Univerzita Karlova
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Dissertation summary

UNIVERZITA KARLOVA
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Studium mechanizmu regulace genové exprese na úrovni funkční organizace chromatinových domén

Dynamika FC/DFC jednotek buněčného jadérka

Study of the mechanism of gene expression regulation at the level of functional organization of chromatin domains

Dynamics of FC/DFC units of cell nucleolus

Ing. Matúš Hornáček

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Školitel: Doc. RNDr. Dušan Cmarko, Ph.D.

Dizertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

CONTENTS

ABSTRAKT

Jadérka (nucleoli) se vytvářejí na základě genů ribozomální DNA (rDNA), nazývaných Nucleolus Organizer Regions (NOR). Základní komponenty jadérek, fibrilární centra (FC) a denzní fibrilární komponenty (DFC) společně tvoří takzvané FC/DFC jednotky. Tyto jednotky jsou centry transkripce rDNA pomocí RNA polymerázy I (pol I), stejně jako raného procesingu, ve kterém hraje podstatnou roli protein fibrilarin. Každá FC/DFC jednotka pravděpodobně odpovídá jedinému transkripčně aktivnímu genu. V naší práci jsme studovali změny FC/DFC jednotek v průběhu buněčného cyklu. Analýza pomocí korelativní světelné a elektronové mikroskopie prokázala, že pozitivní signály pro polymerázu I a fibrilarin v nukleárních kuličkách odpovídají jednotlivým FC/DFC jednotkám. Pozorování *in vivo* prokázala, že v časné S fázi, když byly replikovány transkripčně aktivní ribosomální geny, se počet jednotek v každé buňce zvýšil o 60 až 80%. Během tohoto období jednotky přechodně ztratily pol I ale ne fibrilarin. Poté se až do konce interfáze počet jednotek nezměnil a jejich duplikace se dokončila až po rozdělení buněk v polovině G1 fáze. Tento zvláštní způsob reprodukce naznačuje, že značná podskupina ribozomálních genů zůstává transkripčně tichá od interfáze až po mitózu, ale opět se aktivuje v dceřiných buňkách. Ve výzkumu jsme pokračovali studiem FC/DFC jednotek a zkoumali jsme kinetiku jejich nejdůležitějších proteinů, polymerázy I a fibrilarinu. Po změnách fluorescenčních signálů v FC/DFC jednotkách jsme objevili dva druhy kinetiky: rychlé kolísání s periodami 2-3 minutami a pomalé kolísání intenzit signálů s periody od 10 do 60 min. Naše data naznačují, že komplexní pulzující aktivita transkripce stejně jako raný procesing je pro ribozomální geny běžná. V první teoretické práci jsme se zaměřili na jadérkovou DNA, která je úzce spjata s našimi experimentálními daty o FC/DFC jednotkách a diskutujeme v ní o vlastnostech ribozomální DNA. V druhé teoretické práci jsme se zaměřili na diskontinuální transkripci, která je přímo spojena s daty naší druhé experimentální studie, které naznačují, že ribozomální geny v savčích buňkách jsou přepisovány diskontinuálně. Obě teoretické práce jsou podkladem teoretického úvodu práce.

Klíčová slova: Jadérko, FC/DFC jednotka, rDNA, polymeráza I, fibrilarin, buněčný cyklus, transkripce, replikace, fluktuace.

ABSTRACT

Nucleoli are formed on the basis of genes of ribosomal DNA (rDNA) clusters called Nucleolus Organizer Regions (NORs). The essential structural components of the nucleoli, Fibrillar Centers (FC) and Dense Fibrillar Components (DFC), together compose FC/DFC units. These units are centers of rDNA transcription by RNA polymerase I (pol I), as well as the early processing events, in which an essential role belongs to fibrillarin. Each FC/DFC unit probably corresponds to a single transcriptionally active gene. In our work we study changes of FC/DFC units in the course of cell cycle. Correlative light and electron microscopy analysis showed that the pol I and fibrillarin positive nucleolar beads correspond to individual FC/DFC units. *In vivo* observations showed that at early S phase, when transcriptionally active ribosomal genes were replicated, the number of the units in each cell increased by 60 to 80%. During that period the units transiently lost pol I, but not fibrillarin. Then, until the end of interphase, number of the units did not change, and their duplication was completed only after the cell division, by mid G1 phase. This peculiar mode of reproduction suggests that a considerable subset of ribosomal genes remain transcriptionally silent from mid S phase to mitosis but become again active in the postmitotic daughter cells. In our research we continued the study of the FC/DFC units and examined kinetics of their most important proteins, polymerase I and fibrillarin. Following changes of the fluorescent signals in individual FC/DFC units, we found two kinds of kinetics: the rapid fluctuations with periods of 2-3 minutes and slow fluctuations with periods of 10 to 60 min. Our data indicate that a complex pulsing activity of transcription as well as early processing are common for ribosomal genes. In first theoretical work focused on the nucleolar DNA, which is closely linked to our experimental data about FC/DFC units we discuss the characteristics of ribosomal DNA. In second theoretical work we focused on the discontinuous transcription, and thus directly connected to the data of our second experimental study, which indicate that ribosomal genes in mammalian cells are also transcribed discontinuously. Both theoretical works are the basis of theoretical introduction of thesis.

Key words: Nucleolus, FC/DFC unit, rDNA, polymerase I, fibrillarin, cell cycle, transcription, replication, fluctuation.

1. THEORETICAL BACKGROUND

1.1. General introduction

Nucleolus is a non-membrane nuclear organelle found in all eukaryotes (Lam and Trinkle-Mulcahy, 2015). Nucleolus was formally described by Wagner (1835) and Valentin (1836). In the 1950s, the nucleoli were first isolated (Pederson, 2011). In the 1960s, the nucleolus was described as the site of ribosome synthesis. The average mammal cell nucleolus can produce up to 10,000 ribosomes per minute (Pederson, 2011). Nucleolus is the most prominent and distinctly differentiated nuclear body (Berger et al, 2008). Transcription of ribosomal genes, processing of the transcripts and early stages of assembling of pre-ribosomal precursor particles take place in the nucleolus (Huang, 2002). Initially the nascent pre-rRNA is assembled into an 80–90S nucleolar particle. Structural rearrangement and nucleotide modifications occur as the ribosomal proteins are incorporated, followed with cleavages which ultimately give rise to the mature ribosomal subunits. In addition, 80 or more ribosomal proteins, a surprisingly large number (more than 150) of accessory proteins and dozens of snoRNAs are involved (Nazar, 2004). Nucleoli in mammals have typically tripartite organization: fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC). Nucleolar structure forms around clusters of ribosomal gene at the end of the mitosis, persists in the interphase, and then decays when cells enter mitosis (Prieto and McStay, 2005). Nucleoli are formed on the basis of clusters of ribosomal DNA (rDNA) called Nucleolus Organizer Regions (NORs) coding for RNAs of ribosomal particles. The nucleoli also include a great variety of other DNA regions (Smirnov et al., 2016a).

1.2. Structural organisation of the nucleoli

1.2.1. DNA and chromatin structure in the nucleoli

Eukaryotic genomes associated with histones, other proteins and other non-protein constituents form chromatin (Rickards et al., 2007). In the nucleoli, chromatin exhibits three different levels of organization: compact clusters, fibers with the diameter ranging from 11 to 30 nm, and free agglomerates of loose DNA fibers. Both clusters and chromatin fibers exhibit a nucleosomal organization which is absent in the free agglomerates of loose DNA fibers. These fibers are 2-3 nm thick, just like the double helix DNA molecule. Free agglomerates of DNA filaments are located in fibrillar centers (FC) and apparently correspond to ribosomal DNA (Derenzini et al., 2006). The architecture of the nucleoli during interphase reflects the distribution of the rDNA that is characterized by alternation of clustered and extended genes (Junera et al., 1995).

Electron microscopic analysis has located nuclear transcription, replication and fibrillarin signals in the DFC and at its borders with FC (Pliss et al., 2005). Presence of the transcribed rDNA is necessary to overcome the highly stochastic nucleation step in the formation of nucleoli. In the absence of rDNA, some nucleolar proteins may form aggregates, but these aggregates are very variable in number, location, and time at which they form (Falahati et al., 2016).

Each human cell contains hundreds of copies of rDNA in the form of tandem repeats distributed among the chromosomes. A subset of the ribosomal genes remain permanently silent. Transcription of rDNA generates 45S pre-rRNA, which is then cleaved and processed into 28S, 18S and 5.8S rRNAs of ribosomal particles. The clusters of ribosomal genes are called Nucleolus Organizer Regions (NOR). In human cells, they are located on the short arms of acrocentric chromosomes (#13, 14, 15, 21 and 22) and appear as secondary constriction of metaphase chromosomes. Only some NORs, called competent, are transcriptionally active during the interphase and can be identified by the presence of RNA polymerase I and its transcription factors (Kalmarova et al., 2007, Kalmarova et al., 2008). Specific epigenetic modifications such as histone acetylation and methylation and methylation of DNA regulate the transcription of the rDNA (Cremer et al., 2004; Bartova et al., 2010).

Ribosomal RNA genes also occur in the form of extrachromosomal DNA. During oogenesis of some amphibians, fish, and insects, rDNA is amplified so that many extrachromosomal copies occur (Long and Dawid, 1980).

Ribosomal RNA genes in human cells are predominantly located in dense fibrillar component (DFC), and in the border region between FC and DFC (Hozak et al., 1993). But opinions on the exact localization of active ribosomal genes - in each of the two nuclear subcomponents FC and DFC, still differ (Raska, 2003). Some authors believe that transcription of rDNA takes place in fibrillar centers (FC), and the nascent rRNAs quickly enter the surrounding DFC (Cheutin al., 2002). Data of the electron microscopy identify DFC as a nuclear subcomponent in which rRNA synthesis takes place (Koberna et al., 2002; Melcak et al., 1996). In metabolically active mammalian nuclei, fibrillar centers (FC) and dense fibrillar components (DFC) compose one functional domain for the transcription of rRNA genes, and the nascent transcripts form DFC (Raska et al., 1995, Raska, 2003). The tendency of the NOR bearing chromosomes to associate with the nucleoli correlates with the transcription competence of the NORs. But nucleoli may contain incompetent NORs as well; in such cases the rDNA seems to be connected to the chromosomal territory by a long protrusion (Kalmarova et al., 2007, Kalmarova et al., 2008).

1.2.2. Nucleolar RNAs

Ribosomal RNAs are the most abundant and universal noncoding RNAs in living organisms. In eukaryotes, three of the four nucleolar ribosomal RNAs forming the 40S and 60S subunits are derived from the pre-ribosomal RNA. A complex sequence of processing steps is required to gradually release the mature RNAs from this precursor, concomitant with the assembly of the 79 ribosomal proteins (Henras et al., 2015). Eukaryotic ribosomal RNAs contain numerous modified nucleotides, in vertebrates about 115 methyl groups and some 95 pseudouridines. All but about ten of the methyl groups are methylations of ribose. The remaining ten are on heterocyclic bases. The ribose methylations occur very rapidly upon the primary rRNA transcript in the nucleolus, probably on nascent chains, and they appear to play an important role in ribosome maturation, at least in vertebrates. All of the methyl groups occur in the conserved core of rRNA. Experimental evidence indicates that structural motifs within the snoRNA species do indeed pinpoint the precise nucleotides to be methylated by the putative 2′-O-methyl transferase(s) (Maden and Hughes, 1997).

Eukaryotic cells contain about 150 different species of small nucleolar RNAs (snoRNAs). This surprisingly large number of snoRNAs can be divided into two major classes, designated the box C/D snoRNAs and the box H/ACA snoRNAs, on the basis of conserved sequence elements and conserved predicted secondary structures (Lafontaine and Tollervey, 1998; Balakin et al., 1996; Ganot et al., 1997, Samarsky et al., 1998, Bachellerie et al., 2002). The ACA snoRNAs fold into two hairpin structures connected by a single-stranded hinge region and followed by a short 3' tail. The hinge region carries an evolutionarily conserved sequence motif, called box H. The H box, probably in concert with the flanking helix structures and the ACA box characterized previously, plays an essential role in the accumulation of human U64 intronic snoRNA (Ganot et al., 1997).

A group of lncRNAs coded by the loci upstream of the transcribed rDNA is known as promoter rRNA (pRNA). These RNAs interact with the target site of the transcription factor TTF-I to produce a triplex DNA:RNA that is specifically recognized by DNA methyltransferase DNMT3b. This pathway of RNA-dependent methylation of DNA is an important component of transcription silencing (Schmitz et al., 2010). Under stress conditions lncRNAs transcribed from rDNA spacer regions can bind certain nuclear enzymes and temporarily inactivate them within the nucleoli; this phenomenon is known as “nucleolar detention” (Audas et al., 2012; Jacob et al, 2013). Proteins subject to this detention are recognized by a discrete peptide code, referred to as Nucleoar Detention Sequence (NoDS). Interrupting the NoDS/lncRNA interaction allows proteins to escape from the nucleoli and

restore their function. After dislocation of the intergenic lncRNA, the proteins may be immobilized outside the nucleolus, indicating that these types of ncRNAs can work independently of nucleolar architecture (Audas et al., 2012). Notably, some RNAs produced in the nucleoplasm are then processed in the nucleolus (Pederson, 1998).

1.2.3. Nucleolar proteins

Selected important proteins involved in transcription rDNA and early processing of rRNA:

RNA polymerase I (Pol I, pol I) produces ribosomal RNA (rRNA) (Albert et al., 2011). Transcription of ribosomal RNA by RNA polymerase (Pol) I initiates ribosome biogenesis and regulates eukaryotic cell growth (Engel et al., 2013).

Upstream binding factor (UBF) is a transcription factor for RNA polymerase I which contains several DNA binding motifs. These include a short basic region adjacent to a dimer motif plus five high-mobility-group (HMG) boxes. HMG box 1, in particular, requires association of two double helices before it will bind and, either by itself or in the context of the intact protein, will loop DNA and organize it into higher-order structures (Hu et al., 1994).

Selectivity factor 1 (SL1) is an important component of the pol I transcription machinery. This promoter-binding complex consists of TATA binding protein (TBP) and several TBP associated factors (Heix et al., 1998).

Fibrillarin (Fib, FBL) is a key small nucleolar protein in eukaryotes, which has an important role in pre-rRNA processing during ribosomal biogenesis and would play a critical role in the maintenance of nuclear shape and cellular growth (Amin et al., 2007). Fibrillarin is accumulated in DFC of the nucleoli. The methyltransferase activity of fibrillarin is the primary source of methylation for more than 100 methylated sites during the first steps of pre-rRNA processing (Rodriguez Corona et al., 2015).

Nucleolin is involved in several steps of ribosome biogenesis, including regulation of rDNA transcription, rRNA processing and structural arrangement, but it is not well understood how nucleolin modulates the transcription (Roger et al., 2002, Cong et al., 2012).

Cockayne syndrome group B protein (CSB) plays a role in both transcription-coupled DNA repair and transcriptional regulation of all three classes of nuclear RNA polymerases. The active rRNA genes bind a complex composed of CSB, pol I and histone methyltransferase G9a. G9a methylates histone H3 on lysine 9 (H3K9me2) in pre-rRNA coding regions and facilitates the association of heterochromatin protein 1g (HP1g) with rDNA (Yuan et al., 2007).

Nopp140 is pre-rRNA transcription-processing linking factor and is believed to shuttle between nucleolus and cytoplasm. Nopp140 and pre-rRNA processing factors such as DKC1 and fibrillarin (FBL) associate with r-chromatin during interphase; in mitosis Nopp140, DKC1, and FBL are released from r-chromatin (Ueshima et al., 2014).

C-Myc is transcription factor and protooncogen. C-Myc plays a crucial role in cell proliferation and apoptosis (Li and Hann, 2013).

Transcription termination factor 1 (TTF-1) binds to Sal boxes at the 3'ends of the ribosomal genes and causes termination of the rDNA transcription (Nemeth et al., 2008).

CCCTC-binding factor (CTCF) is a multifunctional factor involved in many cellular processes, such as gene regulation, chromatin isolation and genomic organization. CTCF through epigenetic mechanisms contributes to the regulation of ribosomal gene expression (Hernandez-Hernandez et al., 2012).

1.3. Transcription of ribosomal genes

1.3.1. General characterization

The activities of pol I and pol III dominate cellular transcription, combining to exceed 80% of total RNA synthesis in growing cells (Paule and White, 2000). Different types of cells of the same organism exhibit different numbers of transcriptional units reflecting their differential metabolic activity (Haaf et al., 1991). The number of the active repeats may affect the overall level of rRNA synthesis. (Birch and Zomerdijk, 2008). But nucleolar transcriptional activity does not always correlate with the total number of rRNA genes (Haaf et al., 1991).

Assembly of the RNA polymerase (Pol I) pre-initiation complex (PIC) at ribosomal RNA gene promoters begins with the binding of UBF to the upstream control element (UCE) and core element (CE) of the rDNA promoter, leading to the recruitment of the selectivity factor SL-1. SL1 and other pol I specific factors guide Pol I to the rDNA promotor and contribute to multiple rounds of transcription initiation, promoter escape, elongation and termination. In addition, many accessory factors are now known to assist at each stage of this transcription cycle, some of which allow the integration of transcriptional activity with metabolic demands (Goodfellow and Zomerdijk, 2013). In the course of the transcriptional elongation ribosomal genes with attached nascent transcripts appear on the preparation of spread chromatin as so-called "Christmas trees" (Miller and Beatty, 1969). Transcription elongation plays perhaps a key role in regulating the intensity of rRNA gene transcription (Moss et al., 2006). It is not clear how the various components of the "Christmas trees" are accommodated within the nucleoli *in situ.* But it is believed that the promoter and terminator of

each active rRNA gene are held together spatially throughout the cell cycle, forming a stable core around which the transcribed region is organized (Denissov et al., 2011). Stereological analysis of the clustered transcription signal corresponding to the "Christmas trees" sampled on the surface of thin EM sections suggests that these Christmas trees may be contorted in space and exhibit a DNA compaction ratio on the order of 4-5.5 (Koberna et al., 2002). The interaction of transcription factors with the target genes is highly dynamic. Whether the dynamic nature of these interactions is only the intrinsic property of transcription factors or plays a regulatory role is unknown. Transcriptional upregulation is accompanied by prolonged retention of RNA pol I components in the promoter, resulting in longer promoter dwell time and an increase in the steady-state population of assembling polymerase. As a consequence, polymerase assembly efficiency and, ultimately, the rate of entry into elongation are elevated. (Gorski et al., 2008). Termination of the rDNA transcription has specific features. The termination of mouse rDNA transcription requires the element AGGTCGACCAGATNTCCG (Sal1 box) which is repeated eight times at the 3 'end of the rRNA segment. This sequential motif interacts with a specific nuclear protein that controls the termination of transcription (Grummt et al., 1986; Kuhn et al., 1988). Two out of the five human Sal box elements are functionally inactive due to natural point mutations which damage factor binding. A similar sequence motif with a 10 of 11 base identity with the downstream terminators is located upstream of the human transcription initiation site. The upstream element interacts with the same factor(s) as the downstream terminators and is also capable to stop elongating human RNA polymerase I (Pfleiderer et al., 1990).

1.3.2. Kinetics of rDNA transcription

Recent studies based on new single-cell and single-gene techniques indicate that genes transcribed by pol II are often, perhaps even for the most part, expressed discontinuously, i.e. in pulses alternated by the periods of inactivity (reviewed in Smirnov et al., 2018). It is not clear yet, whether the same applies to the ribosomal genes as well. The kinetics of RNA polymerase I, which transcribes ribosomal genes, is particularly difficult to reveal, since one cell typically contains multiple copies of these genes, and their expression is usually very intensive. But direct measurements of rRNA (ribosomal RNA) production in the entire nucleoli by the label-free confocal Raman microspectrometry showed a pulse-like pattern of the rDNA (ribosomal DNA) transcription. In our work (Hornacek et al., 2017) on tumour-derived cells expressing a GFP-RPA43 (a subunit of pol I) fusion protein, we measured the fluorescence signal upon the nucleolar beads, which are likely to represent individual transcriptionally active genes (Haaf

and Schmid, 1991; Haaf and Ward, 1996; Cheutin et al., 2002; Denissov et al., 2011). Our data, complemented with the measurements of the incorporated fluorouridine signal, suggested that the ribosomal genes are also transcribed in pulse-like manner (Hornacek et al., 2017).

1.4. Replication in the nucleoli

Chromosomal DNA replication in eukaryotes initiates at many sites on each chromosome during the S phase of the cell cycle. Each origin of replication lies in a unique chromosomal environment and can be regulated in different cell types both at the level of utilization and the time of initiation during S phase (Diffley and Stillman, 1990). Replication of genomic material is a process that requires not only high fidelity in the duplication of DNA sequences but also inheritance of the chromatin states (Santoro and De Lucia, 2005).

Replication of the 400 copies of the 43 kb human ribosomal rDNA locus spans most of the S phase (Scott et al., 1997). Expressed rDNA genes are believed to replicate during the first half of S phase. Paradoxically, attempts to visualize replicating rDNA during early S phase have failed. In human (HeLa) cells, early replicating rDNA, which predominantly represents active rDNA units, is well detectable only at the nucleolar periphery. It was suggested that replication of these units takes place on the periphery or even outside the nucleoli (Dimitrova, 2011).

In mammalian rDNA genes replication may start at various points of the transcribed and non-transcribed units (Lebofsky and Bensimon, 2005). But there seem to be two preferable regions of initiation of DNA synthesis. The first one is located upstream of the transcription units and the second one is located at the 3′-end of the coding regions of the ribosomal DNA repeats (Gencheva et al., 1996; Little et al., 1993; Yoon et al., 1995). But because of low efficiency of the RFB some replication forks move in upstream direction (Lebofsky and Bensimon, 2005). Replication of the ribosomal genes is spatially separated from their transcription, and FC/DFC units of the nucleoli may provide a structural basis for that separation (Pliss et al., 2005; Smirnov et al., 2014).

2. THE AIMS OF THE WORK

- To follow cell cycle related changes of FC/DFC units in human sarcoma derived cell lines with stable expression of RFP-PCNA (the sliding clamp protein) and GFP-RPA43 (a subunit of RNA polymerase I, pol I) or GFP-fibrillarin.
- *In vivo* observations of nucleoli at early S phase of cell cycle, when transcriptionally active ribosomal genes are replicated.
- To correlate signals of polymerase I and fibrillarin (processing factor protein) in individual FC/DFC units using correlative light and electron microscopy analysis (CLEM).
- To follow changes of the fluorescent signals in individual FC/DFC units.
- To search types of kinetics of transcription signal intensity and intensity of early processing inside nucleolus.
- To discuss the characteristics of ribosomal DNA: the structure of the rDNA locus, complex organization and functions of the intergenic spacer, multiplicity of gene copies in one cell, selective silencing of genes and whole gene clusters, relation to components of nucleolar ultrastructure, specific problems associated with replication.
- To review current data on the role of non-ribosomal DNA in the organization and function of nucleoli.
- To discuss probable causes preventing efficient visualization of DNA in nucleoli.
- To discuss the occurrence of the transcriptional fluctuations, the techniques used for their detection, their putative causes, kinetic characteristics, and probable physiological significance.

3. MATERIAL AND METHODS

3.1. Cell cultures and cell lines

In our experiments we used these cell cultures: Human derived HEP2, HT-1080, Primary LEP, HeLa cells. We produced 2 cell lines stably expressing:

1.) GFP-RPA43 and RFP-PCNA (Smirnov et al, 2014),

2.) GFP-fibrillarin and RFP-PCNA (Smirnov et al, 2014).

3.2. Plasmids

The plasmid constructs for RFP-fibrillarin and RFP-PCNA were received from Max Planck Institute for Molecular Cell Biology and Genetics, Dresden. GFP-RPA43 and GFP-fibrillarin vectors were received from Laboratory of Receptor Biology and Gene Expression, Bethesda, MD. The constructs were transfected into HT-1080 cells using Fugene (Promega, #E2312) by standard procedure. G418 (GIBCO, #11811031) was used for selection of stable clones with two-colored fluorescence.

3.3. Immunocytochemistry

Primary antibodies against human rRNA polymerase (pol I), human fibrillarin and Upstream Binding Factor (UBF) were kindly provided by Dr. U. Scheer (Biocenter of the University of Wurzburg). We also used polyclonal (rabbit) antiRPA43 (Thermo Fischer Scientific, # PIPA525184). For visualization of fibrillarin in nucleoli, we used antibodies against human fibrillarin or mouse monoclonal fibrillarin (clone 17C12), kindly donated by Kenneth M. Pollard (Scripps Research Institute, La Jolla, CA). Secondary anti-human, anti-rabbit and anti-mouse antibodies were conjugated with Alexa 488 (Invitrogen, #H10120) Alexa 532 (Invitrogen, #A-11002), Cy3 (Jackson ImmunoResearch Laboratories, #109-165-088, #111-165-003, #115-025-062,), or DyLight 488 (Jackson ImmunoResearch Laboratories, #109-005-044, #111-475-003, #115-475-003). After incorporation of 5-fluorouridine (FU), the signal was visualized by a mouse monoclonal anti-BrdU antibody (Sigma-Aldrich, #B8434). Coverslips with the cells were mounted in Mowiol. One drop of mounting medium, approximately 7 µl, per one coverslip.

3.4. Incorporation of nucleotides

For labeling of replication and transcription sites, sub-confluent cells were incubated 5 min with 5-ethynyl-2-deoxyuridine (EdU) at a final concentration of 10 mM and 5-fluorouridine (FU) at a concentration of 100 mM. After incubation with EdU cells were fixed in 2% formaldehyde freshly prepared from paraformaldehyde. After that we permeabilized cells with Triton X100, and processed for FU immunocytochemistry. The replication signal was visualized using EdU Alexa Fluor 647 Imaging Kit. Additionally, we used incorporation of Cy3-dUTP and Cy5-dUTP, which were introduced into the cells by means of the scratch procedure. Each antibodies were dissolved in BSA-PBS.

3.5. Confocal microscopy and FRAP

In our experiments were confocal images of fixed samples acquired using confocal laser scanning microscope Leica SP5 equipped with oil immersion objective. For *in vivo* cell imaging we used a spinning disk confocal system based on Olympus IX81 microscope equipped with Olympus oil immersion objective with CSU-X spinning disk module from Yokogawa company and EM-CCD camera from Andor company and FRAPPA unit for FRAP experimets. The live cells were maintained in glass bottom Petri dishes (MatTek) at 37 °C and 5% CO_2 using a microscope incubator (Okolab).

3.6. Correlative light and electron microscopy (CLEM)

Cells for experiment were grown in glass bottom Petri dishes with grid (MatTek) under standard conditions in incubator. DIC images and confocal z-stacks from the regions of interest were obtained by the confocal microscope with spinning disk Yokogawa. After obtaining images in light microscopy, we fixed cells in glutaraldehyde solution (EMS, #16019) in Sörensen. After fixation, we post-fixed our samples in OsO_4 solution (EMS, #19140). Dehydration was in series of ethanol solutions. After dehydration we embedded our samples in epoxy embedding mixture (EMS, #14130). The blocks containing cells were separated from the cover slips after brief submerging in liquid nitrogen. The region of interest was localized by the a-numeric imprint on the surface of the block. Thin sections made on Leica Ultracut UCT ultramicrotome were mounted on formwar/carbon-coated nickel grids. Grids were contrasted with lead citrate and uranyl acetate. After contrasting were samples examined on the transmission electron microscope Morgagni (FEI company). Comparing the ultrastructural

images with optical sections of the z-stacks, we looked for correspondence between GFP-positive nucleolar beads and FC/DFC units which appeared distinctly on the thin sections.

3.7. Microscopic instrumentation

Electron microscope:

Morgagni (FEI, Eindhoven, Holland) accelerating voltage 100 kV, tungsten cathode, 1K CCD camera Mega View III.

Light microscopes:

Olympus AX 70 Provis (Olympus, Tokio, Japan) epifluorescence microscope, equipped with 10x, 20x and 40x dry lenses and 60x immersion objective and PlanApochromat 100x.

Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 63x PlanApochromat / 1.4 NA and 40x / 1.25 NA oil lens. Lasers: 405 nm diode laser, 488 nm argon laser, 561 nm DPSS laser and 633 nm HeNe laser. Leica incubator was used to provide a suitable cell environment.

Spinning disk microscope based on the Olympus IX 83 microscope and equipped with a 100x / 1.4NA immersion oil lens (Olympus, Tokyo, Japan), a CSU-X spinning disk unit (Yokogawa, Tokyo, Japan) and Ixon Ultra EM-CCD (Andor, Belfast, UK). Okolab incubator (Okolab, Naples, Italy) was used to provide a suitable environment for cells.

3.8. Software and data analysis

For measurement of FC/DFC units in 3D confocal images, we developed a MatLab based software. The program identifies each unit by creating a maximum intensity projection of the confocal stack and blurring the projection with a Gaussian filter, thresholding the blurred image with a value obtained by Otsu's method for automatic threshold selection. MatLab software was used for Fourier analysis of the time series. For measuring signals in the entire nucleoli we used a custom ImageJ plugin (Smirnov et al., 2014), available at https://github.com/vmodrosedem/segmentation-correlation.

4. RESULTS

4.1. Reproduction of the FC/DFC units in the nucleoli

4.1.1. Nucleolar beads correspond to FC/DFC units

At the beginning of our research, which we published under the title "Reproduction of the FC/DFC units in the nucleoli", we were interested in whether there were significant changes in FC/DFC units during a cell cycle, and if so what kind of changes. Our experiments were performed on two stable cell lines based on human sarcoma cells. The first cell line stably expressed RFP-PCNA (sliding clamp protein) and GFP-RPA43 (subunit of RNA polymerase I, pol I) fusion proteins. The second cell line stably expressed RFP-PCNA and GFP-fibrillarin (protein involved in early processing of rRNA transcripts) fusion proteins. The basic method used in our experiments was correlative light-electron microscopy (CLEM). Based on the CLEM observation results, we have been able to confirm that the FC/DFC units of mammalian cells fully correspond to signals of fibrillarin and pol I proteins.

4.1.2. FC/DFC units may lose polymerase I signal but not fibrillarin signal in early S phase

Moreover, *in vivo* observations revealed that during early S phase when transcriptionally active ribosomal genes are replicated, the amount of individual FC/DFC units in each cell increases by 60-80%. Another interesting finding was that during this early S phase, the FC/DFC units temporarily lost signal for polymerase I but did not lose signal for fibrillarin. From middle S phase to the end of interphase, the number of units did not change and their duplication was completed only after the cell division in daughter cells halfway through the G1 phase. Our findings indicate that the reproduction of FC/DFC unit is two-step. From these findings, we come to a conclusion that a significant portion of the ribosomal genes remain transcriptionally inactive (silent) from the mid-S phase to mitosis but again become active in postmitotic daughter cells. FC/DFC units thus play an important role in cellular metabolism by regulating rRNA transcription and early transcription processing. The FC/DFC units undergo fundamental changes during the two interphase intervals: early G1 phase and early S phase. At the beginning of the G1 phase, when the nucleoli is remixed after mitosis, NORs gradually unfold into nucleolar necklaces formed by nucleolar beads. Each NOR produces one necklace. This process is stopped in the mid-G1 phase. Then, the number of beads or

FC/DFC units will not change until the end of the mentioned G1 phase. Another significant changes occur in the early S phase when transcriptionally active ribosomal genes are replicated. The FC/DFC units occasionally lose the pol I signal, but not the fibrillarin signal. We conclude that the disappearance of pol I, which is not observed in other parts of S phase, is directly related to the replication of ribosomal genes. Based on previous research of our scientific group, we believe that transcription is suppressed in FC/DFC units involved in rDNA replication, which provide efficient separation of replication and transcription mechanisms in nucleoli. The results of our experiments confirm this hypothesis. Interesting finding is the fact that fibrillarin, which is associated with processed rRNA but not with rDNA (Dragon et al., 2002), remains in FC/DFC units throughout the S phase. The absence of pol I in FC/DFC units is temporary and lasts relatively short. During this absence, the total number of FC/DFC units with a signal for pol I and fibrillarin is increased. This increase can be observed directly or by counting FC/DFC units before and after the start of the S phase.

4.1.3. Regular changes, duplication and mobility of FC/DFC units

In this work, we assume that all DNA, including ribosomal genes, is duplicated between G1 and G2 phases. However, *in vivo* results of cell cycle observation showed that during S phase the number of FC/DFC units increases by about 70%, not by assumed 100%. Duplication of units was completed only after mitosis, after another sensible grow in the number of units at the beginning of the next G1 phase. Finally, each daughter cell in the mid-G1 phase gained as many units as the parent cell had at the same phase. The results obtained from multiple cell lines, such as HeLa and LEP, are congruent. Currently, it is assumed that FC/DFC units are individual transcriptionally active replications of rDNA (Haaf and Schmid, 1991; Haaf and Ward, 1996; Denissov et al., 2011). The results of our experiments show that approximately 30% of the ribosomal genes remain active from the mid-S phase to the end of G2 phase and re-activated only in the cells of next generation at the start of the G1 phase. The results obtained indicate that this mechanism is responsible for regulating the rDNA transcription intensity and, ultimately, ribosome production. The experiments results show that neither the signal strength of pol I in FC/DFC units nor their average size, measured from electron microscopic images, changed after replication of active rDNA. The transcriptional activity of FC/DFC unit remains at a stable level over most interphase. Silencing of ribosomal genes after replication in mid-to-late S phase is very effective, because there is not increase in the number of pol I positive units and can not be prevented by 5-aza-2'-desoxicytidine (AzdC),

Trichostatin A (TSA) or Roscovitine ((R)-Roscovitine). The permanently inactive ribosomal genes normally found in mammalian cells responded similarly (Santoro, 2005; Santoro, 2014; Conconi et al., 1989). However, the distribution of transcriptional signal intensity showed no tendency to asymmetry either before or after early S phase. This indicates that the original number of active genes per FC/DFC unit (presumably only one gene) will resume shortly after replication. Alternatively, duplication of active genes may be complete but not always followed by duplication of FC/DFC units. In this case, some units (approximately 30% of the entire set in the studied cells) would contain more than one active gene by the end of interphase.

The results of our study also indicate that the restoration of rDNA activity after replication is not always symmetric, which means that one of daughter helixes may be silent. This hypothesis complements older results from our workplace of asymmetric NORs that regularly appear on r-chromosomes and cause mitotic asymmetry (Kalmarova et al., 2008). Reproduction of FC/DFC units in nucleoli follows a special pattern that includes incomplete duplication during early S phase. Our data suggest that a large subset of the ribosomal genes remains transcriptionally silent from late S phase to mitosis but is re-activated in postmitotic daughter cells.

4.2. Fluctuations of pol I and fibrillarin contents of the nucleoli

4.2.1. Quick fluctuations of polymerase I and fibrillarin signal intensities in the FC/DFC units

Based on our previous experimental results, we decided to continue our research by studying the kinetics of the pol I and fibrillarin proteins during the cell cycle. Our findings are described in detail in the attached paper titled "Fluctuations of pol I and fibrillarin contents of the nucleoli". In the light of the latest research in the field of cell biology it starts to believe that many (perhaps even all) genes in the cell are not transcribed continuously, but in pulses of the transcriptional activity separated by periods of inactivity. We assume that pulse-like expression can also be found in ribosomal genes. However, given that the cell transcribes multiple copies of ribosomal genes at the same time, it is difficult to study their individual expression using standard methods, and by using these methods to determine whether it is a continuous or discontinuous process.

In our experiments, we worked with HT1080, HEP2 and HeLa human cell lines that had been transfected with plasmids containing the genes for GFP-RPA43 and RFP-fibrillarin fusion proteins. We also worked with another two cell lines derived from HT1080 cells. The first stably expressed GFP-RPA43 and RFP-PCNA fusion proteins and the second cell line stably expressed GFP-fibrillarin and RFP-PCNA fusion proteins. Both pol I and fibrillarin signals (which look like dots or beads at microscopic observations) as shown in our previous work, correspond to FC/DFC units. Thus, it allowed us to indirectly examine the state of the individual ribosomal genes. Our first observations have shown that some FC/DFC units, probably units currently involved in replication, temporarily lose signal for pol I but not for fibrillarin. However, this is a rare phenomenon that probably reflects transcription pause in units that are involved in replication. In following experiments, we measured the intensity of pol I and fibrillarin signals in individual FC/DFC units in one and two minute intervals. We have found that their intensities fluctuate in an antiphase manner. Pearson correlation coefficient (R_{Δ}) for the intensity of individual signals was variable but always negative, sometimes up to 98%. The pol I signal intensity in neighboring FC/DFC units belonging to the same NOR does not correlate, indicating that these fluctuations can not be attributed to measurement artefact or technical errors. The mismatch of peaks of polymerase I signals intensity in several neighboring FC/DFC units that belonging to same NOR indicates that fluctuations are not due to local changes in pol I concentration. The fact that neighboring FC/DFC units (belonging to the same nucleus and probably to the same chromosomes) are not synchronized is interesting. This means that they are not the result of changes in the surrounding environment. When analyzing signal intensity variations in FC/DFC units, we did not detect any interaction between neighboring units. Previously, we found that the units are in constant motion, so the distance between neighbouring units is changing considerably. The graphs below show clearly that the signal in one unit is not affected by changing the distance to the nearest neighbouring units or by changing the signal strength of these neighbouring units. By using spectral analysis of time series, we identified two main peaks that correspond to second and third minute of the periodogram. However, entire ribosomal gene is not transcribed during this time, and therefore the periods are not directly correlated with transcription.

4.2.2. Slower fluctuations of polymerase I and fibrillarin signal intensities in the nucleoli

By measuring the intensity of signals every 5 minutes, we found that signal fluctuations for pol I and fibrillarin in the same unit do not correlate. Conversely, the pol I signals in different FC/DFC units, even in units belonging to different nucleoli, are synchronized. The same situation after data normalization can be seen. On the other hand, in longer periods there is no full synchronization between the signals for pol I and fibrillarin in the same unit. The periodogram has in this case three phases corresponding to fluctuations with periods of 10, 20 and 60 minutes with only slight variations. Similarly, in this case, the main source of fluctuation could not be a technical error because the intensity of the fibrillarin signal in different FC/DFC units was not synchronized. It seems that our work is related to the recent studies of discontinuous, bursting or pulsating expression of various genes in nucleoplasm (Golding et al., 2005; Chubb et al., 2006; Raj et al., 2006; Suter et al., 2011b).

4.2.3. Exchange rates of polymerase I and fibrillarin are related to their fluctuations in FC/DFC units

It is generally known that the exchange of nuclear proteins is very intense in the cell nucleus. We were interested in the recovery time of pol I and fibrillarin signals after photobleaching one of FC/DFC unit. We used a 2-channel FRAP method with 405 nm and 560 nm wavelengths. From the FRAP results for both proteins, we can conclude that the half-lives ($T_{1/2}$) for polymerase I and fibrillarin are about 20 seconds long and therefore are much shorter than one fluctuation period and also show a tendency to negative correlation. In another experiment we used the FRAP method with prolonged pre-bleach period. The experiment was set up so that at the time of bleaching, the intensity of the pol I signal was increasing and intensity of fibrillarine signal was decreasing, or vice versa. After restoring the signal intensity, in the first case, the signal recovery for pol I was faster than for fibrillarin and in the second case it was inside out. Recovery times are thus modulated by fluctuation of polymerase I and fibrillarin contents in FC/DFC units. The observed changes are implicated in the regular exchange of both proteins in units.

4.2.4. Correlation of polymerase I and fibrillarin signals with transcription signal in the nucleoli

All previous experiments were performed on proteins. In order to link them directly to transcription, we studied co-localization of the incorporated FU (fluorouridine) with polymerase I or fibrillarin signal. The correlation was positive throughout the nucleolus which is not surprising at all, since nuclear transcription takes place in units where both proteins are accumulated. However, if the observation is limited to FC/DFC units alone, the correlation values for fibrillarin are rather low. Co-localization of polymerase I and fibrillarin with incorporated fluorouridine showed that fluctuations in the levels of polymerase I correspond to the fluctuation of transcription intensity. Based on the findings above, we were interested in what happens if we stop transcription with Actinomycin D (ActD). During the first hour of incubation with ActD, signal intensity for pol I continued to fluctuate and even show synchronization between the FC/DFC units. However, signal fluctuations for fibrillarin were suppressed. We conclude that the fluctuation of polymerase I does not depend on elongation, whereas the fluctuation of fibrillarin does and therefore is probably associated with the processing.

5. DISCUSSION

In the introductory part we have shown that FC/DFC units of nucleoli play key role in cell metabolism being centers of ribosomal transcription and early rRNA processing. In the first experimental work included in the present thesis (entitled „Reproduction of the FC/DFC units in nucleoli"), we focused on the dynamics of these units in the course of the cell cycle. Using Correlative light and electron microscopy (CLEM) and specially produced cell lines, we showed that population of the units expressing both pol I and fibrillarin undergoes specific changes during 2 periods of the cell cycle interphase: early G1 and early S.

At early G1 phase, when nucleoli are re-assembled after mitosis, Nucleolar organizer regions (NORs) unfold into nucleolar necklaces with gradually multiplying beads so that each NOR produces one necklace. This process is concluded by mid G1. Then, the number of the beads, or FC/DFC units, does not change until the end of G1. Further significant changes take place during early S phase, when transcriptionally active ribosomal genes are replicated (Berger et al., 1997; Li et al., 2004). FC/DFC units occasionally lost their pol I signal, but not their fibrillarin signal. This lead us to think that such disappearance of pol I, not observed at other stages, is directly connected to replication of the ribosomal genes. According to our earlier hypothesis (Smirnov et al., 2014), transcription is suspended in the FC/DFC units involved in rDNA replicating, which provides a basis for efficient separation of replication and transcription machineries in nucleoli. Our present data agree with this hypothesis. Remarkably, fibrillarin, which is associated with processed rRNA, but not with rDNA (Dragon et al., 2002), is retained by the units throughout the S phase. The loss of pol I must be of a short duration, for at the same period we observe an increase in the total number of pol I and fibrillarin positive FC/DFC units. This increase may be detected directly or by counting the units before and after early S phase. During the rest of S, as well as G2 and most of G1 phase, the number of FC/DFC units do not change.

In this work we assume that all DNA, including the ribosomal genes, is duplicated between G1 and G2 phases. But when we followed progress of individual cells through the cell cycle, we found that in the course of S phase the number of FC/DFC units increased only by about 70%; the duplication was never completed until the end of interphase. But after mitosis, an additional multiplication of the units took place, so that each daughter cell by mid G1 acquired as many units as the maternal cell had at the same stage. Data obtained on HeLa

and diploid LEP cells after synchronization agree with these findings. Since there are good reasons to believe that FC/DFC units correspond, more or less precisely, to individual transcriptionally active repeats of rDNA (Haaf et al., 1991; Haaf and Ward, 1996; Denissov et al., 2011), our data suggest that about 30% of active ribosomal genes remain silent from the middle S till the end of G2 phase, and get re-activated in the cells of next generation, at early G1 phase. This may be important for maintaining optimal level of rDNA transcription. Production of ribosomes is regulated at different levels; it seems that one of the mechanisms prevents excessive production of the rDNA transcription centers after replication. Interestingly, neither intensity of pol I signal on the units, nor their average size measured on electron microscopic preparations, did change after replication of the active rDNA. This suggests that transcription activity per unit remains at a stable level for the most part of interphase. The hypothetical silencing of some ribosomal genes after replication appears to be so efficient, it could not be prevented by 5-aza-2'-desoxicytidine (AzdC) and Trichostatin A (TSA), nor reversed by roscovitine. Likewise, the permanently inactive ribosomal genes, which are usually present in mammalian cells (Santoro, 2005; Santoro, 2014; Conconi et al., 1989), did not respond to the treatment. Alternatively, duplication of the active genes may be complete, but not always followed by duplication of the FC/DFC units. In that case, some of the units (cca 30% of the entire pool in the studied cells), would accommodate more than one active gene until the end of interphase. But distribution of the signal intensity showed no tendency to asymmetry, neither before nor after early S phase. This suggests that an original number (probably just one) of active genes per unit is restored shortly after replication.

Our findings also indicate that restoration of rDNA activity after replication is not always symmetrical, i.e., one of the daughter helices may become silent. This hypothesis complements our data on asymmetrical NORs which regularly appear on certain r-chromosomes and cause a mitotic asymmetry (Kalmarova et al., 2008). Thus reproduction of FC/DFC units in nucleoli follows a peculiar pattern, which includes an incomplete duplication during early S phase. Our data suggest that a considerable subset of ribosomal genes remain transcriptionally silent from late S phase to mitosis, but become again active in the postmitotic daughter cells.

Our second study (entitled „Fluctuations of pol I and fibrillarin contents of the nucleoli“) was focused on the short-term dynamics of the FC/DFC units in the nucleoli of human derived cells. We followed in real time the fluorescent signals of RPA43 subunit of

pol I and fibrillarin with intervals from 1 min to 1h. Since intensity of such signals positively correlates with the actual number of the fusion protein molecules within the region of interest (Golding et al., 2005), our data refer to the changing contents of nucleoli. Measuring the signal intensities of GFP-RPA43 and RFP-fibrillarin in the FC/DFC units, we observed specific fluctuations of two kinds. Firstly, in the short term experiments, when images were taken every 1–2 min, the pol I and fibrillarin signals fluctuated in anti-phase manner, and the intensities of pol I in the neighboring FC/DFC units belonging to the same NOR did not correlate, which shows that these rapid fluctuations cannot be attributed to artifacts or technical errors. Secondly, in the longer observations with intervals of 5 min, correlation of pol I and fibrillarin signals measured in the same unit became uncertain; but we found well defined synchrony of pol I signals in the units belonging to different nucleoli. Repeatability of fluctuations was monitored on a large number of measurements. Here again technical error could not be the chief source of the fluctuation, since the intensities of fibrillarin signal in different FC/DFC units were not synchronized.

Results of this work seems to be related to the recent studies of discontinuous, bursting, or pulsing expression of various genes in the nucleoplasm (Golding et al., 2005; Chubb et al., 2006; Raj et al., 2006; Suter et al., 2011a). Various aspects of this phenomenon are discussed in our review (Smirnov et al, 2018) In the above mentioned studies, irregular pulses of activity, alternated with pulses of silence, were visualized in living cells by inserting into genome, downstream of the promoter, sequences encoding stem loops of RNA, which could be detected with fluorescent bacteriophage coat proteins, such as MS2 or PP7 (Chubb et al., 2006; Bertrand et al., 1998). Alternatively, transcription output has been monitored by single molecule RNA fluorescence *in situ* hybridization (smFISH) (Femino et al., 1998; Mueller et al., 2013). Our work provides an approach to the study of irregular activity of ribosomal genes. Indeed, since we found high correlation of pol I and incorporated FU signals within FC/DFC units, which probably correspond to individual transcriptionally active units of rDNA (Haaf et al., 1991; Haaf and Ward, 1996; Denissov et al., 2011), the observed fluctuations of RPA43 signal follow pulsing activity of ribosomal genes. Remarkably, distribution of pol I and FU signal intensities measured in FC/DFC units showed considerable positive skewness and this agrees with the models of pulse-like, rather than continuous, activity of the promoters (Bahar Halpern et al., 2015).

Different kinetics of the transcription output is peculiar to a specific sort of gene or current status of the cell. Our study indicates that each ribosomal gene may be engaged simultaneously in at least two kinds of fluctuations. In the first kind, which is characterized by periods about 2–3 min, the pol I signals, as well as the corresponding transcription rates, in the neighboring FC/DFC units fluctuated independently. Such behavior, supposedly caused by inherent stochasticity of the transcription, was termed "intrinsic" (Elowitz et al., 2002; Elgart et al., 2011; Gillespie, 1976; Paulsson, 2005; Shahrezaei, 2008; Sherman and Cohen, 2014; Swain, 2002). But the second kind of fluctuations, in which the units belonging to different nucleoli showed a pronounced synchrony, apparently depends on variations in the state of cell nucleus, especially in the levels of pol I, its co-enzymes, and RNA nucleotides. Such kind of pulsation may be related to the "extrinsic" (Elowitz et al., 2002; Elgart et al., 2011; Swain et al., 2002). Our data about the synchronous fluctuations of pol I signal seem to agree with the results of direct measurements of rDNA transcription in the entire nucleoli by label-free confocal Raman microspectrometry (Pliss et al., 2015). Notably, in this work, as well as in ours, the extended periods of silence known from the studies of other genes (Chubb et al., 2006) were not detected. Perhaps such periods just cannot be discovered without monitoring transcription of individual rDNA units. But it is also likely that production of rRNA molecules pauses for no longer than 1–2 min. Several models were devised in effort to understand the nature of transcription bursting discovered in cell populations. Some authors analyzing the results of smFISH (Femino et al., 1998; Mueller et al., 2013; Zenklusen et al., 2008), postulate one state of promoter, whose activity is well characterized by a Poisson distribution. In other models (Raj and van Oudenaarden, 2008; Singer et al., 2014; Sherman et al., 2015), the promoter switches stochastically between an active state, in which mRNA is produced with constant probability, and an inactive state, in which transcription does not occur. The extra state increases the potential variability of output from different cells in population or tissue. A still more complicated model of the bursting gene expression is suggested in recent study of actin gene in myxamoeba *Dictyostelium* (Corrigan et al., 2016). The authors interpreted their data as produced by a wide „spectrum" of activity states with variable rates of initiation. Such kind of model may agree with the complex kinetics of pol I signal intensity in FC/DFC units observed in our work, although in the case of rDNA the multiple genes of similar structure are situated in the same cell nucleus, where some aspects of their activity may be synchronized.

Since the observed fluctuations did not depend on rRNA elongation, and their amplitudes were relatively low, we suppose that the fluctuating components of transcription machinery are accumulated around the promoters, and the observed variations of the gene output depend on a complex periodicity in the assembly of pre-initiation complex. Moreover, the observed correlation between the exchange rate of pol I and the phase of fluctuation in individual FC/DFC units indicates that the rDNA promoter may exist in multiple states with different probabilities of initiation. Our *in vivo* measurements of pol I and fibrillarin signals in FC/DFC units of mammalian cells revealed two kinds of fluctuations, short-term (with periods of about 2-3 min) and long-term (with period up to 60 min); the second kind apparently corresponds to the fluctuations of rDNA transcription and early rRNA processing. Thus our results indicate that the synthetic activity of pol I is discontinuous, and that it follows a complex kinetic pattern.

6. CONCLUSIONS

The studies presented in our published works were designed to contribute to the understanding of the complex nucleolar dynamics. Firstly, we examined the changes of the FC/DFC units in the course of the cell cycle, and we suggested a scheme of their reproduction. Secondly, we found fluctuations of the key enzymes involved in the rDNA expression within the FC/DFC units. Some of these fluctuations, with periods ranging from 10 min to 1 h, probably reflect discontinuity of rDNA transcription and early steps of rRNA processing.

I.) Our study of reproduction of the FC/DFC units showed the following findings:

- FC/DFC units of mammalian nucleoli correspond to the pol I positive foci observed in confocal images of nucleoli.
- Total number of the units in the cell (both pol I and fibrillarin positive) increases by 60-80% in the course of early S phase but does not change from mid S to the end of interphase.
- Duplication of the units is not completed until the end of the interphase. The daughter postmitotic cells replenish the deficit of the units during early G1.
- During early S phase, some of the FC/DFC units lose pol I, but fibrillarin is retained.
- TSA, AzdC, and roscovitine treatment during the middle to late S phase did not increase the number of pol I positive units

Our data suggest that a subset of ribosomal genes remain transcriptionally silent from late S phase to mitosis.

II.) The results of our study of polymerase I kinetics and fibrillarin in FC/DFC nuclear units showed two types of signal intensity fluctuations: rapid and slow

1.) Rapid fluctuations

- The periods are 2 to 3 minutes long.
- Signals of polymerase I and fibrillarin fluctuate therein in an antiphase fashion.
- Signals of polymerase I in the neighboring FC/DFC units are not synchronized.
- Rapid fluctuations correlate with the exchange times of both proteins in FC/DFC units.

2.) Slow fluctuations

- The periods are cca 10, 20 and 60 minutes.
- Polymerase I signal in units belonging to different nucleoli are synchronized.
- The intensity of the polymerase I and fibrillarin signals measured in the same unit do not correlate.
- The intensity of the polymerase I signals strongly correlates with the transcription intensity within the FC/DFC units.

Our data suggest that transcription of ribosomal genes is discontinuous.

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8. LIST OF PUBLICATIONS

Dissertation is based on the following publications (*in extenso*):

Hornáček M., Kováčik L., Mazel T., Cmarko D., Bártová E., Raška I. and Smirnov E. (2017). „Fluctuations of pol I and fibrillarin contents of the nucleoli.“ Nucleus 8(4):421-432. doi: 10.1080/19491034.2017.1306160. IF:3,148.

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Other author's publications (*in extenso*):

Bártová E., Legartová S., Krejčí J., Řezníčková P., Kovaříková A., Suchánková J., Fedr R., Smirnov E., **Hornáček M.** and Raška I. (2018). „Depletion of A-type lamins and Lap2α reduces 53BP1 accumulation at UV-induced DNA lesions and Lap2α protein is responsible for compactness of irradiated chromatin.“ Journal of Cellular Biochemistry *In press*. doi: 10.1002/jcb.26770. IF:3,085.

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