New data concerning the functional organization of the mammalian cell nucleolus: detection of RNA and rRNA by in situ molecular immunocytochemistry

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ABSTRACT
We have investigated the fine spatial distribution of RNA and rRNA within the Ehrlich tumor cell nucleolus by in situ hybridization with a biotin-labeled probe and by two new strategies, the polyadenylate nucleotidyl transferase-immunogold technique and immunolabeling with anti-RNA antibodies. Besides the presence, as expected, of RNA and rRNA in the granular component and the dense fibrillar component, we show, for the first time, significant label over all the fibrillar centers of the nucleoli. When RNA and DNA were detected simultaneously on the same sections, only the fibrillar centers were positive for both. These results throw light on the controversial subject of the precise location of transcribing rRNA genes within the nucleolus. The fibrillar centers, and not the dense fibrillar component, should thus be the site of rRNA synthesis.

INTRODUCTION
As the most prominent feature of eucaryotic interphase cell nucleus, the nucleolus provides us with one of the best opportunities for unravelling the relationships between spatial organization and cell function.

Extensive electron microscopic studies have provided in-depth knowledge of the ultrastructure of this highly specialized region of the nucleus. In addition to condensed chromatin and nucleolar spaces of varying size called interstices or vacuoles, three basic components are usually distinguished: the fibrillar centers, the dense fibrillar component and the granular component (reviews in 1, 2, 3, 4).

Transcription of the rRNA genes as well as processing and initial packaging of their transcripts with ribosomal and non-ribosomal proteins all occur within the nucleolus (reviews in 5, 6, 7, 8, 9). The problem is to assign functions to particular nucleolar components. It is generally agreed that the three nucleolar components correspond in some way to different steps in ribosome biogenesis. Disagreement begins with the fundamental process of rRNA gene transcription.

Until recently, it was unanimously accepted, on the basis of autoradiographic studies of cells pulse-labeled with tritiated uridine, that the dense fibrillar component is the site of rRNA synthesis (reviews in 1, 10, 11, 12, 13, 14).

Over the last few years, however, immunoelectron microscopic techniques have clearly detected RNA polymerase I (15, 16) as well as DNA (17, 18, 19, 20, 21) exclusively in the fibrillar centers and not in the surrounding dense fibrillar component. This argues in favor of rDNA transcription occurring in the fibrillar centers of the nucleolus (reviews in 4, 22, 23).

Recent in situ hybridization studies of rRNA genes have yielded apparently contradictory results. In Ehrlich tumor cells (24, 25) and HeLa cells (26), rDNA was exclusively detected in the fibrillar centers and in some clumps of intranucleolar condensed chromatin, whereas in human lymphocytes (27), human spermatocytes (28) and human Sertoli cells (29), rDNA was essentially detected in the dense fibrillar component.

These data leave a doubt as to the precise location transcribing rRNA genes within the nucleolus. In our opinion, only knowledge of the fine intranucleolar distribution of RNA, especially rRNA, is liable to provide the additional information needed to determine the exact site of rRNA synthesis. There is considerable evidence that rRNA is present in the granular component and in the dense fibrillar component (23, 24, 26), but so far no in situ detection method has revealed a significant amount of RNA in the fibrillar centers.

In the present report, we describe two sensitive methods for precisely locating RNA at the ultrastructural level. The first relies on the appearance of RNA ends at the surface of sections during sectioning. These RNA ends can be specifically labeled with exogenous polyadenylate nucleotidyl transferase (PnT) and biotinylated ATP. Subsequently the labeled sites can be detected by a very sensitive immunogold labeling procedure. The second approach is a Lowicryl postembedding immunogold labeling technique involving two different monoclonal anti-RNA antibodies. We have further examined the ultrastructural location of rRNA in the nucleoli by in situ hybridization with a biotinylated rDNA probe and subsequent detection of hybrids by immunogold labeling and electron microscopy. Finally, to compare the spatial distribution of RNA and DNA, two molecular immunogold labeling techniques were simultaneously applied: the PnT method and the terminal deoxynucleotidyl transferase (TdT) procedure.

Our results consistently show, for the first time, the presence
of RNA and rRNA in the fibrillar centers of the Ehrlich tumor cell nucleolus.

MATERIALS AND METHODS

Cell culture
Ehrlich ascites tumor cells (tetraploid line — ELT) were collected from C57 Bl mice bearing the tumor and cultured as previously described (30).

HeLa cells were grown in Eagle's minimum essential medium (Gibco-BRL, Life Technologies, Gent, Belgium) supplemented with 10% foetal calf serum and 100 U/ml penicillin.

Preparations for electron microscopy
Cultures of both cell lines were scraped off the dishes and centrifuged to form pellets. Small fragments of various pellets were fixed for 60 min at 4°C in 1.6% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4), acetylated as previously described (31) and embedded in Epon. Other fragments of Ehrlich tumor cell pellets were fixed for 15 min at 4°C in 0.2% glutaraldehyde or 4% formaldehyde-0.1% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4), dehydrated through graded ethanol solutions and embedded in Lowicryl K4M as in Roth et al. (32).

Ultrathin sections of the various blocks were either collected in platinum rings (4 mm diameter) formed by a platinum wire (0.1 mm diameter, SA Johnson Matthey NV, Brussels, Belgium) and stored on distilled water until use, or mounted on collodion-coated nickel grids.

In situ polyadenylate nucleotidyl transferase-immunogold procedure
Ultrathin sections of Epon-embedded cells were incubated for 5 min at 37°C on the surface of the following medium: 50 mM Tris HCl, 10 mM β-mercaptoethanol, 10 mM MgCl₂, 2.5 mM MnCl₂, 0.25 M NaCl, 1 mg/ml bovine serum albumin (BSA) (pH 7.9), 25 U/ml E. coli PnT (Gibco BRL, Merelbeke, Belgium), and 0.2 mM biotinyl-17-ATP (Sigma, St Louis, MO). Grids were incubated by floating them, cell sections down, on the same medium.

After five rinses in bidistilled water, the different sections or grids were incubated for 30 min in PBSB (34 mM NaCl, 0.7 mM KCl, 20 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 1% BSA, pH 7.2) containing normal rabbit serum (NRS) diluted 1/30. The next step of the treatment was 60-min incubation at room temperature with goat anti-biotin antibodies (Biosys SA, Compiègne, France) diluted 1/500 in PBS containing NRS diluted 1/50. After four rinses in PBS plus one in PBSB pH 8.2, sections were transferred to an incubation medium containing rabbit anti-goat IgG coupled to gold particles either 5 nm (Janssen Life Sciences, Beerse, Belgium) or 10 nm (BioCell Research Laboratories, Cardiff, UK) in diameter, respectively diluted 1/50 or 1/200 in PBSB pH 8.2. Incubated was for 60 min at room temperature. Samples were then rinsed with PBSB, followed by distilled water.

Considering that only antigenic determinants present at the surface of the sections are accessible to immunoglobulin–gold complexes (33), the in situ PnT immunogold procedure was systematically performed on each of the two faces of ultrathin sections. Once labeled on one side, the sections were mounted on collodion-coated nickel grids and the in situ PnT-immunogold procedure was applied to the second face. To compare the spatial distribution of RNA and DNA on the same section, the very sensitive in situ terminal deoxynucleotidyl transferase-immunogold technique (TdT) for detecting DNA (20, 21) was further applied to ultrathin sections where RNA present at the surface of both faces was revealed by the in situ PnT-immunogold procedure.

The specificity of the PnT reaction was tested in several ways. When PnT or biotinylated ATP was omitted from the PnT medium, the ultrathin sections were devoid of label. When either of the two ions was omitted from the PnT medium, labeling was strongly reduced.

The specificity of RNA labeling was also tested in several ways. When acetylated cell sections were preincubated at 56°C for 120 min with 1 mg/ml of pyrimidine specific RNase (RNase A, Boehringer) in 10 mM Tris HCl (pH 7.4) containing 15 mM NaCl, labeling was strongly reduced. The result was identical when these sections were preincubated at 37°C for 60 min with 500 U/ml of a purine-specific RNase (RNase T₂, Gibco BRL) in 50 mM sodium acetate buffer (pH 4.5) containing 2 mM EDTA. No label was detected on acetylated cell sections pretreated with RNase T₂, followed by RNase A. Preincubation at 37°C for 120 min with 1 mg/ml DNase I (Sigma, type DN-Ep) in PBS (0.14 M NaCl, 6 mM Na₂HPO₄, 4 mM KH₂PO₄, pH 6.8) containing 7 mM MgCl₂, did not prevent labeling. Finally, when RNase T₂ incubation was carried out after the PnT reaction, no labelling occurred.

The immunolabeling specificity was also tested. When the primary antibody was omitted no labeling occurred. Gold lacking the antibody tag did not bind to the sections.

Immunocytochemical technique for RNA
Two mouse monoclonal anti-RNA autoantibodies (D444, BWR5) were used (34, 35). The isotype were IgG3. K. The D444 antibody was specific to a G,C-rich polyribonucleotide sequence, while the BWR5 IgG showed a striking preference for synthetic ds RNA molecules (35).

For labeling, ultrathin sections of Lowicryl-embedded cells were incubated for 25 min in PBSB (pH 7.2) containing normal goat serum (NGS) and NRS, each diluted 1/30, and then for 3h at room temperature in RNA-specific antibodies diluted 1/10 in PBSB containing NGS and NRS, each diluted 1/50. After five rinses in PBSB, the sections were incubated for 30 min with goat anti-mouse IgG3 (heavy chain specific; Sigma, St Louis, MO) diluted 1/100 in PBSB containing NGS and NRS, each diluted 1/50. After five rinses in PBSB (pH 7.2) plus one in PBSB (pH 8.2), sections were transferred to an incubation medium containing rabbit anti-goat IgG coupled to colloidal gold 5 nm in diameter (Janssen Life Sciences) diluted 1/50 in PBSB (pH 8.2). Incubation was for 60 min at room temperature. Samples were then rinsed with PBSB followed by distilled water. This labeling was systematically applied to both faces of ultrathin sections.

Several kinds of control experiments were carried out. When the primary or secondary antibody or both were omitted, the ultrathin sections were devoid of label. When the grids were incubated with antibody-free particles, no labeling occurred. Finally, no label was detected when Lowicryl sections were preincubated at 56°C for 120 min with 1 mg/ml RNase A (Boehringer) in 10 mM Tris HCl (pH 7.4) containing 15 mM NaCl.
In situ rDNA/rRNA hybridization

Fragment C of mouse ribosomal DNA (36) was kindly supplied a 1.95 kb EcoRI-Sall insert in pBR322 = pBRMrC by I. Grummt (University of Würzburg, Würzburg, Germany). The fragment was isolated, eluted (pre-A-gene; Biorad), and then biotinylated by random priming (37) using Boehringer kit with Bio-11-dUTP and Bio-11-dCTP. Purification of the labeled probe was achieved by gel filtration on Sephadex G25 (NAP-5 column; Pharmacia LKB Biotechnology Div., Brussels, Belgium), tracking the DNA with 0.5% blue dextran, and subsequent ethanol precipitation.

Ultrathin sections of Lowicryl-embedded cells were hybridized as previously described (24). Biotinylated hybrids were detected as described above.

The specificity of the reaction was tested in several ways. First, when the probe was omitted from the hybridization medium, no labeling occurred. In the second control, no label was seen when the primary antibody was omitted. Third, when the grids were incubated with antibody-free particles, the ultrathin sections were devoid of label.

Finally the ultrathin sections were mounted on nickel grids and stained with uranyl acetate and lead citrate before examination in a Jeol CX 100 electron microscope at 60 kV.

RESULTS

Location of RNA within the nucleolus

As a first means of identifying the precise location of RNA in the nucleolus, the in situ PnT reaction was used to label the extensively studied Ehrlich tumor cells. Under these conditions (Figure 1), abundant label was found in cell nuclei, especially over the nucleoli which in these cells exhibit a compact structure with large fibrillar centers ensheathed by a discontinuous layer of dense fibrillar component.

Inside the nucleoli (Figure 2a), gold label was always seen over the granular component and the dense fibrillar component, but clearly also over the fibrillar centers. By contrast, the condensed chromatin associated with the nucleolus and the nuclear envelope or scattered in the nucleus was completely devoid of gold particles. This nucleolar RNA labeling pattern was all the more distinct when the in situ PnT-immunogold procedure was applied to acetylated sections, conditions which provide an excellent distinction between the various nucleolar components and which make it possible to visualize condensed chromatin with high contrast. In the nucleus, outside the nucleoli, intense labeling was visualized over the spaces between the heterochromatin blocks, especially over the clusters of interchromatin granules. Intense
Figure 3. Localization of RNA in Ehrlich tumor cells by immunogold electron microscopy using mouse monoclonal anti-RNA autoantibodies (a: D444, b: BWR5). In the nucleolus (a), label is seen in the fibrillar centre (FC) as well as in the dense fibrillar component (D) and the granular component (G). The nucleolar interstices (I) which come into close contact with the fibrillar centres are essentially free of label. Outside the nucleolus (b), an intense labeling is revealed in the cytoplasm (R). A few gold particles are also found to be scattered throughout the interchromatin spaces (S). By contrast, the condensed chromatin (C) is completely devoid of label. NE = nuclear envelope. Bars = 0.1 μm.

labeling was also present over the ribosome-rich cytoplasmic areas and the mitochondria. Several gold particles were also found at the level of nuclear pores.

An identical RNA labeling pattern was observed in the nucleoli of a variety of other cell types: HEP-2 and HeLa (Figure 2b) cells, human lymphocytes, human Sertoli cells.

The diameter of gold particles did not affect the labeling distribution. However, considering that the labeling intensity was lower for 10-nm particles than for 5-nm particles, we have illustrated our results with 5-nm gold particles.

Alongside these experiments, a second approach was used to precisely locate RNA within the nucleolus of Ehrlich tumor cells: postembedding in Lowicryl followed by immunogold labeling involving two monoclonal antibodies directed specifically against RNA. Although this method is inapplicable to acetylated sections and differs in nature from the PnT method, the resulting labeling pattern was the same, including the consistent presence of label over the fibrillar centers (Figures 3a and b). The results were identical with both anti-RNA antibodies. The labeling intensity was lower in the interchromatin areas and over the fibrillar centers with this technique than with the first approach.

Figure 4. Distribution of rRNA in the Ehrlich tumor cell as revealed by in situ hybridization using a biotinylated rDNA probe. Label is found in all the three nucleolar components (a). At higher magnification (b), gold particles are clearly seen over the fibrillar centres (FC) and the dense fibrillar component (D). Outside the nucleolus (c), a few gold particles are visualized in the interchromatin spaces (S), whereas the condensed chromatin (C) is gold-free and, in the cytoplasm (R), the ribosome-rich cytoplasmic regions are strongly marked. G = granular component, NE = nuclear envelope. Bars = 0.1 μm.

Location of rRNA within the nucleolus

After pinpointing the location of RNA within the nucleolus, we investigated the precise nucleolar distribution of rRNA by in situ hybridization with a biotinylated rDNA probe prior to electron microscopy. Under these conditions (Figure 4a), gold particle concentration was particularly high over the nucleolus. Inside the latter, label was obvious over all the fibrillar centers (Figure 4b). Label was also found over the granular component and the dense fibrillar component. By contrast, the condensed chromatin associated with the nucleolus was unlabeled.

In cell nuclei (Figure 4c), beside the intense labeling of the nucleolus, a few gold particle aggregates scattered throughout the interchromatin regions were consistently visualized. Label was also obvious over ribosome-rich cytoplasmic areas (Figure 4c).

Simultaneous nucleolar location of RNA and DNA

To compare the spatial distribution of RNA and DNA within the nucleolus, RNA and DNA were detected simultaneously on the same sections of Ehrlich tumor cells. The methods used were the PnT-immunogold technique for RNA and the powerful TdT-immunogold procedure for precisely locating of DNA in the cells.
tritiated uridine as a precursor often constituted the best technique for demonstrating the presence of RNA in a cytological structure centers of Ehrlich tumor cell nucleoli. The present study decisively demonstrates, for the first time, the presence appreciable amount of RNA and rRNA in the fibrillar centers. Interestingly, no RNA-positive sites were found in nucleolar interstices which interrupt the layer of dense fibrils surrounding the fibrillar centre. S = interchromatin space. Bar = 0.1 μm.

Under these conditions (Figure 5), only the fibrillar centers inside the nucleolus were marked by both procedures. All the other nucleolar components exhibited only one label: the granular component and dense fibrillar component contained RNA-positive sites, while DNA was detected in the perinucleolar condensed chromatin blocks and their intranucleolar invaginations which penetrate the nucleolar body and come into close contact with the fibrillar centers. Interestingly, no RNA-positive sites were found in nucleolar interstices which interrupt the layer of dense fibrils surrounding the fibrillar centers and which enclose small clumps of condensed chromatin.

DISCUSSION

The present study decisively demonstrates, for the first time, the presence appreciable amount of RNA and rRNA in the fibrillar centers of Ehrlich tumor cell nucleoli.

Until quite recently, electron microscope autoradiography using tritiated uridine as a precursor often constituted the best technique for demonstrating the presence of RNA in a cytological structure (38, 39). More recently, a simple approach to specific detection of RNA was based on the possibility of labeling enzyme substrates with enzyme-colloidal gold complexes (40, 41).

Despite their ability to specifically detect RNA at the ultrastructural level, the two above-mentioned techniques present several limitations discussed in recent reviews (3, 22, 42). To avoid these limitations, we have developed a new approach to pinpoint the precise location of RNA within biological material, using a molecular-biology-based procedure. In addition to its high specificity, the PnT method offers higher resolution and is less time consuming than autoradiographic techniques. With respect to the use of enzyme-gold complexes, it shows a more general distribution of RNA in cells and avoids the steric hindrance problem.

Our experiments show that RNA is present in the fibrillar centers of the Ehrlich tumor cell nucleolus. Furthermore, identical results were obtained with other cell types whose nucleoli have less-developed fibrillar centers.

We have confirmed the presence of RNA inside the fibrillar centers of Ehrlich tumor cells by means of two different anti-RNA antibodies. That labeling is weaker in these experiments than with the PnT technique is in keeping with the fact that it is always more difficult to safeguard antigenicity with an immunocytochemical approach than with a molecular cytochemical technique.

The bulk of nucleolar RNA is composed of pre-rRNA and rRNA molecules containing 18S, 5.8S and 28S rRNA sequences as well as 5S rRNA molecules (S). Other RNA species, notably U3 RNA, are also confined to the nucleolus, but these account for a minor proportion of the total RNA content (43, 44, 45, 46, 47).

To ascertain the ribosomal nature of RNA present in the fibrillar centers of Ehrlich tumor cells, we have applied a non-isotopic in situ hybridization technique at the ultrastructural level. The observed labeling is very selective. Very intense labeling is observed over the granular component and over the dense fibrillar component of the nucleolus. This result is in agreement with previous data (23, 24, 26). Sparser labeling scattered throughout the nucleoplasm might indicate transport of preribosomal particles from the nucleolus to the cytoplasm as previously suggested (48, 49, 50, 51). On the other hand, labeling of the nucleolar fibrillar centers is obvious, and not restricted to the peripheral part of these centers, as reported previously (24). This difference might be due to modifications made in the probe. The rDNA fragment was indeed not labeled as previously described by nick-translation (24) but by random priming using two biotinylated nucleotides to produce an uniformly-labeled probe (37).

Now the question is whether rRNAs present in the fibrillar centers are synthesized there. In earlier ultrastructural autoradiographic studies of cells pulse-labeled with tritiated uridine, the first nucleolar structure to be clearly marked by silver grains was the dense fibrillar component (reviews in 10, 52). This is usually taken to mean that transcription of the rRNA genes takes place in this component. In our opinion, it is no longer possible to hold this view, for reasons discussed at length in a recent paper (53). We concluded at the time that the fibrillar centers could not be excluded as a potential site of rDNA transcription.

We here demonstrate the presence of appreciable quantities of rRNA in the fibrillar centers of the Ehrlich tumor cell nucleolus. DNA, including rDNA, has also been found there...
(reviews in 3, 22, 42). Furthermore, our double-labeling experiments point to the fibrillar centers as the only nucleolar component in which significant amounts of both nucleic acids are visualized together. Since RNA polymerase I as well is exclusively located in the fibrillar centers (15, 16), these appear as the only 'meeting place' of all three essential elements in rRNA gene transcription. We conclude that rRNA synthesis occurs in the fibrillar centers of Ehrlich tumor cell nucleoli.

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