Ribosomal genes in focus: new transcripts label the dense fibrillar components and form clusters indicative of “Christmas trees” in situ

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The organization of transcriptionally active ribosomal genes in animal cell nucleoli is investigated in this study in order to address the long-standing controversy with regard to the intranucleolar localization of these genes. Detailed analyses of HeLa cell nucleoli include direct localization of ribosomal genes by in situ hybridization and their indirect localization via nascent ribosomal transcript mappings. On the light microscopy (LM) level, ribosomal genes map in 10–40 fluorescence foci per nucleus, and transcription activity is associated with most foci. We demonstrate that each nucleolar focus observed by LM corresponds, on the EM level, to an individual fibrillar center (FC) and surrounding dense fibrillar components (DFCs).

The EM data identify the DFC as the nucleolar subcompartment in which rRNA synthesis takes place, consistent with detection of rDNA within the DFC. The highly sensitive method for mapping nascent transcripts in permeabilized cells on ultrastructural level provides intense and unambiguous clustered immunogold signal over the DFC, whereas very little to no label is detected over the FC. This signal is strongly indicative of nascent “Christmas trees” of rRNA associated with individual rDNA genes, sampled on the surface of thin sections. Stereological analysis of the clustered transcription signal further suggests that these Christmas trees may be contorted in space and exhibit a DNA compaction ratio on the order of 4–5.5.

Introduction

Approximately 400 copies of ribosomal genes are present in the human diploid genome; they are organized in the form of head-to-tail tandem repeats at well-described positions within five pairs of chromosomes (30–50 repeats per chromosome; Hadjiolov, 1985). Transcriptionally active ribosomal genes of eukaryotic organisms are accommodated in a special nuclear organelle termed the nucleolus, which also represents the site at which the assembly and maturation of preribosomal particles occur (e.g., Scheer and Benavente, 1990). Three basic structural compartments are commonly distinguished within the nucleolar architecture: fibrillar centers (FCs),* dense fibrillar components (DFCs), and granular components (Hadjiolov, 1985).

The molecular organization of ribosomal transcripts in the form of so-called “Christmas trees” was described on spreads of nuclear contents from amphibian oocytes more than 30 years ago (Miller and Beatty, 1969). Similar structures were also reported later in other species (e.g., Trendelenburg et al., 1974; Puvion-Dutilleul et al., 1977). Surprisingly, a description of the morphological equivalent of Christmas trees in in situ systems only recently appeared. Christmas trees in a condensed form were observed in somatic cells of Pisum sativum (Gonzalez-Melendi et al., 2001). In animal cells, similar structures have only been observed in specialized nucleoli of insect cell oocytes (Scheer et al., 1997).

In addition, a schism exists in the nucleolar literature regarding in which nucleolar compartment the rRNA synthesis takes place in somatic animal cells. On one hand, rRNA transcription sites were reported to be localized to the interior of the FC, whereas other authors found them in the DFC, together with the DFC/FC border zone (e.g., Granboulan and Granboulan, 1965; Scheer and Benavente, 1990; Raska et al., 1995; Cmarko et al., 2000; Thiry et al., 2000). The discrepancies in published results can, at least to a large ex-
tent, be explained by a highly compact structure of the nucleolus, particularly of DFC (Stanek et al., 2001). Consequently, in situ hybridization (ISH) and immunocytochemical approaches may fail, and different kinds of cell treatment that provide a more accessible structure (Koberna et al., 1999) may provide clearer signal and less equivocal results.

Keeping the compact nucleolar structure in mind, the problem of in situ organization of active ribosomal genes in human HeLa cells has been addressed using improved methods to label newly synthesized RNA within nucleolar structure at the ultrastructural level, and to compare this with light microscopic analysis. Using a combination of light microscopy (LM) and EM approaches, we show that the pattern of the transcription signal seen in ultrathin nucleolar sections corresponds to marked labeling of the DFC, and further provides evidence of individual compacted Christmas trees detected at high-resolution within the DFC.

**Results and discussion**

**The rDNA coding sequences are localized within multiple fluorescence foci**

The efficiency and pattern of the fluorescence ISH rDNA signal strongly depended on the fixation/permeabilization treatment used (unpublished data). In this respect, the ISH efficiency was highest after methanol/acetone treatment. Although this treatment resulted in apparent ultrastructural changes, these structure alterations were below the resolution limit of LM (Stanek et al., 2001).

At the LM resolution, the ISH signal consisted of 10–40 fluorescence foci per cell nucleus (Fig. 1, A–C). Most of this signal was confined to the nucleoli. Few rDNA foci were found next to the nucleoli, probably in the perinucleolar condensed chromatin. Rare isolated rDNA foci, which were distant from the nucleoli, were also visualized. We identified these foci with so-called micronucleoli (Smetana and Busch, 1974).

Next, we mapped the ribosomal genes at the EM level, by a postembedding labeling procedure. In contrast to preembedding, it enabled the convenient preservation of the ultrastructure. The sensitivity of the postembedding EM ISH was much lower than the preembedding labeling, as most target sequences were hidden in a volume of the section and the ribosomal genes sampled to the surface of thin sections could be revealed at best. However, the sectioning procedure itself could help in exposing a long enough rDNA sequence at the section surface (Raska et al., 1995). In agreement with this previous study in which a different ISH approach with the sense probes to 18-S rDNA was used, the ISH signal in aldehyde-fixed and thin-sectioned nucleoli of HeLa cells consisted only of rare clusters of gold particles. In 25 images tested, 42 gold clusters were detected; only 10 of them were located outside the DFC region, and consequently, ~75% of ISH signal mapped into DFC (Fig. 1 D).

**Figure 1.** Localization of ribosomal genes in HeLa cells. (A–C) Wide-field LM mapping of ribosomal genes. Fluorescence mapping of ribosomal genes (A and C, yellow), phase contrast image (B and C, blue), and the merge image (C) are shown. Ribosomal genes are located in a number of fluorescent foci. Most of the foci are situated in the nucleolus. Two foci are situated in the perinucleolar chromatin (arrowheads). (D) EM mapping of ribosomal genes. The signal (arrowheads) is located in DFC. Bars: (A–C) 10 μm; (D) 200 nm.

**Figure 2.** LM mapping of nucleolar transcription in HeLa cells. (A and B) Wide-field images of nucleolar transcription pattern seen in both hypotonically treated (A) and permeabilized (B) cells. The pattern consists of numerous fluorescent foci. Note that the foci in A are more blurred with respect to the foci in B. (C–E) Light microscopic image of ribosomal genes (C and E, red) with respect to the position of nucleolar transcription sites (D and E, green). The merged image is in E. One confocal section after image restoration is shown. Most of rDNA foci exhibit the transcription activity. An rDNA focus without transcription signal is indicated by the arrow. Bars: (B) 10 μm; (E) 5 μm.
ISH approach was not able to differentiate between transcriptionally active and inactive genes. Thus, the bromouridine (BrU) labeling of growing transcripts, reporting the active genes position exclusively, was performed next.

**Most nucleolar rDNA foci exhibit transcriptional activity**

We mapped the nucleolar transcription by means of BrU, which was incorporated during 5 min into newly synthesized transcripts both in hypotonically treated cells that incorporated BrU in vivo and in permeabilized cells (Fig. 2, A and B). After this time interval, the majority of the signal corresponded in living cells to nascent precursor rRNA (Stanek et al., 2001). The lower rate of the transcription in permeabilized cells incorporating BrU was reported (Jackson et al., 1993), such that in permeabilized cells, the large majority of labeled transcripts had to correspond to nascent transcripts. In both cases, the LM mapping of the nucleolar RNA synthesis provided a pattern consisting of 10–40 fluorescence foci per nucleus. However, some spatially close foci appeared to fuse due to the limited resolution of LM (see below). This phenomenon was also observed in the case of rDNA foci mappings. Both in cells that underwent the hypotonic shift and in permeabilized cells, the nucleolar, but not extranucleolar transcription, as distinguished by phase-contrast microscopy, was abolished by the low concentration of actinomycin D. At the same time, the nucleolar signal was not sensitive to α-amanitin treatment in concentrations sufficient to inhibit RNA polymerases II and III, but not RNA polymerase I (Stanek et al., 2001; unpublished data). Thus, we concluded that the BrU incorporated into the nucleolar RNA corresponded to the synthesis of the rRNA precursors.

The nucleolar signal in permeabilized cells was more prominent with regard to the extranucleolar signal if compared with cells, which underwent the hypotonic shift. We ascribed this phenomenon in permeabilized cells to the fact that, in contrast to the nucleoplasm, the nucleolar structure was loosened/extracted only to a limited extent. In addition, the fluorescence foci in living cells were more blurred than in permeabilized cells (Fig. 2, A and B). This difference was still more pronounced after the prolongation of incorporation time (unpublished data). Results observed in the living cells were compatible with previously reported rapid changes in localization of BrU labeled RNA (Stanek et al., 2001). In permeabilized cells, a prolongation of the incubation time in the transcription buffer led to an increase of signal intensity without influencing the focal pattern of the fluorescence.

Next, we performed the simultaneous mapping of nucleolar rDNA sequences and transcription (Fig. 2, C and D). The observed fluorescence patterns were compatible with the above single labeling experiments. The whole transcription signal within the nucleolar body was colocalized with the rDNA foci, and only a minor part of the nucleolar rDNA foci did not colocalize with the transcription signal. Therefore, in the frame of the resolution of LM, most nucleolar rDNA foci were identified with transcriptionally active foci. We considered the minor cases of nucleolar rDNA foci not exhibiting the transcription activity as pointing to the detection of (transcriptionally) silent rDNA sequences, probably replicating rDNA (unpublished data). The rDNA foci observed outside the nucleolar body did not contain a transcription signal.

**Intense ultrastructural transcription signal in the form of clustered gold particles in nucleoli of permeabilized cells points to the presence of Christmas trees in DFC**

Next, we performed EM mapping of transcription. In cells, which underwent the hypotonic shift, the ultrastructural details of all three nucleolar compartments were preserved in the form of clustered gold particles in nucleoli of permeabilized cells points to the presence of Christmas trees in DFC.
Figure 4. Concomitant LM and EM mapping of transcription signal in a thin section of permeabilized cell. (B) Detailed part of A. The colored insert in A represents the wide-field LM picture of the same area depicting newly synthesized RNA (red) and DNA counterstained with YOYO (green). (C) Merge of an inverse EM image from A in cyan, and a transcription LM signal from the upper insert in red. Note the overlap of the LM and EM transcription signals. Interestingly, because of the consecutive EM and LM labeling performed, an electron-dense stain due to antibodies accumulated at the surface of thin section can be recognized. Bars: (A) 0.5 μm; (B) 0.2 μm.

an excellent way (Fig. 3 A). The signal was observed in DFC and at the FC/DFC border after 5 min of incorporation of BrU, whereas FC was devoid of signal. The clustering of gold particles was recognized frequently.

In permeabilized cells, the ultrastructure of nucleoli was, in contrast to highly extracted nucleoplasm, well preserved, and all the nucleolar compartments could be readily identified. The transcription signal was also confined to DFC and the FC/DFC border after 5 min of BrU incorporation. However, the signal consisted entirely of clusters of up to several tens of gold particles confined to subdomains of DFC (Fig. 3, B–D). The clusters were frequently elongated with a maximum length of 200 nm and a mean width 45 nm. Multiple clusters of gold particles were regularly detected in DFC surrounding individual FC, and even tens of such clusters could be observed in a large nucleolar section encompassing several FC profiles. The pattern of the transcription signal was, to a large extent, insensitive to the prolongation of BrUTP incorporation (unpublished data). If incorporation time was shortened to 1 min, the signal was lowered progressively. Individual gold particles or clusters of a few gold particles were observed in only in some EM profiles in the DFC and DFC/FC border (unpublished data).

The clear-cut and intense EM signal of newly synthesized rRNA sampled to the surface of individual thin sections (Fig. 3) was much higher than the signal revealed by ribosomal genes ISH. Long enough sequences had to be available for successful ISH, and only a very small fraction of them was exposed to the section surface during the postembedding detection. In contrast, there had to be ~100 nascent rRNA chains present in each rRNA gene (Jackson et al., 1998), with many incorporated BrUs in each transcript, so that thousands of brome epitopes had to be present in nascent rRNA synthesized from one gene. This allowed us to suggest that the very high transcription signal in the form of clustered gold particles in nucleoli of permeabilized cells reflected the presence of Christmas trees in the DFC. Our conclusion is in harmony with results of Gonzalez-Melendi et al. (2001) that identified the clustered bromouridine signal with the condensed Christmas trees in plant cells.

Nucleolar BrU focus observed by LM corresponds to individual FC and surrounding DFC on the electron microscopic level

A simultaneous LM and EM mapping of rDNA transcription was performed next in order to correlate the rDNA transcription pattern revealed by fluorescence and EM. The number of FCs was identical to the number of fluorescence foci or was, due to the resolution limit of LM, slightly higher. The superposition of concomitant LM and EM images of the same nucleolar section identified each fluorescence focus with the individual FC (or two or more closely spaced FCs) together with the surrounding DFC labeled by gold particles at the EM level (Fig. 4). The concomitant LM and EM mapping of transcription unambiguously showed that LM alone did not resolve FC from DFC. Within the resolution limit of the LM, individual FC, together with the surrounding rim of DFC, was in most cases visualized as a single focus.

Based on a serial sectioning, a three-dimensional view of the arrangement of focal transcription sites in the nucleolus is shown in Fig. 5. Regarding the arrangement of active ribo-
transcribing ribosomal genes was
mas trees enabled us to contribute to the debate about the
of a transcription signal in permeabilized cells with Christ-
ularly at the 5′end of the transcription unit. The in situ length
lows us to make some approximations as to the compaction
contorted within DFC
Ribosomal transcription units are compacted and
contorted within DFC
The clear visualization of clusters of transcription signals al-

to make some approximations as to the compaction of indi-
vidual rDNA transcription units. The in situ length of the ri-
osomal transcribed unit was shown to be ~300 nm in a pea (%isaum sativum; Gonzalez-Melendi et al., 2001) and
>400 nm in a grasshopper (Locusta migratoria; Scheer et al.,
1997). These lengths corresponded to 6.6- and 7-kb se-
quences of DNA, respectively. The transcription unit size in hu-
man cells is 13 kb (Hadjilov, 1985). As the length of clus-
ters of gold particles observed in nucleoli of permeabili-
zation cells did not exceed 200 nm, the transcription units
may be highly contorted in space. In order to estimate the
length of the transcription unit, a stereological approach
was used. 549 clusters were detected in 200 random nuclear
sections. The total volume occupied by newly synthesized
rRNAs was 0.035% of the nuclear volume. The mean nu-
clear volume as assessed by LM was 730 ± 80 μm³. Keeping
in mind that there were 150 transcriptionally active riboso-
mal genes in the HeLa cell nucleus (Jackson et al., 1993), we
computed a volume of BrU labeled parts of the Christmas
tree. Approximating the shape of the labeled part of the
Christmas tree as a cone, we could roughly estimate its length
as ~800 nm. In other words, the packing ratio of transcribing ribosomal genes was ~5.5. This number was
~4 when the cylindrical shape of the observed clusters was
assumed instead. These numbers should be understood as
lower limits of the legitimate value, as we cannot exclude
that some transcriptional markers were not detected, parti-
cularly at the 5′ end of the transcription unit.

The presence of a transcription signal in the DFC both in
living cells and in permeabilized cells, and the identifica-
tion of a transcription signal in permeabilized cells with Christ-
mas trees enabled us to contribute to the debate about the
position of the active ribosomal genes within the nucleolus
of somatic animal cells. Our results are not in agreement
with findings of Thiry et al. (2000) who in cultured HeLa
cells situated the bulk of the rDNA transcription into the in-
terior of FC. Our results are compatible with the model pre-

cised by the mapping experiments up to now.

Materials and methods
Cell culture and drugs treatment
HeLa cells were cultured as described previously (Koberna et al., 1999). In all ex-
periments, we limited ourselves to cells showing in phase contrast
regular nucleoli, i.e., to truly interphase cells. We also avoided multinu-
clear cells as well as cells with large nuclei possessing apparently highly

elevated genome copies, which were occasionally seen in the culture.

The inhibition of RNA polymerase I transcription or extranuclear tran-
scription was achieved by the addition of actinomycin D or α-amanitin, re-
spectively (Raska et al., 1995; Koberna et al., 1999).

Incorporation of BrU into nascent RNA
Two different approaches for the labeling of rDNA transcripts with BrU were
used: the hypotonic shift enabling the incorporation of BrU in vivo was per-
fomed as described previously (Koberna et al., 1999); for permeabilized cells,
the protocol of Raska et al. (1995) was followed with these modifications:

0.03% Triton X-100 was used for cell permeabilization and the BrU incorpo-
ration was performed in a buffer containing 15% glycerol at 35°C.

In situ hybridization of rDNA
The DNA probe was prepared using an Enzo DNA labeling kit from a Pa
plasmid construct (Erickson et al., 1981), provided by Jo Sylvester (Ne-
mours Children’s Clinic Research, Orlando, FL). This construct involved a

spanning sequence for downstream ~200 nucleotides of 18 S rRNA, inter-
nal transcribed spacer 1, sequence for 5.8 S rRNA, internal transcribed spacer
2 and upstream ~4,500 nucleotides of 28 S rRNA. The probe was
labeled with digoxigenin-11-DUTP by nick translation.

The cells growing on coverslips were washed in cold PBS and fixed/permea-

labeled with digoxygenin-11-dUTP by nick translation.
ml RNase A (Roche) for 2 h at 37°C, gradually dehydrated in ice-cold 30, 50, 70, 96, and 100% ethanol, and air-dried. The denaturation of the cells was performed in 70% deionized formamide/2 × SSC, pH 7.0, at 75°C for 2–3 min. The cells were then placed in ice-cold 70% ethanol, dehydrated, and air dried. The probe was denatured in deionized formamide at 75°C for 6 min and placed on ice. 6 μl of a hybridization mix containing 25 ng of probe, 0.5 mg/ml sonicated salmon sperm DNA, 2 mg/ml Escherichia coli RNA, 50% deionized formamide, 2 × SSC, 0.2% BSA, and 10% dextran sulfate, was used per each coverslip. The ISH was performed overnight at 37°C in a humidified chamber. After the posthybridization washes, coverslips were incubated with 200 U/ml of RNase A (Sigma-Aldrich) at 37°C for 2 h for the elimination of possible RNA–DNA duplexes. The combined fixation of BrU labeled transcripts and ISH was performed as described previously (Haaf and Ward, 1996). After immunolabeling, the cells were postfixed overnight with 3:1 mixture of methanol:acetic acid at −20°C, and then proceeded to hybridization as described above.

LM

Permeabilized cells were fixed either in 2% formaldehyde in PBS for 10 min after transcription, or fixed/permeabilized in methanol at −20°C for 30 min, rinsed in acetone for 20 s, and air-dried. Before immunolabeling, the cells were washed in PBS and incubated for 10 min with 0.5% BSA in order to block nonspecific binding. Subsequently, the cells were incubated with rat anti-BrU antibody (Harlan SeraLab) and donkey anti-rat antibody conjugated with Cy3 fluorochrome (Jackson ImmunoResearch Laboratories).

Specimens were embedded in a ProLong antifade kit (Molecular Probes) and viewed using an AX70 Provis (Olympus) microscope equipped with a charge-coupled device camera (PXL with KAF 1400 chip; Photometrics) or TCS NT (Leica) confocal microscope. In the case of confocal imaging, three-dimensional sets of optical sections 120 nm apart were collected and restored using the maximum likelihood estimation algorithm (Huygens 2; Soft Imaging Systems).

EM and LM on ultrathin sections

The postembedding rDNA ISH was performed as described previously (Wachtler et al., 1992) on lowicryl sections. BrU-labeled RNA was detected on lowicryl sections according to Raska et al. (1995). For the simultaneous visualization of BrU at the LM and EM levels, the sections on the EM grid were incubated with rabbit anti-BrU antibody, and followed by incubation with gold adduct. Then antibody with fluorochrome was restored using the max-