this stage lead to detailed analysis of the chromosomal structures involved in nucleolar synthesis. In the quail, localization of the ribosomal cistrons in the oocyte nucleus can be identified by *in situ* hybridization. Comparison of the results obtained by this technique with those of the electron microscope shows that in the growing nucleolus the ribosomal genes are located in the fibrillar centre (Knibiehler *et al.*, 1977). In human oocytes, cytological and cytogenetic techniques allowed us to demonstrate the appearance of numerous

(1) C. N. R. S.

micronucleoli during diplotene (Stahl *et al.*, 1975b). This observation led us to propose the hypothetical amplification of ribosomal cistrons in the human oocyte, later confirmed by *in situ* hybridization (Wolgemuth-Jarashow *et al.*, 1977) and by electron microscopy (Luciani *et al.*, 1977).

The nucleolus, formed during meiotic prophase progressively displays its characteristic aspect observed in the interphase nuclei of active cells. Analysis of the stages during this evolving process should lead to an increased understanding of both the spatial configuration and the activity of the intranucleolar ribosomal genes. A new approach to these problems was attempted in this study using quail and mouse oocytes.

Material and Methods.

1. *In situ hybridization.* — Ovaries of 1-2, and 3-day old Japanese quails, and those of mouse embryos aged 18, 19 and 20 days (Swiss OF1) were removed and treated according to the technique of Luciani *et al.* (1974), i.e. fixation in methanol-acetic acid (3/1) and dissociation in 45 p. 100 acetic acid. The resulting cell suspension was spread on precooled slides which were then dried over a flame. *In situ* hybridization was performed using tritium labelled 18S and 28S RNA, prepared from cultured Hela cells by the technique of Ascione and Arlinghaus (1970). Specific activity of the ribosomal RNA was 1.4×10^6 cpm/ μ g. Hybridization performed on slides was realized according to the technique described by Gall and Pardue (1969). Preparations were coated by Kodak NTB2 emulsion, exposed for 90 days at 4 °C, then developed using Kodak D19b developer, and finally stained by Giemsa's solution.

2. *Electron microscopy.* — Ovaries of 1-4-day old Japanese quails, those of mouse embryos aged 13 through 20 days, and ovaries of 1-, 2-, and 3-day old mice were removed. The ovaries were fixed for 15 min. at 4 °C by 3 p. 100 glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, containing 0.1 M sucrose. After washing in the buffer solution, the specimens were postfixed by 2 p. 100 osmium tetroxide in identical buffer for 20 min. After dehydration, the ovaries were embedded in Epon and sectioned with a diamond knife in a Reichert OMU2 ultramicrotome. The sections were contrasted with uranyl acetate and lead citrate.

3. *High resolution autoradiography.* — Entire ovaries were incubated 30 min. or 45 min. at 37 °C in 1 ml of 80 p. 100 Eagle and 20 p. 100 calf serum medium containing 100 μ Ci/ml of 3H-uridine (sp. act. 24 Ci/mM, CEA, France). After the above described fixation and before postfixation, the specimens were repeatedly washed during 30 min. in the buffer in order to remove unincorporated radioactive uridine. After dehydration and embedding in Epon, autoradiography was performed according to the technique described by Granboulan (1965) and by Salpeter and Bachmann (1972). The sections transferred to a collodion-coated slide were stained with uranyl acetate and lead citrate. A thin carbon layer was vacuum evaporated over the stained sections. The slides were then coated with a diluted Ilford L4 emulsion by the dipping method. After exposure for 4 months at 4 °C the preparations were developed in Microdol-X (Kodak) for 4 min. at 18 °C and fixed with Kodak Rapid Fixer for 3 min. The specimen was stripped off onto a surface of double-distilled water and copper grids were

placed over the sections. The specimens on the grid were then picked up with a filter paper. After drying, the grids were carefully released from the remnant film. The collodion film was thinned for 2 min. in iso-amylacetate. The preparations were examined with a Siemens Elmiskop 101 microscope at 80 kV.

Observations.

A. Nucleolar synthesis in the quail oocyte.

In quail oocytes, the nucleoli are formed from the euchromatic segment of several microchromosomes having the centromeric heterochromatin contained within a chromocenter (Stahl *et al.*, 1974). Analysis of ³H-uridine incorporation demonstrates that the ribosomal RNAs are elaborated during middle and late pachytene, in the vicinity of numerous microchromosomes (Hartung and Stahl, 1976).

Data from electron microscopy studies in the quail nucleus show that newly synthesized nucleoli are formed during mid- and late pachytene. These data permit precise identification of the chromosomal structures involved in such nucleolar synthesis.

The nucleolus always appears at one extremity of a very short bivalent formed by the pairing of two microchromosomes. The other bivalent extremity is composed of centromeric heterochromatin. The latter is associated with the centromeric heterochromatin of other bivalents, thus constituting a chromocentre which is constantly in contact with the nuclear envelope (fig. 1).

In most cases, the distal end of the bivalent presents an enlargement composed of tightly packed chromatin fibres. Thin fibres which penetrate into the nucleolus can be seen emanating from the enlargement. The nucleolar region penetrated by these fibres invariably corresponds to the fibrillar centre described by Recher *et al.* (1969). A zone of electron-opaque fibrils can always be seen surrounding the fibrillar centre. At the onset of its synthesis, the nucleolus is composed of only the fibrillar centre and an incomplete « corona » of electron-opaque fibrils. Granules appear at the exterior of the dense fibrillar zone at a more advanced stage. At this stage the appearance of the nucleolus corresponds to that of a natural segregation, all the while remaining in a state of active synthesis (fig. 2).

At diplotene, the nucleolus progressively takes on a reticular appearance. Multiplication of the fibrillar centres, which are no longer peripherally situated, can be seen.

Incorporation of ³H-uridine was studied using high resolution autoradiography. Silver grains were almost always observed over the zone of electron-opaque fibrils surrounding the fibrillar centre (fig. 3).

In situ hybridization demonstrates that 4 bivalents contain the ribosomal cistrons. Following autoradiography, silver grains are observed over the euchromatic segment of certain microchromosomes in proximity to the centromeric heterochromatin (fig. 4). This region topographically corresponds to the fibrillar centre of the developing nucleolus.

B. Nucleolar synthesis in the mouse oocyte.

In the pachytene mouse oocyte, the centromeric heterochromatin of several bivalents can be seen to associate thus forming chromocentres. This process is quite comparable to that observed in the quail oocyte. As in the latter case, the chromocentres are in contact with the nuclear envelope. At pachytene stage, new nucleoli are formed in contact with the juxta-centromeric zone of the bivalents which are either isolated or inserted in a chromocentre.

Ultrastructural study of this phenomenon permits analysis of the chromosomal region giving rise to the nucleolus. This region corresponds to a secondary constriction and is characterized by the presence of thin chromatin fibres approximately 50 Å in diameter. These fibres extend from the lateral element of the synaptonemal complex up to the fibrillar centre and penetrate into the latter (fig. 5). At the onset of nucleolar synthesis, the nucleolus is only composed of the fibrillar centre partially surrounded by a layer of electron-dense fibrils. As the nucleolus gradually develops it tends to appear band-shaped. The proximal portion of the nucleolus, facing the bivalent secondary constriction, is constituted by the fibrillar centre incompletely surrounded by dense fibrils. This proximal region is followed by a fibrillar zone of lower electron opacity and finally a fibrillo-granular zone. The distal region of the nucleolus appears essentially granular.

The nucleolus presents a reticular texture at diplotene stage. Several fibrillar centres can be observed, occupying various sites within the nucleolus. These fibrillar centres are invariably surrounded by electron-dense fibrils.

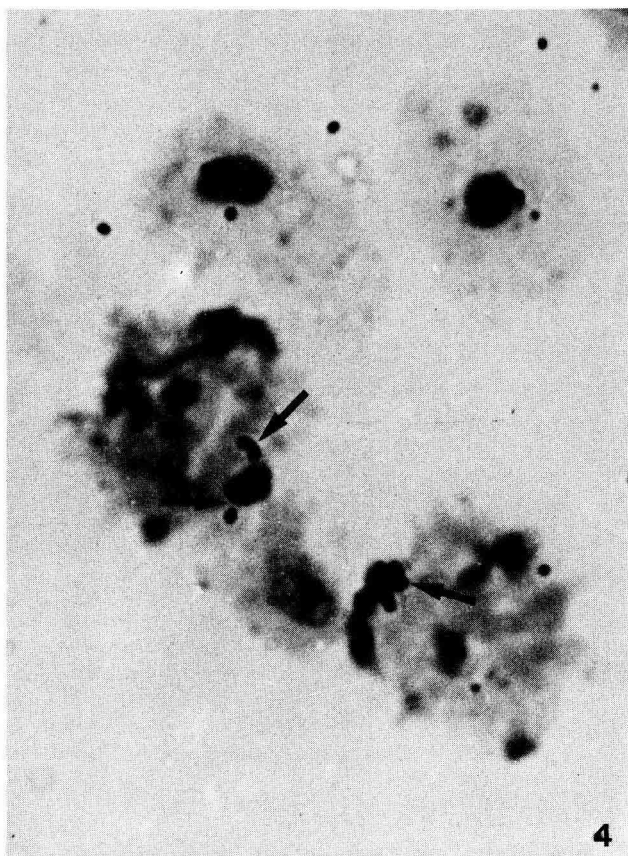
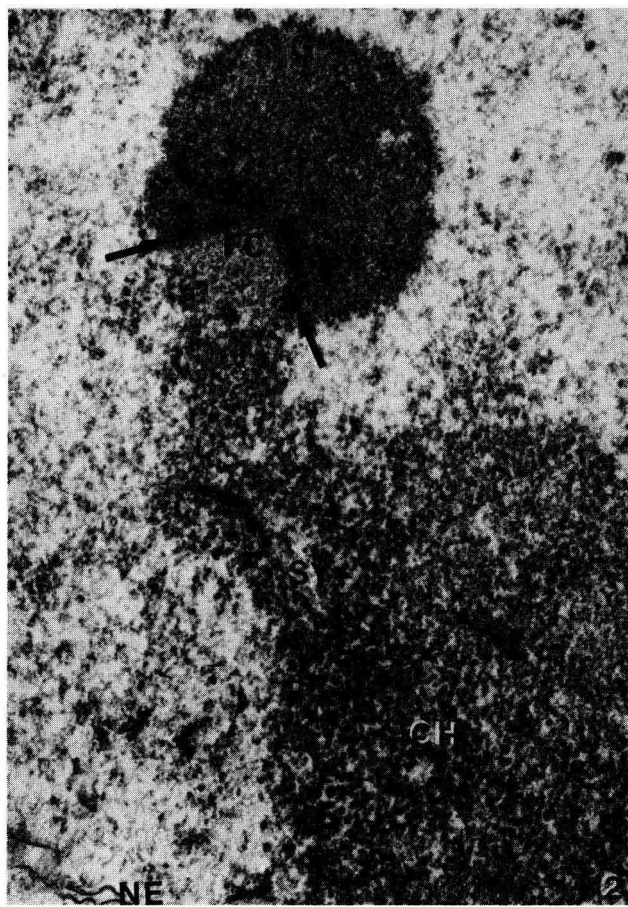
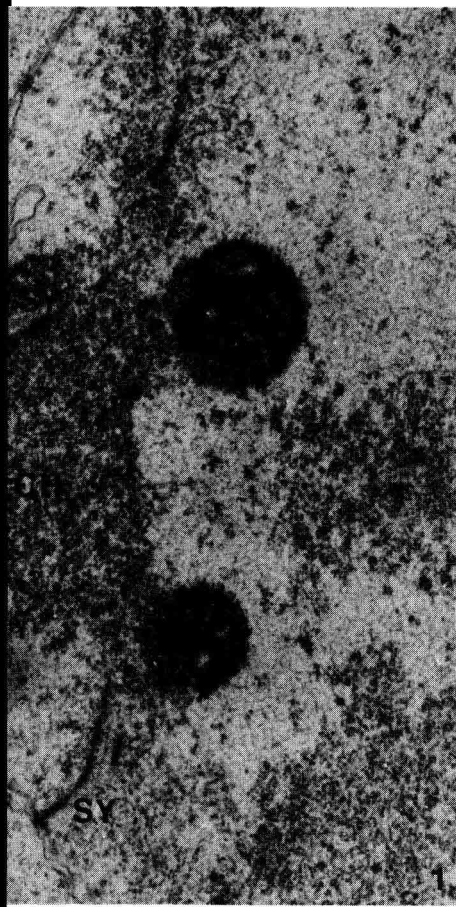
Incorporation of 3H-uridine was studied in the light and electron microscope. After incorporation for 30 min., light microscopy reveals labelling grains over the juxta-centromeric region of certain bivalents the heterochromatin of which generally participates in a chromocentre. High resolution autoradiography demonstrates that the silver grains are localized on the electron-dense fibrils surrounding the fibrillar centre, while the latter is unlabelled (fig. 6).

FIG. 1. — *One-day old quail oocyte at pachytene.* Synthesis of nucleoli occurs in contact with a chromocentre formed by association of the centromeric heterochromatin (CH) from several bivalents. SY : synaptonemal complex. NE : nuclear envelope ($\times 20\ 000$).

FIG. 2. — *One-day old quail oocyte at pachytene.* The newly formed nucleolus presents a fibrillar centre (FC) into which penetrate chromatin fibres emanating from a bivalent. The fibrillar centre is partially surrounded by strands of electron-opaque fibrils (arrows). The distal part of the nucleolus appears granular (G). SY : synaptonemal complex ; CH : centromeric heterochromatin ; NE : nuclear envelope ($\times 40\ 000$).

FIG. 3. — *Autoradiography following 3H-uridine incorporation for 30 min.* Silver grains are located over the fibrillar strands. The fibrillar centres (arrows) are unlabelled. Two-day old quail oocyte at diplotene ($\times 26\ 000$).

FIG. 4. — *Autoradiography after in situ hybridization using tritium labelled 18S and 28S RNA.* In the two pachytene oocytes labelling grains (arrows) are located near a chromocentre, overlying the bivalents containing the two paired nucleolar organizers ($\times 1\ 800$).



In situ hybridization reveals ribosomal cistrons on a maximum of 3 bivalents. These cistrons are situated in a juxta-centromeric region which is both narrow and less highly stained than the remaining bivalent segments. The appearance and location of this region correspond to that of a secondary constriction. In some cells, the localization of labelling grains corresponds to that of the fibrillar centre (fig. 7).

Discussion.

Our observations in quail and mouse oocytes demonstrate that fine chromatin fibres extend from the nucleolar organizer region up to the developing fibrillar centre and penetrate into the latter. The Gogliati and Gautier technique reveals that the fibrillar centre indeed contains numerous DNA fibres (Mirre and Stahl, 1978). This DNA contains ribosomal cistrons as evidenced by *in situ* hybridization.

The presence of DNA in the fibrillar centre of the nucleolus has been shown in various cell types: Ehrlich's tumor cells (Goessens, 1974, 1976), mouse fibroblasts (Pouchelet *et al.*, 1975; Anteunis *et al.*, 1975) and plant cells (Lafontaine and Lord, 1973; Lord *et al.*, 1977). It should be underlined that only *in situ* hybridization is capable of revealing the nature of the genes in such DNA.

In agreement with the conclusions of Goessens using different study material, our observations indicate that the fibrillar centre is the site of the nucleolar organizer.

It is interesting to note that the relations between the chromosomes and fibrillar centre observed in the present study are highly similar to those reported in plant meiocytes (Jordan and Luck, 1976).

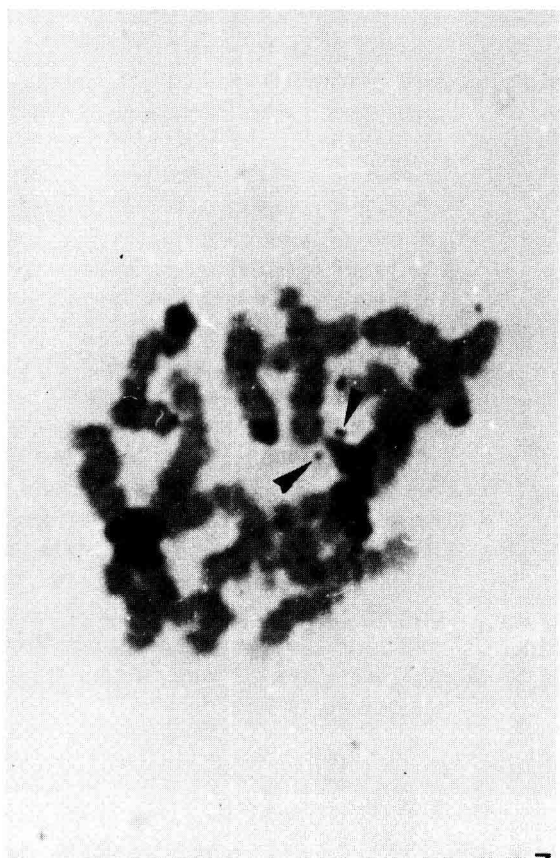
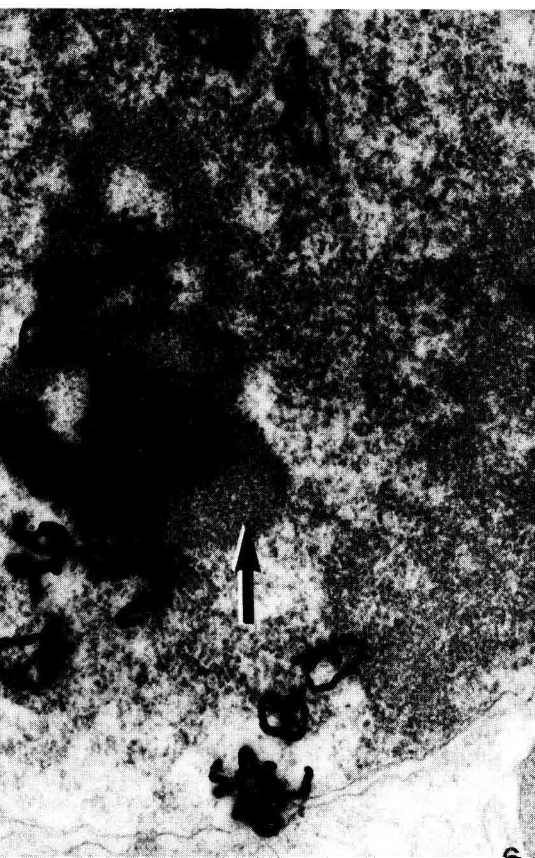
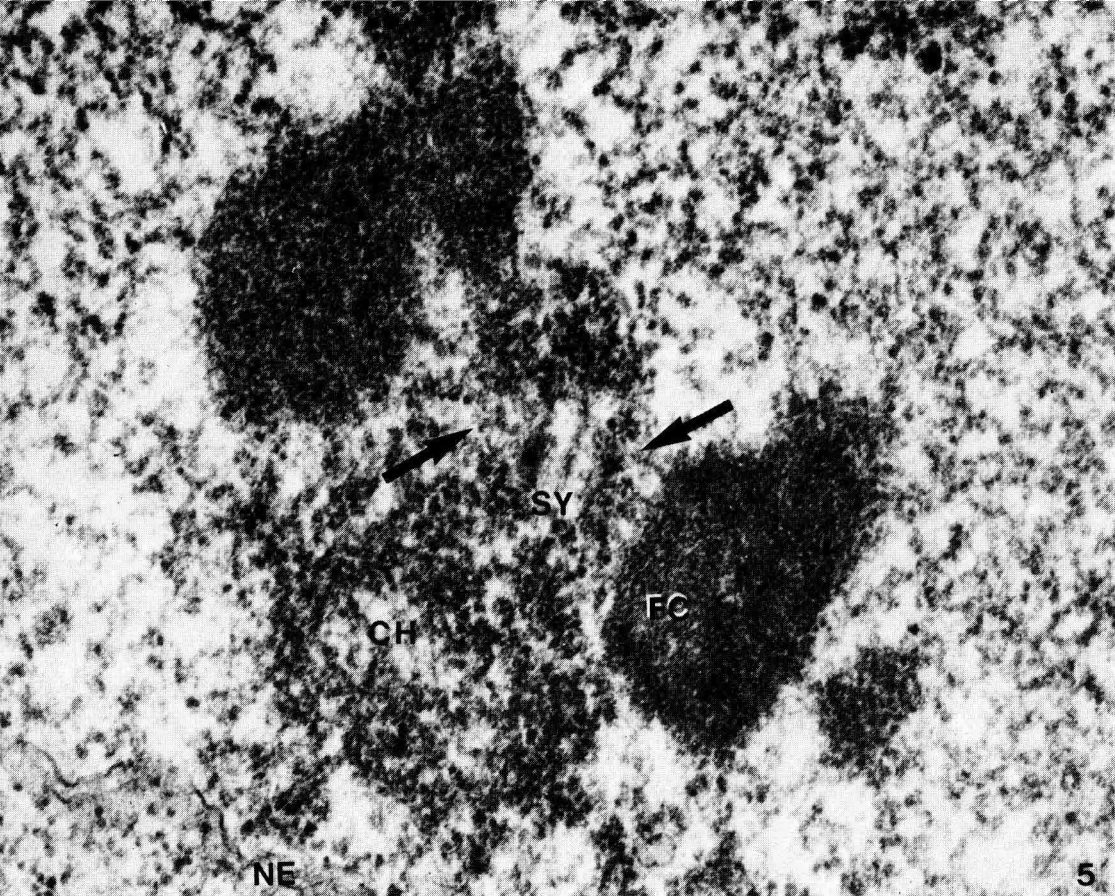
It is probable that the chromatin fibres which penetrate into the fibrillar centre form loops by taking on a spatial configuration allowing rDNA transcription to occur. This configuration has already been proposed by Baker and Franchi (1967) in human oocyte chromosomes at diplotene. Kierszenbaum and Tres (1974) showed that spermatocyte bivalents in the mouse are of the « lampbrush » type with the fibres emanating from either side of the axial region forming lateral loops.

The transcription of rDNA in the fibrillar centre is still controversial (Recher *et al.*, 1976). Our studies using 3H-uridine incorporation reveal that transcription of

FIG. 5. — *Nineteen-day old embryonic mouse oocyte*. Synthesis of the 2 nucleoli is symmetrical in the secondary constriction region (arrows) of a bivalent the centromeric heterochromatin of which (CH) is attached to the nuclear envelope (NE). In the secondary constriction region 50 Å fibres penetrate into the fibrillar centre (FC). SY: synaptonemal complex ($\times 57\ 000$).

FIG. 6. — *Autoradiography following 3H-uridine incorporation for 30 min*. Labelling grains are located over the fibrillar components of the nucleolus. The fibrillar centre (arrow) is unlabelled. Oocyte of 15-day old mouse embryo ($\times 31\ 500$).

FIG. 7. — *Mouse oocyte at pachytene stage*. *In situ* hybridization with tritium labelled ribosomal RNA. Autoradiography after 70 days exposure. The silver grains (arrows) are located near the secondary constriction. This localization corresponds to the fibrillar centre ($\times 2\ 000$).



the ribosomal cistrons essentially occurs at the periphery of the fibrillar centre. A hypothetical shift of the preribosomal RNA following its transcription is difficult to accept under our experimental conditions which excluded chase after ³H-uridine incorporation. Furthermore, since the studies of Miller and Beatty (1969) it is known that the newly synthesized RNA does not immediately detach itself from the rDNA, thus allowing visualization of the transcription units. Consequently, the localization of labelling grains must be accepted as corresponding to the transcription sites of the rDNA. This conclusion leads us to believe that the rDNA fibres emanating from the secondary constriction in the mouse oocyte or from the euchromatin of certain microchromosomes in the quail oocyte, extend through the bulk of the fibrillar centre up to its periphery. It is in this peripheral region that the rDNA is transcribed into rRNA. This notion would thus explain the presence of strands of electron-opaque fibrils directly in contact with the fibrillar centre. Accordingly, these strands would be composed of both DNA and newly synthesized RNP. Our observations thus lead to the conclusion that the fibrillar centre and its surrounding strands of electron-opaque fibrils constitute a functional unit.

Several problems nevertheless remain unresolved : on the one hand, how should we interpret the multiplicity of fibrillar centres characteristic of certain nucleoli, and secondly, how are the ribosomal cistrons arranged in those nucleoli apparently devoid of any fibrillar centre ?

According to Chouinard (1971), the multiple fibrillar centres in the diplotene mouse oocyte represent transverse or oblique sections through a contorted loop of chromatin belonging to the nucleolar organizer. Serial sections of the nucleolus would be necessary to verify this hypothesis. Moreover, any model of the spatial configuration of the ribosomal cistrons must take into consideration the existence of 5 pairs of nucleolar chromosomes in the mouse which apparently contain a variable gene number according to strain (Elsevier and Ruddle, 1975 ; Henderson *et al.*, 1976). If each active organizer gives rise to one fibrillar centre and if the former remains an individual unit, then the multiple fibrillar centres observed cannot be excluded as being individual entities. Certain data indicate that the nucleolar organizers may be independant of one another ; apparently this occurs most often in the peripheral zone of the nucleolus. Indeed, in human fibroblasts *in situ* hybridization reveals that the ribosomal cistrons are arranged in several foci at the periphery of the nucleolus and are separated from each other (Vagner-Capodano *et al.*, 1977).

Finally, several cell types are known to present no fibrillar centre. Following brief ³H-uridine labeling in monkey kidney cells cultured *in vitro*, the silver grains corresponding to newly synthesized RNA are found in both the condensed peri- and intra-nucleolar chromatin and in the fibrillar zones of the nucleolus (Granboulan and Granboulan, 1965). In such nucleoli lacking a fibrillar centre, the ribosomal cistrons appear to be more diffusely arranged, and this configuration may correspond to that of the fibrillar strands. The latter could be equivalent to the layer of dense fibrils which surround the fibrillar centre. Two types of arrangements of the ribosomal cistrons can thus be imagined : a) focal arrangement, evidenced by the presence of a fibrillar centre and characterized by the presence of abundant protein (Recher *et al.*, 1969) ; b) a diffuse configuration where the rDNA would be located within the

fibrillar strands. In this variety, the protein component is lacking. Intermediate types (many small fibrillar centres) could exist between these two extreme configurations.

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Résumé. La localisation, la structure et l'activité des organisateurs nucléolaires ont été étudiées dans les ovocytes de caille et de souris pendant la prophase méiotique, par la microscopie électronique et l'hybridation *in situ* par des ARN 18S et 28S marqués au tritium.

Le nucléole nouvellement formé est constitué d'un centre fibrillaire entouré par une couche de fibrilles denses. Les fibres de DNP qui ont un diamètre de 50 Å s'étendent de l'organisateur nucléolaire au centre fibrillaire et pénètrent dans ce dernier. Au début de la synthèse nucléolaire, la position du centre fibrillaire coïncide avec celle des cistrons ribosomiques révélés par l'hybridation *in situ*.

Après une brève incorporation d'uridine tritiée, le marquage est localisé sur la couche de fibrilles opaques aux électrons, adjacente au centre fibrillaire.

Ces observations indiquent que le rADN est présent à la fois dans le centre fibrillaire et dans la couche fibrillaire dense qui sont toujours associés et que la transcription des cistrons ribosomiques se produit essentiellement dans la couche fibrillaire dense.

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