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Original article

Localization of DNA within Ehrlich tumour cell nucleoli by immunoelectron microscopy

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The distribution of DNA in Ehrlich tumour cell nucleoli was investigated by means of an immunocytochemical approach involving a monoclonal antibody directed against double- and single-stranded DNA. Immunolabelling was performed either before or after the embedding process. The postembedding labelling method allows better ultrastructural preservation than the preembedding labelling method. In particular, the various nucleolar components are well preserved and identifiable. In the nucleolus, labelling is particularly concentrated over the perinucleolar chromatin and over its intranucleolar invaginations, which penetrate the nucleolar body and often terminate at the fibrillar centres. In addition, aggregates of gold particles are found in the fibrillar centres, preferentially towards the peripheral regions. By contrast, the dense fibrillar component is completely devoid of labelling. The results seem to indicate that DNA containing the rDNA genes is located in the fibrillar centres, with a preference for the peripheral regions. This finding suggests that transcription of the rDNA genes should occur within the confines of the fibrillar centre, probably close to the boundary region of the surrounding dense fibrillar component. The results are discussed in the light of present knowledge of the functional organization of the nucleolus.

nucleolus — DNA — monoclonal antibody

INTRODUCTION

The penetration of DNA into the nucleolar body has been well documented [7]. However, its spatial distribution within the nucleolar body has not been clearly established. In particular, the question of whether DNA occurs in both fibrillar components (the dense fibrillar component and the fibrillar centres) is still under discussion.

For the majority of authors, the fibrillar centres (FC) and the dense fibrillar component both contain DNA. This concept is the result of autoradiographic studies using tritiated thymidine or tritiated actinomycin D or cytochemical stains, *e.g.*, the osmium-ammine method (for review see [7]). However, high-resolution autoradiographic studies after pulse labelling of cells with tritiated uridine have demonstrated that the fibrillar centres are generally devoid of silver grains, whereas incorporation of RNA precursors was usually detected first in the dense fibrillar component. Thus, the DNA of the fibrillar centres was considered to represent transcriptionally inactive rDNA regions [4, 7].

For some authors, however, the fibrillar centres do not contain DNA. These researchers interpret the fibrillar centres as skeletal and/or storage structures for proteins involved in rDNA transcription and/or in later steps of ribosome biogenesis. This concept is supported by the observations that the fibrillar centres are composed mainly of proteins, that the FC are sometimes not visible [9], and that during mitosis they could appear as extrachromosomal material [16, 17].

Using a monoclonal antibody that reacts specifically with DNA in both double- and single-stranded forms, Scheer *et al.* [13] recently detected intranucleolar DNA exclusively in the fibrillar centres and not in the surrounding dense fibrillar component. This result confirms and extends their earlier observations based on immunolocalization of RNA polymerase I [15]. Thus, according to these authors, transcription of rDNA takes place in the fibrillar centres of nucleoli.

In the present work, the distribution of DNA in the extensively studied nucleolus of Ehrlich tumour cells was investigated with the same anti-DNA antibodies. Labelling was performed either before or after the embedding procedure.

MATERIALS AND METHODS

Cell cultures

Ehrlich ascites tumour cells (ELT) from the peritoneal cavity of C57B1 mice were cultured as monolayers in Petri dishes according to Lepoint and Bassleer [10]. The monolayer cultures were scraped off the dishes and centrifuged at low speed to form a pellet.

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Antibodies

Monoclonal antibodies (IgM) to DNA were used. The antibody binds to double- and single-stranded DNA but not to RNA. A detailed account of the characteristics of this antibody has previously been presented [13].

Electron microscope immunolocalization

Preembedding method

Cryostat sections 4-μm thick through a pellet of Ehrlich cells frozen in phosphate-buffered saline (PBS: 0.14 M NaCl, 0.009 M Na_2HPO_4, 0.001 M KH_2PO_4, pH 7.2) containing 0.2% bovine serum albumin (BSA; Sigma Type V) and 10% sucrose were laid down on coverslips. After being air-dried, the preparations were dipped in cold (−30°C) acetone for 10 min and air-dried again. The preparations were first incubated for 1 hr with PBS containing goat normal serum diluted 1/30 and 0.5% BSA, and then rinsed with PBS containing 0.5% BSA, followed by incubation with antibodies to DNA (1 mg/ml) containing goat normal serum diluted 1/50 for 6 hr at room temperature. The preparations were thoroughly washed in PBS containing 0.5% BSA and incubated overnight with goat anti-mouse IgM coupled to colloidal gold (5-nm diam, Janssen Life Sciences, Beerse, Belgium) diluted 1/20 with PBS (pH 8.2) containing 0.5% BSA and 0.05% Tween 20. After several washes in PBS, the specimens on coverslips were fixed in cold 2.5% glutaraldehyde in 0.1 M Sørensen's buffer (pH 7.4) for 30 min. Specimens were then processed as previously described by Scheer and Rose [15].

Postembedding method

Small fragments of the pellet were fixed in 4% formaldehyde or in a mixture of 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M Sørensen's buffer (pH 7.4) at 4°C for 60 min. After fixation, the cells were washed in Sørensen's buffer and incubated in 0.5 M NH_4Cl at 4°C for 1 hr, dehydrated through graded ethanol solutions, then processed for embedding in Lowicryl K4M according to the technique of Roth *et al.* [12]. Some other fragments were fixed in 4% formaldehyde in Sørensen's buffer for 30 min, washed in Sørensen's buffer, incubated in 1 mg/ml pepsin (Merck) in 0.1 N HCl for 15 min. After this enzymatic digestion, the cells were incubated in 5% trichloroacetic acid for 15 min at 4°C and fixed again in 0.5% glutaraldehyde in Sørensen's buffer for 2 hr.

After washing in the same buffer, the cells were processed as described above.

Ultrathin sections of the various blocks were cut with a diamond knife and mounted on nickel or gold grids.

For labelling, the grids were incubated by floating them, cell sections down, on a drop of PBS containing goat normal serum diluted 1/30 and 2% gelatin, then rinsed with PBS containing 1% gelatin. The next step of the treatment was an incubation with antibodies to DNA (50 μg/ml) in PBS containing goat normal serum diluted 1/50 and 1% gelatin for 5 hr at room temperature. After washing with PBS containing 1% gelatin, the sections were incubated for 30 min with goat anti-mouse IgM coupled to colloidal gold (5–10-nm diameter, Janssen Life Sciences, Beerse, Belgium) diluted 1/25 with PBS, pH 8.2 containing 1% gelatin, at room temperature. After washing with PBS containing 1% gelatin, the sections were rinsed in deionized water. Some cell sections were preincubated at room temperature in 10 mg/ml pronase (Boehringer Mannheim) in 0.05 M Tris pH 6.8 for 10 min before being mounted on grids. The sections were stained with uranyl acetate and lead citrate before examination in a Jeol CX 100 II electron microscope at 60 kV.

Four different kinds of controls were carried out. First, the monoclonal DNA antibody was omitted. In the second control, the grids were preincubated at 37°C for 3 hr with 1 mg/ml DNase I (Sigma) in PBS containing 7 mM $MgCl_2$. Third, the grids were preincubated in 3.5 N HCl for 20 min at 37°C. In the fourth control, the grids were incubated with gold particles free of antibodies.

RESULTS

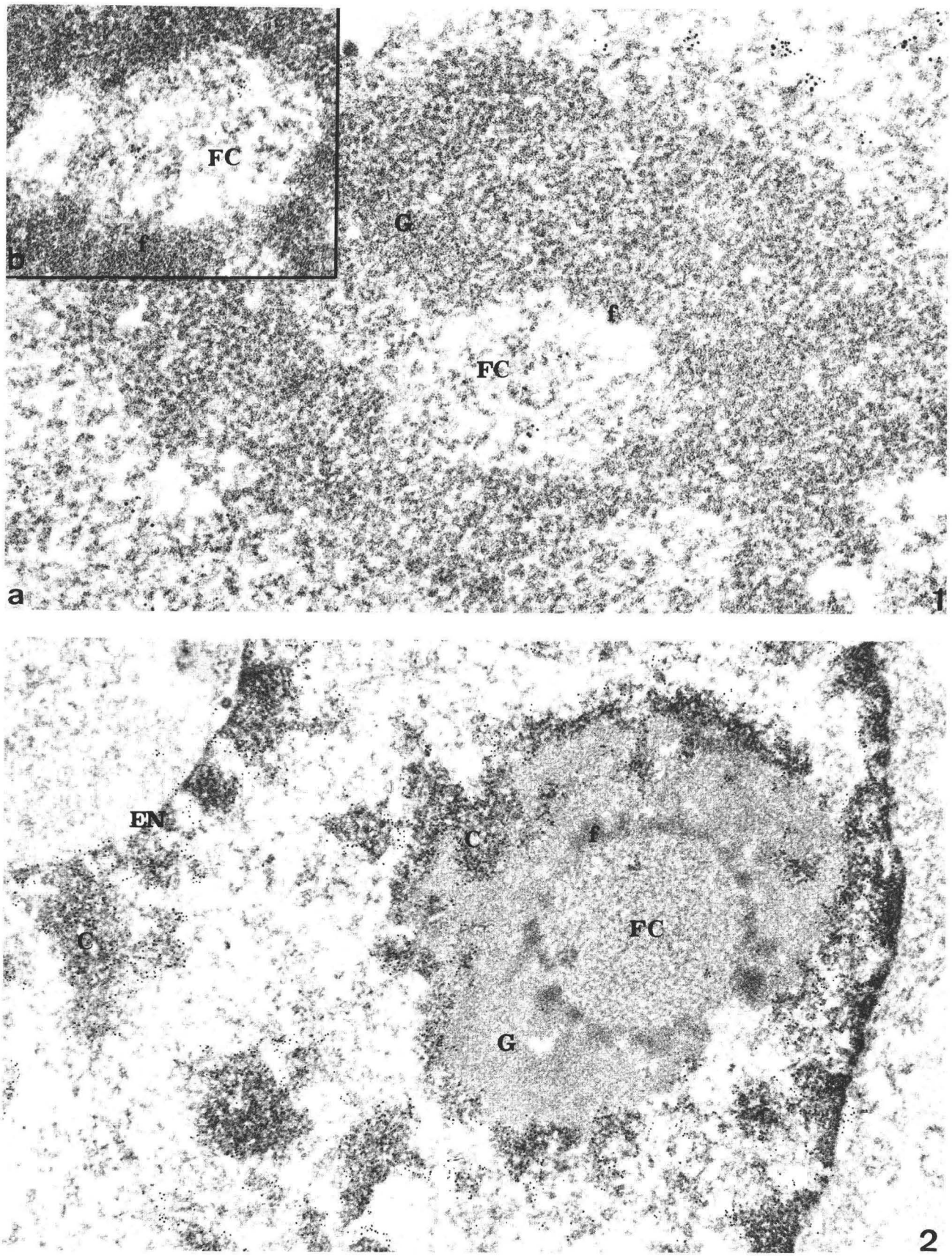
When labelling is performed before embedding, gold markers bind preferentially to the nucleoplasmic chromatin (Fig. 1a). However, this method induces marked ultrastructural changes. Specifically, a decondensation of chromatin is seen and the distinction between the various nucleolar components becomes less clear. In addition, the fibrillar centres appear to be somewhat loosened. In some nucleoli (Fig. 1a), gold particles can be seen inside the fibrillar centres. More precisely, the labelling of the fibrillar centres and the absence of gold particles in the surrounding dense fibrillar component are well visualized (Fig. 1b). Gold particles were found only in relaxed fibrillar centres such as shown in Figure 1. Fibrillar centres with a more compact texture are not decorated.

To improve ultrastructural preservation, immunolabelling was performed on Lowicryl ultrathin sections. This postembedding method allows excellent distinction between the various nucleolar components, as illustrated in Figure 2. Under these conditions, gold particles are located preferentially in the condensed chromatin associated with the nucleolus and with the nuclear envelope (Fig. 2). Large spaces between these heterochromatic blocks remain free of labelling. Over the nucleolus, gold particles are particularly concentrated at the perinucleolar chromatin shell and intranucleolar invaginations. The granular component is totally devoid of gold particles. This situation is especially well displayed when the cell sections are preincubated with pronase (Fig. 3). The main effect of this treatment is to free the nucleolus from the granular component, thus increasing the visibility of intranucleolar clumps of chromatin. Under such conditions gold particles bind to the rim of chromatin that surrounds the nucleolus and to the numerous chromatin invaginations that, in sections, appear as widely separated islands of chromatin (Fig. 3).

Some of the labelled intranucleolar invaginations of chromatin even come into contact with the fibrillar centres and interrupt the layer of dense fibrils surrounding them (Fig. 4). Such chromatin regions are quite conspicuous since they are usually embedded in small nucleolar cavities.

Gold particles are also present in the fibrillar centres. This is especially clearly seen after pronase extraction (Fig. 5). With this treatment, gold particles are observed in the network of fibrils present in the fibrillar centres. In untreated Lowicryl sections, gold particles are preferentially distributed towards the periphery of the fibrillar centres, often in close proximity to nucleolar cavities or interstices (Figs. 6, 7). In some fibrillar centres, a cluster of gold markers is also seen in the central area (Fig. 7 and 8). In this case, the labelled material seems to correspond to specific structures that are characterized by a higher electron density than the surrounding material of the fibrillar centres.

Except for the presence of a few rare gold particles (Fig. 7), the dense fibrillar component surrounding and occasionally also located inside the fibrillar centres is free of gold particles. Even in this last case, however, gold



FIGURES 1 AND 2. – Immunoelectron microscopy of intranucleolar DNA in Ehrlich tumour cells by either the preembedding technique (Fig. 1) or the postembedding technique (Fig. 2). Gold particles (5-nm diam), reflecting the distribution of the monoclonal antibody, are particularly concentrated over the condensed chromatin (**C**) associated with the nuclear envelope (**EN**) and surrounding the nucleolus. In both cases, the fibrillar centres (**FC**) of the nucleolus are labelled and the granular component (**G**) and the dense fibrillar component (**f**) are devoid of labelling. **Fig. 1:** (*a*) Portion of nucleus containing a nucleolus. ×77,000. (*b*) Detail of a fibrillar centre. ×64,000. **Fig. 2:** 4% formaldehyde, pepsin, 5% trichloroacetic acid, 0.5% glutaraldehyde, Lowicryl. ×27,000.

markers are present at the periphery of the dense fibrillar component (Fig. 9).

The specificity of DNA labelling has been tested in several ways. First, there is virtually no labelling if the primary antibody is omitted. Next, if the antigen is specifically removed from the sections by treatment with DNase I, labelling is almost completely abolished. The same result is obtained after a strong hydrolysis (Fig. 10). Finally, when gold lacking the antibody tag is used, gold particles do not bind to the sections.

DISCUSSION

To study the spatial distribution of DNA inside the nucleolus of Ehrlich tumour cells, an immunocytochemical approach at the ultrastructural level was employed. A monoclonal antibody that specifically reacts with DNA in both double- and single-stranded forms [13] was used either before or after the embedding procedure. The preembedding labelling technique has the advantage that the antibody binding reaction can be performed on cell sections without any chemical fixation and before the dehydration and plastic embedding processes. All these preparative steps of embedding methods of biological material have an adverse influence on the antigens [12]. However, as previously pointed out by Priestley [11], a major disadvantage of pre-embedding staining could be the slow penetration of the antibodies into the relatively thick (4 μm) cryosections. The variations in labelling of the fibrillar centres according to their state of preservation is probably related to this problem. In addition, this method leads to quite drastic ultrastructural changes. Specifically, the chromatin appears decondensed and the fibrillar centres are often in a loosened configuration.

In order to improve ultrastructural preservation, immunolabelling was performed on ultrathin sections of cells embedded in Lowicryl K4M. In agreement with the observations of Roth *et al.* [12], the low-temperature embedding method in Lowicryl K4M gave excellent preservation of the ultrastructure. In particular, excellent distinction between the various nucleolar components was obtained. The labelling pattern obtained confirms numerous previous observations, based on a variety of methods, that the shell of perinucleolar chromatin is connected with clumps of intranucleolar chromatin penetrating into the nucleolar body [7]. The fibrillar centres have been shown to be attached to and continuous with this condensed chromatin [1, 6, 19].

In addition, we also demonstrated the presence of DNA in the fibrillar centres. This result is in agreement with recent observations using hepatocytes treated with the same antibody [13]. It also confirms the results of many cytochemical investigations performed by means of enzymatic digestions, preferential or specific stainings, *in situ* hybridization, and autoradiography after incorporation of tritiated thymidine or tritiated actinomycin D into cells [for review see 7].

As judged from the distribution of gold particles, DNA is probably restricted to certain specific regions inside the fibrillar centres. Specifically, DNA-positive regions were preferentially located at the periphery of the fibrillar centres and only rarely found in their central parts. This non-uniform DNA distribution within the fibrillar centres contradicts the results obtained by Derenzini *et al.* [5] by means of the Feulgen-like osmium-ammine reaction according to Cogliati and Gautier [3]. Indeed, Derenzini *et al.* [5] have shown that the fibrillar centres of various cell types contain uniform agglomerates of extended, non-nucleosomal DNA filaments.

The DNA present at the periphery of the fibrillar centres does not constitute a continuous layer, but instead forms several individual foci. Generally, these DNA clumps are included within or situated close to nucleolar interstices. This frequent association between nucleolar cavities and the intranucleolar chromatin located at the border of the fibrillar centres might be of functional significance, as discussed by Chouinard [2].

In none of our preparations, following both the pre- and the postembedding labelling protocols, could we detect antibody binding to the dense fibrillar component surrounding the fibrillar centres. This result is surprising in view of numerous studies based on a variety of experimental approaches which suggested that DNA is present in the dense fibrillar component [for review see 7]. Clearly, one has to ask whether the absence of gold particles in the dense fibrillar component, as observed in the present study, reflects the true absence of DNA in this nucleolar region and not simply such a low local packing density of DNA that it escapes detection by our immunocytochemical approach. In addition, it may also be asked whether those rDNA regions that are engaged in transcriptional activity (*i.e.* covered by polymerase molecules) are accessible to the antibodies to the same extent as transcriptionally inactive DNA stretches. Although these questions cannot be answered for the moment, our data do not support the concept that transcriptionally active rRNA genes are present in the dense fibrillar component. This interpretation is in agreement with autoradiographic studies using tritiated thymidine and tritiated actinomycin D performed on a cell type identical to that used in the present work, which revealed DNA exclusively in the fibrillar centres and not in the dense fibrillar component [8].

Furthermore, the location of RNA polymerase I in the fibrillar centres [14, 15] also supports the notion that rDNA genes occur in the fibrillar centres.

Although the dense fibrillar component does not seem to contain DNA, some DNA-positive regions are found in the border region between the fibrillar centres and the surrounding dense fibrillar component. The similarity of this DNA location and the distribution of RNA, as obtained by autoradiographic studies [8, 20] or by the RNase-gold method [18], suggests that rDNA transcription could occur at the border between the fibrillar centres and the dense fibrillar component. If our interpretation is correct, that active rRNA genes are clustered in the peripheral part of the fibrillar centres, then the functional role of the surrounding dense fibrillar component in the process of ribosome biogenesis remains to be defined again, since the dense fibrillar component has been generally interpreted as the superposition of transcriptionally active rRNA genes and their primary transcription products.

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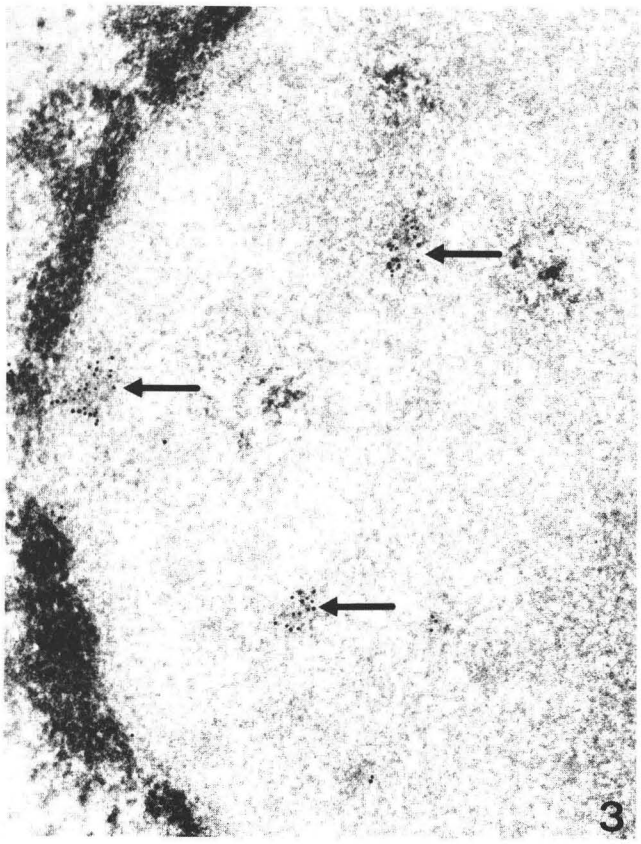
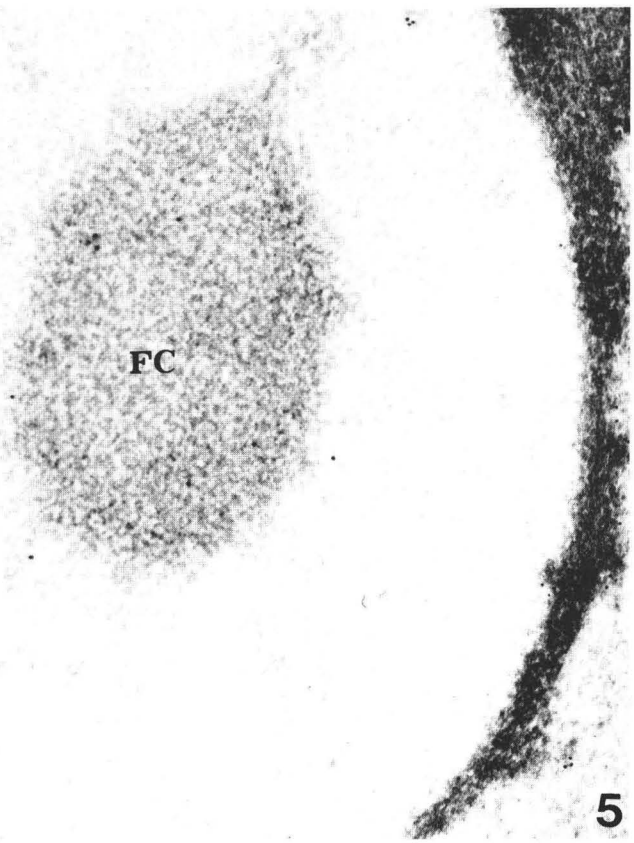
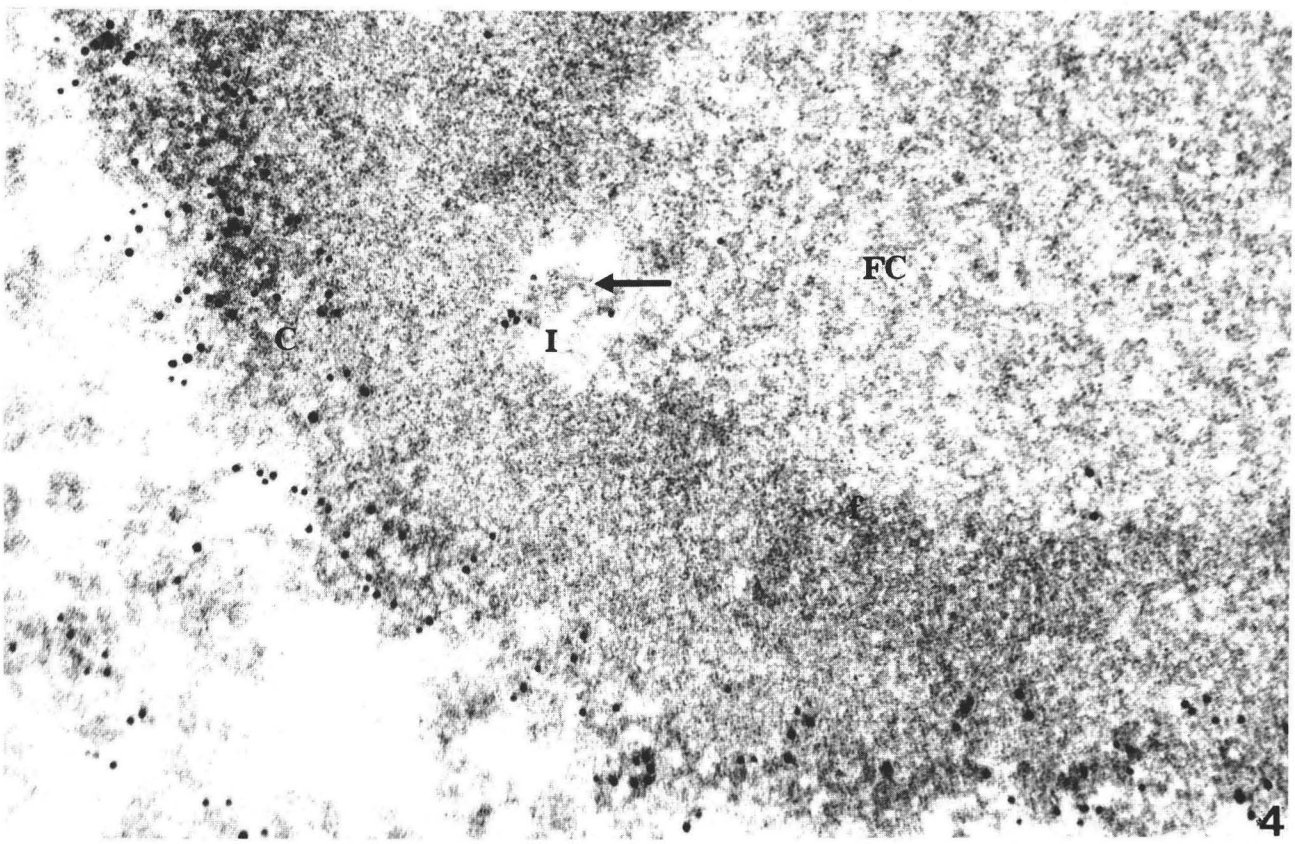
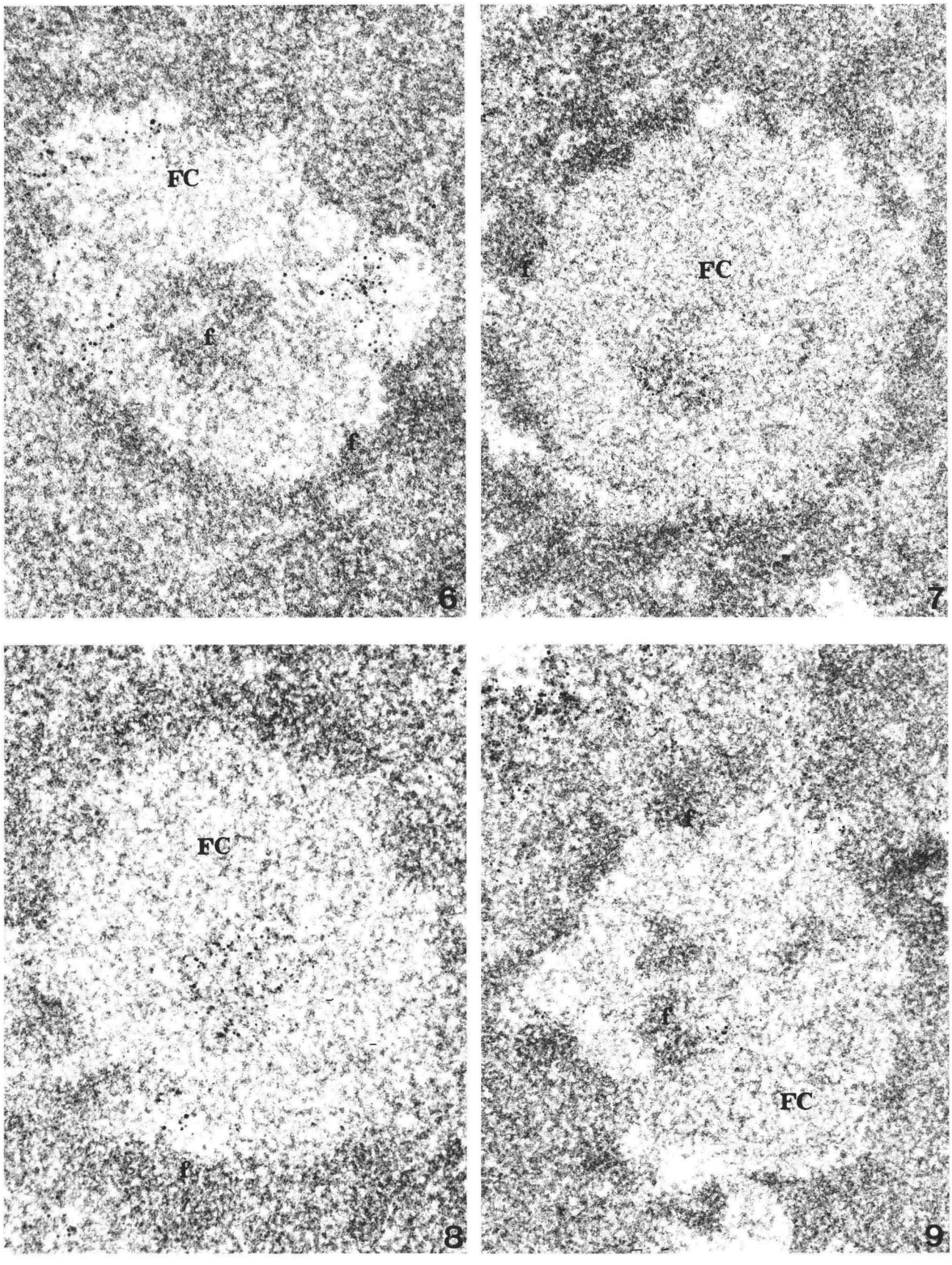






FIGURES 3–5. – Immunoelectron microscopy of intranucleolar DNA on sections of Lowicryl-embedded Ehrlich tumour cells. **Fig. 3:** Preincubation of the thin section with pronase. Gold particles can be localized over the perinucleolar chromatin (**P**) and over their conspicuous intranucleolar invaginations (*arrows*). ×36,000. **Fig. 4:** Some of the labelled intranucleolar invaginations of chromatin (*arrows*) even come into contact with the fibrillar centres (**FC**). These clumps of chromatin are generally situated in an interstice (**i**). **f**, dense fibrillar component; **C**, condensed chromatin. ×80,600. **Fig. 5:** Gold particles are present in the fibrillar centre (**FC**) after pronase extraction. **P**, perinucleolar chromatin. × 37,500.



FIGURES 6–9. – Immunoelectron microscopy of intranucleolar DNA on sections of Lowicryl-embedded Ehrlich tumour cells. Gold particles are present in the fibrillar centres (**FC**), the labelling appears frequently localized at the border of the FC. In some FC (Figs. 7, 8), a cluster of gold markers can also be seen in the central area. The dense fibrillar component (**f**) is almost completely unmarked; however, gold particles are present at its periphery, even when the dense fibrils are located inside the FC (Fig. 9). Fig. 6: ×89,600; Fig. 7: ×66,000; Fig. 8: ×86,800; Fig. 9: ×68,000.

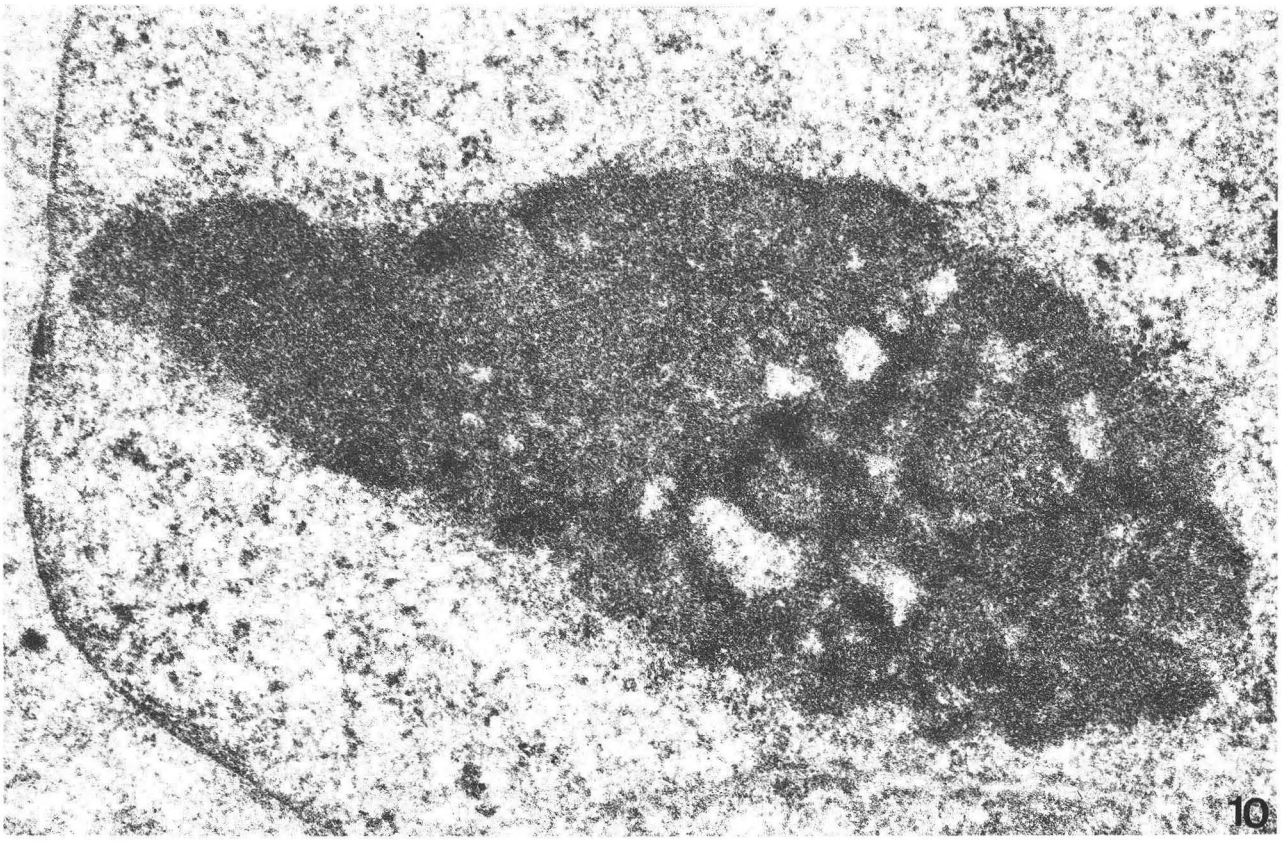


FIGURE 10. – Labelling control on Lowicryl section of Ehrlich tumour cells showing part of a nucleolus. 3.5 N HCl hydrolysis of the section for 20 min before labelling; only a few gold particles are visible over the condensed chromatin of the nucleus. ×31,200.

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