

A CYTOCHEMICAL AND RADIOAUTOGRAPHIC STUDY OF HUMAN TISSUE CULTURE CELL NUCLEOLI

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ABSTRACT

Fine structural aspects of human tissue culture cell nucleoli were studied by cytochemical and radioautographic methods. Ribonuclease and pepsin digestions were carried out on glutaraldehyde-fixed cells that, in some instances, were labeled with thymidine-³H prior to digestion. Double digestion by ribonuclease and pepsin revealed a fine fibrillar reticulum that appears to be the supportive structure of nucleolonemal threads. The nature of the reticulum remains to be determined. The question of whether it may represent a dispersed form of chromatin was raised. Structural findings suggested such an hypothesis but the results of radioautographic studies do not support it. The reticulum showed a striking absence of radioactive labeling following a 3 hr incorporation of thymidine-³H. Only few silver grains were observed occasionally in the fibrillar nucleolonema that may or may not be significant. The radioautographic results are believed to be inconclusive for the various reasons discussed. The possibility that the reticulum is composed of proteins has to be considered. It appears that basic proteins can resist pepsin digestion in aldehyde-fixed cells. Individual chromatin fibrils were found to be associated with the nucleolar reticulum. It is possible that these alone represent the dispersed genetically active chromatin of nucleoli.

INTRODUCTION

Several investigators (8, 15, 16, 26-28) have found Feulgen-positive material within nucleoli. Electron microscopic studies have shown that the chromatin of the nucleolar surface (nucleolus-associated chromatin) extends deep into the nucleolar body in the form of irregular strips or regular lamellae (9). In addition, intranucleolar incorporation of thymidine-³H has been demonstrated in a variety of cells by light and electron microscopy (2, 9, 11, 12). Therefore, little doubt exists that chromatin is an essential component of nucleoli. The term "intranucleolar chromatin" has, in most instances, however, been applied to condensed chromatin located in the meshes of the nucleolonema (1-5, 9, 13, 18, 20, 26, 29, 31, 32).

Littau et al. (17) and Granboulan and Granboulan (10) observed no significant incorporation of radioactively labeled RNA precursor in condensed chromatin and concluded that condensed chromatin is genetically inactive. If this applies to nucleoli, then it is difficult to visualize how they can be actively involved in RNA synthesis, unless one assumes that a dispersed, genetically active form of chromatin is part of their structure. Kasten and Strasser (14) studied the nucleic acid synthetic patterns in synchronized mammalian cells and differentiated two fractions of intranucleolar DNA, both having their own distinctive pattern of renewal. The renewal of the first fraction in the early S phase was accompanied by a simultaneous repression of RNA synthesis, but no such repres-

sion was associated with the synthesis of the second fraction that occurred later in the S phase. The authors (14) therefore suggested that the first fraction may represent a genetically active chromatin, whereas the second fraction is likely to be genetically inactive or repressed ("heterochromatin"). Lettré et al. (16) described Feulgen-positive material in the nucleolonemal threads and interpreted these structures to be functionally active parts of chromosomes. The nucleolonemal threads referred to by these authors (16) appear to correspond to the fibrillar component of nucleoli observed in the electron microscope. Granboulan and Granboulan (10) have shown that the fibrillar component is labeled very early after short pulses of uridine-³H incorporation. This may suggest that genetically active chromatin is located in fibrillar areas.

To further clarify this point, an attempt was

made to demonstrate a dispersed form of nucleolar chromatin by enzyme digestion techniques. Although the results are not conclusive, pertinent observations were made that are summarized in this report.

MATERIALS AND METHODS

Tissue Culture

The rapidly growing CMP cell line (obtained from Dr. D. Rounds, Pasadena Foundation for Medical Research, Pasadena, California) has been used in most initial studies. The cell line derives from a human adenocarcinoma and has been propagated in our laboratory on Eagle's minimum essential medium (MEM), supplemented with 20% calf serum or 30% fetal calf serum. In more recent studies, the cell line ME-180, derived from a metastatic carcinoma of the cervix, was employed. This cell line was established in our laboratory and is being

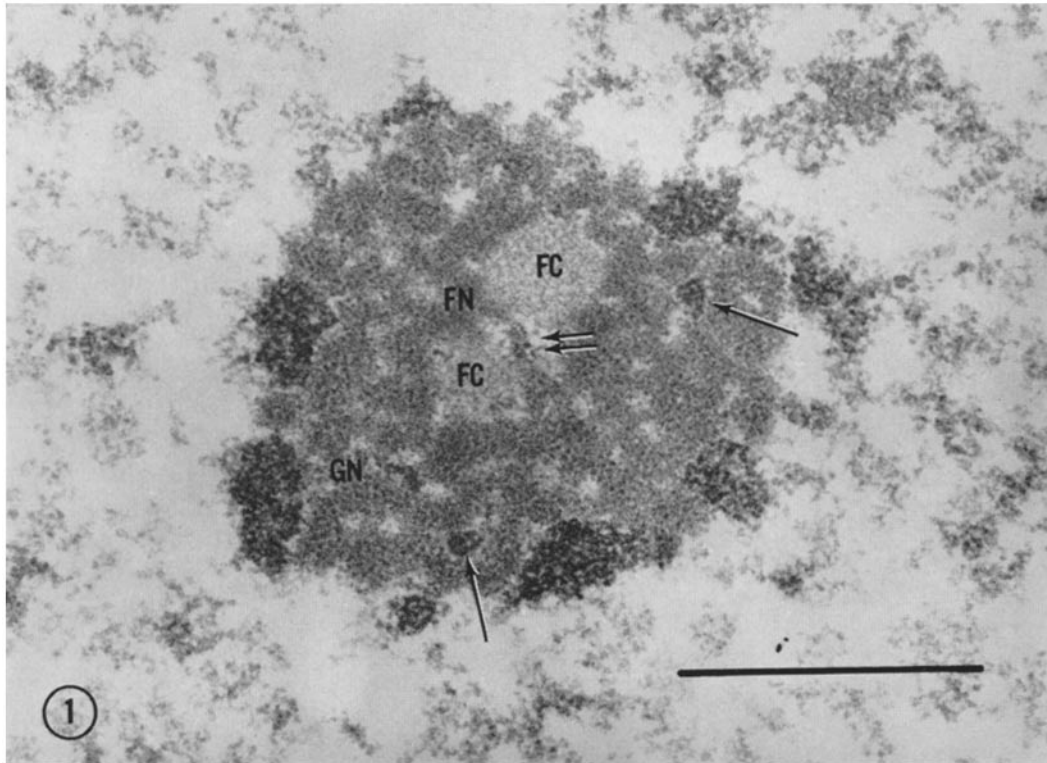


FIGURE 1 Nucleolus of a CMP cell that has been digested with pepsin (0.1 mg/ml) for 1 hr at 37°C. The nucleolonema is well formed and is composed of both granular (GN) and fibrillar (FN) substances. Two fibrillar centers (FC) are present. The intranucleolar (arrows) and nucleolus-associated chromatin is prominent. A small speck of chromatin is located between the two fibrillar centers (double arrow). Fixation: glutaraldehyde prior to digestion; osmium tetroxide following digestion. Marker = 1 μ . \times 40,000.

propagated on Eagle's MEM supplemented with 30% fetal calf serum. The antibiotics streptomycin sulfate and neomycin were added to nutrient media in the following concentrations: streptomycin sulfate, 25 mg/liter; neomycin, 100 mg/liter. Cultures processed for radioautography were labeled with methyl-thymidine- H^3 (100 μ Ci/ml medium, specific activity 10 Ci/mole, Schwarz Bio Research Inc., Orangeburg, N.Y.) for 3 hr at 37°C. All cultures were grown in T-flasks.

Electron Microscopy

The cells were detached with a rubber policeman from the glass surface of the T-flasks, pelleted at 1000 *g*, fixed with cacodylate-buffered 2% glutaraldehyde for 20–30 min, washed with 0.01 M Tris-(hydroxymethyl)-aminomethane (Tris), and then digested for 1–2 hr at 37°C with the following en-

zymes: ribonuclease (lyophilized, phosphate-free, Worthington Biochemical Corp., Freehold, N.J.) or ribonuclease (crystallized five times, Calbiochem, Los Angeles, Calif.) dissolved in 0.01 M Tris adjusted to pH 6.8 (1 mg/ml); pepsin (crystallized two times, Worthington) dissolved in 0.1 N HCl (0.1 mg/ml or 1 mg/ml). In some cases, ribonuclease digestion was followed by incubation in 5% trichloroacetic acid (TCA) for 10 min at 4°C. Cells that were treated with both enzymes were exposed first to ribonuclease, washed with Tris, and then digested with pepsin. Following enzymatic digestions, the cells were washed with Tris or Millonig's buffer, then pelleted in the same buffer, immediately dehydrated with ethyl alcohol, and embedded in Epon, or first post-fixed with 1% osmium tetroxide in Millonig's buffer for 1 hr and then further processed. In recent experiments, cells that had been digested with pepsin or ribonuclease and pepsin were immediately fixed with osmium tetroxide without being washed first with a buffer. It was found that

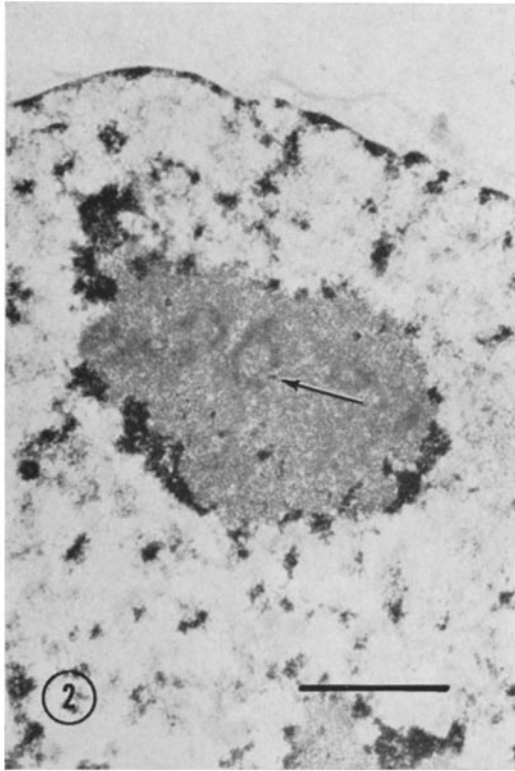


FIGURE 2 Nucleolus of ME-180 cell that has been digested with ribonuclease (1 mg/ml) for 1 hr at 37°C. The fibrillar and granular substances show a loss of electron opacity. Several fibrillar centers are present. A few small specks of chromatin are scattered throughout the nucleolar substance. One speck is interspersed in the fibrillar substance next to a center (arrow). Fixation: glutaraldehyde. Marker = 1 μ . \times 20,000.

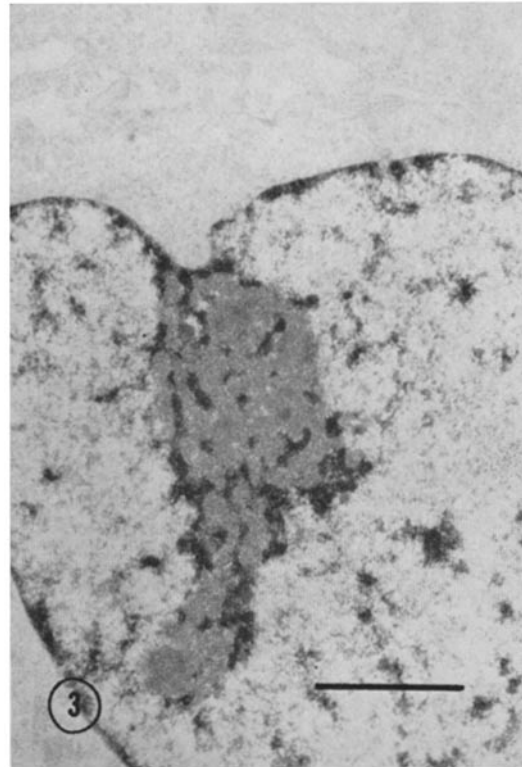


FIGURE 3 Nucleolus of ME-180 cell that has been digested with ribonuclease (1 mg/ml) for 1 hr at 37°C. The fibrillar and granular substances have a low electron opacity whereas the chromatin appears extremely dense. The intranucleolar chromatin is prominent. Fixation: glutaraldehyde. Marker = 1 μ . \times 20,000.

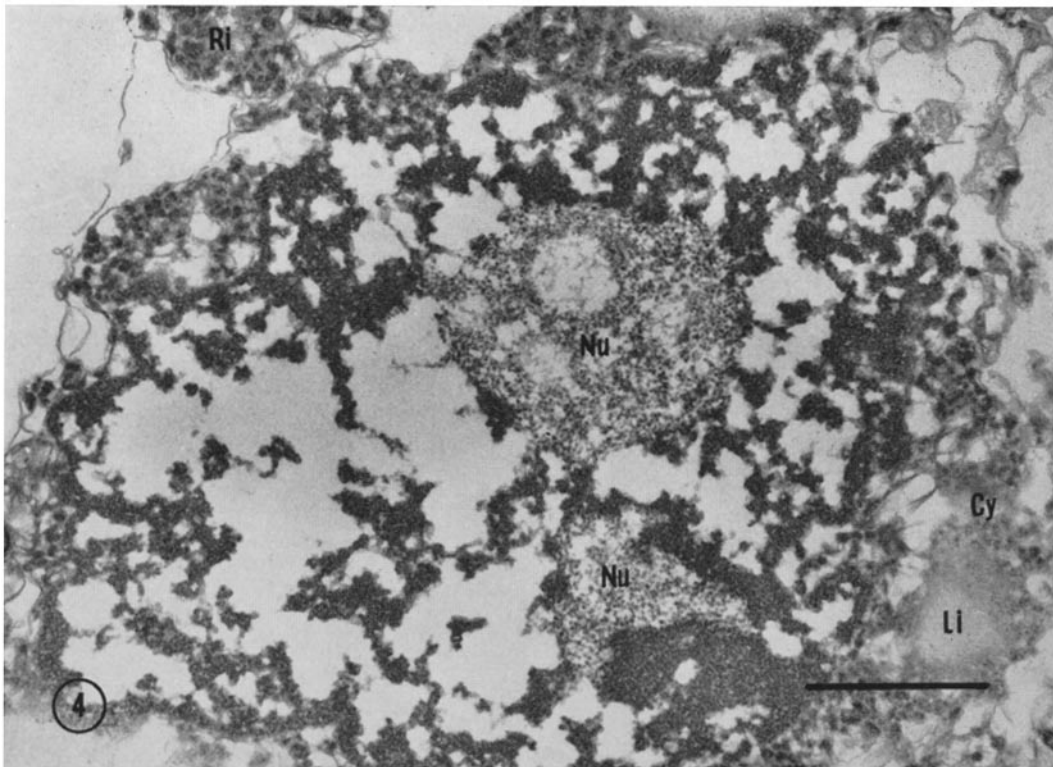


FIGURE 4 ME-180 cell that has been digested with pepsin (1 mg/ml) for 2 hr at 37°C. The chromatin is mostly condensed. The nucleoplasm seems to have been removed. The nucleoli (*Nu*) have a low electron opacity. The cytoplasm (*Cy*) shows neutral fat droplets (*Li*), swollen ribosomes (*Ri*), and essentially intact membranes. Fixation: glutaraldehyde prior to digestion; osmium tetroxide following digestion. Marker = 1 μ . \times 24,000.

immediate fixation prevents the undigested nucleolar structures from aggregating.

Sections were cut on an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) with a diamond knife at a thickness of 500–800 Å. They were collected on Formvar-coated copper screens, stained for 5 min with a saturated solution of uranyl acetate in 50% ethanol, washed with distilled water, and then stained with lead citrate for 3 min. The sections were thinly coated with carbon and examined in a Siemens Elmiskop IA.

For radioautography, sections mounted on Formvar-coated copper or stainless steel grids were routinely stained, carboned, and then coated with Ilford L-4 emulsion by the loop technique described by Caro and van Tubergen (6). Following an exposure of 1–2 wk, the sections were developed in Microdol X for 5 min and fixed with a 20% solution of sodium thiosulfate (hypo).

RESULTS

General

The structural components of nucleoli are easily differentiated in cells that have been subjected to mild pepsin digestion (Fig. 1).

The nucleoli of CMP and ME-180 cells, in most instances, show a well-formed nucleolonema. The granular component (granular nucleolonema) usually forms the bulk of the nucleolonemal mass. The fibrillar component (fibrillar nucleolonema) is largely confined to nucleolonemal threads that are located next to areas of low electron opacity. These latter areas are composed of fine fibrils that are interlaced and form a loose network. They will be referred to as “fibrillar centers” in the forthcoming text. The intranucleolar chromatin is found in the meshes of the nucleolonema and, on occasion, is

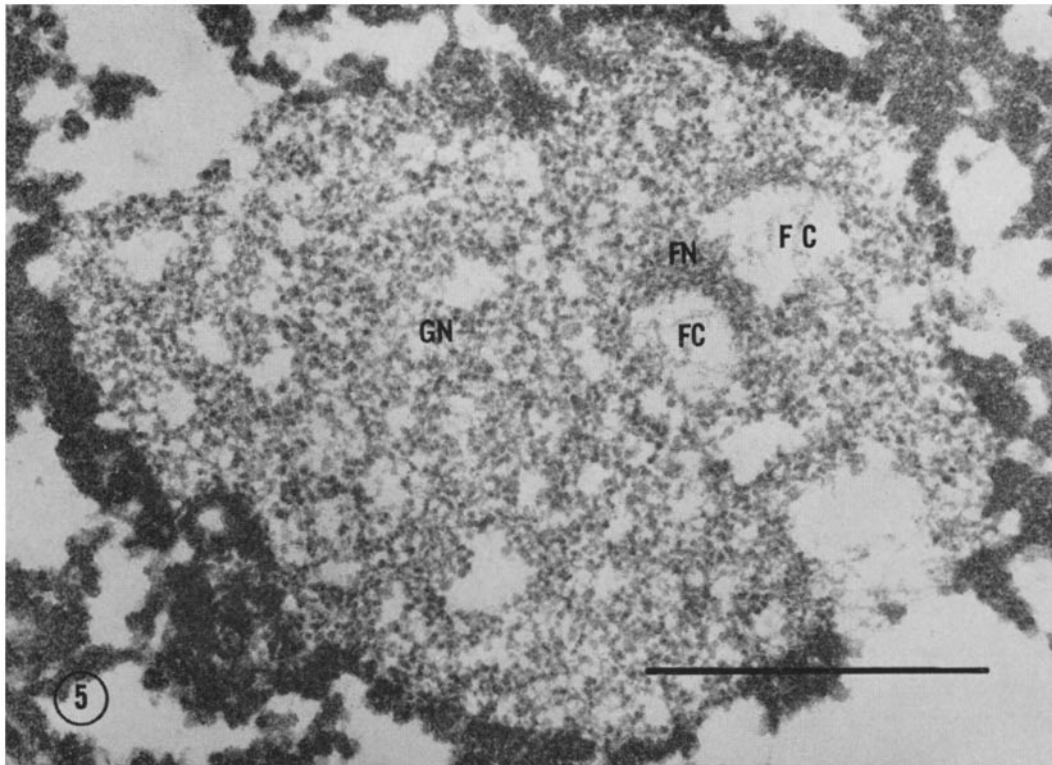


FIGURE 5 Nucleolus of ME-180 cell that has been digested with pepsin (1 mg/ml) for 2 hr at 37°C. The granular substance (GN) forms the bulk of the nucleolonema. The granules average 200 Å in diameter and often show a linear arrangement. The fibrillar substance (FN) is scant and is located next to two fibrillar centers (FC). Note the fine fibrils within the centers. Fixation: glutaraldehyde prior to digestion; osmium tetroxide following digestion. Marker = 1 μ . \times 45,000.

seen to communicate with the nucleolus-associated chromatin.

Enzyme Digestion Studies

RIBONUCLEASE: Ribonuclease digestion for 1–2 hr decreases the electron opacity of the fibrillar and granular nucleolonema (Figs. 2, 3). This loss of electron opacity is usually more evident in cells fixed with glutaraldehyde than it is in cells fixed with glutaraldehyde and osmium tetroxide. Digestion with ribonuclease does not appear to affect the fibrillar centers. The condensed nucleolar chromatin is usually quite prominent, mostly due to the low electron opacity of all other nucleolar components. The amount of the intranucleolar chromatin varies in individual nucleoli but may vary also in different areas of a single nucleolus. In general, areas with large numbers of fibrillar

centers contain only few small specks of condensed chromatin that are often found in the fibrillar substance near centers. Areas with an abundance of condensed chromatin often lack fibrillar centers and are composed primarily of granular substance.

PEPSIN: Pepsin digestion removes the amorphous proteinaceous matrix of nucleoli, leaving behind the formed elements. The degree of digestion depends on the length of the digestion time and the concentration of the enzyme. Fig. 4 illustrates a cell that has been digested with pepsin (1 mg/ml 0.1 N HCl) for 2 hr at 37°C. The amorphous nucleoplasm is largely removed. The chromatin in this instance is aggregated in irregular clumps, but occasionally it is seen to be dispersed. The nucleolus has lost much of its electron opacity. At higher magnification (Fig. 5) the various nucleolar components are readily

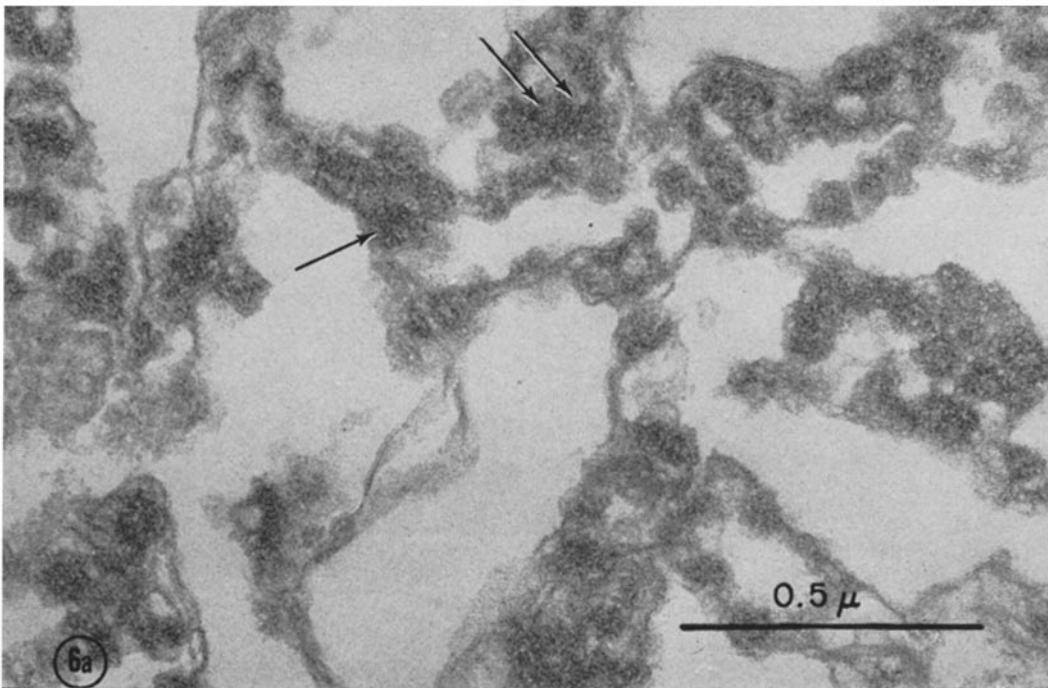
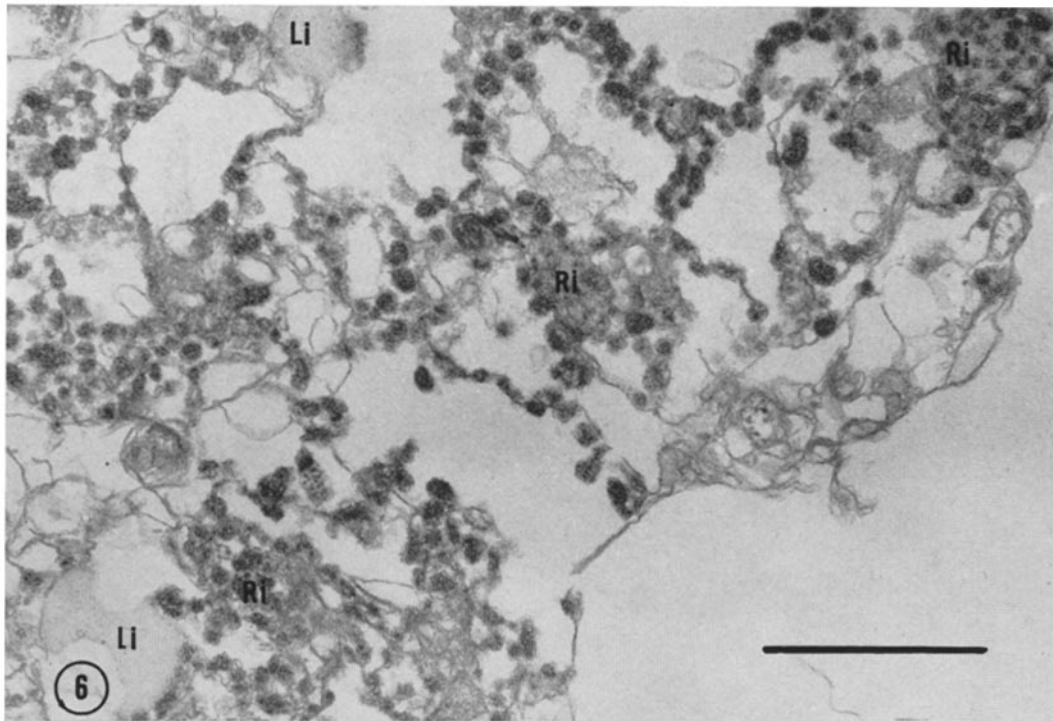


FIGURE 6 Cytoplasm of ME-180 cell that has been digested with pepsin (1 mg/ml) for 2 hr at 37°C. The proteinaceous matrix appears to have been removed completely. The membranes, neutral fat droplets (*Li*), and ribosomes (*Ri*) resisted digestion. The ribosomes (*Ri*) appear swollen and have become confluent. Fixation: glutaraldehyde prior to digestion; osmium tetroxide following digestion. Marker = 1 μ . $\times 30,000$.

FIGURE 6 *a* Same as Fig. 6 at higher magnification. Fine fibrils (arrows) can be observed occasionally in the ribosomal masses. $\times 80,000$.

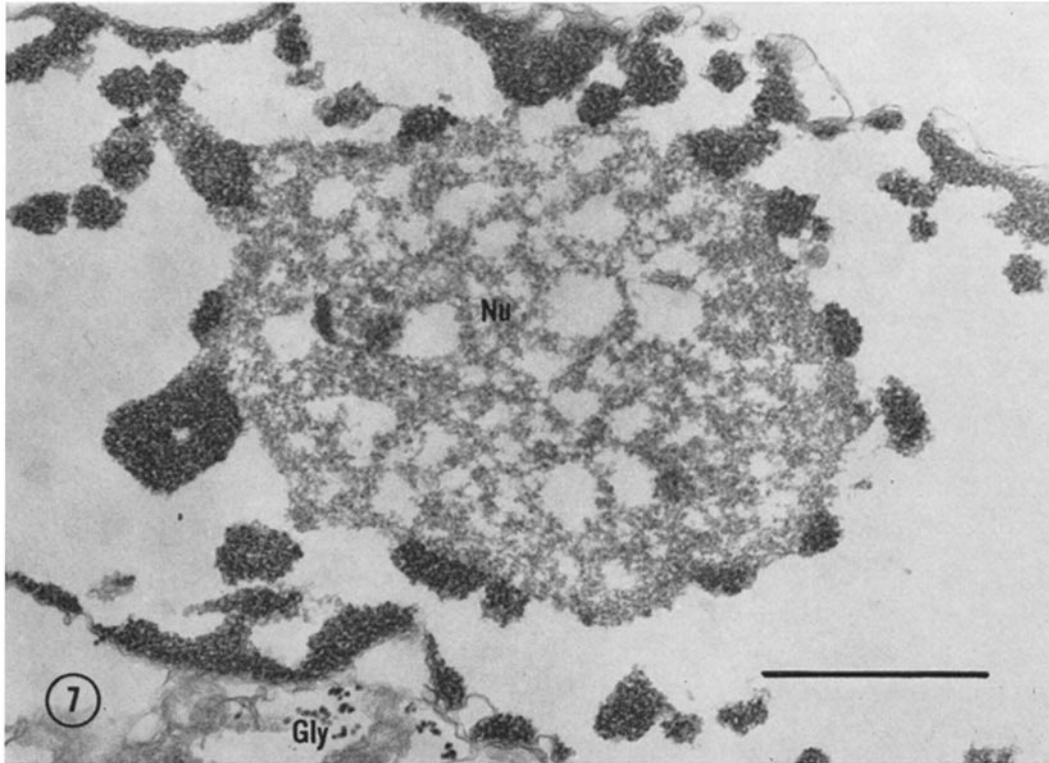


FIGURE 7 Nucleus of CMP cell that has been digested with ribonuclease (1 mg/ml) for 1 hr and pepsin (1 mg/ml) for 1 hr at 37°C. The nucleolus (*Nu*) shows a fine fibrillar reticulum that is patterned after the nucleolonema. The reticulum is continuous with the nucleolus-associated chromatin. The nuclear chromatin is mostly condensed. A few glycogen granules (*Gly*) are seen in the cytoplasm. Fixation: glutaraldehyde prior to digestions; osmium tetroxide following digestions. Marker = 1 μ . \times 30,000.

differentiated. The granules usually form the bulk of the nucleolar mass. They average 200 A in diameter and often appear arranged in a linear fashion as if they were residing on filaments. The fibrillar nucleolonema is primarily found in the vicinity of fibrillar centers. The fibrillar centers contain thin fibrils that average 50–70 A in thickness and appear to be continuous with fibrils of the associated fibrillar nucleolonema. The nucleolus is often framed by condensed chromatin that may or may not extend into the nucleolar substance.

Pepsin digestion also removes the proteinaceous matrix of the cytoplasm (Fig. 6). The membranes, the neutral lipids, the glycogen granules, and the ribosomes remain. The ribosomes show a rather interesting phenomenon. They appear to swell as a result of pepsin digestion and become confluent, forming large rounded masses (Fig. 6). In such

ribosomal masses, tiny fibrils of 20–30 A thickness can be distinguished if high electron opacity does not obscure details (Fig. 6 *a*). The ribosomal changes are seen only in cells that have been subjected to vigorous pepsin digestion.

RIBONUCLEASE-PEPSIN: Double digestion by ribonuclease and pepsin reveals a fine fibrillar reticulum (Figs. 7, 8). The display of the reticulum appears to be patterned after the original nucleolonema. The fibrils average 60–100 A in thickness and often appear to be twisted around each other. The reticulum is usually denser in nuclei that contain a markedly condensed chromatin (Fig. 7) than it is in nuclei that contain a more dispersed chromatin (Fig. 8). The fibrils of the reticulum are continuous with the fibrils of the intranucleolar and the nucleolus-associated chromatin. They form together an intricate system that appears to be the supportive structure of nucleoli. The fibrils

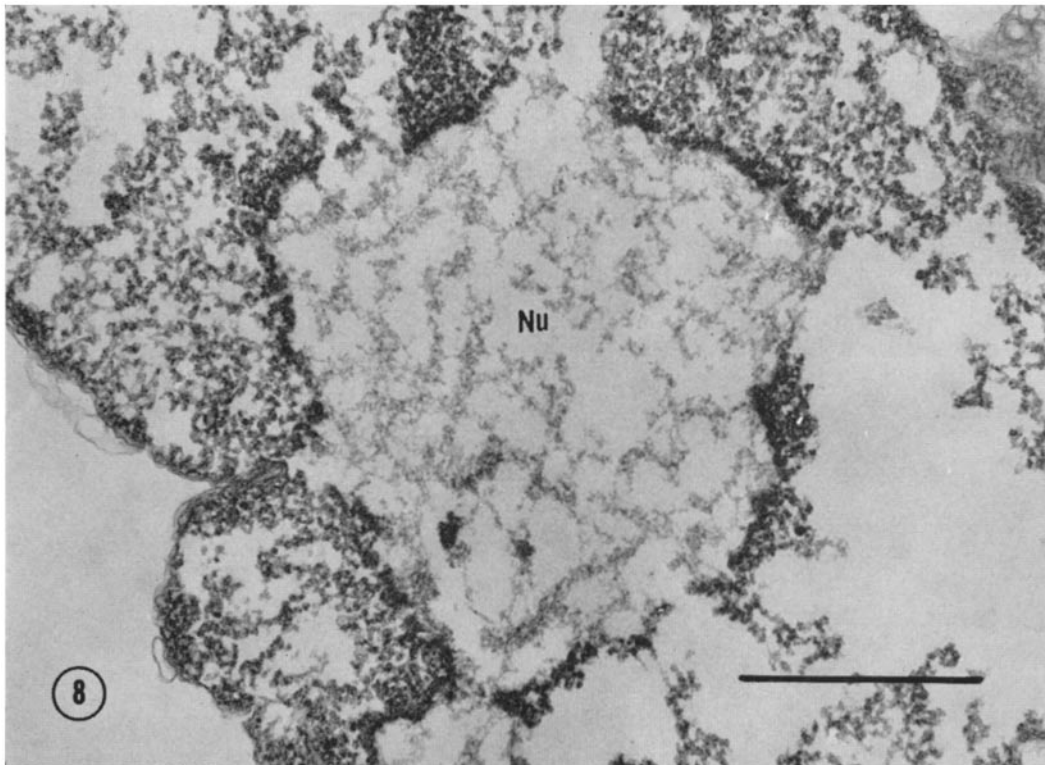


FIGURE 8 Nucleolus (*Nu*) of CMP cell digested with ribonuclease (1 mg/ml) for 1 hr and pepsin (1 mg/ml) for 1 hr at 37°C. The nucleolar reticulum appears thinner than in Fig. 7, and the nuclear chromatin is more dispersed. The electron-opaque zone along the nucleolar border appears to be an artifact. Fixation: glutaraldehyde prior to digestions; osmium tetroxide following digestions. Marker = 1 μ . \times 30,000.

of the reticulum are also continuous with those of fibrillar centers and the associated fibrillar nucleolonema.

Details of the nucleolar reticulum are better resolved if a low concentration of pepsin (0.1 mg/ml 0.1 N HCl) is used and the cells are fixed with osmium tetroxide immediately following digestion. Cells thus processed are illustrated in Figs. 9 and 10: one cell shows a nucleus with a markedly condensed chromatin (Fig. 9), whereas the other cell shows a more dispersed chromatin (Fig. 10). In both illustrations the close relationship of the nucleolar chromatin and the nucleolar reticulum is well demonstrated. Individual chromatin fibrils show continuity with the fibrils of the reticulum, as if they would be one and the same merely representing different stages of unwinding.

It is to be noted that the nucleolar granules observed following pepsin digestion (Fig. 5) are

completely removed if ribonuclease digestion precedes pepsin digestion (Figs. 7–10). The cytoplasmic ribosomes are also removed by double digestion. The membranes, neutral lipids, and glycogen granules remain.

Digestion studies did not furnish the evidence that the fibrils of the nucleolar reticulum contain DNA. Deoxyribonuclease was found to have no effect on cells fixed with glutaraldehyde even following double digestion by ribonuclease and pepsin. A phosphate-buffered formalin fixative was tried in a few instances, but the digestion results have not been satisfactory. Because of these failures, electron microscope radioautography was employed in further studies.

Radioautographic Studies

ME-180 cells were labeled with thymidine-³H and digested with ribonuclease or ribonuclease

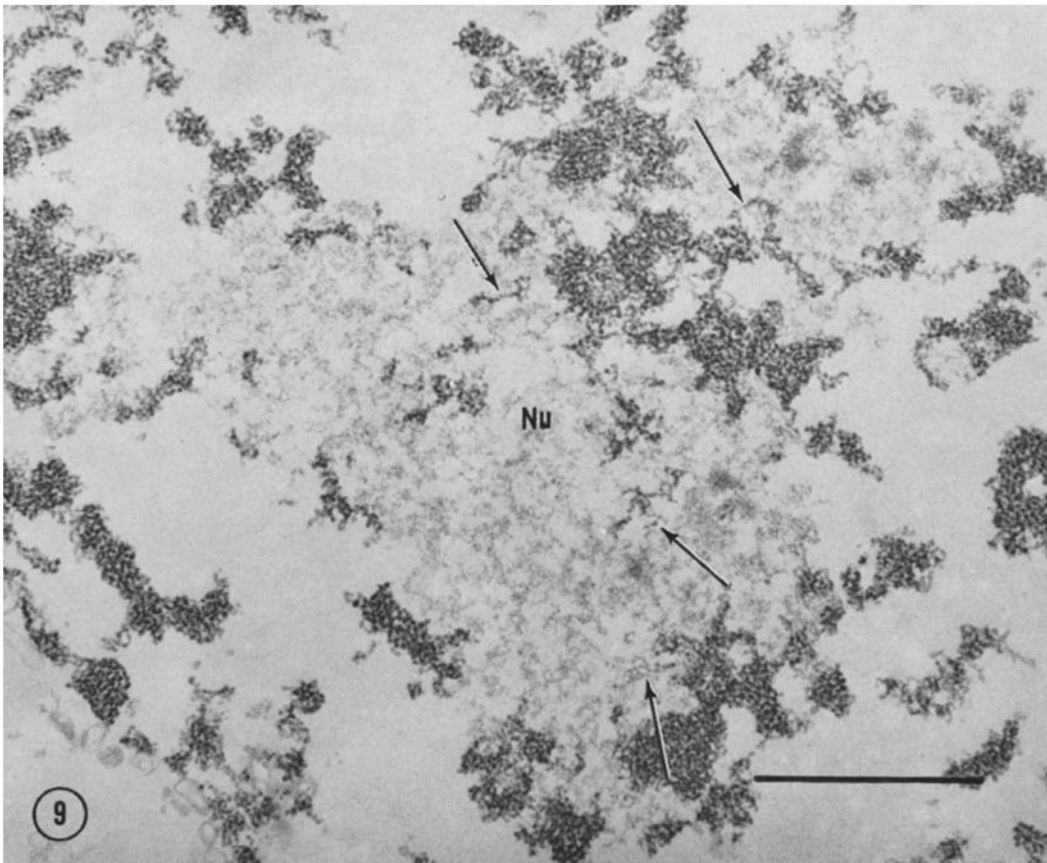


FIGURE 9 Nucleolus (*Nu*) of ME-180 cell that has been digested with ribonuclease (1 mg/ml) for 1 hr and pepsin (0.1 mg/ml) for 1 hr at 37°C. The condensed nucleolar chromatin shows a close relationship with the nucleolar reticulum. Individual chromatin fibrils (arrows) appear to be continuous with those of the reticulum. Fixation: glutaraldehyde prior to digestions; osmium tetroxide following digestions. Marker = 1 μ . \times 30,000.

and pepsin. Digestion with these enzymes results in better visualization of the nucleolar chromatin and exposes the nucleolar reticulum.

Following an incubation time of 3 hr, approximately 45% of the ME-180 cells are radioactively labeled. The amount of labeling varies greatly among individual cells. Cells that are lightly labeled can be classified into two groups, namely those that show scattered labeling of the dispersed chromatin and those that show focal labeling of the condensed chromatin. The former group of cells apparently entered the synthetic phase toward the end of the incubation period, whereas the second group terminated DNA-synthesis early in the incubation period. Heavily labeled cells usually

show labeling of the dispersed and the condensed chromatin.

The intranucleolar condensed chromatin is labeled frequently in cells that show labeling of the condensed chromatin otherwise (Figs. 11, 12). However, nucleoli that lack condensed chromatin show a striking absence of labeling (Fig. 13). Few labels only are found occasionally in the fibrillar nucleolonema adjacent to fibrillar centers (Figs. 14, 15). These are observed usually in cells that show either exclusive or predominant labeling of the dispersed chromatin. In some instances, however, such labels are associated with tiny specks of condensed chromatin interspersed in the fibrillar substance.

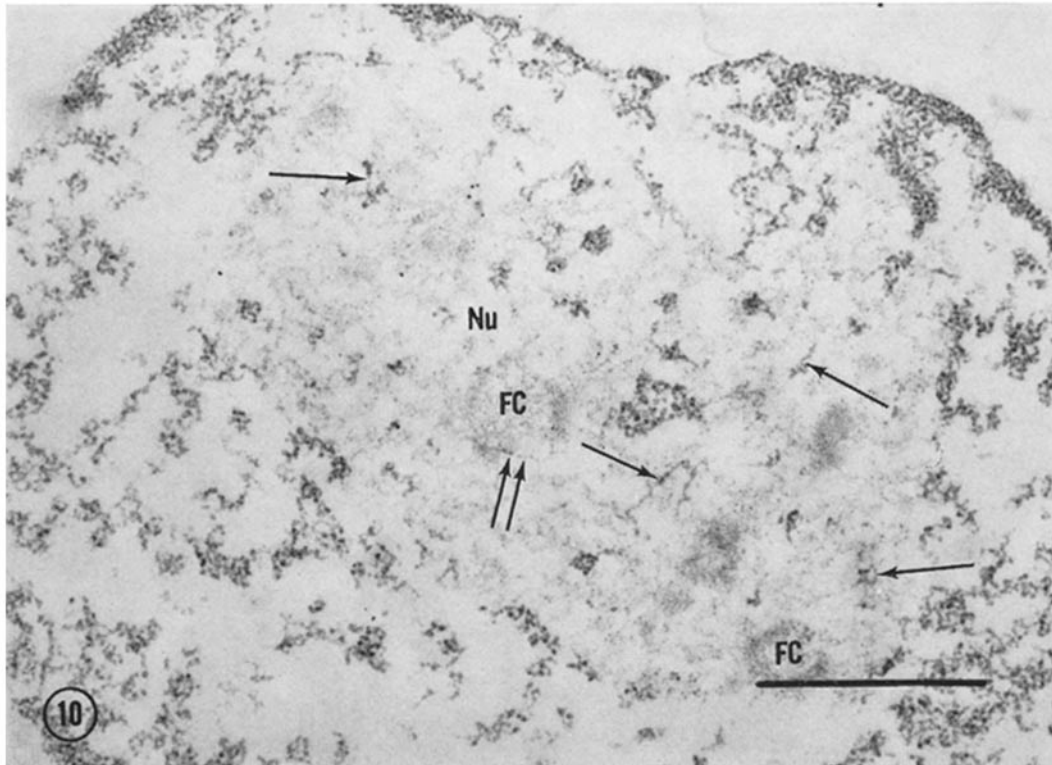


FIGURE 10 Nucleolus (*Nu*) of ME-180 cell that has been digested with ribonuclease (1 mg/ml) for 1 hr and pepsin (0.1 mg/ml) for 1 hr at 37°C. The nuclear and nucleolar chromatin is primarily of the dispersed type. The intranucleolar chromatin shows an intricate relationship with the nucleolar reticulum. Individual chromatin fibrils (arrows) appear to be continuous with those of the reticulum. One chromatin fibril (double arrow) appears to extend into the fibrillar substance next to a fibrillar center (*FC*). Fixation: glutaraldehyde prior to digestions; osmium tetroxide following digestions. Marker = 1 μ . $\times 30,000$.

DISCUSSION

The enzyme digestion studies have shown that the nucleolonemal threads contain a fine fibrillar reticulum. The reticulum is revealed following double digestion by ribonuclease and pepsin. It appears to be the nucleolar skeleton that is anchored on the condensed intranucleolar and nucleolus-associated chromatin. The resistance of the fibrils to ribonuclease and pepsin digestion and their continuity with the fibrils of the condensed intranucleolar and nucleolus-associated chromatin raise the question as to whether they are a dispersed form of chromatin. While ultrastructural findings suggest such a hypothesis, electron microscope radioautographic results do not support it. The nucleolar reticulum shows a rather striking absence of radioactive labeling following a 3 hr

incorporation of thymidine- ^3H . Only few silver grains are observed occasionally in the fibrillar nucleolonema next to fibrillar centers. Whether this lack of labeling means that there is no DNA present in the fibrils is difficult to answer. It is possible that the labeling conditions were not properly chosen. Synchronized cell cultures labeled during the early S phase might possibly be more suitable for this purpose. It also appears that the DNA complementary to ribosomal RNA is but a small fraction of the total nuclear DNA (22) and may be the only actively transcribing DNA of nucleoli. In addition, the ribosomal DNA is known to have a high guanine-cytosine ratio (7). For these reasons, it may not incorporate an amount of thymidine- ^3H that can easily be resolved by radioautography.

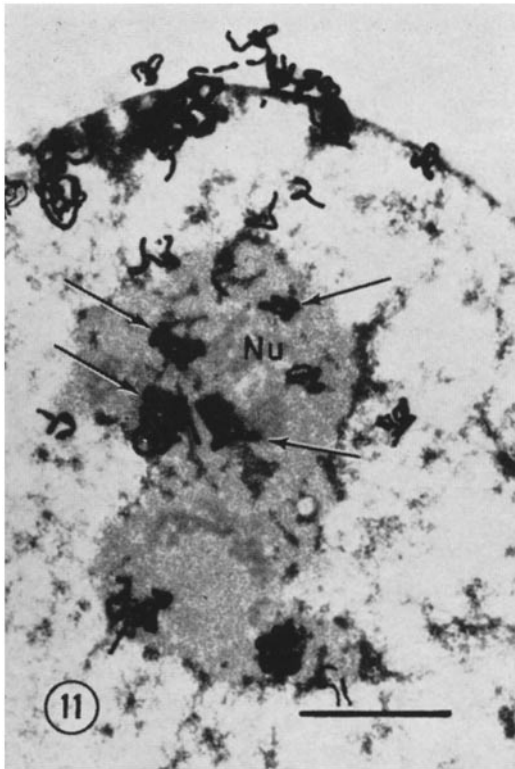


FIGURE 11 Nucleolus (*Nu*) of ME-180 cell that has been labeled with thymidine- ^3H (100 $\mu\text{Ci}/\text{ml}$) for 3 hr at 37°C and digested with ribonuclease (1 mg/ml) for 1 hr at 37°C . Note the focal labeling (arrows) of the condensed intranucleolar chromatin. There is also labeling of the nuclear chromatin. Fixation: glutaraldehyde prior to digestion. Marker = 1 μ . $\times 20,000$.

The occasional labeling of the fibrillar nucleolonema may be significant, since it is known that this component shows incorporation of uridine- ^3H earlier than other nucleolar components (10). One, therefore, would expect an actively transcribing chromatin to be present in this substance. But because of the relative scarcity of labeling and some technical shortcomings (23) inherent to the particular method used in these experiments, the finding is considered to be inconclusive. Tokuyasu et al. (30) found a considerable number of silver grains over the fibrillar nucleolonema in lymphocytes that were stimulated to growth with phytohemagglutinin and labeled with thymidine- ^3H for 2 hr. The authors (30) were also cautious in interpreting the findings by stating "a positive conclusion is precluded by the uncertainty of localizing

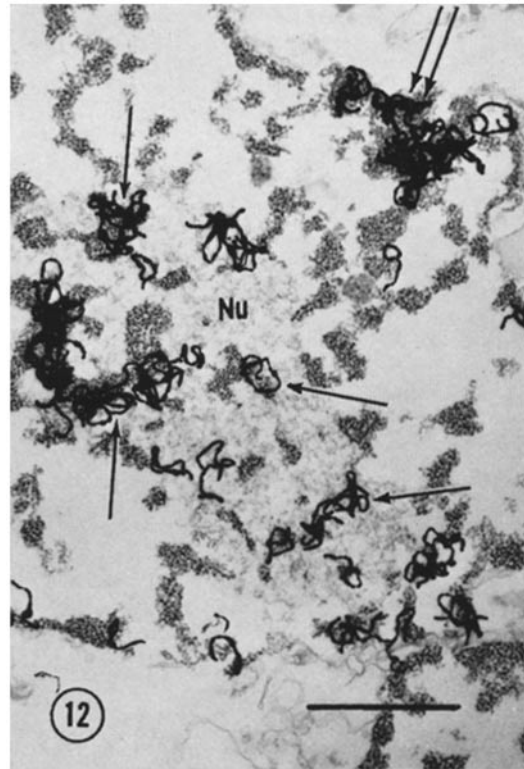


FIGURE 12 Nucleolus (*Nu*) of ME-180 cell that has been labeled with thymidine- ^3H (100 $\mu\text{Ci}/\text{ml}$) for 3 hr at 37°C and digested with ribonuclease (1 mg/ml) for 1 hr and pepsin (0.1 mg/ml) for 1 hr at 37°C .

Note the focal labeling of the condensed intranucleolar and nucleolus-associated chromatin (arrows). There is also focal labeling of the nuclear chromatin (double arrows). Fixation: glutaraldehyde prior to digestions; osmium tetroxide following digestions. Marker = 1 μ . $\times 20,000$.

grains relatively large in comparison to narrow regions concerned."

The nucleolar reticulum described here is primarily composed of fine fibrils of low electron opacity that are not observed in extranucleolar areas. If, therefore, these fibrils should prove to be of chromosomal nature, they would represent a unique form of dispersed chromatin. Chromatin fibrils resembling those of the dispersed extranucleolar chromatin can be associated with the nucleolar reticulum (Fig. 10). It is possible that these alone may constitute the dispersed, genetically active chromatin of nucleoli.

If so, the possibility that the fibrils of the nucleolar reticulum are composed of proteins has to be

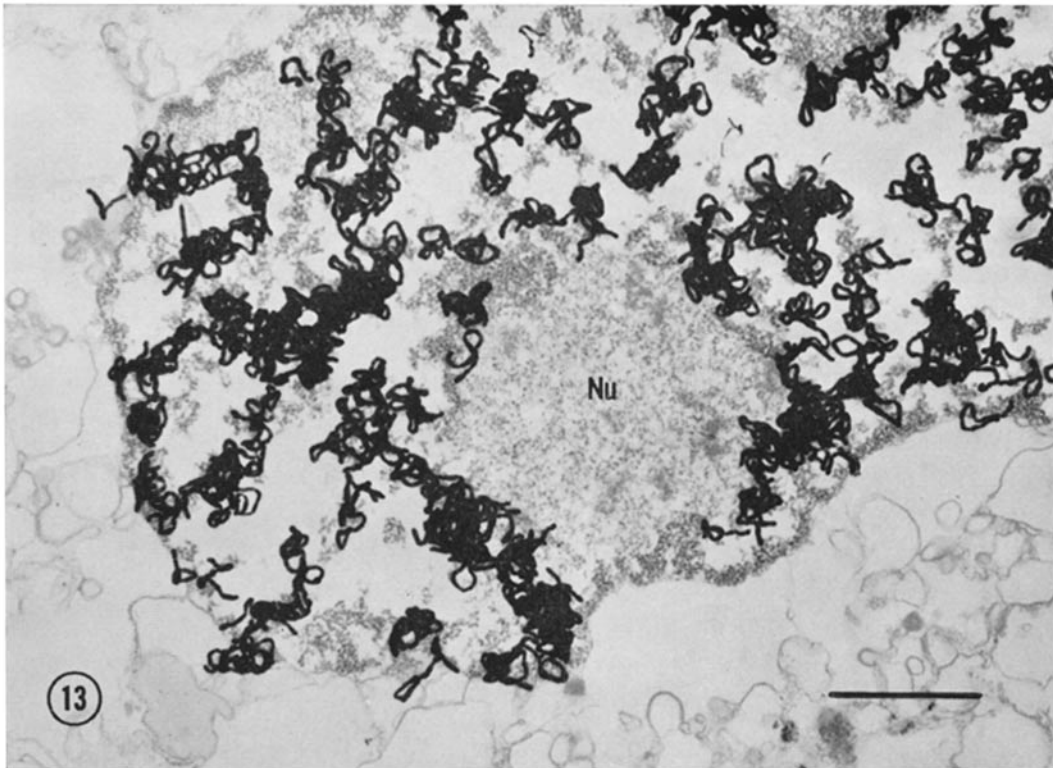


FIGURE 13 Nucleolus (*Nu*) of ME-180 cell that has been labeled with thymidine-³H (100 μ Ci/ml) for 3 hr at 37°C and digested with ribonuclease (1 mg/ml) for 1 hr and pepsin (0.1 mg/ml) for 1 hr at 37°C. Note the total absence of labeling of the nucleolar area while the nucleus is heavily labeled. Fixation: glutaraldehyde prior to digestions; osmium tetroxide following digestions. Marker = 1 μ . \times 20,000.

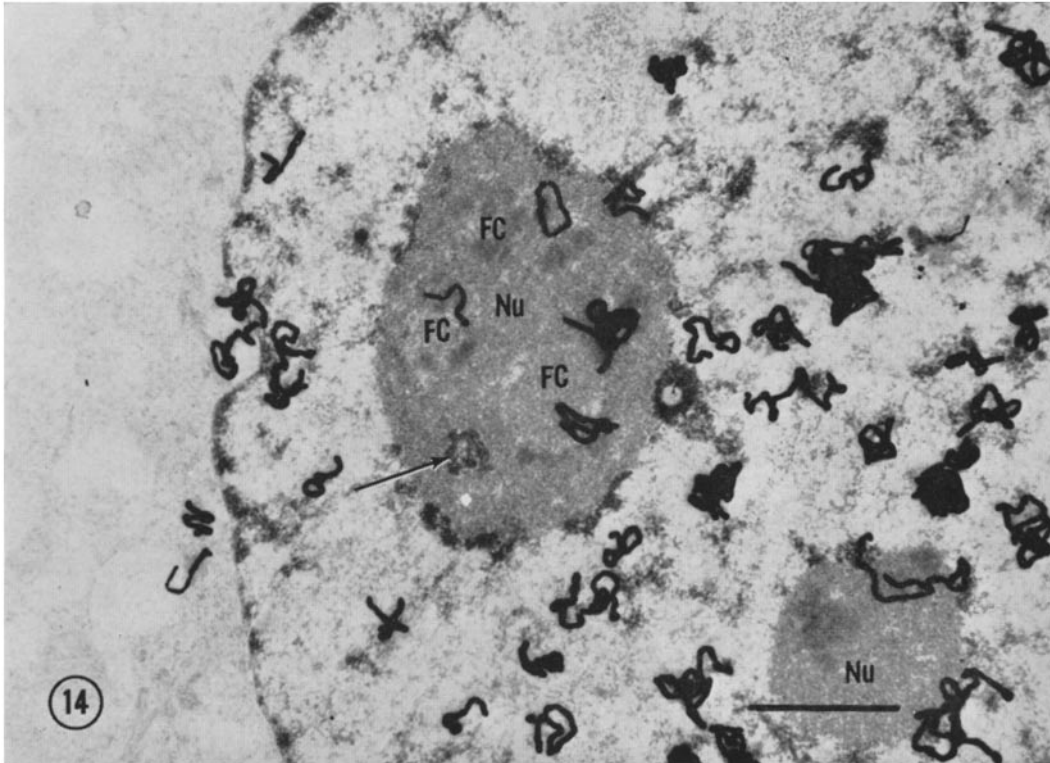


FIGURE 14 ME-180 cell that has been labeled with thymidine-³H (100 μ Ci/ml) for 3 hr at 37°C and digested with ribonuclease (1 mg/ml) for 1 hr at 37°C. One nucleolus (*Nu*) contains four silver grains that are associated with the fibrillar substance near fibrillar centers (*FC*). The intranucleolar chromatin (arrow) and most of the nucleolus-associated chromatin are not labeled. Fixation: glutaraldehyde prior to digestion. Marker = 1 μ . \times 20,000.

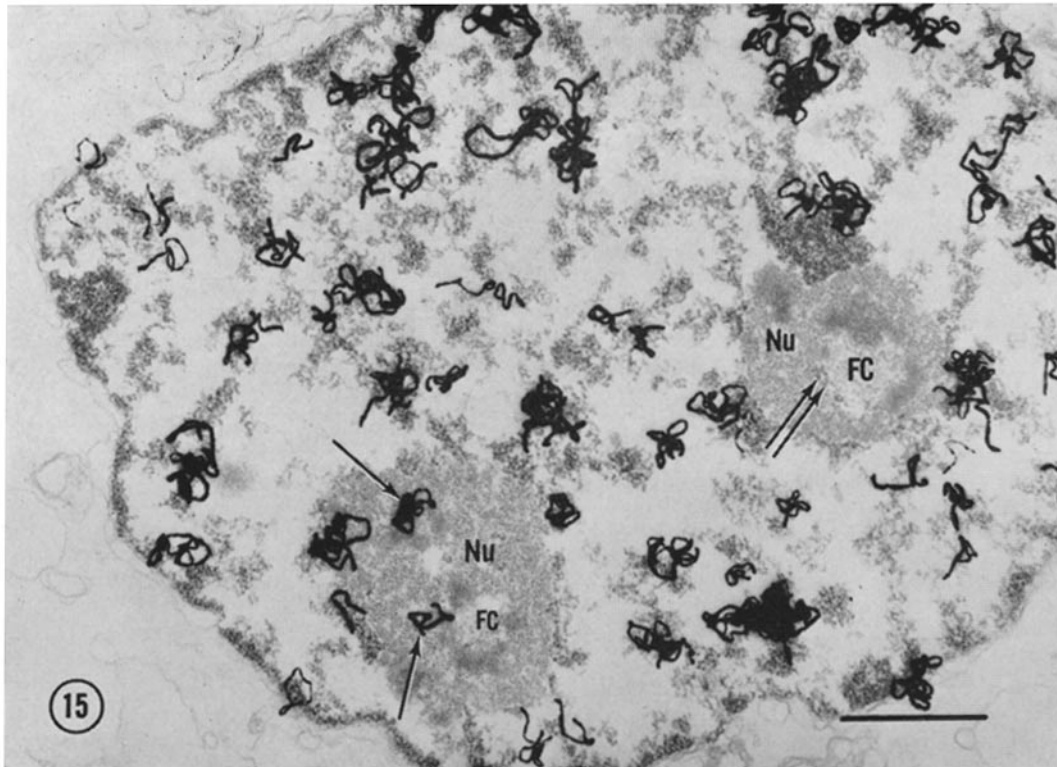


FIGURE 15 ME-180 cell that has been labeled with thymidine- ^3H ($100 \mu\text{Ci/ml}$) for 3 hr at 37°C and digested with ribonuclease (1 mg/ml) for 1 hr and pepsin (0.1 mg/ml) for 1 hr at 37°C . Two nucleoli (*Nu*) are present. One nucleolus (left) shows two silver grains (arrows) in the fibrillar substance next to fibrillar centers (*FC*). The second nucleolus (right) shows no labeling but note the chromatin fibrils (double arrows) at the periphery of the one fibrillar center (*FC*) present. Fixation: glutaraldehyde prior to digestions; osmium tetroxide following digestions. Marker = 1μ . $\times 20,000$.

considered. From the illustrations Figs. 7–10, it is evident that the integrity of the chromatin is largely preserved following double digestion by ribonuclease and pepsin, indicating that the chromatin still contains proteins. If all proteins were removed, the chromatin would have the appearance of naked DNA as seen in bacterial nuclei (25) and mitochondria (21). The presence of pepsin-resistant proteins in chromatin may suggest that similar proteins form the structural elements of the fibrillar reticulum of nucleoli. Such proteins would most likely be basic proteins. Others (19, 24) have suggested that basic proteins may resist pepsin digestion in aldehyde-fixed cells.

The digestion results presented here differ somewhat from the findings reported by Marinozzi (18). He found that double digestion by ribonuclease and pepsin following aldehyde fixation removed

completely the nucleolonemal components, leaving behind only the condensed intranucleolar chromatin. The digestion methods used in these two instances are quite different, making it difficult to compare results. But the question may be raised as to whether the nucleolar reticulum described here might possibly be formed during a structural rearrangement of the substances left after enzyme digestion and thus represent an artifact. Assuming that to be the case, it would be extremely difficult to explain how these substances would rearrange themselves in a pattern that resembles the basic structure of nucleoli and their constituents.

Monneron (20) digested rat tissue with pronase and ribonuclease and found that nucleoli contained a fibrillar material that resisted digestion by these two enzymes. The author (20) suggested that

the material might be a pronase-resistant protein or protein-RNA complex, but she did not rule out the possibility that the material might be DNA.

The cytoplasmic changes that are observed following digestion with ribonuclease, pepsin, and ribonuclease-pepsin appear to underline the specificity of action of the two enzymes. The ribo-

somal changes following pepsin digestion are possibly due to hydrolysis of structural proteins.

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