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Review

Factors Responsible for Modulation of Ribosomal RNA Transcription

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The aetiology of variation in transcription of ribosomal genes is still an open question. Investigations carried out in many organisms showed that the variation depends not only on genetic mechanisms such as rDNA methylation, elimination of rDNA or the position effects. In this review, we discuss the results of the studies on repression of rRNA transcriptional activation from protein factors to conditions of cell culture, which may influence the variation of nucleolar organizer region activity.

Key words: Nucleolus, rDNA, epigenetics, AgNOR proteins.

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The nucleolus is a non-membrane-bound substructure situated inside the eukaryotic nucleus and serves as the site of ribosomal RNA synthesis and ribosomal biogenesis. The nucleolus is formed around the ribosomal DNA (rDNA) repeats, which cluster at chromosomal loci called nucleolar organizer regions and it is the unit in which 28S, 18S, 5.8S ribosomal RNAs are transcribed, processed and assembled into ribosome subunits (LAMOND & EARNSHAW 1998; SCHEER & HOCK 1999). Moreover recent studies have shown that the nucleolus is also involved in the sequestration of proteins which act in cell-cycle regulation (SHOU *et al.* 1999), signal recognition particle biogenesis (JACOBSON & PEDERSON 1998), p53 metabolism (ZHANG & XIONG 1999), cellular aging (STRAIGHT *et al*. 1999), small RNA processing, mRNA export and degradation as well as telomerase activity (PEDERSON 1998).

The number of nucleoli in the nucleus is determined by the number of chromosomes with active nucleolus organizers (NOR). The chromosome pairs with NORs are species-specific. In some species, there are two NORs around which two nucleoli are generated and in other species there are several pairs of NORs in which case several NOR-bearing chromosomes participate in the building of one nucleolus.

NORs can be detected with silver (AgNOR) staining techniques as well as in situ hybridization techniques using rDNA probes. In contrast to the silver staining used to distinguish between active and inactive rRNA gene clusters, the FISH approach detects all rDNA loci, irrespective of their transcriptional status. Therefore only in situ hybridization (ISH) can detect 5S rDNA genes. The size and number of the silver deposits on the NOR is thought to be positively correlated to the degree of transcriptional activity of the cell (HUBBELL 1985; DERENZINI *et al*. 2000).

AgI staining has shown the variation in the transcriptional activity of rDNA in many organisms. Variation in the intensity and frequency of silver deposits on NOR-bearing chromosomes in many animals fulfils the criteria of polymorphism. The numerical polymorphism of AgNORs is frequently observed at cellular, individual and population levels and the staining pattern appears to be inherited according to the Mendelian laws (DE CAPOA *et al*. 1991; SŁOTA *et al*. 1996; DANIELAK-

-CZECH *et al*. 1999). However, the precise etiology of such variation is not always clear.

The number of genes is positively correlated with the level of function. On the other hand, sometimes chromosomes which show a very large number of rRNA gene copies do not display the expected increased level of activity. There is no doubt that the level of NORs activation (level of ribosome production) depends on the efficiency of nucleolar mechanisms such as transcription and processing of rRNA and also on the number of active rDNA genes. A detailed analysis of ribosomal transcriptional regulation will require identification of both the proteins and the DNA sequences involved in this process.

Generally, activation of transcription is correlated with the relaxation of chromatin structure as well as sites specifically recognized by transcription factors.

Recently investigation of the repression of transcriptional activation in NORs suggested that at least three different genetic mechanisms exist that are responsible for the inactivation of nucleolus organizer regions: elimination of rDNA, DNA methylation, position effects induced by heterochromatin or telomeres (GUILLEN *et al*. 2004).

Elimination of rDNA

The first mechanism for the repression of transcriptional NORs activation is related to the presence of non-heterochromatic DNA arrays consisting of β-satellite DNA and other repetitive sequences. Unequal crossing-over can induce loss as well as tandem duplication of rDNA regions between homologous chromosomes, and non-homologous recombination can result in such rearrangements between non-homologous chromosomes. Non-homologous recombination relieves interlocking between nonhomologous chromosomes in rDNA or distal heterochromatin association. Human rDNA structure consists of non-heterochromatic reiterated DNA sequences such as β-satellite DNA and other repetitive sequences like Alu elements. Chimpanzees have heterochromatic DNA arrays consisting of different sequences at NOR regions, and in addition occupied by rDNA sequences. Therefore human rDNA, which is outside the non-crossing over zones (NCOZ), may move to other chromosomes, while the chimpanzee rDNA cannot, as it is within the NCOZ. NORs appear to form associations independent of links between other terminal repetitive arrays at early stages of meiosis. NORs tend to induce not only loss and duplication but also homogenization of DNA sequences among NOR loci. As a result of this mechanism rDNA may be eliminated from a particular locus (GUILLEN *et al*. 2004). In the wasp *Trypoxylon* (Trypargilum) *albitarse* (solarity haplodiploid wasp $n_{males}=16$; $2n_{females}=32$), there is a single rDNA cluster on chromosome 14, with a heterochromatic short arm showing size variability. The absence of males carrying three chromosome variants with lower amounts of rDNA indicates that these variants are lethal in this sex. This suggests the existence of a threshold marking the minimum amount of rDNA which is tolerable in haploidy (ARAUJO *et al*. 2002). In contrast, an increase in rRNA gene number should be well tolerated. A study carried out on the grasshopper (*Stauroderus scalaris*) revealed the first case of an extreme amount of inactive ribosomal DNA. These genes make up most of the paracentromeric heterochromatin, which constitutes about half of the total chromatin in the genome of the species. However, AgI staining showed the presence of a single active NOR (LOPEZ-LEON *et al*. 1999). The inactivity of the remaining rDNA clusters could be explained by NOR activation sequentially achieved in order of size (ZURITA *et al*. 1998). This particular NOR is able to provide enough rRNA to satisfy cell demands and there is no need to express any of the rRNA genes in the remaining chromosomes (LOPEZ-LEON *et al*. 1999).

Inactivation NORs by methylation

Methylation, an epigenetic event, plays an important role in regulation of gene expression. Methylation may change the affinity of DNA to transcription factors, array nucleosomes in specific regions and interaction between histones and DNA. Vertebrate DNA is frequently modified at the dinucleotide CpG by a covalent addition of a methyl group to the 5′-carbon of cytosine in a CpG dinucleotide (resulting in m^5CpG) (GRUENBAUM *et al*. 1981). BROCK & BIRD (1997) used restriction enzymes to cleave DNA demonstrating a mosaic pattern of CpG methylation in the majority of human rDNA repeat units. In man, the 13.3 kb transcribed region is apparently free of methylation, whereas the NTS, which lies between consecutive transcribed regions is highly methylated at ~ 300 tested CpG sites. The transition between methylated and non-methylated domains appears to be sharp and occurs near the boundaries of the transcribed region. Close examination showed that the major part of the chromosomal rDNA repeat unit might be methylated in most if not all vertebrates. Another group, using antibodies to 5-methylocytosine, revealed that expressed variants of chromosomes with amplified copies of rDNA showed less rDNA than expected on the basis of the number of gene copies and that the NORs were highly methylated (DE CAPOA *et al*. 1991).

Methylation is the most likely candidate for the absence of AgNOR proteins in the regions with rDNA. Similar results were obtained by GULLIEN *et al*. (2004) who used *in situ* nick translation with the *Hpa*II restriction enzyme. However, in this study inactive NORs that did not undergo methylation were also observed. In rats CpG methylation was observed mainly in enhancer and promoter regions of inactive rRNA gene copies. The methylation of one single *Hpa*II site, located in the promoter region, showed particularly strong correlation with transcriptional activity (STANCHEVA *et al.* 1997). The phenomenon of DNA methylation is correlated with the phenomenon of histone acetylation. Methylation decreases the degree of histone acetylation and induces more packing of chromatin structures in the methylated regions of chromosomes. Histones in heterochromatin are mostly not acetylated in contrast to acetylated histones in functional domains. Acetylation decreases the affinity of histones to DNA and very likely reduces interactions between histones (EDEN *et al*. 1998).

Recent studies showed that silencing of rDNA is mediated by the chromatin remodeling NoRC (nucleolar remodeling complex). NoRC consists of Tip5 (TTF-I-interacting protein 5) and Snf2H. NoRC mediates histone H4 deacetylation, histone H3-Lys9 dimethylation and *de novo* DNA methylation, thus establishing heterochromatic features at the rRNA gene promoter (SANTORO & GRUMMT 2005). NoRC silences rDNA transcription through recruitment of histone-modifying and DNA-methylating activities to the rDNA promoter, thereby establishing and/or maintaining a repressive higher-order chromatin structure. NoRC is associated with silent rRNA gene copies, and over-expression of TIP5 represses rRNA polymerase I transcription (SANTORO *et al*. 2002; ZHOU *et al*. 2002). Over-expression of NoRC silences rDNA transcription, reduces the size and number of nucleoli, impairs cell proliferation and resets replication timing from early to late (LI *et al*. 2005). NoRC interacts with the N-terminal part of TTF-I, and this interaction enables TTF-I to bind to its cognate sequence upstream of the gene promoter. NoRC binds near the promoter and subsequently represses rDNA transcription (STROHNER *et al*. 2004**)**.

Position effect of heterochromatin and telomeres

The specific localization of eukaryotic loci of ribosomal DNA near heterochromatin blocks suggests that heterochromatin influences rDNA function and/or maintenance. In eukaryotes, rDNA is usually associated with transcriptional silencing of nearby genes and also the suppression of recombination. A similar effect by telomeres has been described.

The position effect was described many times in *Saccharomyces cerevisiae* (GOTTSCHLING *et al*. 1990) and *Drosophila melanogaster* (YAMAMOTO & MIKLOS 1978; SPRADLING & RUBIN 1983; GREWAL & ELGIN 2002). In *S. cerevisiae* (GOTTSCHLING *et al*. 1990; FRITZE *et al*. 1997) and *Drosophila* (WALLRATH 1998; BRISCOE Jr. *et al*. 2000) translocation of euchromatic genes into heterochromatic regions results in transcriptional silencing of these genes.

Silent information regulator (Sir) proteins are required for heterochromatin silencing in *S. cerevisiae*. Proteins Sir1, Sir2, Sir3 and Sir4 are required for efficient silencing of the HM loci while the telomere position effect requires products of genes SIR2, SIR3 and SIR4. (SMITH *et al*. 1999). Telomeric and cryptic mating-type loci (HML and HMR) silencing also share requirements for Rap1, histones H3, H4 and several other factors. The rDNA silencing is mediated by RENT (regulator of nucleolar silencing and telophase) complex, which contains Sir2 and the protein phosphatase Cdc 14 (SHOU *et al*. 1999). Sir2 is a silencing protein at all heterochromatic loci and is a NAD-dependent histone deacylase (TANNY & MOAZED 2001). *SIR2*-like genes are present in all three kingdoms (BRACHMANN *et al*. 1995). In yeast, mutations in this gene lead to the inability to form heterochromatin, resulting in an altered chromatin structure at loci exhibiting position effects, such as the cryptic mating-type loci and telomeres (RINE & HERSKOWITZ 1987; APARICIO *et al.* 1991).

The position effect has also been described in chimpanzees, while in man the position effect is not strong in a distal region due to a lack of the distal C-band block (GUILLEN *et al*. 2004). Obviously highly condensed heterochromatin regions repress transcription by restricting the ability of sequence-specific gene activator proteins to access their DNA target sites. However, recent studies show that heterochromatin structure is inherently dynamic, and that sequence-specific regulatory proteins are able to bind to their target sites in heterochromatin (CHEN *et al*. 2005).

The AgNOR proteins remain associated with NORs

The staining protocol leads to labeling of non-histone proteins of transcriptional machinery that remain associated with rDNA genes (UBF, SL1, RPI), processing pre-rRNA (nucleolin, fibrillarin, RNAase, Rpp29) and ribosome assembly (ROUSSEL *et al*. 1994; CHEN *et al*. 2004). The other proteins involved in rDNA transcription are shown in Table 1.

Table 1

Other proteins involved in transcription of rDNA

Protein	Function/functions
DNA topoisomerase I	involved in the general transcription process, but its association with rDNA transcription is particularly prominent
transcription termination factor (TTF-1)	mediates termination of rDNA transcription and remodeling of the promoter (SIRRI *et al*. 1999; NEMETH *et al*. 2004)
70 kDa protein (p70)	requires stable binding to the core promoter the SL1-fraction. p70 has an essential role in the initiation of mammalian rDNA transcription as shown by a reconstituted *in vitro* transcription assay (YAMAMOTO *et al*. 2000)
Rubin	encoded by a message complementary to rRNA, which interacts with the Pol I factor UBF and is capable of modulating ribosomal transcription and cell proliferation. Its cellular level correlates with the growth state of the cells (KERMEKCHIEV & IVANOVA 2001)

The Pol I transcription machinery, including the polymerase complex, and the Pol I–specific transcription factors, such as upstream binding factor (UBF) and selective factor 1 (SL1), bind to NORs throughout mitosis (metaphase and anaphase) when rDNA transcription is inactivated (SUMNER 1990; ROUSSEL *et al.* 1993; GEBRANE-YOUNES *et al*. 1997). Pol I transcription machinery is generally species-specific (GRUMMT 1999) and is responsible for the positive signal of silver staining.

UBF and SL1 are two DNA-binding factors that interact with each other and with DNA to form a stable preinitiation complex at the rDNA promoter. These factors are associated with RNA polymerase I and connected by RRN3. RNA polymerase I is involved in transcription of 18S, 5.8S and 28S ribosomal RNA genes (rDNA). However 5S rDNA is transcribed by RNA polymerase III, which also transcribes snRNA genes.

Cytological analysis performed on mitotic cancer cells revealed two additional proteins. Their distribution during mitosis was compared with the distribution of the silver staining protein. The proteins were identified by specific immunostaining and silver staining and were described as nucleolin (initially called C23 and often described as a 100-110 kDa protein) and B23 (also called NO38, nucleophosmin/NPM, or numatrin) and now they are acknowledged as the major AgNOR proteins detected during active transcription and proliferation.

Nucleolin is composed of several structural domains, which allows for the interaction of nucleolin with different proteins and RNA sequences. Nucleoline can regulate transcription of the rDNA genes and maturation of the pre-rRNA and nucleocytoplasmic transport (GINISTY *et al*. 1999). Studies carried out in *Xenopus* oocytes suggested that the interaction of nucleolin with the rDNA sequence and with nascent pre-rRNA leads to the blocking of RNA polymerase I (RNA pol I) transcription (ROGER *et al*. 2001).

B23 is a multifunctional nucleolar phosphoprotein. Among others, the B23 interacts with nucleolar proteins such as nucleolin, which may represent a nucleolar-targeting mechanism in which B23 acts as a nucleolar-localization signal-binding protein (LI *et al*. 1996).

During mitosis, nucleolin is localized at the NORs and distributed diffusely throughout the mitotic cytoplasm, appearing to sheath the condensed chromosomes. Protein B23 is predominantly localized at the periphery of chromosomes (SPECTOR *et al.* 1984; DOUSSET *et al*. 2000; SIRRI *et al*. 2000).

The AgNOR proteins such as C23 and B23 are used as markers of tumor pathology because there is a close relationship between their expression and the rapidity of cell proliferation (DERENZINI *et al*. 1989; TRERE 1998; CORTES-GUTIERREZ *et al*. 2001). Moreover, a recent study aiming at evaluation of AgNORs in normal, post hepatitic, alcoholic cirrhosis and malignant liver tissue showed that AgNORs can act as a good marker in diagnosis of liver diseases, especially in differentiating post-hepatitic and alcoholic cirrhosis (MISRA *et al*. 2003).

Regulation of activity of NOR-proteins

The analysis of rDNA transcription in mitosis showed that this process is still active in prophase, inhibited from prometaphase until early anaphase, and active again in late anaphase. The mechanism of gene inactivation can be regulated at either the level of the transcription machinery by dissocia-

tion or modification or at the level of chromatin by condensation or modification. Thus, considering all of these conditions, dissociation of the transcription machinery cannot explain mitotic arrest of rDNA transcription (GEBRANE-YOUNES *et al*. 1997; RASKA *et al*. 2004).

Activity of proteins is often modulated by methylation, phosphorylation, ADP-ribosylation as well as interaction with other proteins. There are universal mechanisms responsible for controlling the cell cycle and metabolism in live organisms.

The upstream binding factor is modulated by posttranslational modifications. UBF contains multiple phosphorylation sites (VOIT *et al*. 1995; VOIT *et al*. 2001). The phosphorylation state of UBF appears to determine its ability to activate transcription but not its ability to bind to DNA (VOIT *et al*. 1992). The C-terminal domain of UBF, which is regulated by phosphorylation, interacts with two subunits of TIF-IB/SL1, namely TBP and TAF_I48. Dephosphorylation abolishes the binding of UBF to TIF-IB/SL1 and prevents transcriptional activation (TUAN *et al*. 1999).

The transcription initiation factor (TIF)-IB/SL1 is also modulated by phosphorylation and is inactivated during mitosis by cdk2/ cyclin B-directed phosphorylation of TAF_I110. Mitotic phosphorylation is used as a molecular switch to prevent preinitiation complex formation and rDNA transcription (KLEIN & GRUMMT 1999). Cdk2/ cyclin B induces a similar effect in TTF-1 (VOIT *et al*. 1999). Phosphorylation seems to play a key role in the inactivation of rDNA transcription.

Recently it was shown that TAF_I68, the second largest subunit of the TATA box-binding protein (TBP)-containing factor TIF-IB/SL1, may target histone acetyltransferase and that resulting acetylation enhances binding of TAFI68 to the rDNA promoter (MUTH *et al*. 2001).

Activity of nucleolin is controlled by high phosphorylation (OLSON *et al*. 1975; RAO *et al*. 1982), methylation (LISCHWE *et al*. 1982), and ADP-ribosylation (LEITINGER & WESIERSKA-GADEK 1993). Modulation of protein activity is also often observed as a response to stress caused by the different agents. For example, protein B23 is more ADP-ribosylated and phosphorylated after exposure to radiation (RAMSAMOOJ *et al*. 1995).

Protein activity may be altered by interaction with other cellular proteins. UBF may be modulated by the product of the retinoblastoma susceptibility gene, which binds and represses UBF and in consequence inhibits rDNA transcription *in vitro* and *in vivo* (CIARMATORI *et al*. 2001). This transcriptional inhibition is due to a physical interaction of pRb with UBF, which involves the C-terminal part of pRb and a region of UBF harboring.

Factors coordinating transcription activity of rDNA

Changes in rDNA chromatin structure and alterations in amount, localization and activity of AgNOR proteins are associated with hormone administration, nutrient starvation, viral infection, nucleolus-specific drugs and the tumor suppressor proteins p53 and Rb (BUDDE & GRUMMT 1999; GRUMMT 1999; HANNAN *et al.* 2000).

Hormones coordinate interactions between various kinds of cells, regulate all physiological processes and undoubtedly modulate activity of NORs.

Insulin may regulate several components of the rDNA transcription machinery. Two of these targets are UBF and the RNA polymerase I-associated factor 53 (PAF53). PAF53 may interact with UBF helping to recruit the core RNA polymerase I to the rDNA promotor (HANADA *et al*. 1996; HANNAN *et al*. 1998). RUBIO *et al*. (1997) showed that vasopressin changes three parameters: nucleus area, Ag-NOR area, and mean number of Ag-NORs. Transcription of rDNA is also stimulated by androgens, which induce an increase of the quantities of the components of the rDNA transcription system (KABLER *et al*. 1996).

Nutrient starvation leads to significant nucleolar size reduction in yeast and mammalian cells. This state inhibits the target of the rapamycin (TOR) protein, which is a conserved regulator of ribosome biogenesis. TOR leads to site-specific deacetylation of histone H4. This suggests that TOR acts via a chromatin-mediated mechanism (TSANG *et al*. 2003). Moreover, examinations carried out on yeast showed that nutrient deprivation inactivates TIF-IA and impairs the association of TIF-IA with Pol I (YUAN *et al*. 2002).

Another mechanism modulating NOR activity is associated with viral infection. Many findings indicate that viral infection is accompanied by unequivocal activation of rDNA transcription. For example, cytomegalovirus (CMV) infection of human diploid embryo fibroblasts *in vitro* induces an increase of nucleolar size, augments the number of intranucleolar foci binding the specific RNA-polymerase I transcription factor (UBF), enhances the Ag-NOR staining, activates 3H-uridine incorporation to the nucleoli and changes in the ultrastructure of the nucleolus (ZHARSKAIA *et al*. 2003). Analysis of NORnumber, and NOR area distribution in HIV-infected patients showed that these parameters were consistently increased (CANNAVO *et al*. 2001).

Many antitumor drugs localize to nucleoli or exert their effects on nucleoli. Though it is sometimes difficult to define the precise target(s) of a particular drug, it is a well known fact that many drugs affect rDNA transcription inactivation and segregation of the rRNA transcription machinery (HERNANDEZ-VERDUN & ROUSSEL 2003).

When studies are carried out using cells in cultures, it should not be forgotten that these cells might behave in many ways distinctly from the manner of cells *in vivo* (HALLIWELL *et al*. 1999). Some components of cell culture media are responsible for the generation of reactive oxygen species (ROS) that may alter the redo status of the cell (HALLIWELL 1996; GRZELAK *et al*. 2001; BAJT *et al*. 2002; HALLIWELL 2003). We suggest that some components of cell culture media may modulate nucleoli activity, due to their ability to respond to oxidative stress.

Another problem arises from possible serum deprivation of cultured cells. Analysis of the expression, localization and phosphorylation of UBF in a Chinese hamster ovary cells culture in response to serum deprivation has shown that serum deprivation shuts off ribosomal RNA synthesis (SCHNAPP *et al*. 1990). Later examination demonstrated that cells have a significantly reduced degree of phosphorylation of UBF in response to serum deprivation (O'MAHONY *et al*. 1992).

Colchicine also has some effects on the number of NORs. The concentrations of colchicine used for cell arrest influence the degree of satellite association (RAVIA *et al*. 1985).

Conclusion and perspective

The nucleolus is a multifunctional substructure, which is among others involved in transcription of rRNA genes. This process is characterized by self dynamics and may be modulated on many levels. Moreover, recognition factors which coordinate the activity of the nucleolus may be helpful in understanding the cell physiology from beginning to death. Recently, an examination performed on old cells of *S. cerevisiae* revealed that there is a relationship between telomere-binding proteins, rDNA and promotion of longevity (GUARENTE 1997). Therefore future studies on nucleolus proteomics (AgNOR proteins) may give information about new targets in tumor therapy as well as the reasons for ageing.

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