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Separation of replication and transcription domains in nucleoli

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ABSTRACT

In mammalian cells, active ribosomal genes produce the 18S, 5.8S and 28S RNAs of ribosomal particles. Transcription levels of these genes are very high throughout interphase, and the cell needs a special strategy to avoid collision of the DNA polymerase and RNA polymerase machineries. To investigate this problem, we measured the correlation of various replication and transcription signals in the nucleoli of HeLa, HT-1080 and NIH 3T3 cells using a specially devised software for analysis of confocal images. Additionally, to follow the relationship between nucleolar replication and transcription in living cells, we produced a stable cell line expressing GFP-RPA43 (subunit of RNA polymerase I, pol I) and RFP-PCNA (the sliding clamp protein) based on human fibrosarcoma HT-1080 cells. We found that replication and transcription signals are more efficiently separated in nucleoli than in the nucleoplasm. In the course of S phase, separation of PCNA and pol I signals gradually increased. During the same period, separation of pol I and incorporated Cy5-dUTP signals decreased. Analysis of single molecule localization microscopy (SMLM) images indicated that transcriptionally active FC/DFC units (i.e. fibrillar centers with adjacent dense fibrillar components) did not incorporate DNA nucleotides. Taken together, our data show that replication of the ribosomal genes is spatially separated from their transcription, and FC/DFC units may provide a structural basis for that separation.

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1. Introduction

Replication and transcription enzymatic complexes run at high speed and for long distances, which apparently compels the cell to employ a special strategy in order to prevent collision of the DNA and RNA polymerases. This need seems to be particularly urgent in the nucleoli, owing to certain aspects of their organization. In mammalian cells, nucleoli are formed on the basis of Nucleolus Organizer Regions (NORs), i.e. the clusters of ribosomal genes coding for 18S, 5.8S and 28S RNAs of the ribosomal particles (Henderson et al., 1972; Long and Dawid, 1980; Puvion-Dutilleul et al., 1991; Raška, 2003; Raška et al., 2006a,b; Cmarko et al., 2008; Sirri et al., 2008). When the cell enters S phase, the transcription activity of these genes continues and may even increase (Gorski et al., 2008). Moreover, replication of rDNA may be initiated in the transcribed as well as non-transcribed loci and proceed in both directions (Little et al., 1993; Yoon et al., 1995). Then how are the two machineries segregated?

So far this intriguing problem has not drawn special attention partly because of the specific structure of nucleoli in which individual rDNA repeats are "hidden" and the chief structural elements are represented by fibrillar centers (FCs), dense fibrillar components (DFCs), and granular components. All pol I dependent transcription in the nucleoli seems to take place in the DFC or in the border region between the DFC and the FC (e.g. Raška et al., 1983a,b, 1995, 2006a,b; Cmarko et al., 1999, 2000; Melčak et al., 1996; Malinský et al., 2002; Koberna et al., 2002; Casafont et al., 2006). As for rDNA replication, it is still not clear whether it occurs in the same regions as transcription. It has been established that the transcriptionally active genes, which in cycling cells represent about a half of the entire rDNA (Warner, 1999; Grummt and Pikaard, 2003), are replicated in early S phase, and replication of the silent genes is postponed until late S phase (Berger et al., 1997; Li et al., 2004), so that the problem of collision is chiefly confined to the early S phase. But there is yet another complication: trying to detect nucleolar replication *in situ* by incorporation of labeled nucleotides, one finds that the intensity of the signal is usually very low, especially in early S phase. The causes of this

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phenomenon are not clear. According to one hypothesis (Dimitrova, 2011), rDNA may move to the nucleolar periphery, or even to the surrounding nucleoplasm, replicate there, and then pull back to its original position.

In the present work we focused on two aspects of the problem: (1) the spatial separation of replication and transcription foci in nucleoli; (2) relocation of nucleolar DNA in the course of S phase. To investigate these problems, we measured the spatial correlation of various replication and transcription signals in the nucleoli in cells of human and murine origin using specially devised software for analysis of confocal images. We also studied colocalization of replication and transcription foci employing a super-resolution single molecule localization microscopy (SMLM) method (Křížek et al., 2011; Ovesný et al., 2014). To observe the relocation of nucleolar DNA, we labeled it in early S phase with Cy5-dUTP in a cell line expressing RFP-PCNA and GFP-RPA43.

2. Methods

2.1. Cell culture and plasmids

Human derived HeLa and HT-1080 (human fibrosarcoma) cells were cultivated at 37 °C in Dulbecco modified Eagle's medium (DMEM, Sigma, #D5546) containing 10% fetal calf serum, 1% glutamine, 0.1% gentamycin, and 0.85 g/l $NaHCO_3$ in standard incubators. Mouse NIH 3T3 cells were maintained in DMEM supplemented with 10% calf serum and 1 mg/ml penicillin/streptomycin.

We produced a cell line stably expressing two plasmid constructs: RFP-PCNA vector was received from the Max Planck Institute for Molecular Cell Biology and Genetics, Dresden; and GFP-RPA43 vector was received from Laboratory of Receptor Biology and Gene Expression Bethesda, MD (Dunder et al., 2002). Both constructs were transfected into the HT-1080 cells using Fugene (Qiagen) and stable clones were selected with G418 (GIBCO). One clone with bright two-colored fluorescence was chosen and expanded into the cell line.

2.2. Incorporation of DNA and RNA nucleotides

For simultaneous labeling of replication and transcription sites, sub-confluent cells were incubated 5 min with a mixture of 5-ethynyl-2′-deoxyuridine (EdU) (Invitrogen) at a concentration of 10 μM, and 5-fluorouridine (FU) (Sigma) at a concentration of 10 μM.

The cells were fixed in 2% formaldehyde freshly prepared from paraformaldehyde, permeabilized with Triton X-100, and processed for FU immunocytochemistry using a mouse monoclonal anti-BrdU antibody (Sigma). After that the replication signal was visualized using EdU Alexa Fluor 647 Imaging Kit (Invitrogen). Additionally, we visualized the replication signal using Cy3-dUTP and Cy5-dUTP using the scratch procedure (Schermelleh et al., 2001).

2.3. Immunocytochemistry

Primary antibodies against human rRNA polymerase (pol I) and Upstream Binding Factor (UBF) were kindly provided by Dr. U. Scheer (Biocenter of the University of Wurzburg). We also used a monoclonal (mouse) anti-UBF antibody (Santa Cruz Biotechnology, Inc.).

For visualization of fibrillarin in nucleoli, we used monoclonal antibodies against human fibrillarin or mouse fibrillarin (clone 17C12), kindly donated by Kenneth M. Pollard (Scripps Research Institute, La Jolla, CA). Secondary anti-human and anti-mouse antibodies were conjugated with Alexa 532 (Invitrogen), Cy3, or DyLight 488 (both from Jackson ImmunoResearch Laboratories). Coverslips with the cells were then mounted in Mowiol and allowed to harden overnight before imaging.

2.4. Microscopy

Confocal images were acquired using a SP5 (Leica) confocal laser scanning microscope equipped with a 63×/1.4NA oil immersion objective. Live cell imaging was performed with a spinning disk confocal system based on an Olympus IX81 microscope equipped with an Olympus UPlanSApo 100×/1.4NA oil immersion objective, CSU-X spinning disk module (Yokogawa) and Ixon Ultra EMCCD camera (Andor). For live cell imaging cells were maintained at 37 °C and 5% CO_2 using a microscope incubator (Okolab). For live cell microscopy, cells were cultured in glass bottom Petri dishes (MatTek).

For single molecule localization microscopy, we used an Olympus IX70 microscope equipped with an Olympus planapochromatic 100×/1.45NA oil immersion objective and a front-illuminated EMCCD camera (Ixon DU885, Andor). The back-projected pixel size in the sample was 80 nm. For imaging of Alexa 647 labeled EdU, we used a 630 nm, 500 mW diode laser, while for imaging of Alexa 532 labeled fluorouridine, we used a 532 nm, 1000 mW DPSS laser (both from Dragon laser, ChangChun, China). The laser light was delivered to the microscope using a multimode optical fiber (M29L02, Thor Labs) and a custom-made critical illumination setup which imaged the end face of the fiber into the sample. Power densities at the sample for both lasers were approximately 2 kW/cm^2. Fluorescence was isolated using a custom dual band filter set for SMLM (emission bands 569–610 nm and 667–736 nm, Chroma).

2.5. Software and data analysis

For colocalization analysis of the confocal image stacks, we developed a custom ImageJ plugin (available at https://github.com/vmodrosedem/segmentation-correlation). Using images from both channels, the program identifies the regions occupied by nucleoli and calculates Pearson's correlation coefficient (PCC) and Spearman's rank coefficient (SRC) between the signals corresponding to the two channels within the volume of nucleoli. Additionally, the program measures the areas (in pixels) occupied by the nucleoli and the average intensities of both signals within these areas.

To achieve this, the software first identifies areas with nuclei (in cells positive for EDU replication signals) by creating a maximum intensity projection of the confocal image stack, then blurring the projection with a Gaussian filter ($\sigma = 8–10$ pixels), and finally thresholding the blurred image with a value obtained by Otsu's method for automatic threshold selection. The obtained mask of segmented nuclei is subjected to a hole filling operation using a binary, 4-way recursive flood fill algorithm. If several nuclei are found in a single image, they are identified by labeling of connected components. To segment nucleoli within each nucleus, we blurred each 2D confocal image (FU signal) with a Gaussian filter ($\sigma = 3$ pixels), followed by automatic threshold selection using Otsu's method. Regions with an area smaller than a user-specified value (300–400 pixels) were discarded to ignore nucleoplasmic signals. The two channels and their respective masks were then combined to identify nucleoli within each nucleus. All images were inspected visually to ensure that the segmentation was accurate. The calculation of PCC and SRC between the signals was limited to the intersection of the nuclear and nucleolar masks and was performed on the raw data without any background subtraction or normalization.

For SMLM data processing, we used ThunderSTORM (Ovesný et al., 2014), an open-source plugin for ImageJ designed for

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