Advances in Biotechnology

Chapter 2

Ubiquitous Role of Ribosomal DNA in the Viability of Living Cells and Organisms

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Abstract

Ribosomes are among the most ancient and important substructures of the cell, retaining the organization of common features in all living organisms. The genes responsible for the synthesis of nucleic acids and proteins that form ribosomes, as well as those that serve the processes of these genes, the maturation of transcription products, and the transition of mature products to the active state, together form the largest polygenic complex, the coordinated work of which depends on the viability of individual cells and the whole body generally. In humans, tandem rDNA clusters are located on the short arms of five acrocentric chromosomes and form the so-called nucleolar organizers (NORs), specific sites of chromosomes where nucleoli form during mitotic telophase. Ribosomal DNA, by its very nature, is susceptible to recombination. Recombination events can result in a change of the repeat copy numbers or in DNA mutations. To prevent these, multiple DNA repair mechanisms are in place to retain rDNA repeat integrity. Here, we examine the literature and our own data on various aspects of

studying the structure and functions of rDNA, particularly the regulatory elements of rRNA transcription; intrachromosomal, interchromosomal, and evolutionary variability; and the characterization of extended genome regions adjacent to rDNA clusters in NOR. In addition, we present new findings on the association of nucleolar stress, cellular dysfunction, and human diseases, including cancer, cardiovascular, neurodegenerative, and autoimmune disorders; and infectious and metabolic disorders. Considerable attention is focused on rDNA in its native state, and on mechanisms providing its variability.

Keywords: Ribosome; Ribosomal DNA; Ribosomal Intergenic Spacer; Nucleolar Organizer; Ribosomal RNA Transcription and Regulation; Chromosomal Abnormalities; Nucleolar Functions and Human Diseases

1. Introduction

The growth and division of the bulk of human body cells are tightly connected. Ribosome and protein synthesis are necessary for cell growth and, in return, the control of cell growth is inevitably connected with the control of ribosome formation. rDNA is formed by two zones, one of which encodes the ribosomal RNA (rRNA) precursor (pre-rRNA), and the other the ribosomal intergenic spacer (rIGS). Today, it is clear that the rIGS is a highly complicated structure consisting of functionally specific segments that can be easily transported to different genomic regions. Although the structure and function of rDNA repeating units are rather similar in all vertebrates, the extent of certain features is specific to humans and higher primates [1, 2]. Considerable attention has recently been devoted to rDNA as a whole, and on RNAs being coded in the rIGS. It has turns out that rDNA, while being a factory producing rRNAs, also carries out many important functions, and damage to rDNA can lead to the development of different pathologies.

2. Structural and Functional Organization of Human rDNA

In the human genome, rDNA clusters are placed on acrocentric chromosomes 13, 14, 15, 21, and 22, forming so-called nucleolar organizers (NORs). Structurally, the nucleolus consists of a fibrillary center (FC) surrounded by and associated with dense fibrillar components (DFCs). This construction is embedded in a granular component (GC) composed of granules surrounding the fibrillary components. Many segments similar to rDNA can also be detected on NOR^- chromosomes [3]. The peculiarities of ribosome biogenesis and nonribosomal nucleolar functions have become much clearer over the last few years. A number of studies have indicated that the nucleolus has functional activities in ribosomal as well as nonribosomal processes, such as development, aging, cell cycle, gene stability, lifespan regulation, and progeria, to name but a few. Damage to the nucleolar structure and function (nucleolar stress) is often connected with human diseases.

In all vertebrate genomes, rDNA exists in the form of abundant discrete clusters. Tandemly arranged rDNA repeats comprise NORs—specific chromosomal regions where nucleoli form during mitotic telophase. Each rDNA unit consists of a coding region for the rRNA precursor (pre-rRNA) and ribosomal intergenic spacer (rIGS) **(Figure 1).**

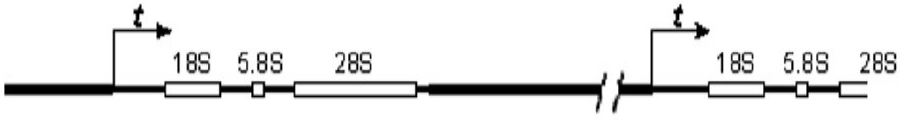

Figure 1: Ribosomal DNA repeat unit organization in mammalian genomes [3].

In **Figure 1**, the rDNA regions coding 18S, 5.8S, and 28S rRNA are represented as white rectangles. Transcribed spacers and rIGS are respectively shown as thin and thick black lines. The transcription start point is denoted as ***t***.

The coding region being formed by external transcribed spacer (5'-ETS)–18S rDNA–internal transcribed spacer1 (ITS1)–5.8S rDNA-internal transcribed spacer 2 (ITS2)–28S rDNA–(3'-ETS) is transcribed by RNA polymerase I (Pol I) in the nucleolus as an inextricably linked extensive (40S–47S) pre-rRNA. The mature rRNA molecules (28S, 18S, and 5.8S) are formed after a series of specific endo- and exonucleolytic cleavage reactions. Ribosomal RNA accounts for no less than 80% of total steady-state cellular RNA. In the course of evolution, the length of 18S and 28S rDNA genes and transcribing spacers has increased, but common principles of the structural organization of the transcribing regions has remained invariant [4,5].

In a genome, apart from rDNA clusters, there exist so-called orphans, which are discrete elements similar to rDNA which have diverged from the major rDNA clusters. Evidence in flies and mammals shows that rIGS-like and 18S-like regions form long tandem blocks outside of rDNA clusters [6]. Several rDNA-like sequences were detected on human chromosome 22 at the distal position relative to the rDNA cluster area that included 28S rDNA-like and rIGS similar segments. At the same time, there was an absence of segments homologous to rDNA at the proximal area of the human chromosome 22 rDNA cluster [7, 8].

It is universally accepted that rDNA tandem repeats at five pairs of human chromosomes homogenize as a result of recombination and gene conversion. However, switching on both of these mechanisms can lead to opposite results: the correction of new sequence variants, as well as their propagation over all individual clusters among homologous and nonhomologous chromosomes, and also during the course of interbreeding of different populations. Many scientists have tried to elucidate the question of which mechanism dominates the homogenization process, gene conversion or nonequal crossover, and what exchanges occur most often: intrachromatid, between sister chromatids, or between homologous or nonhomologous chromosomes.

Various contradictory results were obtained. Data were obtained that verify the existence of interchromosomal exchange as a result of nonequal crossover, leading to the origination of variability in the rDNA spacers on nonhomologous chromosomes [9-12] which is enhanced over generations [13]. On the other hand, there are data concerning intrachromosomal exchanges of human rDNA leading to imbalance over generations [14], familiar inheritance of spacer variants according to Mendel [15], and the presence of synteny in the inheritance of human rDNA spacer variants [16]. Today synteny is defined as the conservation of blocks of order within two sets of chromosomes that are being compared with each other.

As mentioned earlier, the transcribing rDNA regions are divided by rIGS. It turned out that rIGS length can vary in the genomes of discrete individuals, just as inside the genome [17-20]. These differences are conditioned by the different numbers of repeats in clusters entering into the rIGS that are generated by different elementary monomers: from thousands of nucleotides to 2–6 nucleotides (microsatellite clusters). Highly polymorphic markers, such as microsatellites (mcs), appear to be the major instruments for making up genetic chromosomal maps and for mapping of disease loci [21-23] **(Table 1).**

Table 1: Frequency of occurrence of seven microsatellite motifs in the ribosomal DNA (rDNA)

Motif	M_p/M	L	M	Q
TCC	0.84	21	124	60
GACA	0.78	23	113	56
GA	0.69	26	110	50
CAC	0.50	36	72	36
GACT	0.42	43	60	30
TCG	0.14	260	10	10
GATG	0.01	2600	1	1

M, total number of cosmid clones; M_p, number of clones hybridized with microsatellite (mcs) probes; L, average spacing between loci (tpn); M, number of concrete loci in rDNA region; Q, number of rDNA copies containing the motif of interest.

Polymorphism of the rDNA nucleotide sequences is defined by the presence of variable zones in the rIGS, as well as by mutations in the transcribing region of rRNA genes. The obtained results suggest relative autonomy of the evolution of nucleotide sequences belonging to the nucleolar organizer. A number of data show that the extent of representation of some functionally important repeats in the pre-promoter region can affect the expression of rRNA genes and, accordingly, the power of the protein synthesizing cell system and, consequently, the common organism status [24, 25].

The other widely distributed type of polymorphism is represented by point mutations, often occurring in the rDNA pre-promoter region. This type of polymorphism has long been

known, and was detected for the first time as a polymorphism of the restriction fragment length manifesting as the result of different restrictions on endonuclease cleavage [26–28]. The high extent of nucleotide replacement in the regions connected with rDNA transcription management changes the specificity of the transcription factors, and provides the molecular mechanisms of species formation during evolution**.**

Our sequencing study of the 5'- and 3'-ends of recombinant rDNA inserts from the cosmid library of human chromosome 13 revealed that *Sau*3A sites on DNA depleted of protein were not digested stochastically, but that some of them appeared to be hypersensitive to endonuclease attack [29]. A similar phenomenon was observed early on SV40 DNA depleted of protein, but the reason remained unclear [30].

Analysis of the sequences adjacent to hypersensitive digestion sites indicated that, as a rule, they are either incorporated into *Alu* retroposon sequences or are adjacent to them. Many regulatory elements have been identified, including the promoter and terminator sites for RNA polymerase III (Pol III) and the points often used for in vivo rDNA recombination [31–33].

In performing blot hybridization analysis of cosmid clones containing different rIGS fragments of chromosome 13, we detected two clones with huge (10 and 26 tbp) deletions in the rIGS (Figure 2) [34, 35]. The deletion mapping of recombinant inserts in cosmid clones 36G10 and 47H2 after sequencing shows that in both cases, the cut points were located in microsatellite clusters $(TC)_{n.}$, which probably represent unstable genomic loci.

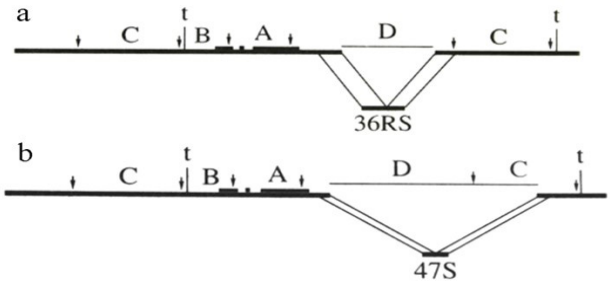

Figure 2: Graphical representation of two clones with huge deletions from the ribosomal intergenic spacer (rIGS) of human chromosome 13 [34, 35].

Comparative fluorescence in situ hybridization (FISH) of genomic DNA with native rIGS fragments and with DNA of the deleted clone 47H2 was also performed. It showed that in mild hybridization conditions, 47H2 binding was observed on all five pairs of acrocentric chromosomes, whereas when more stringent washing-off conditions were used, hybridization only occurred with chromosomes 13 and 21 [36]. This points to the presence of rDNA repeats carrying deletions in the rIGS in the human genome.

The results obtained are evidence of the frequent intrachromosomal homologous and nonhomologous exchanges between coding rRNA and regulatory and distal rDNA regions. There are a number of factors that favor such frequent exchanges between these regions

beyond the dependence of the exchange mechanism. First, the protein nucleolin, which takes an active part in transcription, pre-rRNA processing, and ribosomal maturation, can favor recombination [37, 38]. Second, hypomethylation of the coding and regulatory rDNA regions is also favorable to the activation of rDNA chain exchange [39–42]. Warmerdam et al. [2] have previously proposed that the observed HR-mediated loss of repeats after breaks in the rDNA occurs in trans, through recombination between sisters' chromatids or rDNA repeats on different chromosomes. Unlike in S/G2, homology-dependent repair in G1/G0 might be more prone to occur in cis, by using unrepaired repeats on the same tandem array **(Figure 3).**

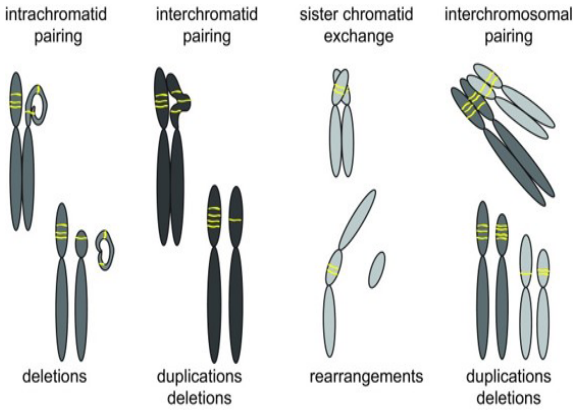

Figure 3: Interactions between chromosomes with repeats that can cause structural chromosomal rearrangements [2]. Repair of repeats can result in repeat expansions, contractions, and structural chromosomal aberrations.

It is also known that human rDNA contains a great many consensus sites for topoisomerase I (TOPO I), particularly in nucleoli on TC-enriching rIGS chains during active rRNA transcription in complexes with Pol I [43, 44]. Recombination under TOPO I participation can assist in homogenization of the regions between TOPO I sites. Also important is the factor of extensive rDNA saturation by $(CT)_n$, $(CTTT)_n$, $(TG)_n$, poly-G, and GC-enriched segments, which is typical for hotspots of recombination [45–47]. It seems reasonable to say that coding and regulatory rDNA regions are homogenized uniformly along the entire length by patching modified segments ("patch-like" manner), whereas rIGS homogenization reveals local characteristics favorable for the formation of homogenous subclusters. Subsequent homologous nonequal exchange between sister chromatids or between homologous and nonhomologous chromosomes can lead to the fixation, increase, or decrease in the number and a loss of blocks containing specific rIGS variants. Clusters in the vicinity of $(CT)_n/(AG)_n$ can assist in fragment homogenization, since it was shown that these sequences can initiate recombination and gene conversion [48].

Evolutionary conservation of the sequences surrounding rDNA clusters suggests that the NORs occupy a chromosome scope beyond proper rDNA, representing a significant capture of subtelomeric space. By cloning the X/21 translocation DNA fragment that is responsible for the development of Duchenne muscular dystrophy, the clones were obtained containing the frontier segment (DJ) between rDNA and adjacent nonribosomal DNA. Using these clones, it was found that transcription from the rDNA cluster on acrocentric chromosome occurs in the

direction of the centromere [49, 50]. The study of DJ-containing clones allowed to determine the primary structure of 8.3 kbp of nonribosomal DNA adjacent to the 5'-end of the head repeat in the rDNA cluster on chromosome 21 [51]. On human chromosome 22, a proximal connecting link between the 3'-end of the rDNA cluster and nonribosomal DNA was also estimated [52]. It enters into the ITS1 and represents a unique 68 bp sequence followed by a tandemly organized cluster formed by 147 bp monomers. This cluster is detected in all acrocentric chromosomes and participates in the formation of genomic repeates of higher order (6.4–6.8 kbp).

3. rDNA Polymorphism and Chromosomal Abnormalities

Variability in the lengths of human rDNA monomers was originally demonstrated by electron microscopy [53]. A link was detected between rRNA gene copy numbers, their transcription activity, and the survival of individuals with a variety of rDNA chromosomal abnormalities and different diseases [54–57]. A number of active ribosomal genes (AcRGs) were estimated to be the cause of chromosomal abnormalities (CAs) in conditions such as Down syndrome (DS), Robertsonian translocation (RT), Klinefelter and Turner syndrome, trisomy X, and disomy. In the control selection of individuals (n = 318), a dozen AcRGs varied at between 119 and 190 copies per diploid genome. In all CA carriers, with the exception of new DS, a dozen AcRGs were not beyond these limits [57]. It was shown earlier that cellular homeostasis and the survival of organisms are only supported when AcRG copy numbers are within these limits, whereas embryos are eliminated when their genomes are characterized by copy numbers outside these limits [56]. It was shown that bearers of structural chromosomal abnormalities can survive only with a relatively high number of AcRGs. These data suggest that the problem of rDNA variability and transcriptional activity correlation with the status of the human body, and this significant observation requires further research for verification.

4. Regulatory Elements of rRNA Transcription

Binding of the rDNA promoter by two Pol I-specific factors, upstream binding factor (UBP) and promoter selectivity factor (SL1), leads to an assembly of the specific multiprotein complex containing Pol I and a number of auxiliary proteins necessary for the initiation of rRNA transcription. UBF activates rRNA transcription by bringing Pol I to the promoter, stabilizing TIF-IB/SL1, and replacing histone H1 [5–7]. UBF can also control promoter release [58] and transcription prolongation [59]. Very often, the rate of cancer cell proliferation correlates with the UBF level [60, 61]. The rate of rRNA transcription is connected with the compatibility between the positive (ERK, mTOR, CBP, c-Myc) and negative (p. 53, Rb, PTEN, ARF, AMPK, GSK3) regulatory pathways of its synthesis [4, 5]. The amount of UBF1 binding to rDNA loci corresponds to the number of transcriptionally active repeats. About 40%–50% of the tandem rDNA repeats are transcribed in normal diploid cells, whereas the rest are present in a state of inactive heterochromatinization. Active and inactive rDNA repeats contain different

histone markers. Inactive rDNA repeats contain repressive markers associated with constitutive heterochromatin. rDNA heterochromatinization is regulated by spacer promoter from the rIGS. The first considerable review devoted to the transcription regulation of ribosomal genes and the assembly of multiprotein complex on rDNA promoter was published in 1995 by Jacob [62]. The number of newly discovered factors in the composition of this multiprotein complex increased markedly, but the essential principles of rRNA transcription regulation have remained unchanged.

Variable rDNA regions can differ according to size, and nucleotide substitutions in both different as well as within the same species. The site of transcription initiation of the 45S pre-rRNA lies within the major core promoter. There is also a spacer promoter upstream of the core promoter (about 2000 bp before), and a spacer terminator (To) adjacent to the core promoter [62–67].

As mentioned above, the joint binding of two Pol I-specific factors with the rDNA promoter, UBF, and SL1 leads to formation of the preinitiation complex [63]. Once more, a group of proteins (TAFI) provides specific binding between the rDNA promoter, UBF, and SL1 that is necessary to support the connection between the Pol I complex and a number of associated proteins (such as actin, myosin, NM1, TIF-IA, PAF53, and TOPOI, as well as IIα, Ku70/80, SIRT7, CK2, and others) [63-71]. These associated proteins can modulate rRNA synthesis in response to various cellular conditions, such as in the case of TIF-IA activity modulation under the action of mTOR. This protein can inactivate TIF-IA, decreasing Ser44 phosphorylation and enhancing Ser199 phosphorylation. The changes in TIF-IA phosphorylation influence transcription complex formation [69-71].

A deficiency of nutrition or growth factors in mammalian cells can decrease significantly (up to 80-90%) rRNA transcription. Various mechanisms are involved to reestablish normal ribosomal biogenesis. Some proteins can modify Pol I to make it incapable of specific initiation [4–13]. One of the proteins involved in this process is Rrn3 [72], which available for reversible association with Pol I. Only 10% of the Pol I molecules in a cell contain Rrn3, which makes connection between Pol I and SL1 [71,72]. Sirtuin 7 (SIRT7) is an NAD-dependent protein deacetylase that is a member of the sirtuin family comprising key mediators of cellular proliferation and oncogenic activity. It influences the rRNA syntesis by monitoring the cell cycle at the control points during diseases and exchange homeostasis, stress tolerance, aging [73,74].

If, in normal cells, rRNA transcription activity is tightly connected with nutrient availability, tumor cells become independent owing to the specific kinase activation, regardless of external signals. It was shown that some protein kinases, such as CK2, ERK, and mTOR, are hyperactivated in carcinogenesis. Pol I-associated CK2 phosphorylates some components of

the Pol I transcription complex, such as TIF-IA, UBF, SL1/TIF-IB, and TOPO I. MAPKs have been observed to activate rRNA synthesis through initiation factors TIF-IA and UBF. Since mTOR inhibition by rapamycin inactivates TIF-IA, it is reasonable that mTOR inhibitors can act as powerful tumor-suppressing substances [70,71]. Rapamycin also affects 18S and 28S rRNA processing, although it remains unknown whether the disruption of rRNA synthesis leads to cell transformation or plays a secondary role in the transformation process.

Analysis of the mouse rIGS region 2000 bp upstream from the transcription start point allowed to detect noncoding a Pol I transcript 150–200 nucleotides, denoted as promoter RNA (pRNA). This molecule binds with TIP5, the large subunit of nucleolar remodeling complex NoRC, suppressing rRNA synthesis [75-77]. Although mammalian rDNA promoters are almost devoid of homology, NoRC-dependent suppression of Pol I transcription was not found to be species-specific. On the contrary, the overexpression of mouse TIP5 suppressed Pol I transcription in mice, as well as in human cells, which was unexpected when considering that NoRC activity is dependent on binding between TIP5 and pRNA complementary to the rDNA promoter [75].

Figure 4 shows a comparison of the promoter and pre-promoter regions of several mammalian ribosomal genes.

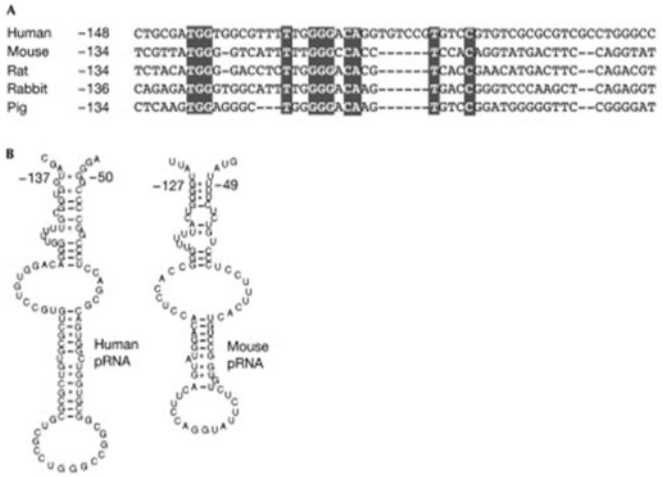

Figure 4: (**A**) Alignment of promoter region of various mammalian ribosomal genes. A black background indicates conserved nucleotides. Numbers indicate the positions of nucleotides relative to the transcription start point. The alignment was performed using CLUSTAL W (1.74). (**B**) Secondary structures of human (left) and mouse (right) promoter RNAs (pRNAs) predicted using RNAalifold [75].

5. rDNA Modification and rRNA Transcription

There is a link between the methylation status of the rDNA promoter and rRNA transcriptional activity, which was experimentally verified in various systems [78–82]. What is surprising is that in cells with amplified rDNA, rRNA synthesis activity was not increased, but is elevated upon rDNA demethylation under 5-aza-C action [82]. It was shown that the CpG methylation status in rat rIGS 145 nucleotides upstream of the transcription start point (tsp) can serve as an indicator of gene activity, while CpG methylation in the −133 position in

mouse rIGS impedes it's binding with UBF. It is interesting that in human rIGS, CpG is also present precisely in the positions −145 and −135 [62]. In recent years, age alterations of the ribosomal gene number and the characteristics of their methylation were studied in a number of systems. The age reduction of rDNA quantity in brain and heart tissues was demonstrated using Southern hybridization [83, 84]. The rate of rRNA transcription activity was found to decrease in human fibroblasts in connection with a person's age [85]. This was determined by the calculating the number of Ag-stained NORs. Scientists have shown that cells taken from patients with Werner syndrome grew more slowly and perished, completing only a few divisions, unlike the control [86]. The increased methylation level of rDNA in the cell cultures of patients was considerably higher than in the culture of control cells [87–91]. Thus, rDNA-specific methylation patterns can be used as a marker of a disease or its progression.

The protein coding gene C9ORF72 contains many intronic sequences enriched with microsatellites that are able to expand significantly. The expansion of a GGGGCC repeat in the intronic sequence from 2–22 to 700–1600 copies in transcripts of this gene is associated with 9p-linked amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (PMID: 21944778, 21944779). Hexanucleotide expansion resulting in the selective stabilization of repeat-containing pre-mRNA and the accumulation of insoluble dipeptide repeat protein aggregates are most probably pathogenic in FTD/ALS patients [92, 93]. The results obtained suggest that two C9ORF72 gene translation products—GRN from the sense transcript and PRN from the antisense transcript—act to alter information from DNA to mRNA, to protein-poisoning of pre-mRNA splicing, and in the biogenesis of ribosomal RNA. These translation products penetrate cells, bind nucleoli, and kill cells. Exposure of cultured cells to the GR20 and PR20 translation products of C9ORF72 affects both pre-mRNA splicing and the biogenesis of ribosomal RNA. Computational analysis of RNA-seq data also revealed changes in the abundance of a subset of cellular RNAs under the action of the PR20 peptide. Nine PCR primer pairs were designed to study the synthesis and processing of rRNA in detail [92, 93]. Three primer pairs were used to monitor the levels of mature 18S, 5.8S, and 28S rRNAs, and the other six primer pairs were designed to monitor the 45S rRNA precursor. In the cells treated with PR20 peptide, slight reductions in 28S rRNA were observed. Surprisingly, the level of 5.8S rRNA was reduced by 70% under these conditions. These data indicate that peptide treatment of cells presents a severe hazard to ribosomal RNA maturation. Similar results were obtained with brain tissue derived from patients carrying repeat expansions in the C9ORF72 gene. Nucleolar disorders have also been revealed, including delayed processing of the 45S ribosomal RNA precursor. This might mean that these GR20 and PR20 peptides of the hexanucleotide repeats from different disease variants of the C9ORF72 gene behave similarly to cytotoxins that hinder pre-mRNA splicing and the biogenesis of ribosomal RNA [92–94].

6. Disruption of rRNA Biogenesis and Some Human Disorders

We will now discuss the progress made in the latest studies of initiation, mechanisms, and the development of different diseases, and elucidate the participation of ribosomal DNA in these processes.

6.1. Treacher Collins syndrome

Treacher Collins syndrome (TCS) is an autosomal dominant disorder of craniofacial development with a number of features. TCS is caused by mutations in the gene, which encodes the nuclear phosphoprotein treacle. It was detected that a 5 bp deletion in exon 22 of the TCOF1 gene (3469del ACTCT) gives rise to premature stop codon formation [94–96]. Many craniofacial disorders are determined by heterozygous mutations in the regulators of ribosome biogenesis. Genetic perturbations caused by heterozygous mutations in components of the Pol I transcriptional machinery or its cofactor TCOF1 lead to relocalization of nucleolar RNA helicase (DDX21) from the nucleolus to the nucleoplasm, inhibition of rRNA processing, and downregulation of ribosomal protein gene transcription, as well as its loss from chromatin targets. At the molecular level, it was demonstrated that impaired rRNA synthesis occurs as a consequence of the DNA damage response. This means that rDNA damage results in tissue-selective and dose-dependent effects on craniofacial development. The TCOF1 gene product is also involved in pre-rRNA methylation. Defects in craniofacial development result from mutations in the TCOF1 gene. Nucleolar phosphoprotein treacle encoding by the TCOF1 gene interacts with upstream binding factor (UBF) and affects the transcription of rDNA [96]. Treacle participates in 2′-*O*-methylation of pre-rRNA. A comparison of rRNA isolated from wild-type mouse embryos and from strains with a lethal phenotype showed significant reduction in 2′-*O*-methylation at nucleotide C463 of 18S rRNA. The function of treacle in pre-rRNA methylation is most likely mediated by its immediate interaction with NOP56, a component of the ribonucleoprotein methylation complex. Although treacle co-localizes with UBF throughout mitosis, it also co-localizes with NOP56 and fibrillarin, a putative methyltransferase, only during telophase when rDNA gene transcription and pre-rRNA methylation are known to interact.

6.2. Hodgkin’s lymphoma

Hodgkin’s lymphoma (HL) is a type of lymphoma in which the cancer originates from lymphocytes. In the last few years, a greater understanding of the peculiarities of different Hodgkin’s lymphomas has come to light [97–99]**.** The morphofunctional characteristics of lymphoma node cells from patients with Hodgkin’s disease were obtained by measuring silver-stained nucleolar organizer regions (AgNORs). According to the Rye histological classification of Hodgkin’s disease, 3 cases were lymphocyte predominant (LP), the most favorable type, 14 were nodular sclerosing (NS) guarded type, and 15 were mixed cellularity (MC) type [99].

The nucleolar activity of Hodgkin and Reed–Sternberg (HRS) cells was greater in the NS type than in the MC type. The authors explain that the highest expression of interphase rRNA cistrons was found in NS HRS cells by their high proliferative activity and elevated production of transforming growth factor 1, the most effective cytokine present in the NS subtype [97].

6.3. Bloom syndrome

Bloom syndrome [BS] results from mutations in the BLM gene that lead to mutated DNA helicase protein formation [100−102]. The downregulation of cytidine deaminase against the background of genome instability is usually a marker of Bloom syndrome. Synthetic lethal interaction between cytidine deaminase (CDA) and microtubule-associated Tau protein was detected with the use of a genome-wide RNAi screen and transcriptomic profiling [102]. In Tau-deficient HeLa cells, high levels of H2A histone family member X (γ-H2AX) and a large number of induced 53BP1 foci were observed in all cells. Tau downregulation decreases upstream binding factor recruitment and ribosomal RNA synthesis, and affects ribosomal DNA stability. The accompanying depletion of both Tau and CDA proteins intensifies genetic instability and is lethal. This can cause an early cellular response in the induction of double-stranded breaks. Thus, the mechanisms underlying synthetic lethality involve impaired DNA repair and replication processes, and Tau may be regarded as a multifunctional protein whose precise role depends on its localization. As nucleolar regions contain rRNA genes, an important role for Tau in rRNA synthesis was proposed. It was shown that a decrease in Tau expression decreased the level of 45S pre-RNA by attracting UBTF to the rDNA repeats. This allows for the conclusion that Tau binds throughout rDNA loci, with a preference for the promoter and transcriptionally active regions. The high GC content of rDNA loci might make them prone to the formation of G-quadruplex structures, causing arrest or a slowdown in rDNA replication [100]. The mechanisms regulating localization of BLM to the nucleolus are currently under investigation [101,102].

6.4. Senescence

In humans, rDNA instability is caused by a high level of genomic recombination and rearrangements that are involved in premature aging disorders such as Werner and Bloom syndromes [103]. SIRT7 is a member of the mammalian sirtuin family of proteins, acting as a multifunctional chromatin regulator via deacetylation of histone H3K18ac [104]. It can be activated by double-steanded DNA breaks. Nucleolar SIRT7 is implicated in the regulation of rDNA transcription [104−106]. SIRT7-dependent heterochromatin silencing protects against cellular senescence [103].

Transcription of rDNA loci is essential for cell viability in mammals [105], and SIRT7 activates this process. In a new study, Paredes et al. [107] show that SIRT7 can have an opposite effect under the action of SNF2H, a member of the ATP-dependent chromatin remodeling

complex SWI/SNF. This interaction leads to specific silencing of rDNA promoter regions. Cells lost about 50% of their rDNA copies and depletion of SIRT7 led to increased rDNA damage and overall disruption of nucleolar integrity, and more rapid senescence. Subsequent studies have shown that another mammalian sirtuin, SIRT6, also interacts with SNF2H at sites of double-stranded breaks (DSBs), promoting DNA repair [108]. SIRT7 acts on H3K18 and SIRT6 acts on H3K56, influencing their localization. This demonstrates a critical role for SIRT7 in complex with SNF2H in maintaining heterochromatic regions to protect rDNA loci from the rearrangements that are connected with aging and related pathologies. These findings identify rDNA instability as a driver of mammalian cell senescence and implicate SIRT7-dependent heterochromatin silencing in protecting against this process [108,109].

6.5. Cancer

The transcription of rRNA genes and maturation of rRNA are important in the intricate processes of cell growth and proliferation control. It has become clear that changes in rDNA activity can be an important cause of tumorigenesis. This means that cancerous tumors may be attacked using strategies aimed at specific targets. The use of anticancer medicines at the stage of Pol I transcription may become necessary in the immediate future. One increasingly important strategy involves the development of new substances that selectively suppress Pol I transcription in rapidly proliferating cells without injuring healthy tissues.

Recent studies have highlighted the role of ribosomal pathologies in a variety of animal models for diseases associated with cell growth and cell cycle control, and many other diseases. It is now clear that the disruption of ribosome biogenesis causes nucleolar stress that triggers the p53 signaling pathway. Two alternatives have been studied over ca. 20 years regarding the relationship between the priority of cancer transformation and nucleolar function. It is known that protein synthesis consistently increases during cellular neoplastic transformation [62,110–114]; transformed cells produce oncogenic proteins and tumor-suppressor proteins (P53, C-MYC, etc.) [69–71]; and polymerase I transcription of rRNA genes is negatively regulated by tumor oncogenes and suppressor genes [72, 73]. Recently, a number of studies were dedicated to the nucleolar functions in tumors. As long as the majority of cancer cells display a large size and/or increased numbers of nucleoli, nucleolar size can be used for many cancers as a parameter for predicting the prognosis of a tumor, with increasing size corresponding to worse prognosis [62]. rDNA copy number fluctuations and their instability has been shown to result from a disruption in H3.3 deposition and, thus, a failure in heterochromatin formation at rDNA repeats as a result of the absence of ATRX (ATP-dependent helicase ATRX, X-linked helicase II). ATRX-depleted cells have a reduced output of rRNA transcription. Such cells display increased sensitivity to the RNA polymerase I (Pol I) transcription inhibitor CX5461 [112–114]. In addition, human alternative lengthening of telomeres (ALT)-positive cancer cell lines are also more sensitive to CX5461 activity [115-119].

6.6. Neurodegenerative diseases

Several studies performed in the past two decades have indicated that diseases associated with deregulated ribosomal biogenesis result from functional mutations in the nucleolar constituent of the ribosome or factors closely associated with polymerase I (Pol I), collectively named ribosomopathies [31,32]. The most important issue is to identify factors influencing disease onset and progression.

Nucleolar proteins such as nucleolin (Ncl), nucleostemin (NS), nucleophosmin (NPM), and fibrillarin (Fbl) are involved in both cell homeostasis and disease. NS protein, for example, plays a pivotal role in processes such as maintenance of stem cell pluripotency, regulation of telomere length, inhibition of stem cell differentiation, senescence, control of pre-rRNA processing, cell death, and others [120,121]. Neuronal nucleolar stress, a cause of neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease, Huntington disease, and many others, has been investigated by numerous researchers [122–127]. In neurodegenerative diseases, nucleolin plays an important role because depriving the rRNA promoter of this histone chaperone downregulates rRNA transcription [128–133]. Abnormal interaction with nucleolin also accounts for oculomotor apraxia type 1, a neurological disorder caused by mutated aprataxin. Abnormalities in the nucleolus are also a cause of spinocerebellar ataxia, an inherited disorder caused by CAG or CTG expansion [134,135]. In these neurodegenerative diseases, nucleolin appears to play a role because rRNA transcription downregulation arises from depriving the rRNA promoter of this histone chaperone by CAG RNAs [136,137]. Several studies have shown that the transcription of rRNA genes is altered in Huntington disease (HD). A protein inhibiting the transcription of both rRNA and ribosomal protein genes (Rrs1) was highly expressed in a presymptomatic HD mouse model [132]. The level of factor UBF1 is tightly connected with RNA polymerase I, and was decreased in cellular and animal HD models [133]. In post-mortem specimens of human HD cases, insoluble aggregates of huntingtin were found in the nucleolus [134].

DNA-damaging response (DDR) proteins, which operate in the nucleus, also play a role in nucleolar repair. The number of studies on nucleolar stress in neurodegenerative diseases is rapidly growing [124]. Silencing of rDNA during the early stage of AD plays a role in AD-related ribosomal deficit with subsequent dementia [122,128]. Differential methylation activity of rDNA is a possible mechanism causing decreased rDNA gene expression in AD patients. A specific methylation pattern could be used as a marker for AD detection and for tracing the course of the disease. Post-mortem brain tissue revealed the disruption of dopaminergic neurons in Parkinson's disease [122,123].

During the passing of neural lineage, there is no direct evidence that rRNA transcription is inhibited, but there is indirect evidence that nucleolar-correlated mechanisms could take

part in this process. For example, in brain and retina, the level of the nucleolar protein nucleostemin-a controller of pre-rRNA processing-is rapidly reduced prior to cell cycle exit and neural differentiation [123]. As far as impaired mitochondrial activity affects nucleolar function, it supports the concept that nucleolar stress may occur at early disease stages, contributing to pathogenesis. It also takes place in the nucleolus in spinocerebellar ataxia, an inherited disorder caused by CAG or CTG expansion [134,135].

Recently, mouse models were developed in which nucleolar function was specifically impaired by genetic removal of TIF-IA**,** a conserved transcription factor essential for the recruitment of Pol I to rRNA promoters. Since its identification, it became evident that TIF-IA activity is strongly dependent on external signals [138]. Today, it is known that TIF-IA is regulated by a variety of protein kinases at distinct serine residues: ERK and RSK in response to mitogenic signals [139], S6K in response to growth stimuli, JNK2 in response to oxidative stress [140], AMPK in response to cellular energy status, and PERK-dependent phosphorylation in response to endoplasmic reticulum stress [141]. It is also known now that proteasome activity is necessary for pre-rRNA synthesis, and TIF-IA may represent a potential link between proteasomes and rRNA genes.

7. rDNA and Epigenetic Genome Regulation

With a general increase in interest in the organization and functional role of rDNA in the genome, different research groups are focusing on various aspects of this role. Above, we discussed the significance of rDNA representation in genomes in the development of such diseases as Down syndrome, Treacher Collins syndrome, Bloom syndrome, neurodegenerative diseases, cancer, CA abnormalities, to name but a few. Nuclei also perform important functions such as in cell cycle regulation and stress response. In particular, several stress-induced loci localized in the rIGS produce noncoding nucleolar RNAs specific for the state of stress. By mapping the 5′- and 3′-ends of such responsible IGS (srIGS) segments scattered across NOR^+ chromosomes, we found that the links in srIGSs most frequently subjected to breaks are adjacent to or overlap with stress-induced loci [3].

Recently, Churikov and co-authors revealed connections between the hotspots of double-stranded breaks (DSBs) in rDNA and gene expression in chromosomal domains [142]. The authors showed that double-stranded DNA breaks are involved in coordinated local gene expression. These 50–150 tbp DNA domains (designated as forum domains) can be visualized by separating nondigested genomic DNA in agarose pulsed-field gel electrophoresis [143] and used for large-scale mapping of DSBs in the genome. Nine DSB hotspots were found in human rDNA genes, which coincided with the binding sites of the CTCF and H3K4me3 markers [144], implying the participation of DSBs in active transcription. In this work, DSBs were mapped in the chromosomes of human HEK293T cells, and a bioinformatics analysis

of the data in Gene Expression Omnibus (number GSE53811) was carried out in accordance with the data from [145]. The findings show that H3K4me3 markers often coincide with DSB hotspots in HEK293T cells and that mapping of these hotspots is important for the research on genomes affected by cancer. Data of 4C analysis of rDNA show that the regions containing DSB hotspots can often bind to specific regions of different chromosomes, including the pericentromeric regions, the binding regions of some histone markers, as well as the binding sites of CTCF, ChIA-PET, and RIP signals. The results suggest a close relationship between chromosome breaks and some mechanisms of epigenetic regulation of gene expression [146-148].

As noted above, eukaryotic cells contain several hundred rRNA genes, some of which are disabled by epigenetic mechanisms. Extensive research has revealed the details of these mechanisms and shown how DNA methyltransferase and histone-modifying enzymes work consistently with chromatin remodeling complexes [149,150]. It was also possible to identify the participation of noncoding RNAs in the mechanism of formation of a specific chromatin structure which determines the transcriptional status of rRNA genes. These studies indicate the existence of complex links between various participants in epigenesis when the chromatin structure changes during the process of activating and deactivating genes. It has become clear that constitutive heterochromatin (GC) is a dynamic and transcriptionally active part of the genome that is important for its stability. In human cells, the main GC clusters are localized at centromeres, telomeres, and rRNA genes. Despite differences in biological function, telomeres, centromeres, and rRNA genes possess characteristic heterochromatic features necessary to maintain nuclear structure and function under normal conditions [151–153].

It has been shown that the switch between active and inactive states of rRNA genes is regulated by NoRC, a chromatin remodeling complex that includes the ATPase SNF2h and the large TIP5 subunit. The direction of NoRC to rDNA establishes de novo histone modifications and DNA methylation, which leads to chromatin compaction and transcriptional silencing of the rDNA repeat fraction. Thus, NoRC combines several enzymatic processes that fix repressive chromatin structures on the rDNA promoter [153]. The consolidation of such chromatin structures at centromeres and telomeres is vital for the integrity of the kinetochore and protection of the ends of the chromosomes; in other words, the protection of chromosome stability. Considering the structural similarity of centromeres, telomeres, and rRNA genes leads to the conclusion that the functions of the NoRC complex are not limited to suppressing the activity of rRNA genes, and that it can play an important role in organizing higher-order chromatin structures at other clusters of repeating sequences. This assumption is confirmed by the results of immunoprecipitation and FISH experiments, which show that, in addition to the nucleoli, NoRC is localized at the ends of the chromosomes and at centromeric repeats. Tip5 knockdown resulted in reduced histone modification in pericentromeres, telomeres, and

subtelomeres, allowing for the definitive conclusion that NoRC has a direct effect on chromatin in these repeating sequences. Although further research is needed to study the mechanism and the results of NoRC function in the regulation of chromatin structure and genomic stability, the fact that NoRC participates in the formation and maintenance of the repressive chromatin conformation in the main genomic clusters of repeating sequences implies its important role in maintaining the structure and function of the genome [149–151].

Noncoding RNAs also play a key role in the formation of functional subcompartments of the nucleus [152]. Using fluorescence microscopy and deep sequencing, it was shown that the nucleolus is enriched with Pol II transcripts of intron *Alu* elements (aluRNA). Inhibition of RNA polymerase II or exhaustion of aluRNA by antisense oligos led to the destruction of nucleolus structure and disrupted the transcription of rRNA genes dependent on RNA polymerase I. On the contrary, overexpression of aluRNA resulted in an increase in the size of the nucleolus and the level of pre-rRNA, which suggests a functional relationship between aluRNA and nucleolus integrity and rRNA synthesis. It is also shown that aluRNAs interact with nucleolin and direct individual segments of the genome to the nucleolus. The obtained results suggest the existence of a mechanism that ensures the interaction of RNA polymerases I and II with the participation of aluRNA and modulates the nucleolus structure and the production of rRNA [152–155].

8. Conclusions

Until 2000, the nucleolus had been considered as an organelle solely involved in ribosome biosynthesis. Ribosomes, the molecular factories that carry out protein synthesis, are essential for every living cell. At the same time, due to the discovery of more nucleolar functional activities, researchers all over the world have provided new insights into the role of the nucleolus as a signaling hub that is important in maintaining cellular homeostasis and lifespan and that may also cause human diseases. The variable rDNA regions differ in size and sequence among organisms and within individual species. A trend has appeared in recent years to develop many novel findings concerning various aspects of ribosome biogenesis in cell growth and cell cycle control. Defects in ribosome biogenesis have also been linked to human diseases. It is now clear that the disruption of ribosome biogenesis causes nucleolar stress that triggers a p53 signaling pathway, thus providing cells with a surveillance mechanism for monitoring ribosomal integrity. The association of nucleolar stress, cellular dysfunction, and human diseases, including cancer; cardiovascular, neurodegenerative, and autoimmune disorders; and infectious and metabolic disorders has become an important research topic. In addition, identifying potentially new risk factors may also help in the development of novel therapeutic lines of attack and new therapeutic approaches for the treatment of diseases.

9. Funding

This work was supported by the Russian Academy of Sciences molecular and cell biology program.

10. Conflicts of Interest: The authors declare no conflict of interest.

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