NUCLEOLAR ORGANIZER STRUCTURE 
AND ACTIVITY IN A NUCLEOLUS WITHOUT 
FIBRILLAR CENTRES: THE NUCLEOLUS IN 
AN ESTABLISHED DROSOPHILA CELL LINE

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SUMMARY
Classical electron-microscopic techniques (enzymic digestion, EDTA regressive staining) 
allied with autoradiographic studies after [3H]uridine incorporation or after RNA synthesis 
initiated by an exogeneous RNA polymerase in the presence of tritiated GTP, enabled us to 
describe the fine structure and activity of the nucleolus in an established Drosophila cell line. 
This nucleolus is composed of a large central multilobed core containing proteins, RNA 
molecules and a DNA-containing component. This core is surrounded by and connected to 
large clumps of dense fibrillar nucleolus-associated chromatin, which are intermingled with 
fibrillogranular ramifications extending from the core towards the nuclear envelope. These 
ramifications are covered by granules of ribosomal ribonucleoprotein. As shown by EDTA 
regressive staining the nucleolar core contains a ribonucleoprotein network, which unravels 
and ramifies within a fibrous matrix. RNA synthesis takes place at the level of this network in 
the internal part of the core. The molecules synthesized are associated with proteins and are 
exported out of the core in the form of granules. Although it is composed of the same con-
stituents as other nucleoli, the nucleolus of Drosophila cells seems to be less organized, in that 
it never displays fibrillar centres, which have been referred to as the nucleolar counterparts of 
the nucleolus-organizers in a wide variety of organisms.

INTRODUCTION
In comparison to other genes, the ribosomal cistrons are unique in that their 
transcription occurs in a chromosomal complex: the nucleolus (for a review, see 
Busch & Smetana, 1970). The fine structure and size of the nucleolus are very sensitive 
to changes in ribosomal activity. The nucleolus can therefore serve as a cytological 
indicator of ribosomal RNA gene transcription (and in this respect is analogous to the 
puffs of the polytene chromosomes) (Ghosh, 1976).

The nucleoli of mammalian somatic cells can be classified into three types, ‘ring-
shaped’, ‘nucleolonema-containing’ and ‘compact nucleoli’, according to their 
appearance in the electron microscope. They are all composed of three main compo-
nents: nucleolar chromatin, a granular component containing premature ribosomal 
particles and a fibrillar component composed of ribonucleoprotein material (Busch & 
Smetana, 1970; Smetana & Busch, 1974). In addition to these classical components,
in a large variety of cells the nucleoli also contain clear fibrillar zones, which Recher called 'fibrillar centres' (Recher, Whitescarver & Briggs, 1969). These fibrillar centres have been referred to as corresponding to the nucleolar counterparts of the chromosomal nucleolar-organizer regions (Goessens & Lepoint, 1979).

In *Drosophila melanogaster*, the nucleolus-organizing regions (NORs) are located in the proximal centromeric heterochromatin of the X chromosome, and on the short arm of the Y chromosome (Kaufman, 1936; Ritossa & Spiegelman, 1965). According to Ritossa & Spiegelman (1965) about 0.25% of the DNA in the haploid genome of *D. melanogaster* is used to transcribe the 18S and 28S rRNA molecules. From these data they calculated that there are approximately 130 cistrons for the 18S and an equal number for the 28S rRNA in each sex chromosome.

Developmental and ultrastructural studies were made on the nucleoli of *Drosophila* ovarian nurse cells (Dapples & King, 1970) and of *Drosophila* primary spermatocytes (Meyer & Hennig, 1974). These studies have shown that changes in nucleolar morphology and activity could be used as a rapid means of identifying transient stages of cellular activity. Nevertheless, no precise ultrastructural identification of the nucleolar components was made, rendering it very difficult to interpret the morpho-functional changes observed.

In this paper we report ultrastructural studies on *Drosophila* KCo cell nucleoli based on a combination of classical electron-microscopic observations allied with autoradiographic studies after tritiated uridine incorporation, or after RNA synthesis initiated in vitro by an exogeneous RNA polymerase of *Escherichia coli* in the presence of tritiated GTP.

**MATERIALS AND METHODS**

*Drosophila* KCo cells were grown in D22 medium without foetal calf serum in 5 cm² Falcon flasks at 22 °C (Echallier & Ohanessian, 1970). All the electron-microscopic observations were made with a Siemens Elmiskop 101 microscope at 80 kV.

**Routine electron microscopy**

The cells were harvested on ice and washed with phosphate-buffered saline (PBS). They were treated by double fixation: (1) 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), containing 0.1 M sucrose for 15 min at 6 °C. (2) After washing with the buffered solution, the cells were postfixed in 2% osmium tetroxide in identical buffer for 20 min. After dehydration in a graded series of acetone the cells were embedded in Epon. Thin sections were collected on copper grids and contrasted with uranyl acetate and lead citrate.

**Bernhard's (1969) regressive EDTA staining**

The cells were fixed only in 3% glutaraldehyde in 0.1 M phosphate buffer, without sucrose for 1 h at 4 °C. Fixation was followed by washing for at least 1 h in the same buffer. After embedding in Epon, thin sections were collected on grids and contrasted with uranyl acetate. EDTA (0.2 M) was allowed to act for 45 min. The sections were contrasted with lead citrate.

**Enzymic studies**

After simple fixation in 3% glutaraldehyde in 0.1 M phosphate buffer, the cells were embedded in glycol methacrylate (GMA, Polysciences Inc.) according to Leduc & Bernhard...
Sections of gold interference colour were collected on gold grids and treated with the following enzymes: (1) Pronase, 0.3% aqueous solution, pH 7.4 for 10 min at 40°C. (2) RNase (after Pronase, 0.3%, 2 min), 0.7% solution in 2x SSC (SSC is 0.15 M-NaCl, 0.015 M-Na citrate), pH 7.2, 3 h at 37°C. (3) DNase (after Pronase, 0.3%, 2 min) 0.4% solution in 7 mM-MgSO₄, pH 6.8 for 3 h at 37°C.

Staining was carried out with both uranyl acetate and lead citrate.

RNA synthesis initiated by an exogeneous RNA polymerase

The technique used was that described by Geuskens (1977). Ultra-thin sections of KCo cells embedded in glycolmethacrylate (GMA) as described above, were incubated for 2 h 30 min at 37°C on the surface of the following medium: 100 μM-ATP, CTP, UTP; 150 μM-[³H]GTP (NEN, 17 Ci/mmol); 40 mM-Tris.HCl, pH 7.9; 10 mM-MgCl₂; 0.1 mM-EDTA; 0.1 mM-dithiothreitol; 0.15 M-KCl; 0.5 μg/ml bovine serum albumin; 10 units/ml E. coli RNA polymerase holoenzyme. After incubation, the sections were washed in 0.15 M-NaCl for 5 min and then in three baths of 5% trichloracetic acid containing 2% Na pyrophosphate for 10 min at 4°C. After washing in distilled water, the sections were harvested on Formvar-coated carbon grids and covered with Ilford L₄ emulsion. The autoradiograms were developed after exposure for 3 months with Kodak D₁₉b developer.

Ultrastructural autoradiography

The cells were incubated in the culture medium in the presence of 100 μCi/ml of [³H]uridine (CEA, 29 Ci/mmol) during 5 min, and either followed or not by a 30 min chase in unlabelled medium. They were immediately double-fixed with glutaraldehyde and osmium tetroxide, as described above. Medium gold sections were placed on Formvar-coated grids. Each grid was covered with the golden zone of Ilford L₄ emulsion in a platinum loop as described by Bouteille, Dupuy-Coim & Moyne (1975).

Exposure was for three months at 4°C. Sections were developed with Kodak D₁₉ developer and fixed with Kodak rapid fixer.

RESULTS

Nucleolar morphology

In most cases, only one large nucleolus was observed in each KCo cell nucleus. In certain cells we have also observed two smaller individual nucleoli, which are associated with it.

The nucleolus is always located at the vicinity of the nuclear envelope on which it is attached by its associated chromatin. Depending upon growing conditions or cell-cycle stage the morphology of the KC cell nucleolus is variable: at the ultrastructural level it can display a compact aspect or a less compact reticulated structure with, however, several intermediary morphologies. Three main nucleolar morphologies can be observed:

1) The nucleolus shown in Fig. 1A resembles that observed at the onset of nucleolar development during the blastoderm stage of *Drosophila* embryogenesis (Knibiehler, unpublished data). This nucleolus displays two types of components: a central fibrillogranular structure, flanked by two greatly condensed masses of fibrillar material, which could correspond to chromosomal heterochromatin; it is surrounded by clumps of nucleolus-associated chromatin.

2) The compact nucleolus shown in Fig. 1B corresponds to an inactivated state of nucleolar activity, since it was observed during cell starvation. This nucleolus comprises a round central grainy structure, surrounded by a small electron-opaque layer
Drosophila nucleolus

of fibrillar granular material. A segregated crown of dense perinucleolar chromatin envelops this structure.

(3) The multilobed or reticulated nucleolar morphologies shown in Fig. 2A and B are found in KCo cells during exponential growth. They correspond to the nucleolar expression of ribosomal RNA synthetic activity. They are the most frequent types of nucleolar morphology that we found and were considered to be the 'normal' nucleolar organization in KCo cells.

The nucleolar mass, which can cover up to one-third of the nuclear area, is always attached to the nuclear envelope by large heterochromatin segments (Fig. 2B). Sometimes, cytoplasmic invaginations, where numerous ribosomal particles are visible, penetrate the nucleoplasm to reach the nucleolar area as large channels.

It is very difficult to describe the fine organization of such a nucleolus since all the components appear to be intermingled. In the nucleolus of glutaraldehyde-fixed, uranyl-stained cells we can distinguish: (1) a large central fibrillar granular mass, which we have called the 'nucleolar core', displaying an irregular multilobed heterogeneous structure; (2) several clumps of dense fibrillar material constituting nucleolus-associated chromatin that flank this core and are connected with it; (3) numerous ramifications that seem to extend through the nucleolus-associated chromatin towards the nuclear envelope.

Enzymic digestions

All the intermingled nucleolar components appear, after treatment with Pronase (10 min), in a lightly stained background, which allows a better understanding of their organization (Fig. 3).

The clumps of nucleolus-associated chromatin that are located at the periphery of the nucleolus appear more contrasted than the nuclear chromatin with which they are connected, suggesting that they are inactive heterochromatin. Several small masses of heterochromatin are also visible intermingled with the internal components of the nucleolus.

The internal nucleolar core displays a more or less multilobed structure. Its internal...
Drosophila nucleolus

part is composed of electron-opaque fibrillar material, which becomes granular at the periphery. The core extends fibrillogranular ramifications throughout the nucleolus-associated chromatin.

Digestion by ribonuclease (3 h) after mild treatment with Pronase (2 min) markedly diminishes the contrast of the cytoplasm, while nuclear and nucleolar chromatin structures are less affected and remain well contrasted (Fig. 4).

The central nucleolar core has lost some of its contrast. After RNase digestion it displays a heterogeneous structure: darker threads of RNase-resistant material can be seen embedded in a bleached homogeneous component. Granules are no longer visible over the edges of the core, and the fibrillogranular ramifications have disappeared, suggesting that the nucleolar core and its extending ramifications are partially composed by RNA molecules and proteins.

Following digestion with deoxyribonuclease (3 h) after mild treatment with Pronase (2 min), both nuclear and nucleolar chromatins are completely bleached, so that the nucleolar core appears more contrasted (Fig. 5). The latter seems to be less affected by DNase, although it displays a more homogeneous grainy texture after DNase treatment than after RNase digestion. This observation confirms the results described above: the central nucleolar core is made up of RNA molecules and proteins (resistant to DNase), but also by a DNA-containing component (resistant to RNase), which could represent the intranucleolar chromatin.

Regressive colouration by Bernhard's EDTA technique

After simple fixation and staining by Bernhard's (1969) regressive EDTA method, the ribonucleoprotein can be seen as electron-opaque structures within nuclear as well as nucleolar chromatin that has lost its contrast. (Fig. 6).
The dense heterochromatin masses of the nucleolus-associated chromatin appear, after EDTA regressive staining, as bleached homogeneous fibrillar structures devoid of ribonucleoproteins. The large central nucleolar core is partially bleached by the EDTA treatment and its heterogeneous structure is revealed: it is composed of a homogeneous fibrous matrix embedded with darker threads of ribonucleoprotein, which is related to the bleached fibrillar masses of the nucleolus-associated chromatin. This ribonucleoprotein ramifies as a network within and at the periphery of the nucleolar core. On the edges of the core numerous small granules of ribonucleoprotein can be observed, but none were resolvable within the confines of the core (Fig. 6). The ramifications extending out of the core are in continuity with this ribonucleoprotein network and their granular texture is more apparent: in their proximal portions they are composed of fibrillar material covered with small discrete granules of ribonucleoprotein (RNP). As they penetrate the bleached nucleolus-associated chromatin towards the nuclear envelope, they appear to be covered with larger granules of ribonucleoprotein, looking like preribosomal particles. They become completely granular in their distal portions, so that several individual granules of rRNP are observed at the vicinity of the nuclear envelope (Fig. 7).

RNA synthesis initiated by an exogeneous RNA polymerase

According to Geuskens (1977), the labelling observed in ultrathin sections incubated in vitro in a medium containing E. coli RNA polymerase holoenzyme and all four ribonucleoside triphosphates (GTP being tritium-labelled) probably results from a limited synthesis of RNA initiated in vitro on the chromatin DNA contained in the sections, whatever the specificity of this initiation may be.

Fig. 8 shows a KCo cell incubated under these conditions. The labelling is restricted to clumps of nuclear chromatin and, at the nucleolar level, to the central nucleolar core. Neither the nucleolus-associated chromatin, nor the rRNP fibrillar granular ramifications are labelled.

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Fig. 7. This peripheral section of a nucleolus reveals, after EDTA regressive staining, the fibrillar granular (arrows) and also granular (arrowheads) nature of the RNP ramifications extending from the periphery of the nucleolar core towards the nuclear envelope (ne) through the bleached, fragmented heterochromatin masses (nh). x 52,000.

Fig. 8. Ultrastructural autoradiography after RNA synthesis initiated on ultrathin sections by E. coli RNA polymerase in the presence of tritiated GTP. The label is restricted to the internal parts of the nucleolar core (fgc). The clumps of nucleolus-associated chromatin intermingled with the ramifications extending from the core are devoid of silver grains. ne, nuclear envelope. x 27,000.

Fig. 9. Ultrastructural autoradiography after 5 min of [3H]uridine incorporation. The labelling is restricted to the central nucleolar core. The heterochromatin masses associated with the nucleolus are devoid of silver grains, as well as the ramifications extending from the nucleolar core (arrows). x 22,000.

Fig. 10. Ultrastructural autoradiography after pulse-labelling with tritiated uridine (5 min) followed by a 30-min chase. We observed (as compared to Fig. 9) a shift of the label from the internal part of the central nucleolar core (fgc) towards its periphery and the proximal portions of the ramifications. x 25,000.
This result demonstrates the presence, within the nucleolar core, of an intra- nucleolar chromatin DNA that is accessible to the RNA polymerase, and thus available for transcription in vivo. It confirms the result obtained by enzymic digestions, that the nucleolus-associated chromatin is in a supercoiled state and thus inaccessible to RNA polymerase.

Ultrastructural autoradiographic studies

Following incorporation of tritiated uridine for 5 min, the labelling is more prominent over the nucleolar structures than over the nucleoplasm where some clusters of silver grains are localized over clumps of chromatin (Fig. 9). This result demonstrates the high level of nucleolar RNA synthetic activity (rRNA) in the KCo cells chosen for their 'normal' nucleolar morphology.

At the nucleolar level, the labelling is restricted to the central nucleolar core and to a lesser extent to the proximal portions of the fibrillogranular ramifications that develop through the nucleolus-associated chromatin. The dense fibrillar masses of the nucleolus-associated chromatin are devoid of silver grains, demonstrating, as previously postulated, its inactivity in transcription.

If the pulse-labelling was followed by a 30 min chase in unlabelled medium (Fig. 10) we observed a shift of the label from the internal parts of the nucleolar core towards its periphery and the proximal portions of the ramifications. This result demonstrates that RNA molecules are synthesized within the confines of the nucleolar core, and as their maturation proceeds they migrate out of this structure towards the nucleopores.

DISCUSSION

The single nucleolus of Drosophila KCo cells appears as a large dense complex structure, always attached to the nuclear envelope by large clumps of condensed associated chromatin. It can cover up to one-third of the nuclear area.

Depending upon cell-cycle stage or growing conditions, the morphology of this nucleolus is very variable but in normal growing conditions it displays a multilobed structure at the ultrastructural level.

The multilobed morphology described here corresponds to an active transcription of the ribosomal cistrons. We think that it represents an intermediate nucleolar type between the compact nucleolus observed at the onset of nucleolar formation in Drosophila embryos during the blastoderm stage (McKnight & Miller, 1976; Knibiehler, unpublished data) and the segregated inactivated nucleolus of starved Drosophila KCo cells shown in Fig. 1B.

The internal organization of such a nucleolus is not obvious, since its components appear to be intermingled. The use of classical ultrastructural techniques (enzymic digestion, EDTA regressive staining) in correlation with autoradiographic observations after tritiated uridine incorporation enabled us to describe better the fine ultrastructural organization and activity of the multilobed nucleolus of Drosophila KCo cells.
We can distinguish three main intermingled nucleolar components:

1. A large central fibrillar granular mass displaying a lobed structure: the nucleolar 'core'. Its dense heterogeneous structure resembles that described in another insect nucleolus (Locke & Huie, 1980). Studies by enzymic digestion with Pronase, RNase and DNase have shown that the nucleolar core is composed of a proteinaceous fibrous matrix embedded with both RNA and DNA molecules.

2. Several dense homogeneous fibrillar masses, which contain electron-opaque DNA fibres and are devoid of RNA molecules. They are present at the periphery of the nucleolar core and constitute the nucleolus-associated chromatin. Drosophila KCo cells are derived from female embryonic cells and display an XX or XXX caryotype (Best-Belpomme & Courgeon, 1977). The electron-opaque masses of the nucleolus-associated chromatin could correspond to inactive heterochromatin segments surrounding the nucleolus organizer regions (NORs), which are located on the X chromosomes of Drosophila.

3. Intermingled with the clumps of nucleolus-associated chromatin, large ramifications extend from the nucleolar core towards the nuclear envelope. These ramifications are covered with numerous granules; they are composed of proteins and RNA molecules.

Autoradiographic studies of RNA synthesis initiated in ultrathin sections of KCo cells by an exogeneous RNA polymerase show that the labelling is restricted to the central part of the nucleolar core. According to Geuskens (1977), the results obtained by this technique 'could provide a useful tool for studying, in situ, the accessibility to RNA polymerase of DNA'. We thus interpret our results as demonstrating the presence within the confines of the nucleolar core of an intra-nucleolar chromatin, which is accessible to RNA polymerase and thus available for transcription in vivo. These results also confirm that nucleolus-associated chromatin is in a supercoiled state, preventing the access of RNA polymerase molecules to the DNA template.

Ultrastructural observations after EDTA regressive staining, allied with autoradiographic studies after a pulse-labelling with [3H]uridine, allowed us to confirm these results and to specify the functional organization of the nucleolar components.

EDTA regressive staining reveals a pattern of darker threads, which are made up of ribonucleoprotein and ramify, as a network, within the bleached fibrous matrix of the central nucleolar core. Autoradiographic studies after pulse-labelling with [3H]uridine demonstrate that active RNA synthesis takes place within the core. We thus conclude that the DNA of the intranucleolar chromatin, which unravels in the central nucleolar core, is ribosomal DNA. The ribosomal RNA molecules synthesized are associated with proteins after their transcription; together they comprise the internal ribonucleoprotein network. No individual granule of RNP is resolved within the confines of the core, the ribonucleoprotein network becomes granular at its periphery where some discrete granules of rRNP can be seen in an electron-opaque layer (Bernhard, 1966). The ramifications extending out of the nucleolar core are continuous with the internal ribonucleoprotein network and are covered with granules of rRNP. They are less or not labelled after 5 min of [3H]uridine incorporation; but, after a 30 min chase in unlabelled medium, a shift of the label is observed from the
internal part of the nucleolar core towards its periphery and the proximal portions of the ramifications.

These results are in agreement with those described for RNA transcription and maturation in most nucleoli (for a review, see Fakan & Puvion, 1980). As their maturation proceeds, the rRNP particles enlarge and are exported from the periphery of the nucleolar core towards the nucleopores.

Preliminary ultrastructural observations of the nucleolus in cells of the imaginal wing discs of \textit{D. melanogaster} (Martynova, 1979), suggest that the nucleolar organization that we describe here for \textit{Drosophila} KCo cells is characteristic of this insect; it is also similar to that described for another insect, \textit{Calpodes} (Locke & Huie, 1980).

Although the \textit{Drosophila} KCo cell nucleolus is composed of the same classical components (peri- and intra-nucleolar chromatin that is granular and fibrillar), its ultrastructural organization does not resemble those of mammalian, avian and plant nucleoli. When active in rRNA synthesis it is not of the nucleolonemal type (Mirre & Stahl, 1981), neither does it display a ring-shaped morphology (Mirre & Stahl, 1976). Its compact structure is not similar to that described in a variety of cells after natural or artificial inactivation of the nucleolar activity (Smetana & Busch, 1974).

Moreover, one of the most interesting ultrastructural features of the \textit{Drosophila} nucleolus is the absence of fibrillar centres. These structures were found in a large variety of cells, in both animals and plants, in normal cells as well as in tumour cells, \textit{in vivo} and \textit{in vitro} (for a review, see Goessens & Lepoint, 1979). In some cellular types they are not obvious, but they become more conspicuous under some experimental conditions (Goessens, 1976). The fibrillar centres contain DNA and proteins but no RNA molecules, they are most commonly seen adjacent to or embedded in the fibrillar component and are frequently associated with chromatin (Mirre & Stahl, 1976, 1981).

The fibrillar centres have often been interpreted as corresponding to the nucleolar counterparts of the active nucleolus-organizing regions of the chromosomes (Goessens & Lepoint, 1979). However, no transcription occurs within them but it is initiated at their periphery (Lafontaine & Lord, 1973; Goessens & Lepoint, 1974; Mirre & Knibiehler, 1981); so that, as pointed out by Wachtler, Ellinger & Schwarzacher (1980), this correspondence does not prove identity. Though active organizers could be obscured by the products of transcription and maturation (Miller & Beatty, 1969; Franke \textit{et al.} 1979) in active KCo nucleoli, the fact that the \textit{Drosophila} nucleolus never displays such structures, even in inactivated states, favours this hypothesis.

The absence of fibrillar centres in the \textit{Drosophila} KCo cell nucleolus is neither relevant to the low number of nucleolar organizers, since \textit{Macacca fascicularis} nucleolus displays fibrillar centres whereas this organism has, like \textit{Drosophila}, one pair of nucleolar organizers (personal observation), nor to the number of ribosomal RNA cistrons (130 per haploid genome; according to Ritossa & Spiegelman, 1965).

Our results indicate that the fibrillar centres do not seem to be essential structures of nucleoli. However, they have been demonstrated to contain specific argyrophilic proteins that have been associated with nucleolar activity (Hubbell, Rothblum & Hsu, 1979; Bourgeois, Hernandez-Verdun, Hubert & Bouteille, 1979). We think that
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fibrillar centres could correspond to storage structures for these proteins and/or to skeletal elements of a specific matrix structure.

The Drosophila KCo cell nucleolus might be less organized. For polytene chromosomes, Rodman (1969) suggested that DNA from the chromocentre extends into the nucleolus like a puff. The organization we observe here could be of the same type: the intranucleolar chromatin, in continuity with the nucleolus-associated chromatin and originating from the nucleolus-organizer regions (NORs), extends and unravels as a puff within the nucleolar core matrix where it is transcribed.

In order to clarify this morphofunctional organization we are now studying the effects of the moulting hormone ecdysone, which controls insect metamorphosis (Doane, 1973), on the ultrastructure and activity of Drosophila KCo cell nucleoli.

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