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REVIEW

Nucleolar DNA: the host and the guests

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Abstract Nucleoli are formed on the basis of ribosomal genes coding for RNAs of ribosomal particles, but also include a great variety of other DNA regions. In this article, we 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. We also review current data on the role of non-ribosomal DNA in the organization and function of nucleoli. Finally, we discuss probable causes preventing efficient visualization of DNA in nucleoli.

Keywords Nucleolus · rDNA · Transcription activity · Replication · NADs · DNA staining

Nucleoli have been known since the works of Wagner (1835) and Valentin (1836) as the most conspicuous components of cell nucleus. A century later, it was found that these nuclear bodies are assembled around certain chromosomal loci, termed afterward "nucleolus organizer regions (NORs)" (Heitz 1931; McClintock 1934). Subsequent findings indicated that structure and the main function of nucleoli are based upon transcription of ribosomal genes. In recent studies, multiple other genomic regions have been found within and closely adjacent to the nucleoli. This review is focused on peculiarities of ribosomal DNA and on the role of non-ribosomal DNA sequences in organization and function of nucleoli.

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Ribosomal DNA (rDNA)

rDNA locus

Ribosomal DNA is responsible for production of ribosomal RNAs. Thus, in mammalian cells, there are three kinds of coding regions which produce 18S RNA of the small ribosomal subunit, as well as 28S and 5.8S RNAs of the large ribosomal subunit (Gonzalez and Sylvester 1995). Accordingly, each transcription unit includes three genes, separated by internal transcribed spacers, ITS1 and ITS2, and flanked by external spacers, 5′ ETS and 3′ ETS (Fig. 1). These spacers vary significantly in composition and size in different species (reviewed in Nazar 2004).

The transcription units of rDNA locus are separated from each other by non-transcribed, or intergenic, spacers, NTS or IGS (Fig. 1). In yeast and infusoria, the spacers are rather uniform (Philippsen et al. 1978; Wild and Gall 1979). But generally, structure and length of the IGS vary to a large extent not only from species to species, but also within the species, and even within a single individual (Lewin 1980; Wellauer and Dawid 1977; Reeder et al. 1976). The satellite contents of the DNA are particularly variable (Gonzalez et al. 1992a; Gonzalez and Sylvester 1995, 2001; Maden et al. 1987; Sasaki et al. 1987). Most frequently, the small repetitive arrays get increased or reduced in number as a result of slipped-strand mispairing and other errors associated with replication (Tautz et al. 1986; Levinson and Gutman 1987). Human IGS includes various kinds of DNA repeats, both tandemly and non-tandemly arranged; they include simple sequence motifs,

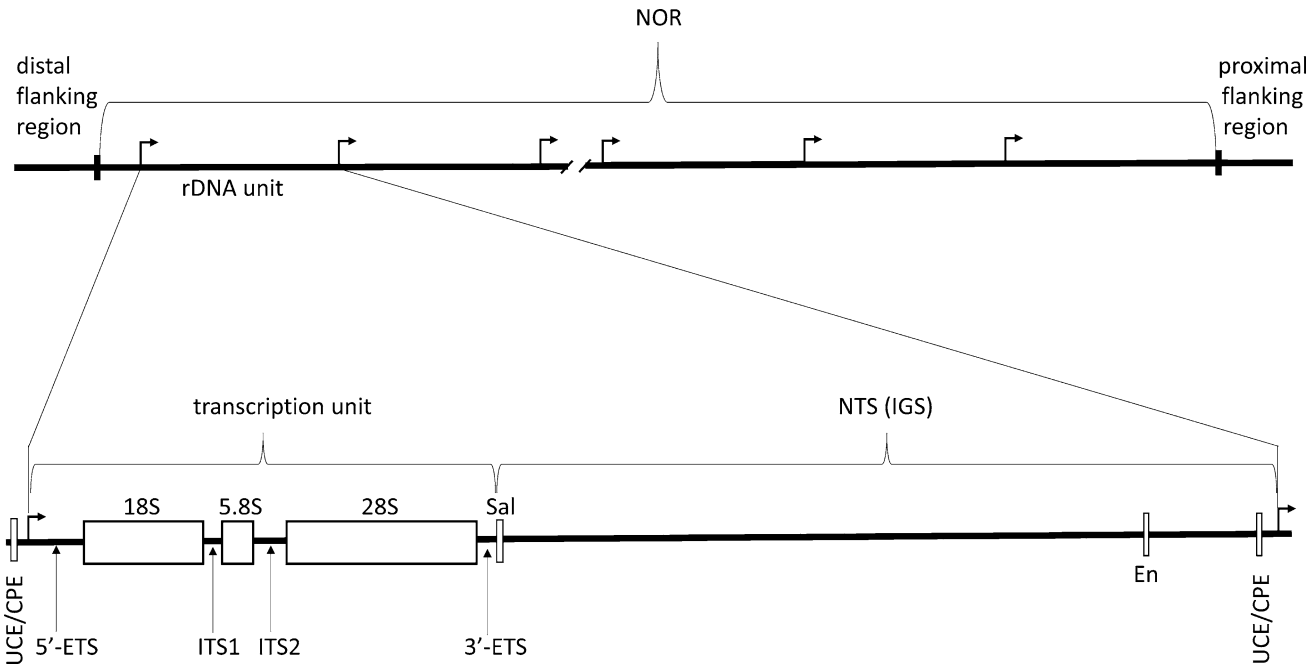

Fig. 1 Organization of rDNA in mammalian cell. *NOR* nucleolus organizer region, the cluster of rDNA loci, *NTS (IGS)* non-transcribed (intergenic) spacer, *5′-ETS, 3′-ETS* external transcribed spacers, *ITS1, 2* internal transcribed spacers, *UCE/CPE* promoter including upstream control element (UCE) and core promoter element (CPE), *Sal* Sal box, the sequence serving as transcription terminator, *En* enhancer

microsatellites (2–6 bp), long repeats (cca 2 kb), as well as transposable elements. Among the latter, *Alu*, belonging to the short interspersed elements (SINE), are predominant (Tautz et al. 1986; Gonzalez et al. 1989; Gonzalez and Sylvester 1995). Some of rDNA repeats also contain nucleotide substitutions, supplementary microsatellite clusters, and, more seldom, extended deletions (Ryskov et al. 1993; Braga et al. 1995; Kupriyanova et al. 2015).

It has long been known that the rDNA spacers include regulatory sequences. Thus, the promoter of each ribosomal gene is partly or entirely situated in IGS, upstream of the start codon. Typically, it consists of two parts: core domain and upstream control domain (Haltiner et al. 1986; Clos et al. 1986; Paule 1994; Reeder 1992; Doelling and Pikaard 1995). Another essential part of the spacer is the terminator. It may be absent in rDNA of *Xenopus* and *Drosophila*, and in these cases, the transcription is ceased by a kind of processing (Labhart and Reeder 1986; McStay and Reeder 1986; Tautz and Dover 1986; De Winter and Moss 1986). In mammalian cells, transcription of rDNA is terminated at the 3′ end of each gene, at a sequence motif called "Sal box" with the length of 18 bp in mouse and 11 bp in human (Grummt et al. 1985; Kuhn et al. 1988). Sal box binds the TTF-1 (transcription termination factor 1) protein, which is essential for arresting RNA polymerase I (pol I) (Grummt et al. 1985, 1986; La Volpe et al. 1985; Bartsch et al. 1987; Pfleiderer et al. 1990; Diermeier et al. 2013; reviewed in Németh et al. 2013). But it seems that one such site is not sufficient, for each human or murine rDNA unit is provided with 10 terminators, $T_{1–10}$ (reviewed in Diermeier et al. 2013).

Other sites regulating the expression of rDNA have been found in the non-transcribed spacers. A usual component of IGS is the enhancer; enhancers appear as clusters of repeating sequences distanced from the regulated region (Pikaard et al. 1990; Moss et al. 1985). In mammalian cells, there are also one or more reduced transcription units situated ~2 kb upstream of the core promoter. Such units include spacer promoter, spacer terminator, and a minigene producing transcripts of ~150 bp, termed promoter-associated RNAs (pRNAs), which are involved in rDNA silencing (Sylvester et al. 2003; Mayer et al. 2006, 2008; Moss et al. 2007; McStay and Grummt 2008; Santoro et al. 2010; Anosova et al. 2015).

Until recently, extensive IGS regions of mammalian cells had been regarded as receptacles of useless, "junk" sequences. But this idea will probably follow the fate of the general "junk DNA" theory. Remarkably, RNA-seq analysis of human and murine rDNA revealed a specific pattern of low-abundance expression over the entire IGS region (Zentner et al. 2011, 2014; reviewed by Jacob et al. 2012), indicating that the spacer has a complex functional organization. The same idea is suggested by the data of Chip and Chip-seq analysis which show a regular distribution of pol

I and its transcription factors throughout the intergenic region (Copenhaver et al. 1994; Hu et al. 1994; O'Sullivan et al. 2002; Zentner et al. 2011, 2014). A special role belongs to the IGS in the recent theory of "nucleolar detention," according to which nucleoli may serve as a kind of lumber room for useless proteins and other cell components (Audas et al. 2012a). The process is triggered when RNAs produced by loci situated within the IGS recognize and "capture" proteins furnished with a "detention signal" (Audas et al. 2012a, b; Jacob et al. 2012; Diermeier et al. 2013; Padeken and Heun 2014; reviewed in Lam and Trinkle-Mulcahy 2015).

Repetitive arrays

The presence of numerous ribosomal gene copies in one cell is one of the most remarkable characteristics of rDNA. There are exceptions to this rule. For instance, some species of *Protozoa* and *Myxomycetes* have only one chromosomal gene which is amplified into a set of extrachromosomal inverted repeats in the course of somatic growth (Lewin 1980). But generally, metazoan genomes contain several hundred ribosomal gene copies (Birnstiel et al. 1971); in plants, this number often reaches several thousands (Rogers and Bendich 1987).

Usually, rDNA is arranged in clusters of tandem repeats, nucleolus organizer regions (NORs). These regions were first discovered as secondary constrictions of mitotic chromosomes (Heitz 1931; McClintock 1934). But later, it was found that some of the NORs make no secondary constrictions; such clusters are transcriptionally silent and may appear both within and without nucleoli (Sullivan et al. 2001; Strohner et al. 2001; Kalmárová et al. 2007).

In certain cases, rDNA is organized as inverted repeats (Bergold et al. 1983). In amphibia (Birnstiel et al. 1971; Bird 1978), insects (Birnstiel et al. 1971), and fungi (Butler and Metzenberg 1993), ribosomal genes are amplified into numerous extrachromosomal copies (reviewed in Moss and Stefanovsky 1995). Human diploid genome contains about 400–600 copies of a 43-kbp unit (Moss et al. 2006; Stults et al. 2009). Human NORs with an average size of 3 Mbp are situated on the short arms of the acrocentric chromosomes 13, 14, 15, 21, and 22 (Henderson et al. 1972; Long and Dawid 1980; Puvion-Dutilleul et al. 1991).

The abundance of ribosomal gene repeats not only enables the cell to regulate the production of ribosomal RNA more efficiently, but also increases the frequency of recombination. The number of repeats varies as a result of unequal homologous exchange, and this may cause damage to the cell (La Volpe et al. 1984; Mroczka et al. 1984; Erickson and Schmickel 1985; Sylvester et al. 1986, 1989; Cassidy et al. 1986; Dumenco and Wejksnora 1986; Tower et al. 1989; Stults et al. 2009). The partial silencing of rDNA seems to be an important factor in maintaining stability of the loci (Peng and Karpen 2007). Gene conversion is regarded as an additional stabilizing process, since it reduces the variability (Gonzalez and Sylvester 1995; Elder and Turner 1995). Tandem arrangement of rDNA increases the risk of inappropriate transcription; therefore, isolation of each repeat from its neighbors on the DNA strand seems necessary. Such demarcating function is ascribed to insulators (Valenzuela and Kamakaka 2006). Association of CTCF protein with human and murine rDNA at the spacer promoter region suggests the presence of an insulator element here (Torrano et al. 2006; van de Nobelen et al. 2010; Zentner et al. 2011). Remarkably, CTCF depletion leads to disorganization of nucleolar structure and overexpression of ribosomal genes (Hernández-Hernández et al. 2012).

Studies of restriction products show that the repeats within NORs vary in length and structure (Kominami et al. 1981; Gonzalez et al. 1985, 1990; Maden et al. 1987; Sasaki et al. 1987). Variants with tissue-specific expression were found among murine rDNA repeats (Tseng et al. 2008). In situ hybridization on the preparation of isolated DNA fibers, "molecular combing" (Bensimon et al. 1994; Michalet et al. 1997; Anglana et al. 2003; Caburet et al. 2005; Tseng et al. 2008) revealed high percentage of non-canonical, including palindromic, sequences (about one-third of the repeats), and great variability in the length of IGS (from 10 to 50 kb) in several types of human cells (Lebofsky and Bensimon 2005; Caburet et al. 2005). It seems that the variability in the length of NORs provides each person with a unique rDNA electrophoretic karyotype, a kind of "fingerprints" (Stults et al. 2008).

The palindromic structures may cause fork stalling and/or arrest by forming hairpin structures during lagging-strand synthesis, which apparently results in significant slowing down of rDNA replication. Thus, in HeLa cells, the average speed of replication fork for the whole genome is 1.7 μm/min, but only 1 μm/min for rDNA (Lebofsky and Bensimon 2005). If the palindromes are pseudogenes (Caburet et al. 2005), they must be non-functional by definition (Mighell et al. 2000). But it is still unknown whether the length and composition of IGS have any impact on transcription of the adjacent genes.

The multiplicity and high sequence similarity of rDNA repeats greatly hinder their study. For that reason, NORs were excluded from the initial sequencing and analysis of the human genome.

Active and silent rDNA

It is typical for the clustered rDNA that its transcriptionally active genes are interspersed by transcriptionally silent repeats (Conconi et al. 1989; Santoro 2005, 2014; Zillner et al. 2015). The active genes are characterized by

hypomethylation of CpG sites and histone modifications generally associated with transcriptionally active nucleoplasmic chromatin (i.e., H3K4me3 and H3K9ac), whereas transcriptionally silent rDNA is condensed, hypermethylated, and marked with repressive histone modifications (i.e., H3K27me3 and H4K20me3) (Heintzman et al. 2007; McKeown and Shaw 2009; Zentner et al. 2011; Zillner et al. 2013; reviewed in McStay and Grummt 2008; Shaw and McKeown 2011). Key role in the silencing scheme belongs to the nucleolar remodeling complex (NoRC) (Strohner et al. 2001). Targeting NoRC to rDNA leads to repositioning of a promoter-bound nucleosome, changes in histone modifications, increase in DNA methylation, and silencing of rRNA genes (Zhou and Grummt 2005; Li et al. 2006; Mayer et al. 2006, 2008; Schmitz et al. 2010; Anosova et al. 2015). On the other hand, nucleosome remodeling and deacetylation complex (NuRD) creates chromatin state in which rDNA is poised for transcription, though not yet transcribed (Xie et al. 2012).

It has been established that the active ribosomal genes form loops in which a promoter is joined to the terminator. Transcription termination factor 1 (TTF-1) and protooncogene c-Myc seem to be particularly important for this connection (Németh and Längst 2008; Pontvianne et al. 2013; Li and Hann 2013). Both proteins regulate the association of epigenetically activated rDNA genes with the nucleolar matrix (Shiue et al. 2014). TTF-1 binds to an upstream site, termed T_0, located 170 bp upstream of the transcription start site (Clos et al. 1986). This is required for efficient transcription initiation and for the recruitment of chromatin remodeling complexes that establish distinct epigenetic states of rRNA genes. Interaction of TTF-1 with CSB (Cockayne Syndrome protein B), NoRC, or NuRD leads to the establishment of active, silent, or poised state of chromatin, respectively (Strohner et al. 2001; Santoro et al. 2002; Yuan et al. 2007; Xie et al. 2012; Diermeier et al. 2013).

In steadily cycling cells, chromatin structure of ribosomal genes is maintained through multiple rounds of cell division (e.g., Li et al. 2006, reviewed in Birch and Zomerdijk 2008; Santoro and De Lucia 2005; Guetg et al. 2012). From prophase to late anaphase, the gene activity is efficiently blocked by cdc2/cyclin B-directed phosphorylation of SL-1 and other transcription factors (Heix et al. 1998; Voit et al. 2015). Nevertheless, the components of pol I transcription machinery, including the upstream binding factor (UBF) and promoter selectivity complex (SL1), can be detected on certain NORs even in metaphase (Babu and Verma 1985; Moss et al. 1985; Weisenberger and Scheer 1995; Jordan et al. 1996; Roussel and Hernandez-Verdun 1994; Roussel et al. 1996; Gebrane-Younes et al. 1997; Sirri et al. 1999, 2008; O'Sullivan et al. 2002; Leung et al. 2004; Prieto and McStay 2005). Such NORs, termed "transcriptionally competent" or just "competent" (Dousset et al. 2000; Savino et al. 2001), are transcribed, while the other, "non-competent" NORs remain silent during interphase (Weisenberger and Scheer 1995; Roussel et al. 1996; Gebrane-Younes et al. 1997). The competence, which can be revealed by UBF or silver nitrate staining, is regularly distributed among the different chromosomes (Héliot et al. 2000; Smirnov et al. 2006). After S phase, some NORs may become "asymmetrical," when only one of the daughter chromatids acquires the competence signal. The presence of such NORs causes mitotic asymmetry (Kalmárová et al. 2008).

Organization of rDNA in the nucleolus

Structure of nucleoli is based upon transcriptionally active rDNA (Henderson et al. 1972; Long and Dawid 1980; Puvion-Dutilleul et al. 1991; Raska 2003; Raska et al. 2006a, b; Cmarko et al. 2008; Sirri et al. 2008). Crude versions of that structure appear on ectopical loci in the form of "pseudo-NORs" or "neo-NORs" produced experimentally on the basis of simple UBF binding arrays (Mais et al. 2005; Prieto and McStay 2007; Grob et al. 2014).

It has been known that nucleoli are usually formed at the end of mitosis around competent NORs, which gradually unfold and fuse into a few bodies. But the organization of rDNA in the interphase is still not understood. On the one hand, hypotonically isolated and spread ribosomal genes appear as so-called Christmas trees, in which the "tree stem" represents a single DNA fibril, from which the transcripts grow like branches (Miller and Beatty 1969; Trendelenburg et al. 1974; Scheer and Zentgraf 1982; Trendelenburg and Puvion-Dutilleul 1987; Mougey et al. 1993; Scheer et al. 1997; Albert et al. 2011). On the other hand, electron microscopical studies show that transcription of rDNA and the first steps of rRNA processing take place in the FC/DFC units, i.e., fibrillar centers (FC) surrounded by dense fibrillar components (DFC) (Fig. 2). The transcribed part of rDNA as well as the transcription signal after pulse labeling has been observed in the DFC or at the border between DFC and FC (Raška et al. 1983a, b, 1995; Ochs et al. 1985; Raska et al. 1989, 2006a, b; Scheer and Benavente 1990; Hozák et al. 1993, Cmarko et al. 2000; Melcák et al. 1996; Koberna et al. 2002; Casafont et al. 2006; Shaw and McKeown 2011). But it proved to be very difficult to find out how the elements of "Christmas trees" are accommodated among the elements of nucleolar ultrastructure.

There are reasons to believe that each FC/DFC unit typically accommodates one transcriptionally active rDNA repeat (Haaf et al. 1991; Haaf and Ward 1996; Denissov et al. 2011), which forms multiple coils passing through DFC and adjacent FC area (Reeder and Lang 1997; Cheutin et al. 2002; Puvion-Dutilleul et al. 1991; Derenzini et al.

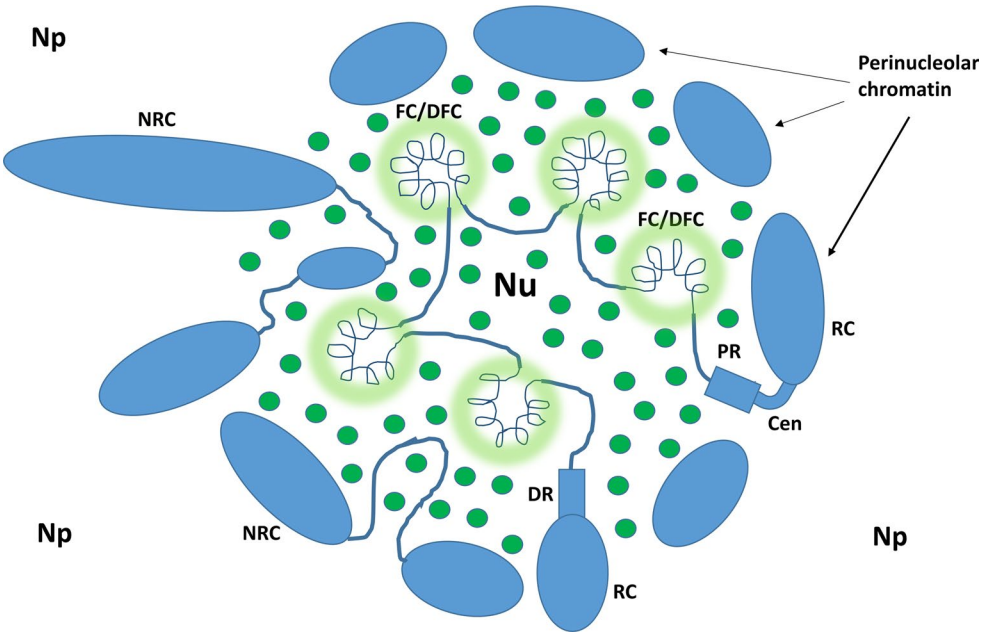

Fig. 2 A schematic representation of nucleolus-associated DNA. *Nu* nucleolus, *Np* nucleoplasm, *RC* chromosome carrying ribosomal genes (ribosomal chromosome), *Cen* centromere, *PR* proximal flanking region, *DR* distal flanking region, *NRC* non-ribosomal chromosome, *FC/DFC* FC/DFC unit—the center of rDNA transcription consisting of fibrillar center (FC) surrounded with dense fibrillar component (DFC). *Green dots* represent granular component of the nucleolus

2006; McStay and Grummt 2008). Pictures of osmium amine staining show the presence of some DNA in the FC, but the status and composition of this DNA still have not been determined (Derenzini et al. 2006, 2014). Further studies are needed to establish the position of inactive rDNA repeats, as well as the "poised genes" (reviewed in Németh and Längst 2011). The latter, together with silent genes, may be localized in the fibrillar center but, upon activation, move toward the DFC (Raska et al. 2006b).

According to the data of chromatin capture analysis, the promoter of each active gene is joined to the respective terminator and may be close topographically to several loci of the gene (Grewal et al. 2005; Arabi et al. 2005; Gomez-Roman et al. 2006; Grandori et al. 2005; Németh et al. 2008; Németh and Längst 2008; Shiue et al. 2009, 2014; Denissov et al. 2011; Lykke-Andersen et al. 2011; Xie et al. 2012; Diermeier et al. 2013; reviewed in Németh and Längst 2011). Based on such data, and considering increased binding of promoter selectivity complex SL1 over the entire region, Denissov et al. (2011) proposed a "core-helix model." According to it, a single ribosomal gene occupying the FC/DFC unit assumes the form of rotating cylindrical solenoid. The transcribing pol I complexes driven by actin revolve around the SL1-containing core, which is situated in the FC and serves as an anchor for both the promoter and the terminator of the rDNA repeat; the nascent rRNAs exit radially into the DFC. Remarkably, this chiefly speculative model seems to be the only hypothesis describing organization of rDNA in the interphase nucleoli. So far, it is not even known whether replication of ribosomal genes occurs within FC/DFC units or in other nucleolar structures.

Reproduction of rDNA

Replication of rDNA should be viewed in connection with two circumstances: the great number of the gene copies in the cell and the ongoing transcription, which may even intensify during S phase (Gorski et al. 2008). Accordingly, each cycling cell must have means to avoid two significant dangers. On the one hand, the multiple tandemly repeated rDNA arrays may be recombination hotspots and thus present a potential source of genomic instability (Stults et al. 2009; Ide et al. 2010). This risk is probably diminished by alternation of silenced and active repeats in each array (Santoro 2005, 2014).

On the other hand, collision of the swiftly running replication and transcription machineries (reviewed in Magdalou et al. 2014) must be prevented, which requires a special spatio-temporal arrangement of replication. Thus, in yeast cells, each rDNA repeat has one potential origin of replication, and clusters of synchronously firing origins are separated by a few units with silent origins (Pasero et al. 2002). Besides, there is an efficient fork barrier situated at the 3′ end of each transcription unit; it arrests the upstream moving of the forks, which prevents collision of replication and transcription complexes (Brewer and Fangman 1988; Linskens and Huberman 1988; reviewed in Rothstein et al. 2000).

In human cells, rDNA replication may be initiated all over the IGS and even upon the genes (Lebofsky and Bensimon 2005), though the potential origins situated upstream of the transcribed region are used more frequently (Little et al. 1993; Yoon et al. 1995; Gencheva et al. 1996; Scott et al. 1997). The replication forks may terminate and

converge at variable sites throughout the rDNA repeat (Little et al. 1993). Fork barriers exist (Gerber et al. 1997; Akamatsu and Kobayashi 2015), but often fail to stop the progress of DNA polymerase complex, so that replication proceeds at far distances in both directions (Edenberg and Huberman 1975; Lebofsky and Bensimon 2005). Neighboring origins fire within 60 min of each other; the distance between them varies from tens of kilobases to a couple hundred kilobases, with an average of 80 kb (Lebofsky and Bensimon 2005). In such conditions, additional mechanisms must be engaged to protect genome stability. Separation of rRNA gene transcription and replication domains probably could make up for the relaxed timing and spacing (Pliss et al. 2005). Such separation may be achieved by regulation at the level of FC/DFC units; indeed, in vivo observations indicate that transcription is suspended in the units involved in the replication (Smirnov et al. 2014; also our new data prepared for publication).

Non-ribosomal DNA in nucleoli

Clusters of rDNA repeats, which include the transcribed and non-transcribed parts, are usually regarded as the founders of nucleoli. But this role may be shared by the regions of the same chromosomes adjacent to NORs (Gonzalez et al. 1989, 1992; Kaplan et al. 1993) (Fig. 2). In human cells, these regions have a very similar structure upon all five acrocentric chromosomes (Floutsakou et al. 2013). The proximal flanking sequences, which are positioned in the neighborhood of centromeres, consist largely of satellite DNA, frequently undergo recombination, and have numerous analogues in other parts of the genome. In contrast, the distal sequences, which are situated closer to the telomeres, exhibit low segmental duplication, but contain chromatin signature characteristic of promoters, as well as putative genes, interspersed among marks associated with heterochromatin. These sequences may regulate the activity of the NORs and participate in the structural organization of the nucleoli by anchoring rDNA to perinucleolar chromatin (Floutsakou et al. 2013).

Microscopic studies have shown that various other parts of the genome may regularly or occasionally find their way to the nucleoli. In different cell types and species, satellite DNA of centromeres is a common component of the perinucleolar shell of condensed late replicating chromatin and appears also in the interior of nucleoli (Comings 1980; Manuelidis 1984; Manuelidis and Borden 1988; Haaf and Schmid 1989, 1991; Bartholdi 1991; Billia and Deboni 1991; Ochs and Press 1992; Léger et al. 1994; Carvalho et al. 2001; Wong et al. 2007). Particularly, centromeres of chromosomes with a lower content of G-dark bands tend to be localized at the nucleolus (Carvalho et al. 2001). Telomeres, together with telomerase components (Rawlins and Shaw 1990; Vourc'h et al. 1993; Armstrong et al. 2001; Zhang et al. 2004), territories of human chromosomes 1, 9, and Y (Stahl et al. 1976; Léger et al. 1994), as well as parts of acrocentric chromosomes (Kalmárová et al. 2008; Pliss et al. 2015), are often found within or very close to nucleoli. Data of 3C analysis suggest that ribosomal genes may interact with repetitive sequences belonging to other chromosomes (O'Sullivan et al. 2009). Functional significance of this interaction is not clear.

Abundant data have been recently obtained by sequence analysis of nucleolus-associated domains (NADs), which represent the entire DNA content of isolated nucleoli (Németh et al. 2010; van Koningsbruggen et al. 2010). In the studies of human cells, it was found that NADs, not counting the ribosomal genes, constitute about 4 % of the genome and include sequences from all chromosomes. The bulk of these domains consists of AT-rich sequences, satellite repeats (mainly alpha-, beta-, GAATG/CATTC types), members of the zinc-finger, olfactory receptor defensin and immunoglobulin protein-coding gene families, transcriptionally active 5S rRNA genes, and tRNA genes (Matera et al. 1995; Thompson et al. 2003; van Koningsbruggen et al. 2010; Németh et al. 2010). Analysis of the transcriptional status and chromatin feature showed that NADs contain mainly inactive chromosomal regions (Németh and Längst 2011; van Koningsbruggen et al. 2010).

There is still an uncertainty about the composition of NADs. The often used term "nucleolar association" is somewhat ambiguous; it embraces sequences of two essentially different compartments, nucleolar interior and nucleolar periphery, since these are swept together in methods based on the isolation of nucleoli. Besides, this isolation requires breaking of NOR-bearing and perhaps some other chromosomes, which may introduce further errors. This problem has been partly solved by using in situ hybridization to confirm the data of deep sequencing (van Koningsbruggen et al. 2010).

Factors directing various DNA sequences toward the nucleolus, as well as consequences of the perinucleolar positioning, have become a focus of intensive study recently (reviewed in Padeken and Heun 2014; also reviewed in Matheson and Kaufman 2015). Localization of NADs in the perinucleolar region is correlated with heterochromatin formation and transcriptional silencing (Zhang et al. 2007; Pandey et al. 2008; Mohammad et al. 2008; Fedoriw et al. 2012; Jakociunas et al. 2013; Yang et al. 2015). Thus, the inactivating center of X chromosome (Xic) is associated with nucleoli (Zhang et al. 2007). Deletion of Xic locus reduces this association (Csankovszki et al. 2001). Remarkably, admittance to the perinucleolar region is not guaranteed by the DNA sequence, for only

inactive X chromosome is found among the NADs (Zhang et al. 2007).

The proteins involved in NAD localization often also regulate rDNA transcription and/or nucleolar structure. For example, CTCF (Yusufzai et al. 2004; van de Nobelen et al. 2010; Huang et al. 2013), NCL (Roger et al. 2003; Rickards et al. 2007; Cong et al. 2012), NPM1 (Murano et al. 2008), and Ki-67 (Rahmanzadeh et al. 2007; Booth et al. 2014) all regulate transcription of ribosomal genes. Likewise, depletion of modulo (NCL) in flies disrupts nucleolar structure as demonstrated by immunofluorescence (Padeken et al. 2013), and after depletion of Ki-67 in human cells, their nucleoli become fewer and smaller (Booth et al. 2014).

Long noncoding RNAs (lncRNAs) seem to be essential for regulation of structure and function of the perinucleolar region (Mohammad et al. 2008; Jacob et al. 2013; Padeken and Heun 2014; Matheson and Kaufman 2015). One of such lncRNAs produced by a locus situated on inactive X (Xi) chromosome was named Firre (Yang et al. 2010). Firre is required for normal perinucleolar positioning of the mouse Xi (Yang et al. 2015). This protein binds CTCF, which may regulate both the silencing of Xi and its association with nucleolus (Hacisuleyman et al. 2014; Yang et al. 2015).

By attracting various segments of chromatin, constraining their mobility, and removing them from the transcriptionally active environment, nucleoli, together with nuclear periphery, play an essential part in the dynamic organization of the genome (Berger et al. 2008; Matheson and Kaufman 2015). Clustering around nucleoli might contribute to a more stable positioning of the DNA elements (Padeken and Heun 2014). Experiments with late replication labeling (Cremer and Cremer 2001) and GFP-tagged histones (van Koningsbruggen et al. 2010) indicate that after mitosis, perinucleolar chromatin partly returns to the nucleoli and partly moves to the nuclear lamina. Thus, composition of the perinucleolar region shows a degree of stability, though it may exchange components with lamina-associated domains (LADs) (van Koningsbruggen et al. 2010; Németh and Längst 2011; Kind et al. 2013).

"Invisibility" of nucleolar DNA

It can be seen from the aforesaid that nucleoli contain different sorts of DNA in both loose and condensed states (Fig. 2). But for some reason, we do not observe in nucleoli the alternation of dense and sparse chromatin foci which is typical for nucleoplasm. Moreover, it is well known that on preparations of cells stained with DAPI or other DNA markers, nucleoli usually appear as dark holes (Fig. 3). The intranucleolar signal is extremely weak, though exceeding the background. We find a similar pattern in distribution of various histones revealed by antibodies or as GFP constructs (e.g., Müller et al. 2007). This shows a striking contrast with results of in situ hybridization staining of transcribed and non-transcribed rDNA (e.g., Junera et al. 1995) or immunostaining for such DNA-binding proteins as UBF or TTF-1. Replication signal observed after incorporation of various nucleotides also has an amazingly low intensity within nucleoli (e.g., O'Keefe et al. 1992), especially during early S phase, when transcriptionally active ribosomal genes are replicated. Dimitrova (2011) attempted to explain this phenomenon by suggesting that certain parts of rDNA may leave nucleoli, get replicated in nucleoplasm or at nucleolar periphery, and afterward return to their former positions. But study of incorporated nucleotides in vivo (Smirnov et al. 2014) revealed no significant exchange of DNA between nucleoli and nucleoplasm or nucleolar periphery.

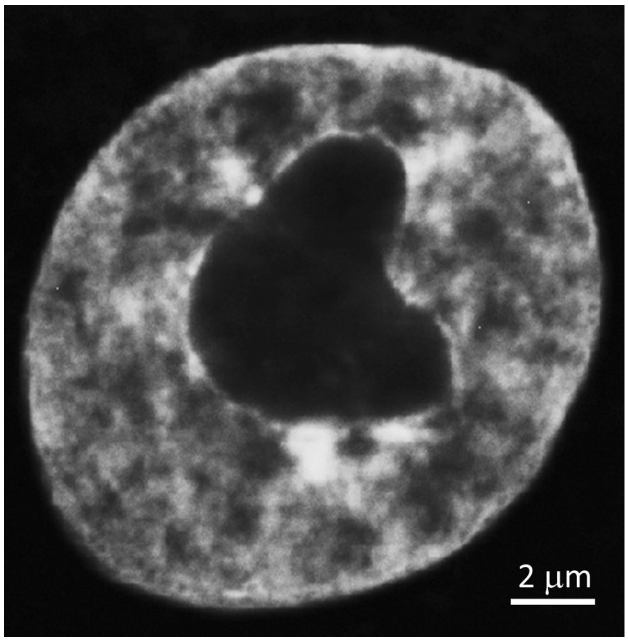

Fig. 3 DAPI staining of a HeLa cell nucleus. Confocal section. The signal is hardly detectable in the entire nucleolus and in some small areas of nucleoplasm

Even electron microscopy studies fail to clarify the matter. Thus, osmium amine reaction on ultrathin sections (Derenzini et al. 2006) reveals patches of condensed DNA in nucleoli, comparable with those in nucleoplasm. But only a pale homogenous staining appears in the area of fibrillary centers; the supposedly coiled DNA of active ribosomal genes is scarcely detected.

Bearing these data in mind, we will consider the following hypotheses:

1. Special biophysical properties of nucleoli.

Since the intranucleolar space is characterized by extremely crowded condition, all processes in it are strongly influenced by short-range entropic forces which compel macromolecules to "crystallize" into nanostructures (Hancock 2014). One consequence of this may be redistribution of electric charges and low permeability of the nucleolus for DNA staining reagents, as well as certain antibodies, GFP constructs, etc. This hypothesis appears to be the easiest to verify experimentally.

2. Peculiar structure of chromatin in the nucleolus.

The state of rDNA chromatin may be "unusual." At any rate, standard ChIP protocol proves to be rather inefficient when applied to nucleolar components (Prieto and McStay 2008). This hypothesis, as well as the previous one, suggests that DNA is present in nucleoli in sufficient quantity, but for some reason eludes detection.

3. Extremely low concentration of DNA in the nucleolus.

It would be the simplest explanation of the phenomenon: There is just too little DNA to be detected. But does this view agree with the facts? For one thing, DNA is not homogeneously distributed in the nucleolar volume, but forms foci of variable density, which may be seen on the electronograms. On the other hand, the average concentration of DNA in the nucleoli does not seem to be particularly low. As we have seen, cca 4 % of human genome belong to NADs. Although the larger part of this may be represented by DNA sequences in the perinucleolar region (Fig. 2), NORs alone constitute roughly 1 % of human genome (about 30 Mb) (Németh and Längst 2011). To this, we must add those non-ribosomal NADs which are known to be intranucleolar, e.g., Alu, Kpn elements, pentameric arrays of chromosome 15 (Kaplan et al. 1993), centromeres (Ochs and Press 1992), together with the adjacent regions. "Core nucleoli" obtained from isolated nuclei by centrifugation and extensive nuclease treatment are deprived of ribosomal genes, yet still contain about 1 % of the total nuclear DNA (Bolla et al. 1985). Thus, nucleoli will claim about 2 % of human genome. But how much is the volume occupied by this DNA? Since mammalian cells typically contain 2–3 nucleoli with average diameter of 1–3 μm (e.g., Smetana et al. 2006), and average diameter of the nucleus in such cells is about 6 μm (Alberts et al. 2002), the ratio of nucleolar volume to the volume of the nucleus lies between 4 and 40 %. These estimates indicate that mean value of DNA concentration in nucleoplasm is generally higher than in nucleoli. Nevertheless, the differences are not so great as to account for the extraordinary low intensity of DAPI or replication labeling.

Thus, none of the three examined hypotheses seems conclusive. Perhaps, their combination will provide a solution in the future. But for the present, the invisibility of DNA in the nucleolus still remains a riddle.

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