INTRODUCTION

In most active nucleoli, four morphologically distinct components can be recognized: fibrillar centres, a dense fibrillar component, a granular component and nucleolar chromatin. The first two components, both somewhat fibrillar in structure, have been established to play a crucial role in the transcription of rRNA. However, the functional organization of nucleoli, and the exact localization of rRNA transcription in particular, still remain under question. Autoradiographic detection of radiolabelled RNA precursors have not given results with sufficient spatial resolution. Ultrastructural immunocytochemistry was able to show the presence of enzymes necessary for rRNA transcription (polymerase I and topoisomerase I) in fibrillar centres and in the dense fibrillar component, but could not discriminate between active and pool molecules (for reviews see Derenzini et al., 1990; Raška et al., 1990; Reeder, 1990; Scheer and Benavente, 1990; Hernandez-Verdun, 1991; Jordan, 1991 and Thiry et al., 1991). Likewise, the rDNA distribution was studied by DNA-DNA in situ hybridization without any universally accepted conclusion. In the few cell types studied so far, one group of papers claims the fibrillar centres to be the exclusive site of rDNA (Thiry and Thiry-Blaise, 1989; Puvion-Dutilleul et al., 1991; Thiry et al., 1991), whereas the second group found the dense fibrillar component to contain most of the rDNA (Wachtler et al., 1989; Ghosh and Paweletz, 1990; Stahl et al., 1991; Wachtler et al., 1992).

There may be several explanations for these discrepancies. The nucleolar morphology is highly variable in miscellaneous cell types and obviously depends on rRNA transcription intensity: the size and number of fibrillar centres and the spatial distribution of individual nucleolar components are different among different types of cells. In DNA-DNA in situ hybridization experiments, the fine morphology is not always easy to recognize in ultrathin sections, therefore it is important to use cell types with a well established and stable nucleolar morphology. Also, it can not be excluded that the fibrillar centres and/or the dense fibrillar component, although appearing morphologically homogeneous in electron micrographs, contain functionally different zones, and that different nucleoli may have different structure-function organizations.

In most approaches to the study of structure-function relationships in nucleoli, the localization of distinct molecules in nucleolar components was performed. In this study, we take the opposite route, by characterizing the molecular contents of a defined nucleolar structure, to more specifically test the nature of the DNA contained in the fibrillar centres and in the dense fibrillar component, in human LEP cells, and to characterize more closely this DNA. We used
nucleolar disintegration, induced by a brief hypotonic treatment, followed by the isolation of the two fibrillar parts (the fibrillar complex) of the nucleolus. Using this procedure, nucleolar fibrillar complexes can be isolated with a high level of integrity (Hozák et al., 1990; Hozák et al., 1992). The DNA found to be present in the fibrillar complexes was extracted, tested by Southern blotting with a 28 S rDNA probe, and with a probe for alphoid satellite DNA, which was known to be a non-ribosomal sequence present in nucleoli (Kaplan et al., 1992), and itself used as a probe for DNA-DNA in situ hybridization. Morphometric analysis and statistical evaluation were performed in order to obtain precise information on the location of hybridization signals with respect to nucleolar components.

MATERIALS AND METHODS

Cells

For the isolation of DNA from nucleoli, the human diploid cell line LEP, originating from embryonic lung epithelium (ÚSOL, Czech Republic), was used. The cells were grown in MEM supplemented with 10% fetal calf serum, glutamine and antibiotics.

For in situ hybridization studies, three cell types were used: human lymphocytes from healthy 25 year old male donors, after 72 h of phytohaemagglutinin stimulation; human Sertoli cells and human spermatogonia A. The testicular samples originated from patients who underwent orchidectomy without any clinical treatment prior to surgery; the testicular histology was normal.

Conventional electron microscopy

Samples of LEP-cells or cell fractions (see below) were fixed in 2.5% glutaraldehyde in 0.1 M Sörensen buffer (SB), pH 7.2 for 1 h at 4°C; testicular tissue was fixed in 2.5% glutaraldehyde and 0.5% paraformaldehyde, in 0.2 M phosphate buffer, pH 7.2. Samples were postfixed in 1% OsO₄, dehydrated in a graduated series of ethanol rines and embedded in Epon.

Preparation of cells for in situ hybridization

For light microscopy, spreads of stimulated lymphocytes were prepared using a standard cytogenetic protocol (Schwarzacher and Wolf, 1974). For electron microscopy, the testicular tissue was fixed in 2.5% glutaraldehyde and 0.5% paraformaldehyde, in 0.2 M phosphate buffer pH 7.2, at 4°C, dehydrated in a graded series of ethanol rinses and embedded in LR White medium according to the manufacturer’s instructions. Ultrathin sections were mounted on gold grids.

Isolation of DNA from nucleolar fibrillar complexes

The LEP cells were rinsed briefly in PBS, harvested by trypsinization, washed twice in 0.1 M SB, pH 7.2, 37°C and incubated for 5 min in 0.035 M SB at 37°C. All of the following procedures were carried out at 0-4°C in the presence of 0.05 mM MgCl₂, 0.1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml peptastin and 1 µg/ml aprotinin. Cells were spun down at 400 g for 5 min and homogenized in 40 volumes of 0.05 M SB with 0.4% Nonidet P-40 by 20 strokes in a Dounce homogenizer. The fraction containing the nuclei was collected by 100 g centrifugation and purified by centrifugation on a saccharose gradient (0.25 M/0.88 M). Nuclei were then sonicated in 0.34 M sucrose, on ice, three times for 3 s (UZD 21, A=40, 20% power output). Released fibrillar complexes were purified by spinning through a 0.88 M saccharose solution at 2500 g for 10 min. All individual steps of the isolation procedure were monitored by electron microscopy. The DNA was extracted by the standard phenol/chloroform method and precipitated by ethanol and sodium acetate.

Characterization of fibrillar complex DNA

DNA extracted from nucleolar fibrillar complexes was restricted with EcoRI and, following electrophoresis, transferred onto a membrane, then probed for the presence of 28 S rDNA using a pA probe (Erickson et al., 1981) and alphoid satellite DNA, originating from human chromosome 21 and known to be at the centromere of chromosomes.

DNA-DNA in situ hybridization on cells

Three different probes were used: (1) DNA extracted from nucleolar fibrillar complexes and digested with EcoRI, (2) human total genomic DNA, prepared by EcoRI and HaeII digestion of placental DNA type XIII (Sigma), and (3) a probe for the transcribed part of human rDNA namely an EcoRI-BglIII fragment of the transcription unit (includes parts of 18 S and 28 S rDNA, 5.8 S rDNA and both internal transcribed spacers; for details see Wachtler et al., 1989). All probes were labelled by biotin or digoxigenin, using a nick-translation kit (Boehringer) according to the manufacturer’s instructions.

The hybridization protocol was as described in Wachtler et al. (1989). Briefly, the slides, or ultrathin sections were pre-treated with RNase A (100 µg/ml in 2xSSC, 1 h at 37°C) and with protease K (0.5 µg/ml in 20 mM Tris-HCl, 2 mM CaCl₂, pH 7.4, 7 min at 37°C), then washed and the hybridization mixture (consisting of 2 ng/µl of the probe, 100 ng/µl salmon sperm DNA, 100 ng/µl yeast RNA and 10% dextran sulphate in 50% deionized formamide in 2xSSC) was placed onto them. DNA denaturation of the probes and the specimens was carried out simultaneously by incubation at 90°C for ten min followed by an overnight hybridization at 37°C, in a moist chamber. In the case of chromosome preparations, hybridization was performed simultaneously with both the DNA probe originating from DNA isolated from fibrillar complexes and labelled with digoxigenin; and with the DNA probe for the transcribed part of the rDNA, labelled with biotin. In order to remove DNA/RNA hybrids, preparations were, after washing, treated with RNase H, 40 µg/ml, according to the manufacturer’s instructions (Boehringer).

Detection of the hybridization signal

For simultaneous detection of different probes and amplification of the signal, after repeated washes, the slides were incubated in 4xSSC with 0.05% Tween 20, 5% non-fat dry milk, 5 µg/ml avidin-FITC (Vector) and 50 µg/ml rhodamine-labelled anti-digoxigenin Fab fragments (Boehringer), for 30 minutes at room temperature. Following a few more washes, slides were incubated with 5 µg/ml biotinylated goat anti-avidin (Vector) and rhodamine-labelled rabbit anti-sheep IgG (50 µg/ml, Biomedica), then washed again and finally incubated with avidin-FITC and rhodamine-labelled goat anti-rabbit IgG (Southern Biotechnology Associates). The slides were mounted in a hydrophilic (0.1 µg/ml) medium containing 0.1 µg/ml DAPI (Boehringer) and observed in a Leitz epifluorescence microscope, using three optical channels selective for rhodamine (digoxigenin-labelled probes), FITC (biotin-labelled probes) and DAPI (total DNA). For ultrastructural studies, the sections were incubated with a goat anti-biotin antibody (Vector Anti-Biotin, affinity purified, 1:300) followed by an incubation with a rabbit anti-goat antibody (1:20) coupled with 5 nm gold particles (Amersham).

Controls

In all hybridization experiments, two control hybridizations were carried out in parallel: one with a biotinylated plasmid DNA of...
similar GC-content to the human rDNA probe (pGEM, Promega) and another with no DNA probe. The light microscope analysis system was tested by applying both the digoxigenin and the biotin detection systems to slides hybridized with one probe only; a possible leak between the two optical channel was tested. No inappropriate signal could be detected in all these checks. Treatment with RNase H after in situ hybridization did not affect the intensity or distribution of the signal.

**Statistical evaluation of morphometric results**
The densities of gold particles over the nucleolar components, the non-nucleolar area of the nuclei, and the cytoplasm were determined using the software Sigmascan (Jandel-Scientific), in 15-35 cells for each set of experiments. The statistical significance of the results was assessed using Student’s two-sided t-test.

**RESULTS**

**Isolated fibrillar complexes**
LEP cell nucleoli possess well defined nucleolar components with a typical spatial arrangement: the fibrillar centres are surrounded by dense fibrillar component layers and together, they are embedded in the granular component (Fig. 1). After isolation, the resulting fraction is highly enriched in fibrillar complexes, although small residues of other nuclear material can occasionally also be found (Fig. 2).

**Characteristics of DNA extracted from fibrillar complexes**
In order to assess the chromosomal localization of the DNA that we had extracted from isolated fibrillar complexes, a double-labelling in situ DNA hybridization was performed on human metaphase chromosomes. Fig. 3 gives a comparison of the hybridization signal of the DNA probes for the transcribed part of the human rDNA (Fig. 3A), and for the DNA extracted from the fibrillar complexes (Fig. 3B). The signal consists mostly of double dots; when compared to total DNA staining (Fig. 3C), these doublets can be localized to the secondary constrictions of acrocentric chromosomes. Within the limits of light microscopy, the distribution of the signal for the ribosomal genes and for the DNA from the fibrillar complexes is identical.

Further proof that the fibrillar complex DNA contains ribosomal gene sequences was obtained by the fact that a probe for 28S rDNA hybridized to it, but not one for alphoid satellite DNA (data not shown).

**Nucleolar distribution of different DNA probes in Sertoli cells and in spermatogonia A**
Sertoli cell nucleoli exhibit a very stable and unique spatial organization. They contain, usually, one large fibrillar centre; the dense fibrillar component surrounds the fibrillar centre, forming a coat with radial protrusions. Next to the fibrillar centre, a large block of granular component can be found. Some dense fibrillar component branches are also frequently found at the periphery of the granular block, especially on the opposite side to the fibrillar centre (Fig. 4A).

The label from all three DNA probes used for the in situ hybridizations were shown by electron microscopy to be distributed in a similar way over the nucleoli. The probes for total genomic DNA (Fig. 4B), DNA from fibrillar complexes (Fig. 4C), and for the transcribed part of ribosomal genes (Fig. 4D) hybridized predominantly to the dense fibrillar component. Gold particles are visible in different zones of the dense fibrillar component; they are seen in the layer around the fibrillar centre. An intense label is also

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**Fig. 1.** The LEP cells possess nucleoli with well distinguishable fibrillar centres (C), dense fibrillar component (D), and granular component (G). Magnification, ×20,800.

**Fig. 2.** Fibrillar complexes, isolated after short hypotonic treatment of LEP cells. The electron micrograph shows a high level of purity of isolated fraction. Magnification, ×35,000.
found associated with the trabeculae of the dense fibrillar component located far away from the fibrillar centres. On the contrary, the fibrillar centres were almost devoid of gold particles; few were seen on their periphery (Fig. 4D). The label over the fibrillar centres was not significantly different from background values (Fig. 6). In addition, when the probe for total genomic DNA was used (Fig. 4B), clusters of gold particles were visible throughout the chromatin outside of nucleolus.

Human spermatogonia A nuclei also have a very stable and characteristic structure (Hartung et al., 1990). The prominent fibrillar centres are always attached to the nuclear envelope and are surrounded by meandering threads of the dense fibrillar component that extend into the nuclear interior. Typically, some granular component is found as a continuation of the strands of the dense fibrillar component (Fig. 5A). Again, if hybridized with the DNA probe from fibrillar complexes, most of the label is found in the dense fibrillar component attached to the fibrillar centres. However, it is more difficult than in Sertoli cells to see the border line between the fibrillar centre and the dense fibrillar component; nevertheless, there is virtually no label in the interior of the fibrillar centre. Significant labelling is found in those threads of the dense fibrillar component that are remote from the fibrillar centres (Fig. 5B).

DISCUSSION

DNA distribution in nucleoli

Condensed nucleolar DNA can be seen morphologically as peri- or intranucleolar chromatin clumps. Using cytochemical or immunoelectron microscopy approaches, decondensed DNA has been localized in both the fibrillar centres and the dense fibrillar component of different cell types. All papers presented so far are in agreement on the presence of DNA in the fibrillar centres, although the intensity and localization of label varies and, in some cell types, only the peripheral zone of the fibrillar centres was found to contain a significant amount of DNA. However, contradictory results were obtained regarding the dense fibrillar component: some authors did not detect DNA in this component in Ehrlich tumour cells or in mouse hepatocytes, but others detected DNA predominantly in the dense fibrillar component of Sertoli cells or in stimulated lymphocytes (for reviews see Wachtler et al., 1989; Ghosh and Paweletz, 1990; Stahl et al., 1991 and Wachtler et al., 1992). This discrepancy has not yet been sufficiently explained. It was shown recently that the DNA found in nucleoli is not exclusively ribosomal DNA. For example, long interspersed (LINE) KpnI elements which are absent from the ribosomal gene repeat, and a tandemly-repeated simple sequence cluster derived from the short arm of chromosome 15, were found, using light microscopy, in human lymphocyte nucleoli (Kaplan et al., 1992). Centromeres were also identified as a frequent part of nucleoli (Ochs and Press, 1992). Until now, the exact localization and function of non-ribosomal DNA in nucleoli was unknown. In our experiment, a difference in the distribution of hybridization signal between total genomic DNA and transcription unit DNA probes would indicate that, besides rDNA, other DNA is involved. However, we did not detect such a difference in Sertoli cells; both probes hybridized predominantly and significantly to the dense fibrillar component and not to the fibrillar centres. Our results confirm previous conclusions about predominant rDNA distribution in the dense fibrillar component in human Sertoli cells (Wachtler et al., 1989; Wachtler et al., 1992) and extend this conclusion, at the ultrastructural level, to human spermatogonia A. However, these results do not necessarily indicate an absolute absence of DNA from fibrillar centres. It was occasionally possible to find small clusters of gold particles in the border region between the two components in question. Also, the concentration of DNA in the relatively large volume of fibrillar centres could be very low and not all of the DNA accessible to the probes. As a consequence, small amounts of
DNA could escape detection by in situ hybridization, given the low signal/background ratio of this technique at the electron microscopic level. We conclude therefore that ribosomal RNA genes in human Sertoli cells and spermatogonia A are predominantly associated with the dense fibrillar component, including the

Fig. 4. Nucleoli of human Sertoli cells. (A) General electron microscopic view; (B,C,D) after in situ hybridization to sectioned material with three different DNA probes: (B) for total genomic DNA, (C) for DNA isolated from the fibrillar complex of nucleoli, (D) for the transcribed part of rDNA. The gold particles are found almost exclusively over the dense fibrillar component of the nucleolus (arrowheads). Magnification, (A) ×21,000; (B) ×32,000; (C) ×24,000; (D) ×24,900.
border region between the fibrillar centres and the dense fibrillar component.

**The nature of DNA in the fibrillar complex**

The hypotonic isolation procedure allowed us to study the DNA contained in the two nucleolar fibrillar components. Southern blot hybridization with the 28 S rDNA probe indicated that the isolated DNA contained ribosomal RNA genes, but it did not hybridize with a probe for alphoid satellite DNA, known to be a non-ribosomal sequence present in nucleoli (Kaplan et al., 1992). The results show that we were predominantly isolating the fibrillar complexes and that the method we used is a promising tool for their further study.

If hybridized in situ, fibrillar complex DNA hybridized mainly to chromosomal regions recognizable as nucleolar-
organizing regions at the light microscope level, and to the dense fibrillar component at the electron microscope level. This can be interpreted (regarding the discussed problems above) in a similar way to that in the previous paragraph and points again to the ribosomal RNA genes as being the main, if not exclusive, DNA type present in the fibrillar complex and, more specifically, in the dense fibrillar component.

In conclusion, we would like to stress that it is probably impossible to explain easily all of the current discrepancies in the localization of rRNA transcription in various cell types. It is questionable as to how much we can rely on the apparent morphological homogeneity of individual nucleolar components. For example, if studying silver staining or a variation in DNA hybridization signal, usually, we do not see a diffuse labelling pattern. More typically, areas with a diverse labelling intensity in the fibrillar centres and the dense fibrillar component can be found. Also, the borderline between these two components is variable; it can be sharp but also more or less gradual. We speculate therefore that the two fibrillar nucleolar components could have a zonal arrangement, possibly with a different activity or function. Moreover, there are several types of nucleoli; whether it is possible to reduce all varieties to one universal model still remains to be answered.

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REFERENCES


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