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The Epigenetics of rRNA Genes: From Molecular to Chromosome Biology

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NOR, nucleolus, rDNA, RNA polymerase I, NoRC, CSB, chromatin remodeling, histone modifications, DNA methylation, transcriptional silencing

Abstract

In eukaryotes, the genes encoding ribosomal RNAs (rDNA) exist in two distinct epigenetic states that can be distinguished by a specific chromatin structure that is maintained throughout the cell cycle and is inherited from one cell to another. The fact that even in proliferating cells with a high demand of protein synthesis a fraction of rDNA is silenced provides a unique possibility to decipher the mechanism underlying epigenetic regulation of rDNA. This chapter summarizes our knowledge of the molecular mechanisms that establish and propagate the epigenetic state of rRNA genes, unraveling a complex interplay of DNA methyltransferases and histone-modifying enzymes that act in concert with chromatin remodeling complexes and RNA-guided mechanisms to define the transcriptional state of rDNA. We also review the critical role of the RNA polymerase I transcription factor UBF in the formation of active nucleolar organizer regions (NORs) and maintenance of the euchromatic state of rRNA genes.

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INTRODUCTION

Growing cells require continuous rRNA synthesis to ensure that subsequent generations contain the ribosome supply necessary for protein synthesis. Ribosome biogenesis is a major cellular undertaking that occurs in distinct nuclear compartments, the nucleoli. A nucleolus forms around clusters of repeated rRNA genes (rDNA) that encode rRNA, the scaffold and catalytic heart of the eukaryotic ribosome. The number of rDNA repeats varies greatly among organisms, ranging from fewer than 100 to more than 10,000. In growing cells, rRNA synthesis accounts for the majority of transcriptional activity to meet the demand for ribosome production and protein synthesis. Practically all signaling pathways that affect growth in response to nutrient and growth factor avail-

ability or during the cell cycle directly regulate rRNA synthesis, their downstream effectors converging at the RNA polymerase I (Pol I) transcription machinery. These topics have been reviewed in the past, and readers are referred to some recent articles for further reading (Grummt 2003, Mayer & Grummt 2005, Moss et al. 2007).

Each rRNA gene encodes a precursor transcript (45S pre-rRNA) that can be processed and posttranscriptionally modified to generate one molecule each of 18S, 5.8S, and 28S rRNA. Transcription of rDNA by Pol I requires the formation of a preinitiation complex on the promoter, including binding of UBF (upstream binding factor) and the promoter selectivity factor, termed SL1 in humans and TIF-IB in the mouse (Clos et al. 1986, Grummt 2003, Learned et al. 1986, Moss et al. 2007,

Paule & White 2000, Russell & Zomerdijk 2005). UBF affects Pol I transcription at multiple levels, functioning as a transcription activator (Bell et al. 1988, Panov et al. 2006), as an antirepressor (Kuhn & Grummt 1992, Pelletier et al. 2000), and as a regulator of transcription elongation (Stefanovsky et al. 2006), and it has been implicated in large-scale chromatin condensation (Chen et al. 2005, Mais et al. 2005, Wright et al. 2006). Promoter specificity is conferred by SL1/TIF-IB, a ~300-kDa protein complex that contains TBP (TATA box binding protein) and at least three Pol I-specific TBP-associated factors (TAF_Is), TAF_I110/95, TAF_I68, and TAF_I48 (Comai et al. 1992, Heix et al. 1997, Zomerdijk et al. 1994). Recently investigators have identified two more TAF_Is, TAF_I41 and TAF_I12, both of which are required for specific and efficient Pol I transcription initiation (Denissov et al. 2007, Gorski et al. 2007). TAF_Is perform important roles in transcription complex assembly, mediating specific interactions between the rDNA promoter and Pol I. They interact with UBF and recruit Pol I to rDNA by binding to TIF-IA, a basal regulatory factor that is associated with the initiation-competent subpopulation of Pol I (Pol I β). The interaction of SL1/TIF-IB with TIF-IA, the mammalian homolog of yeast Rrn3 that mediates growth-dependent control of rDNA transcription (Bodem et al. 2000, Miller et al. 2001, Yuan et al. 2002), drives the assembly of productive transcription initiation complexes.

Given the repetitive nature of rRNA genes, two strategies for regulating rRNA synthesis are conceivable. Pol I transcription may be controlled either by changing the rate of transcription from each active gene or by adjusting the number of genes that are involved in transcription. Although there is evidence for both options, the majority of short-term regulation affects the rDNA transcription cycle, e.g., preinitiation complex assembly, initiation, promoter escape, and transcription elongation or termination (for review, see Russell & Zomerdijk 2005). Moreover, posttranslational modifications influence the activity and func-

tional interplay of transcription factors, adding further complexity and fine-tuning to transcriptional regulation in response to external signals that affect cell growth and proliferation (for review, see Grummt 2003, Russell & Zomerdijk 2005).

The number of active rRNA genes varies between different cell types, indicating that the fraction of active gene copies changes during development and differentiation (Haaf et al. 1991). Thus, long-term changes in rDNA transcription can be achieved by regulating the number of rRNA genes that are transcriptionally active. Some recent reviews summarize our current knowledge of the epigenetic mechanisms that mediate silencing and epigenetic control of rDNA (Grummt 2007, Grummt & Pikaard 2003, Lawrence & Pikaard 2004, McStay 2006). This review provides an update on recent advances in the epigenetic mechanisms that regulate the balance between active and inactive rDNA repeats in mammalian cells. In general, we restrict our review to work concerning mammalian rRNA genes; however, key experiments from other systems are discussed where appropriate.

ORGANIZATION OF MAMMALIAN rRNA GENES

Structure of Mammalian rDNA Transcription Units

Mammalian rDNA transcription units are large, comprising ~43 kb in humans and ~45 kb in mice (Gonzalez & Sylvester 1995, Grozdanov et al. 2003, Sylvester et al. 2004). Sequences encoding pre-rRNA (13–14 kb) are separated by long intergenic spacers (IGSs) of approximately 30 kb. Regulatory elements, including gene promoters, spacer promoters, repetitive enhancer elements, and transcription terminators, are located in the IGS (**Figure 1**). The rDNA promoter has a bipartite structure, consisting of a core promoter element adjacent to the transcription start site and an upstream control element (UCE) approximately 100 nucleotides further upstream (Haltiner

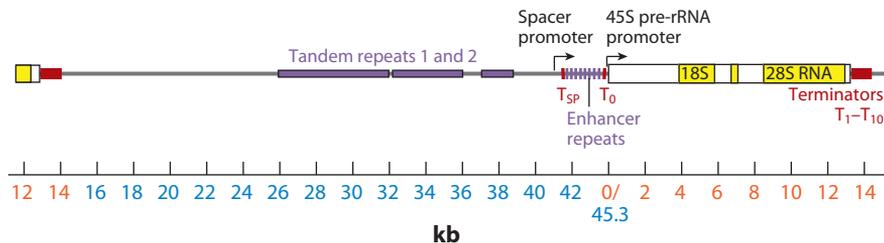


Figure 1

Structural organization of mouse rRNA gene. Graphic of mouse rRNA genes is derived from Genbank accession number BK000964. The sites of transcription initiation of the 45S pre-rRNA and transcripts from the intergenic spacer promoter are indicated by arrows. Scale bars (in kb) are shown below; 0 kb indicates the 5' end of the pre-rRNA. Terminator elements located downstream of the transcription unit (T_1 – T_{10}), downstream of the spacer promoter (T_{SP}), and upstream of the gene promoter (T_0) are marked by red bars. Repetitive enhancer elements (*purple*) located between the spacer promoter and major gene promoter of the mouse gene promoter are also indicated.

et al. 1986, Learned et al. 1986). Mammalian rDNA transcription units are flanked at their 5' and 3' ends by one or more terminator elements that are recognized by TTF-I (transcription termination factor), a specific DNA binding protein that stops elongating Pol I and serves an important role in epigenetic regulation of rRNA genes (Grummt et al. 1985, 1986a; Henderson & Sollner 1986; McStay & Reeder 1986). The major part of the IGS appears to be devoid of regulatory elements, comprising a high density of simple sequence repeats and transposable elements (for review, see Sylvester et al. 2004).

Tissue-Specific Expression of rRNA Gene Variants

The current concept is that arrays of mammalian rRNA genes are composed of identical repetitive transcription units that are clustered on specific chromosomal loci. So far, only one complete mouse rDNA transcription unit has been sequenced (Grozdanov et al. 2003), and therefore rDNA loci constitute major gaps in the human and mouse genome. Contrary to the notion that all rDNA repeats are identical, early cytogenetic studies have documented that individual chromosomal rDNA loci are not equally active in different human cells (de Capoa et al. 1985). Consistent with the exist-

tence of sequence polymorphism and cell-type-specific regulation of rDNA variants, mouse cells contain seven mouse rDNA variant types (v-rDNA) that vary in the length of the IGS and exhibit sequence polymorphism both in the variable region of 28S rRNA and in the 5'-terminal part of the transcription unit (Tseng 2006, Tseng et al. 2008, S. Zhang et al. 2007). Analysis of the copy numbers, expression profiles, and methylation pattern in multiple mouse tissues revealed that v-rDNAs are not regulated in concert, but independently and, in some cases, in a tissue-specific manner. Three v-rDNA types were expressed in all tissues (constitutively active), two were expressed in some tissues (selectively active), and two were not expressed (silent). The finding that rDNA exist in genetically distinct subdomains, which can be regulated individually in different tissues, suggests a heretofore-unappreciated complexity in mammalian rDNA structure and regulation.

In multicellular organisms, cells differ in their requirements for rRNA. The developing mouse oocyte, for example, doubles the activity of Pol I to accumulate rRNA without amplifying the number of rRNA genes. Instead, these cells use basanuclin, a transcriptional regulator that is expressed in highly proliferative cells and tissues, e.g., in keratinocytes and reproductive germ cells, to enhance rDNA transcription (Iuchi & Green 1999, Tian et al. 2001, Tseng &

Green 1992, Tseng et al. 1999, S. Zhang et al. 2007). Another cell-type-specific regulator of rRNA synthesis is Runx2, a factor that controls bone lineage commitment and cell proliferation (Young et al. 2007). Both baso-nuclin and Runx2 localize in nucleoli, are associated with rDNA throughout the cell cycle, and affect Pol I transcription, baso-nuclin acting as an activator of Pol I transcription and Runx2 as a transcriptional repressor. These results indicate that tissue-specific factors may regulate a subset of rDNA variants and suggest that a one-size-fits-all model for regulation of rDNA expression is probably an oversimplification.

Nucleolar Organizer Regions

In situ hybridization experiments have revealed that clusters of rDNA repeats, termed nucleolar organizer regions (NORs), are located on the short arms of the five human acrocentric chromosomes, chromosomes 13, 14, 15, 21, and 22, in a telomere-to-centromere orientation (Henderson et al. 1972). In the mouse, NORs are on chromosomes 12, 15, 16, 17, 18, and 19 (Figure 2a) (Dev et al. 1977). The positioning of NORs on the short arms of acrocentric chromosomes isolates them from genes transcribed by Pol II and Pol III. This isolation is further reinforced by adjacent heterochromatic repetitive satellite DNA. The repetitive nature of both rDNA and adjacent sequences has precluded sequencing of mouse and human NORs. Despite this drawback, other techniques have uncovered interesting and unexpected features of human NORs. For example, pulse-field gel electrophoresis of genomic DNA digested with enzymes that do not cut human rDNA, such as EcoRV and Sse83871, revealed a major rDNA band of 3 Mb as well as several minor bands of 1 and 2 Mb (Sakai et al. 1995). This implies that most human NORs are composed of ~70 copies of rDNA repeats and demonstrates that NORs contain solely rDNA rather than other sequences.

Concerted evolution of rDNA clusters is mediated by interchromosomal recombination between NORs on different chromosomes.

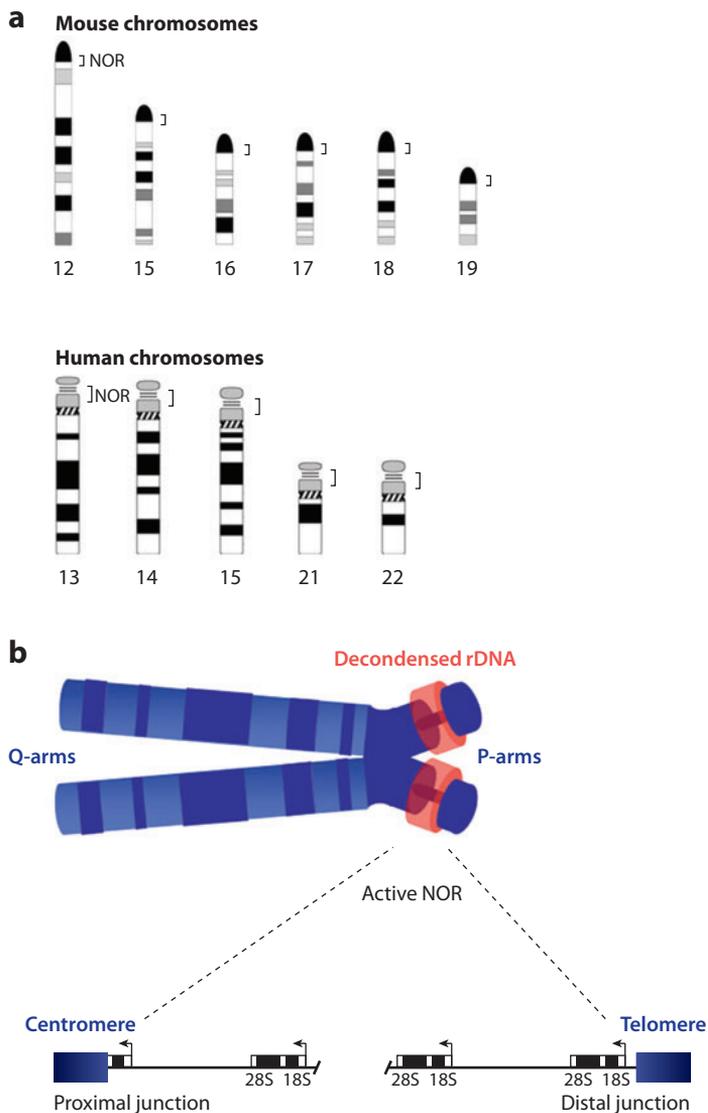


Figure 2

Positioning and organization of nucleolar organizer regions (NORs). (a) The positions of NORs on ideograms of mouse and human chromosomes are indicated by brackets. Ideograms were obtained from the homepage of the University of Washington, Department of Pathology (<http://www.pathology.washington.edu/research/cytopages>). Chromosome identities are indicated below each ideogram. (b) A diagram depicting DAPI-stained human chromosome 15. Dec condensed rDNA (red) is shown around the NOR of each sister chromatid. The organization of rDNA within the NOR is shown below (see text for further details).

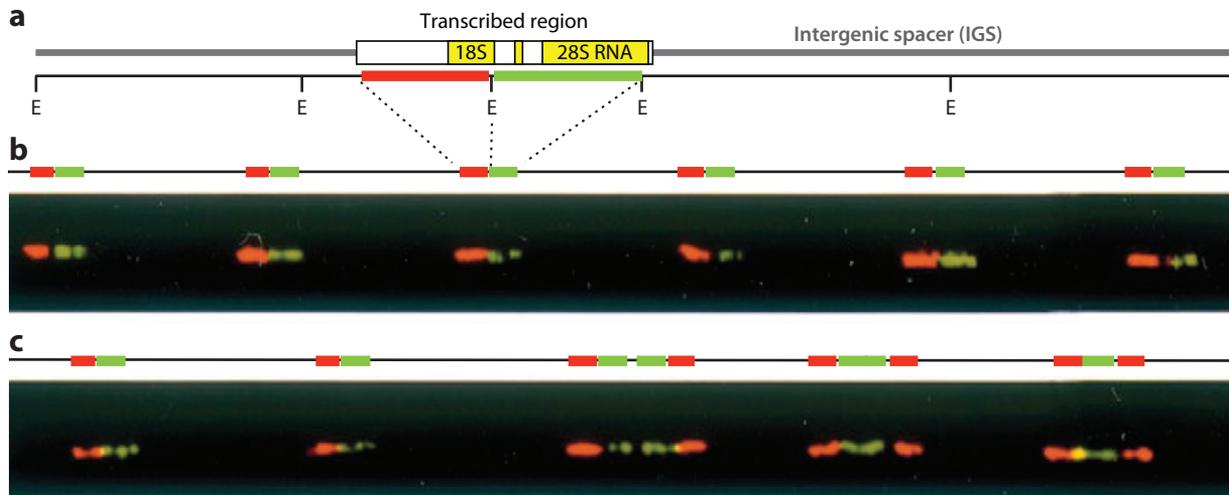


Figure 3

Analysis of the human rDNA locus by molecular combing. (a) Scheme depicting the localization of molecular probes used to identify rDNA transcription units on combed human DNA by two-color hybridization. The red probe (detected with Texas Red) hybridizes to the 5' part of the pre-rRNA coding region; the green probe (detected with FITC) hybridizes to the 3' part of the pre-rRNA coding region. (b) The image displays canonical rDNA units in tandem, each composed of a dual fluorescent signal and the adjacent intergenic spacer (IGS). (c) The image displays a region containing two canonical units (*left*), followed by three palindromic units, each half joined by its 3' region and separated by short IGS sequences. The arrangement of the transcription units and the position of EcoRI restriction sites (E) is illustrated in the schemes above the individual images (adapted from Caburet et al. 2005 with permission from Cold Spring Harbor Laboratory Press).

Such interchromosomal crossover events also result in conservation of sequences distal to the rDNA cluster, a prediction based on the finding that sequences abutting rDNA on the distal end of the NOR are conserved among all five acrocentric chromosomes (Gonzalez & Sylvester 1997, Worton et al. 1988). Moreover, a most surprising observation relates to the organization of rDNA repeats within the NORs. Until recently rRNA genes were thought to be organized as a uniform head-to-tail tandem array, although this hypothesis was unproven owing to difficulties in cloning and sequencing of repetitive DNA. Single-DNA-molecule analysis by molecular combing, however, has revealed that NORs comprise a mosaic of canonical and non-canonical rDNA repeats (Caburet et al. 2005). As many as one-third of rDNA repeats are noncanonical, apparently forming palindromic structures (Figure 3). One would predict that these noncanonical repeats are nonfunctional and that the cell has to silence noncanonical re-

peats to avoid the possibility of base pairing of antisense transcripts to pre-rRNA, which could seriously compromise ribosome biogenesis.

Active and Silent Nucleolar Organizer Regions

Active NORs remain undercondensed during mitosis and have a distinct chromatin structure that is evident as secondary constriction on metaphase chromosomes (Figure 2b). rDNA in active NORs is approximately tenfold less condensed than the adjacent satellite DNA (Heliot et al. 1997). This undercondensation results in reduced dye binding when chromosomes are stained, giving rise to an apparent gap in the chromosome. Often, an axis of condensed AT-rich DNA is found within the secondary constriction; the identity of these sequences is uncertain (Saitoh & Laemmli 1994). The most persuasive evidence that secondary constrictions correlate with the transcriptional

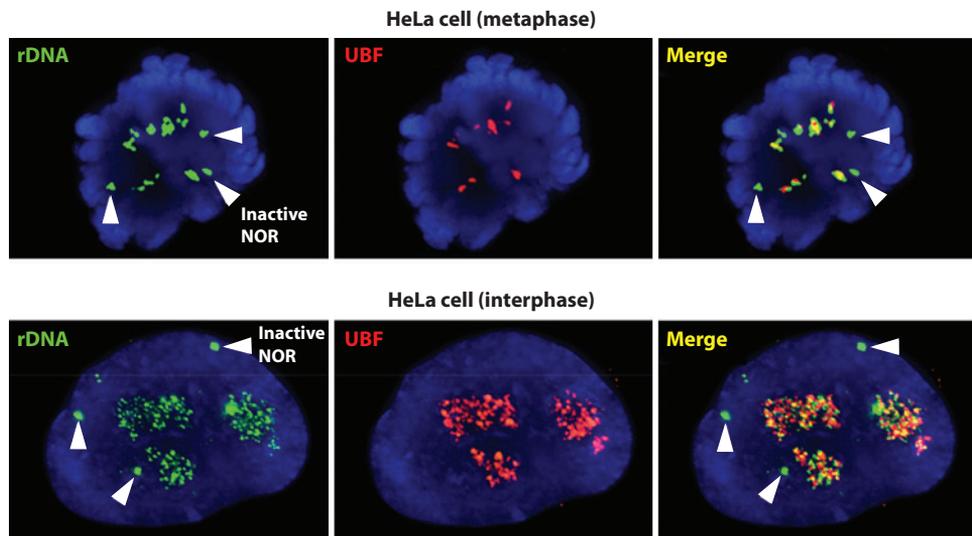


Figure 4

A fraction of nucleolar organizer regions (NORs) are silent throughout the cell cycle. HeLa cells in metaphase (*upper panels*) and interphase (*lower panels*) were subjected to combined immunostaining and fluorescent immunohybridization (immuno-FISH) to show the localization of rDNA and the Pol I-specific upstream binding factor (UBF). rDNA was visualized by hybridization to a probe from the intergenic spacer (*green*), and UBF was visualized with antibodies coupled to rhodamine (*red*). Inactive NORs, devoid of UBF, are indicated by arrowheads. Chromosomes and nuclei were visualized by DAPI staining (*blue*).

competence of rDNA is that Pol I together with Pol I-specific transcription factors, such as UBF, SL1/TIF-IB, and TTF-I, remain associated with NORs on mitotic chromosomes (Roussel et al. 1993, 1996). Because components of the Pol I transcription machinery contain acidic/argyophilic domains, this also explains why metaphase NORs can be easily visualized by silver nitrate (McClintock 1934).

On inactive NORs, rDNA appears to be packaged in a form that is indistinguishable from the surrounding heterochromatin. Silent NORs can be visualized as condensed foci of rDNA that lack associated Pol I and Pol I-specific factors and the secondary constriction that characterizes the decondensed, open state of rDNA (see **Figure 4**). Thus, it appears that in organisms containing multiple NORs, there are mechanisms that silence entire NORs, thereby maintaining this specific epigenetic state throughout the cell cycle and propagating it from one cell generation to the next.

Psoralen Cross-Linking Identifies Active rDNA Repeats

The proportion of rRNA genes that are actively transcribed can be identified by their susceptibility to the DNA cross-linking agent psoralen (Sogo & Thoma 2004). Psoralen is a drug that intercalates in double-stranded DNA and generates covalent interstrand links upon UV irradiation. Chromatin of active, euchromatic genes is accessible to psoralen cross-linking, whereas silent genes exhibit a compact heterochromatic structure that is not cross-linked by psoralen. Because cross-linked DNA migrates more slowly than non-cross-linked DNA in agarose gels, psoralen cross-linking can discriminate between nucleosomal and nonnucleosomal chromatin conformations. Psoralen cross-linking assays in a variety of organisms have shown that two classes of rRNA genes coexist in growing cells. Active genes are free of regularly spaced nucleosomes and are associated with nascent pre-rRNA (Conconi et al. 1989). Inactive gene copies are inaccessible

to psoralen, display regularly spaced nucleosomes, and are not associated with transcription factors and Pol I. The IGS is constitutively nucleosomal in both active and silent gene copies (Conconi et al. 1989, Dammann et al. 1993). In mouse cells, psoralen-accessible and -inaccessible rRNA genes are typically found in similar proportions; approximately half of rDNA repeats are active, and the other half are transcriptionally silent. The ratio of psoralen-accessible to psoralen-inaccessible rRNA genes is tissue-specific and stably propagated through the cell cycle (Conconi et al. 1989). Because more than 50% of NORs in most mammalian cells are active, both active and inactive rRNA genes likely associate with each other to form three-dimensional, higher-order structures within nucleoli.

With regard to the chromatin structure of active genes, electron microscopic studies and psoralen cross-linking experiments suggested that transcribed genes are devoid of nucleosomes in the pre-rRNA coding sequences (Sogo & Thoma 2004). However, recent studies have demonstrated that histones and chromatin remodeling activities are associated with transcriptionally active rRNA genes, indicating that Pol I is capable of elongating through chromatin (Jones et al. 2007, Tongaonkar et al. 2005, Yuan et al. 2007). However, it is unknown whether the nucleosome density is similar at transcribed and nontranscribed rDNA sequences and whether canonical histone octamers are present within the pre-rRNA coding region.

ACTIVE AND SILENT rRNA GENES EXIST IN DISTINCT EPIGENETIC STATES

DNA Methylation

DNA methylation at cytosine residues located 5' to a guanosine in a CpG dinucleotide is an epigenetic mark associated with gene silencing. Specific DNA methylation is mediated by DNA methyltransferases DNMT1, DNMT3a, and DNMT3b, and proper propagation of the

respective CpG methylation through cell division is critical for development and differentiation (Klose & Bird 2006, Meehan et al. 2001).

Regulatory elements and transcribed sequences in vertebrate rDNA are unusual in that they are both rich in CpG dinucleotides and densely methylated. Initial studies used methylation-sensitive and -insensitive restriction enzymes, such as *HpaII* and *MspI*, to determine the methylation status of CpGs within the sequence CCGG of mouse or rat rDNA (Bird et al. 1981, Santoro & Grummt 2001). These studies revealed an intriguing correlation between the proportion of active and inactive versus unmethylated and methylated rRNA genes, the fraction of methylated sequences corresponding to silent repeats (**Figure 5**). Moreover, cross-linking experiments demonstrated that methylated sites are predominantly present in the promoter and enhancer of inactive genes (Stancheva et al. 1997). Consistent with the results of psoralen cross-linking assays, ~40% of rRNA genes were resistant to cleavage with *HpaII* both in mouse liver and NIH3T3 cells. The intriguing correlation between promoter methylation and transcriptional silencing was further strengthened by the finding that treatment of cells with 5-aza-2'-deoxycytidine (aza-dC), a nucleotide analog that inhibits cytosine methylation, stimulated rDNA transcription, suggesting that lack of DNA methylation alleviates transcriptional repression of rDNA (Santoro & Grummt 2001). Importantly, methylation did not impair transcription on naked rDNA templates. However, when assembled into chromatin, methylated templates were not transcribed, indicating a mechanistic link between DNA methylation and chromatin-based processes. Analysis of wild-type and mutant templates in both transfection and in vitro transcription experiments revealed that methylation of one CpG dinucleotide at position -133 is sufficient to impair binding of UBF to nucleosomal rDNA, thereby preventing transcription complex assembly on preassembled chromatin templates. This finding suggests that cytosine -133 is exposed on the surface of the positioned

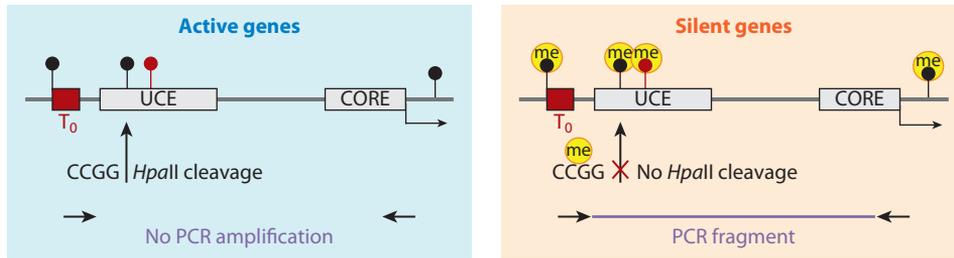


Figure 5

Active (*left*) and silent (*right*) rRNA genes can be distinguished by cleavage with methylation-sensitive restriction enzymes. The scheme illustrates the organization of the murine rDNA promoter, highlighting the position of the upstream terminator T_0 , the upstream control element (UCE), and the core promoter element (CORE). The transcription start site is marked by an arrow. The lollipops indicate the position of CpG residues at nucleotides -167 , -143 , -133 , and $+8$; the critical cytosine at -133 (see text for details) is colored red. If the CpG residues are unmethylated, the CCGG sequence at -143 is sensitive to *HpaII* digestion and cannot be amplified by PCR using a primer pair that covers sequences from $-160/-140$ (forward) and $-21/-1$ (reverse). On silent genes, the CpG residues are methylated and resistant to *HpaII* cleavage.

nucleosome and that the addition of a methyl group may represent an unfavorable sterical hinderance for UBF binding. These results imply that transcriptional silencing in mammals can be mediated or reinforced through an effect of DNA methylation on essential protein-DNA interactions that are needed for transcription initiation complex formation.

In human cells, the methylation status of rDNA, with 25 CpGs residing within the promoter, is more complex. Usually, human rDNA promoters exhibit a mosaic methylation pattern; i.e., they are neither completely methylated nor unmethylated but show methylation of a few to most CpGs (Ghoshal et al. 2004). Analysis of the methylation profile of human hepatocellular carcinomas or the colon cancer cell line HCT116 showed significant hypomethylation of the rDNA promoter in tumors compared with matched normal tissues, consistent with the elevated rRNA synthetic activity of rapidly proliferating cells (Ghoshal et al. 2004, Majumder et al. 2006). Importantly, hypomethylation of rRNA genes correlates with decreased genomic stability, suggesting that silencing entails the assembly of a generally repressive chromatin domain that is less accessible to the cellular recombination machinery.

Bisulfite sequencing of rDNA recovered from chromatin immunoprecipitations (ChIPs)

using antibodies directed against components of the Pol I transcription machinery revealed two classes of hypomethylated rDNA promoters. The first is an active fraction that is unmethylated over the entire promoter and is associated with both UBF and Pol I. In the other fraction, only the core promoter is unmethylated, and this fraction is associated with UBF but not with Pol I (Brown & Szyf 2007). Apparently, marking individual rDNA transcription units by specific methylation contributes to the stable propagation of a subset of active genes during cell proliferation and differentiation. Finally, the finding that in human cells approximately one-third of rDNA repeats exhibit a noncanonical arrangement (Caburet et al. 2005 and **Figure 3**) raises the possibility that noncanonical repeats may constitute a major fraction of methylated rRNA genes.

Histone Modifications Distinguish Active from Inactive rDNA Repeats

Modification of histones has become a key issue in our understanding of gene regulation. The core histones that make up the nucleosome are subject to numerous posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation. Most modifications localize at

specific positions within the N- and C-terminal histone tails. Some of the functional outcomes of these modifications are clear. Whereas lysine acetylation correlates with chromatin accessibility and transcriptional activity, lysine methylation can have different effects, depending on which residue is modified. For example, lysine 4 in histone H3 is trimethylated (H3K4me3) at the 5' ends of active genes, whereas trimethylation of H3K9 and H3K27 marks the promoter of heterochromatic, transcriptionally silent genes (for review, see Wang et al. 2007). These key modifications distinguish silent heterochromatin from permissive euchromatin and correlate with the activity status of rDNA repeats.

The ChIP technique has provided valuable insights into specific histone modifications associated with active and silent rDNA repeats. This technique, combined with the digestion of precipitated DNA with methylation-sensitive restriction enzymes, can be used to link particular histone modifications with transcriptional activity. Hypomethylated, active genes are associated with acetylated histones H4 and H3 as well as with H3K4me3 (Earley et al. 2006, Lawrence et al. 2004, Santoro & Grummt 2005, Santoro et al. 2002, Zhou et al. 2002). The promoter of hypermethylated, silent genes, in contrast, is associated with methylated H3K9, H3K20, and H3K27. As we discuss below, there is not always a strict division between active and repressive modifications, as for example for H3K9me3, indicating that the function of specific chromatin marks is more complex than previously thought. The current view is that histone modifications lay down positive- or negative-acting marks that recruit effector proteins such as heterochromatin protein 1 (HP1), causing structural changes to chromatin that affect the transcriptional outcome. Likewise, DNA methylation recruits repressors that specifically bind sites containing methylated CpG dinucleotides. DNMTs and DNA binding proteins that specifically recognize methylated cytosine residues interact with histone deacetylase corepressors and histone methyltransferases (for review, see Klose

& Bird 2006). As discussed below, methylation represses rDNA transcription by participating in the recruitment of histone deacetylases via methyl-CpG binding proteins or members of the methyl-CpG binding domain (MBD) protein family (Brown & Szyf 2007, Ghoshal et al. 2004).

TTF-I BOUND TO THE PROMOTER-PROXIMAL TERMINATOR RECRUITS CHROMATIN MODIFIERS TO rDNA

During replication, chromatin is erased and the epigenetic state has to be reestablished on the newly replicated daughter strands. This raises the question how the active state and the silent state of rRNA genes are established and maintained throughout cell division. A key player in the establishment and inheritance of a given epigenetic state at specific subsets of rDNA repeats is TTF-I (Bartsch et al. 1988, Grummt et al. 1986b). TTF-I is a multifunctional protein that binds to specific terminator elements downstream of the rDNA transcription unit and mediates transcription termination and replication fork arrest (Gerber et al. 1997, Grummt et al. 1986b). A similar terminator element, termed T₀ in mammals and T3 in frogs (Reeder 1999), is also present upstream of the transcription start site (**Figure 1**). The conservation of a binding site for a Pol I transcription terminator protein adjacent to the gene promoter suggested that TTF-I may also exert some essential function in transcription initiation. Indeed, binding of TTF-I (or the frog homolog Rib2) to the promoter-proximal terminator stimulates Pol I transcription *in vivo* (Henderson & Sollner 1986, McStay & Reeder 1990). Subsequent *in vitro* studies showed that TTF-I binding to the upstream terminator triggered structural alterations of the chromatin on preassembled nucleosomal templates, and these changes in chromatin structure correlated with activation of Pol I transcription *in vitro* (Langst et al. 1997, 1998). These results indicate that in the vicinity of the promoter, TTF-I influences

nucleosome positioning in such a way that allows transcription initiation to proceed. Furthermore, these findings suggest that TTF-I may recruit chromatin remodeling activities to rDNA that modify the promoter-bound nucleosome, thereby facilitating the access of transcription factors and Pol I.

COCKAYNE SYNDROME PROTEIN B: A CHROMATIN REMODELER THAT PROMOTES rDNA TRANSCRIPTION

Given that both TTF-I binding to the upstream terminator and nucleosome remodeling are required for activation of Pol I transcription on chromatin templates, it is reasonable to predict that TTF-I recruits a remodeling complex(es) and coactivators that establish euchromatic features at active rDNA repeats. A candidate for such a chromatin remodeler is CSB (Cockayne syndrome protein B), a DNA-dependent ATPase that is capable of chromatin remodeling and of disrupting protein-DNA interactions at the expense of ATP hydrolysis (Beerens et al. 2005, Citterio et al. 2000). Defects in CSB lead to the genetic disorder Cockayne syndrome (Laine & Egly 2006, Venema et al. 1990), and transcription is markedly reduced in cells from Cockayne syndrome patients in whom the CSB protein is mutated (Balajee et al. 1997, Dianov et al. 1997). CSB localizes in the nucleolus at sites of active rDNA transcription (Figure 6) and is part of a protein complex that contains Pol I, TFIIH, and basal Pol I transcription initiation factors (Bradsher et al. 2002). CSB interacts with TTF-I, a finding that suggests that TTF-I targets CSB to active rRNA genes. In support of CSB activating transcription on chromatin templates, overexpression of CSB stimulates rDNA transcription, whereas siRNA-mediated depletion of CSB impairs the assembly of transcription complexes and inhibits pre-rRNA synthesis (Yuan et al. 2007). Activation of Pol I transcription requires the ATPase activity of CSB, indicating that the chromatin remodeling activity of CSB promotes transcription through chromatin. Sig-

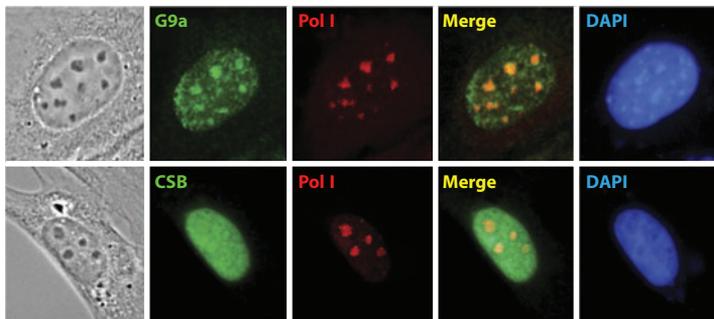


Figure 6

CSB (Cockayne syndrome protein B), G9a, and RNA polymerase I (Pol I) colocalize within nucleoli. The indirect immunofluorescence images show the localization of G9a, CSB, and Pol I in NIH3T3 cells. Phase contrast images are shown at the left. This figure is reproduced from Yuan et al. (2007) with permission from Elsevier.

nificantly, transcription activation by CSB depends on binding of TTF-I to the promoter-proximal terminator T_0 , a finding that demonstrates the synergism of TTF-I and CSB in chromatin-mediated processes and underscores the functional relevance of TTF-I in targeting positive-acting chromatin modifiers to rDNA.

Importantly, CSB-mediated activation of rDNA transcription requires association with G9a, a histone methyltransferase that is responsible for mono- and dimethylation of H3K9, and facilitates binding of heterochromatin protein 1 γ (HP1 γ), a protein containing a chromodomain that recognizes H3K9 methylation (Tachibana et al. 2002). Methylation of histone H3 at lysine 9 and HP1 association have well-established roles in heterochromatin formation (Bannister et al. 2001, Lachner et al. 2001, Peters et al. 2003, Rice et al. 2003). Therefore, the finding that G9a is associated with CSB and is required for Pol I transcription suggested additional surprising functions for H3K9 methylation and HP1 γ recruitment in chromatin-based processes. Notably, H3K9me2 and HP1 γ are present within the transcribed region of active rDNA repeats, and both H3K9 methylation and association of HP1 γ with rDNA are dependent on ongoing Pol I transcription (Yuan et al. 2007). This demonstrates that components of heterochromatin, such as

H3K9me2 and HP1 γ , play additional dynamic roles in establishing a chromatin structure that characterizes actively transcribed genes. This notion is in accord with recent studies that reveal novel roles for H3K9 methylation and HP1 γ in transcription activation. H3K9 di- and trimethylation occur in the transcribed region of all active mammalian Pol II genes examined. H3K9 methylation and HP1 γ binding are dynamic and require active transcription, increasing during transcription activation and being rapidly removed upon gene repression (Hediger & Gasser 2006, Piacentini et al. 2003, Vakoc et al. 2005). This finding demonstrates that the function of these chromatin marks is more complex than previously thought and suggests that these marks may serve distinct functions in transcription, depending on the context of other posttranslational histone modifications.

Important questions are how the active state of rRNA genes is maintained and how demethylated promoters may coexist with hypermethylated transcribed regions; such coexistence has been frequently observed in tumors (Yan et al. 2000). An interesting possibility is the involvement of specific proteins that either protect the promoters from methylation or target them for demethylation. A protein that may serve this function(s) is MBD3, a member of the MBD proteins, which bind to methylated DNA. MBD3 has two amino acid substitutions in the MBD domain that abolish binding to methylated DNA. Interestingly, MBD3 is associated with the rDNA promoter, and bisulfite mapping revealed that the fraction of rDNA bound to MBD3 is unmethylated (Ghoshal et al. 2004). Overexpression of MBD3 decreased methylation of the rDNA promoter, whereas knockdown of MBD3 increased methylation and decreased pre-rRNA synthesis (Brown & Szyf 2007). These results suggest that MBD3 plays an important role in maintaining rDNA promoters in an unmethylated state. In addition, silent rDNA repeats may be converted into active ones by demethylation. DNA demethylation can occur either

by a passive mechanism owing to progressive loss of methylated cytosines with each round of replication or by a process that actively removes the methyl group from CpG residues. An expression screen for an active demethylase has identified Gadd45a, an 18-kDa histone-fold protein that is involved in regulating proliferation, genomic stability, DNA repair, cell cycle, and apoptosis (Barreto et al. 2007). Overexpression of Gadd45a activates transcription of methylation-silenced reporter genes and induces global DNA hypomethylation in mammalian cells. Strikingly, Gadd45a also triggers demethylation of the rDNA promoter (K.-M. Schmitz, N. Schmitt, A. Schäfer, C. Niehrs, I. Grummt & C. Mayer, unpublished data). Preliminary results have shown that Gadd45a is associated with active rDNA copies and that DNA damage induces Gadd45a recruitment to rDNA, resulting in hypomethylation of the rDNA promoter and transient increase of Pol I levels. Conversely, depletion of Gadd45a or XPG, a component of the NER (nucleotide excision repair) machinery, leads to hypermethylation of rDNA and transient transcription silencing. Notably, rDNA demethylation depends on active Pol I transcription, emphasizing the importance of Gadd45a in maintaining the active state of rDNA.

Together, the available experimental evidence reveals a complex and coordinated interplay of the chromatin remodeler CSB, the histone methyltransferase G9a, and the methylation-sensitive proteins MBD3 and Gadd45a in the establishment of an epigenetically active, euchromatic structure at the rDNA promoter. CSB and G9a may promote Pol I transcription elongation by depositing a specific histone modification pattern that is recognized by other chromatin-modifying activities or by elongation factors that are required for transcription through chromatin. MBD3 and Gadd45a, in contrast, may protect the promoter from de novo methylation by DNMTs and therefore may be the key players that maintain the unmethylated state of rDNA.

NoRC: A CHROMATIN REMODELING COMPLEX THAT SILENCES rDNA

NoRC Establishes Heterochromatic Features at a Subset of rDNA Repeats

A yeast two-hybrid screen searching for TTF-I-interacting proteins that have the potential to alter the chromatin structure of the rDNA promoter has identified a chromatin remodeling complex, termed NoRC (nucleolar remodeling complex), which induces nucleosome sliding in an ATP-dependent and a histone H4 tail-dependent fashion (Strohner et al. 2001). NoRC is composed of two subunits, the ATPase SNF2h and a 205-kDa protein termed TIP5 (TTF-I-interacting protein 5). TIP5 shares a number of important protein domains with the large subunits of human SNF2-containing chromatin remodeling complexes ACF, WCRF, CHRAC, and WICH (Bochar et al. 2000, Bozhenok et al. 2002, Ito et al. 1999, LeRoy et al. 1998, Poot et al. 2000). Such shared domains include AT hooks; BAZ1, BAZ2, and WAKZ motifs; a C-terminal PHD (plant homeodomain); and a bromo-

domain (Figure 7). The bromodomain and an adjacent PHD finger form a cooperative unit that has been found in several transcriptional corepressors (Schultz et al. 2001). Consistent with a repressor function, NoRC inhibits Pol I transcription on preassembled chromatin templates in vitro (Strohner et al. 2004), and overexpression of TIP5 in human and mouse cells repressed rDNA transcription in a concentration-dependent manner (Santoro et al. 2002). Transcriptional repression did not occur in the presence of inhibitors of DNMTs and histone deacetylases, such as aza-dC and TSA, implicating NoRC in repressing rDNA transcription by inducing DNA methylation and histone deacetylation. Indeed, subsequent biochemical studies have demonstrated that NoRC physically interacts with DNMT1 and DNMT3 as well as with the Sin3 corepressor complex, which contains the histone deacetylases HDAC1 and HDAC2 (Santoro et al. 2002, Zhou et al. 2002). As a consequence of NoRC interacting with DNMTs and specific corepressors, a subset of rDNA repeats is silenced, and specific epigenetic marks are propagated throughout cell divisions. This indicates that

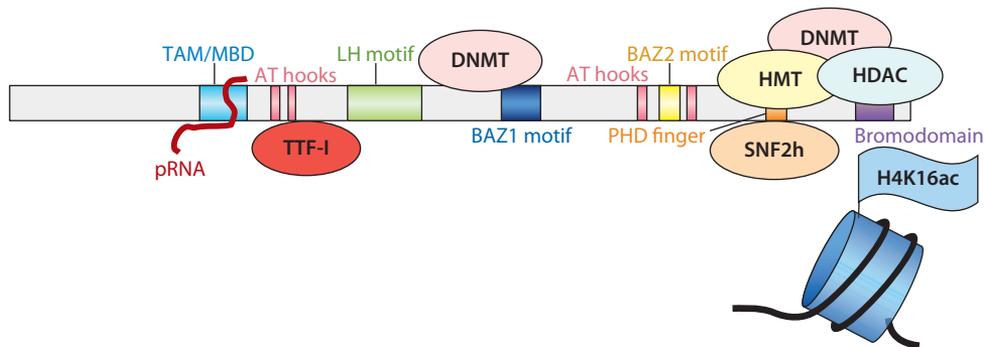


Figure 7

Modular organization and domains of TIP5 (TTF-I-interacting protein 5). Scheme illustrating the modular organization and localization of sequence motifs in TIP5 that have been associated with functions in chromatin structure and function. The domains of TIP5 (colored boxes) that interact with proteins involved in the epigenetic control of gene expression are illustrated. The C-terminal part of TIP5 contains a PHD (plant homeodomain) finger that interacts with SNF2h and with histone methyltransferases (HMTs) and a bromodomain that interacts with histone deacetylases (HDAC1 and -2) and with histone H4 acetylated at lysine 16 (H4K16ac). DNA methyltransferases (DNMTs) interact with both the internal and the C-terminal part of TIP5. The MBD (methyl-CpG binding domain)-like TAM (TIP5/ARBD/MBD) domain is required for association with small intergenic transcripts (pRNA) that are required for nucleolar remodeling complex (NoRC)-mediated heterochromatin formation.

NoRC serves as a scaffold, coordinating the activities of macromolecular complexes that modify histones, methylate DNA, and establish a closed heterochromatic chromatin state.

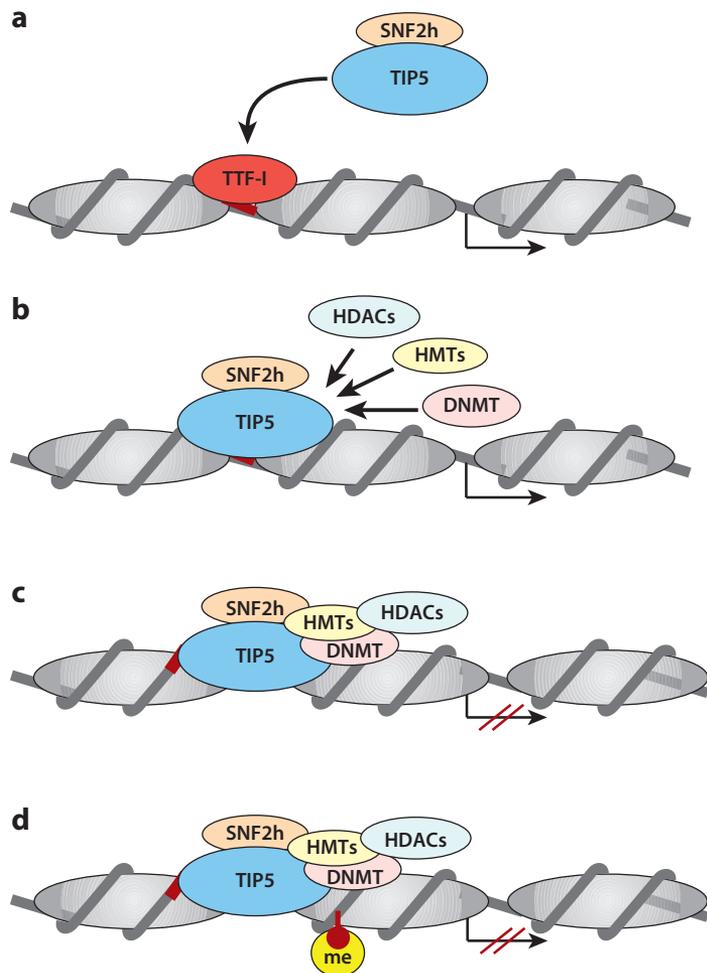


Figure 8

Model depicting individual steps of rDNA silencing. (a) First, NoRC is recruited to the rDNA promoter by TTF-I bound to the promoter-proximal terminator T_0 . (b) In a subsequent step, NoRC interacts with the Sin3 corepressor complex, leading to deacetylation of histones H3 and H4 and with histone methyltransferases (HMTs) that methylate H3K9, H3K20, and H3K27. (c) These heterochromatic histone modifications may act as a signal for the ATPase SNF2h to shift the promoter-bound nucleosome 25 nt further downstream into a translational position that is unfavorable for preinitiation complex formation. (d) The action of SNF2h may either relieve a steric constraint or expose CpG at -133 to methylation by DNA methyltransferases (DNMTs). Methylation of CpG at -133 in the context of chromatin impairs UBF binding and preinitiation complex assembly.

Figure 8 illustrates a model depicting the current view of the individual steps and the complex interplay of multiple protein complexes in the formation of the epigenetically silent state of rRNA genes. As a first step, TTF-I bound to the promoter-proximal terminator T_0 interacts with TIP5, and this interaction targets NoRC to the rDNA promoter. NoRC in turn recruits histone modifiers and DNMTs to rDNA, leading to repressive histone modifications and specific methylation of the promoter. The resulting modifications alter the nucleosome surfaces, which then may recruit other regulatory proteins, for example, HP1, leading to spreading of heterochromatic marks into the body of the gene. Moreover, methylation of a critical CpG residue within the rDNA promoter (at position -133) prevents binding of the basal transcription factor UBF to chromatin, which leads to impaired preinitiation complex formation and repression of Pol I transcription (Santoro & Grummt 2001, Santoro et al. 2002). Although attractive, this model is probably an oversimplification because of the interdependence and functional interplay of different epigenetic layers affecting the transcriptional output. In addition to requiring histone deacetylation and DNA methylation, repression of previously active rDNA repeats would be predicted to require a histone H3K4 demethylase activity. Indeed, recent work has demonstrated that the histone demethylase JHDM1B is located in the nucleolus and triggers H3K4 demethylation and transcriptional repression (Frescas et al. 2007). As yet there are no clues to how JHDM1B is targeted to rDNA or to how it relates to NoRC-mediated silencing.

NoRC Alters the Translational Position of Nucleosomes at the rDNA Promoter

Active and silent rDNA copies are characterized both by distinct epigenetic marks and by different nucleosome positions. At potentially active genes, a nucleosome occupies sequences from -157 to the transcription start

site, whereas at silent genes the nucleosome covers sequences from -132 to $+22$, indicating that specific nucleosome positions determine the transcriptional readout of rRNA genes (Li et al. 2006). This is consistent with several studies demonstrating the importance of nucleosome positioning in the organization of nucleoprotein complexes at promoters and regulatory elements (Simpson 1991). Positioned nucleosomes may either occlude or facilitate binding of basal transcription factors to chromatin, thereby repressing or activating transcription. In some cases, nucleosomes are positioned as a consequence of specific factor binding, whereas in other cases certain DNA sequences can position nucleosomes *in vitro* (Rando & Ahmad 2007). However, most of the sequences identified *in vitro* fail to precisely position nucleosomes *in vivo*, suggesting that in addition to DNA structure and flexibility, other mechanisms define nucleosome positioning in cellular chromatin.

With regard to silent rDNA copies, NoRC is the remodeling complex that shifts the promoter-bound nucleosome into the silent position (Li et al. 2006). In the silent position (covering sequences from -132 to $+22$), both the UBF binding site and the functionally important CpG residue at nucleotide -133 are placed into the nucleosomal linker region. The core element, in contrast, has been moved inside the nucleosome, and the relative alignment of the DNA element with respect to the histone octamer surface has been changed. As a consequence, the core promoter is less accessible for binding of transcription factors. Thus, whereas at active genes the nucleosome juxtaposes the core promoter and the UCE, both sequence elements are separated at silent genes, prohibiting the cooperative binding of UBF and SL1/TIF-IB, the factors that nucleate preinitiation complex assembly. The identification of NoRC as the major determinant of the silent nucleosome position suggests that remodeling complexes are the major determinants of chromatin dynamics and are capable of defining a specific chromatin structure. This dual function may also explain

why remodeling complexes are so diverse and abundant in the cell. Differential gene regulation by specifically positioned nucleosomes is an attractive mechanism that would allow the cell to keep a high signal-to-noise ratio of DNA-dependent processes and to reduce the complexity of regulation by establishing chromatin structures that allow or prevent binding of transcription factors to regulatory sequences. NoRC—the key player in epigenetic silencing of rDNA—coordinates several enzymatic processes, including histone deacetylation and methylation, ATP-dependent chromatin remodeling, and DNA methylation, to establish a closed chromatin structure and block initiation complex formation.

INTERGENIC RNA IS REQUIRED FOR rDNA SILENCING

Recent analyses of mammalian transcriptomes have revealed that the majority of the genomes of mammals and other complex organisms are transcribed into noncoding (nc)RNAs. ncRNAs have an important role in the epigenetic control and in the modulation of gene expression, tissue-specific patterning, and cell fate specification. To date, siRNAs and miRNAs have received the broadest attention owing to their universal applicability in regulating gene expression. However, there is increasing evidence that intergenic and antisense transcripts control gene expression via sequence-specific interactions with regulatory proteins. In mouse cells, transcripts originating from a promoter within the IGS that is located ~ 2 kb upstream of the pre-rRNA transcription start site have been shown to play an important role in heterochromatin formation and rDNA silencing (Mayer et al. 2006, Moss et al. 1980). Transcripts from the IGS are synthesized from a subfraction of rDNA repeats by Pol I (R. Santoro & I. Grummt, unpublished results) and usually do not accumulate *in vivo* (Kuhn & Grummt 1987, Morgan et al. 1983, Paalman et al. 1995). Presumably, these < 2 -kb transcripts are processed into shorter intermediates that are either rapidly degraded or shielded from further

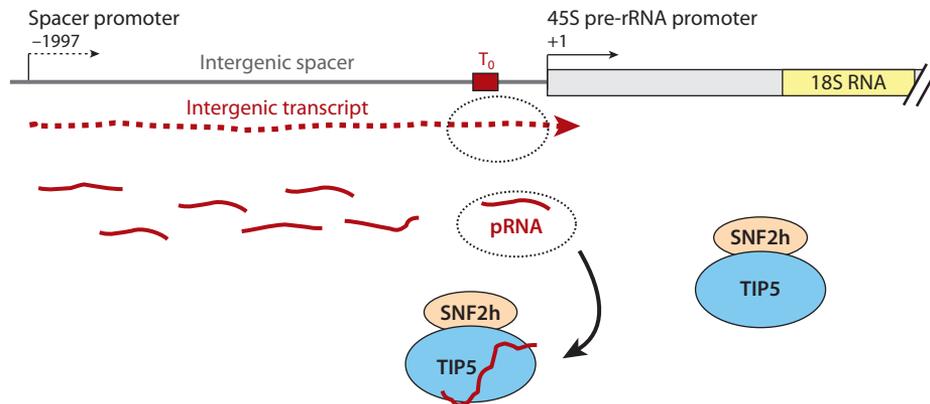


Figure 9

Model depicting the role of promoter-associated RNA (pRNA) in rDNA silencing. Intergenic transcripts (*dotted line*) are synthesized from a spacer promoter located ~2 kb upstream of the major 45S pre-rRNA promoter. The primary intergenic transcripts are degraded or processed by an as-yet-unknown mechanism. Transcripts of 150–300 nt that match the rDNA promoter (pRNA) bind to TIP5 (TTF-I-interacting protein 5) via the MBD (methyl-CpG binding domain)-like TAM (TIP5/ARBD/MBD) domain. Association with pRNA is required for NoRC-mediated heterochromatin formation.

degradation by binding to NoRC. In support of this, a population of processed 150–300-nt IGS transcripts, whose sequence matches the rDNA promoter and therefore have been dubbed pRNA (promoter-associated RNA), is stabilized by binding to TIP5, the large subunit of NoRC. RNase treatment and RNA replenishment experiments have demonstrated that pRNA is an important determinant for NoRC function because it is indispensable for heterochromatin formation and rDNA silencing (**Figure 9**).

The interaction of NoRC with RNA is mediated by the TAM (TIP5/ARBD/MBD) domain of TIP5, a motif that exhibits sequence homology to the MBD in proteins that recognize methyl-CpG. Mutations in the TAM domain abrogate the interaction of TIP5 with RNA, impair NoRC binding to chromatin, and prevent heterochromatin formation. Surprisingly, however, mutant TIP5 can still recruit DNMTs, leading to *de novo* CpG methylation and transcriptional silencing. This finding demonstrates that DNA methylation rather than repressive chromatin modifications causes silencing. Moreover, it indicates that the establishment of repressive histone

marks and transcriptional silencing can be uncoupled.

Regarding the function of pRNA in rDNA silencing, depletion of pRNA by antisense LNA/DNA oligonucleotides had several severe consequences, leading to displacement of NoRC from nucleoli, decreased rDNA methylation, and enhanced pre-rRNA synthesis (Mayer et al. 2006). Moreover, recent results indicate that pRNA has an architectural capacity, playing a role either as a scaffold or an allosteric effector of NoRC in the epigenetic control of rDNA transcription. pRNA folds into a phylogenetically conserved secondary structure that is recognized by TIP5 and is required for both localizing NoRC to nucleoli and for rDNA silencing. Mutations that disrupt the stem-loop structure impair binding of NoRC to pRNA and abolish targeting of NoRC to nucleoli, whereas the introduction of compensatory base changes restores the interaction with TIP5 (Mayer et al. 2008). Thus, NoRC recognizes a specific secondary or tertiary RNA conformation rather than specific sequence information. These results reveal an RNA-dependent mechanism that targets NoRC to chromatin and facilitates the interaction with

corepressors that promote heterochromatin formation and silencing. RNase footprinting and protease sensitivity experiments suggest that TIP5 binds pRNA in an induced-fit mechanism, resulting in structural changes that may facilitate NoRC function. Another possibility that must be considered is that NoRC, by virtue of its MBD-like domain, may aid in guiding pRNA to recruit chromatin-modifying enzymes that silence the rDNA locus. In this scenario, pRNA would direct chromatin modifications by base pairing with complementary DNA sequences.

Future studies will show whether the role of intergenic RNA is restricted to NoRC-dependent silencing or whether RNA cooperates with TTF-I to target transcription activators, such as CSB or WSTF (Percipalle et al. 2006), to rDNA. As TTF-I recruits both NoRC and CSB to the rDNA promoter, this factor is the key player triggering the chain of events by which the active or silent state of rRNA genes

is established (Figure 10). Whether the epigenetic state is spread into adjacent genes is unknown. Given that the epigenetic balance between active and silent rRNA genes is crucial not only for rRNA synthesis but also for genomic stability, it is probable that the interaction of TTF-I with NoRC and CSB (and possibly other factors) is efficiently regulated to avoid cellular transformation and malignancy.

THE ROLE OF UBF IN MAINTAINING A SPECIFIC CHROMATIN STRUCTURE

As discussed above, active rRNA genes display a specialized decondensed chromatin structure that is associated with the presence of a secondary constriction at active NORs in most or all eukaryotes (McStay 2006). This decondensed chromatin retains the Pol I transcription machinery during mitosis, thereby facilitating the rapid resumption of ribosome

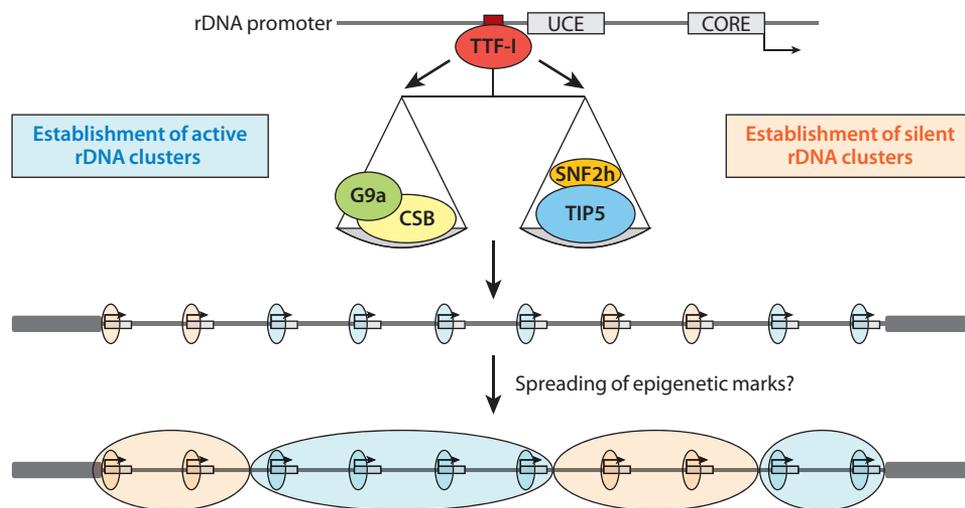


Figure 10

Cockayne syndrome B protein (CSB) and nucleolar remodeling complex (NoRC) establish the balance of active and silent clusters of rRNA genes. TTF-I bound to its target site T_0 upstream of the gene promoter (red box) interacts with either CSB or TIP5 (TTF-I-interacting protein 5), thereby recruiting CSB—together with associated G9a—or NoRC to rDNA. The balance of CSB and NoRC association with rRNA genes determines the ratio of euchromatic active (blue) to heterochromatic silent (orange) genes. Once the epigenetic state is established at the 5'-terminal part of the transcription unit, the respective chromatin structure is propagated throughout the rDNA repeats by an as-yet-unknown spreading mechanism. CORE, core promoter element; UCE, upstream control element.

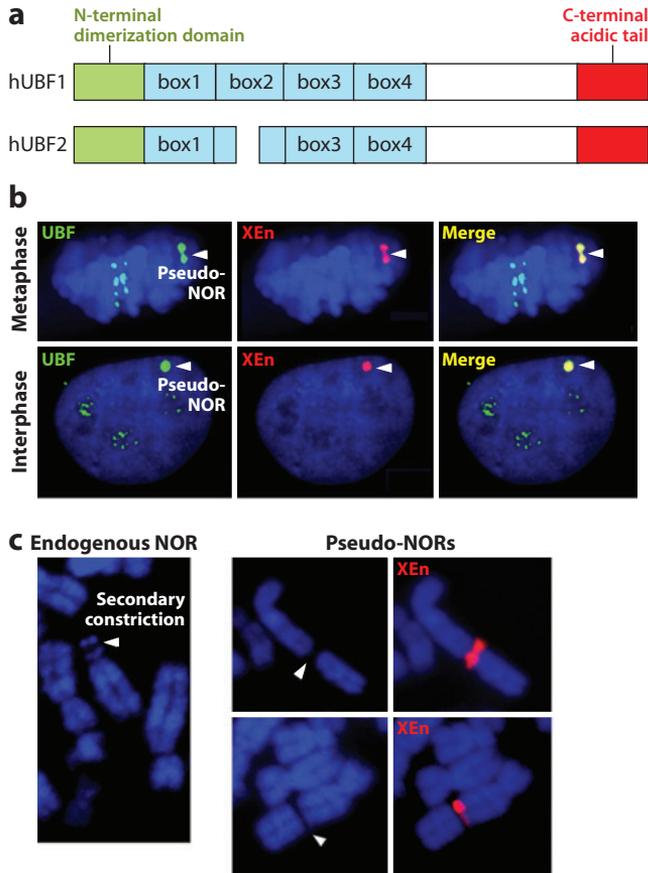


Figure 11

Extensive upstream binding factor (UBF) binding underpins rDNA undercondensation at active NORs. (a) Depiction of human UBF1 and -2 (hUBF1 and hUBF2, respectively) showing HMG (high mobility group) boxes 1–4, which mediate DNA binding; the N-terminal dimerization domain (green); and the C-terminal acidic tail (red). (b) Pseudo-NORs are bound by UBF throughout the cell cycle. A human cell line (3D-1) containing a 1.4-Mb array of a heterologous UBF binding sequence, *Xenopus* enhancer (XEn), on chromosome 10, was subjected to combined immuno-FISH. XEn DNA was visualized by hybridization with a spectrum-red-labeled probe derived from the intergenic spacer. UBF was visualized with antibodies coupled to FITC (green). Upper panels show a cell in metaphase, and lower panels a cell in interphase. Nuclei were visualized by DAPI staining (blue). For details, see Mais et al. (2005). (c) Pseudo-NORs are ectopic UBF binding site arrays that appear as novel secondary constrictions during metaphase. Chromosome spreads from the above cell line were probed with XEn DNA (red) and stained with DAPI. Note the lack of staining (secondary constrictions, indicated by arrowheads) observed over XEn sequences in the panels on the right. For comparison, a single panel on the left shows an acrocentric chromosome with a secondary constriction.

biogenesis as cells enter the G1 phase of the cell cycle. Apparently, the association of Pol I and transcription factors, such as UBF, gives active repeats a unique conformation that resists condensation to the same degree as do adjacent chromosomal regions (Prieto & McStay 2007).

UBF is an abundant protein that persists at NORs throughout metaphase and is absent from transcriptionally silent NORs (Roussel et al. 1993, Wright et al. 2006). UBF is a member of HMG (high mobility group) proteins, containing six HMG box DNA binding motifs, at least four of which are involved in DNA binding (Figure 11a) (Jantzen et al. 1990, 1992; McStay et al. 1991; Reeder et al. 1995). A characteristic feature of the HMG box DNA binding motif is its ability to bend DNA. Multiple HMG boxes present in a dimer of UBF can organize naked, i.e., nucleosome-free, DNA into a 360° loop, establishing a structure that resembles the core nucleosome in both mass and DNA content (Bazett-Jones et al. 1994, Putnam et al. 1994). In vitro DNA binding assays have failed to identify a consensus other than a preference for binding to GC-rich sequences (Copenhaver et al. 1994). This apparent lack of sequence specificity contrasts greatly with its highly specific targeting to rDNA repeats throughout the cell cycle.

UBF binds throughout the IGS and the pre-rRNA coding region, suggesting that UBF plays an important structural and functional role on active NORs (O'Sullivan et al. 2002). This hypothesis has been supported by the demonstration that arrays of heterologous binding sites for UBF trigger the formation of ectopic secondary constrictions when integrated into novel, non-NOR-bearing human chromosomes (Mais et al. 2005). The largest of these arrays were ~2 Mb in length, approximating the size of endogenous NORs. These ectopic UBF binding sites, termed pseudo-NORs, were associated with UBF throughout the cell cycle and adopted the key morphological features of active NORs during metaphase; i.e., they were undercondensed, appearing as achromatic regions on DAPI-stained chromosomes (Figure 11b,c). Notably, pseudo-NORs

were transcriptionally inert but positive in silver staining. Thus, the appearance of secondary constrictions at NORs on metaphase chromosomes is not due to rDNA transcription or certain structural features impeding chromosome condensation, but rather to binding of argyrophilic proteins to rDNA. In accord with this finding, depletion of UBF by siRNA led to loss of secondary constrictions and silver staining at pseudo-NORs (Prieto & McStay 2007). Thus, besides its role in Pol I transcription, UBF plays an important role in promoting undercondensation of active NORs and maintaining the active chromatin structure through cell divisions. Strikingly, although pseudo-NORs do not contain rDNA promoters and do not support transcription, they can recruit the entire Pol I transcription machinery and components of the pre-rRNA processing machinery (Mais et al. 2005, Prieto & McStay 2007). These results suggest that UBF binding establishes a chromatin structure that facilitates the rapid reformation of nucleoli around active NORs as cells exit mitosis.

That UBF occupancy is observed across the entire rDNA repeat raises the question as to whether UBF binding and the presence of nucleosomes are compatible. Clearly, the resolution of ChIP experiments is not sufficient to distinguish whether UBF binds to nucleosomal DNA or whether cooperative binding of UBF loops DNA into a structure, termed enhanceosome, that is free of nucleosomes (Stefanovsky et al. 2001). However, several lines of evidence support the view that UBF binds to nucleosomal DNA *in vivo*. First, a positioned nucleosome that encompasses the UBF binding sequence is located on active promoters (Langst et al. 1998, Li et al. 2006). Second, the UBF binding sequences that form pseudo-NORs yield a classical nucleosomal ladder when digested with micrococcal nuclease (Wright et al. 2006). Moreover, pseudo-NORs can be readily visualized when cells are stained with antibodies against acetylated histone H4, consistent with pseudo-NORs exhibiting a euchromatic structure (J. Wright & B. McStay, unpublished observation).

Regarding the mechanism by which the secondary constriction characteristic for active NORs is generated, there is evidence that UBF competes with histone H1, leading to chromatin decompaction. The interaction of the linker histone H1 with nucleosomes stabilizes compact higher-order chromatin structures and impedes the access of regulatory factors (for review, see Catez et al. 2006). UBF can displace histone H1 from histone octamers *in vitro*, thereby promoting decompaction of chromatin (Kermekchiev et al. 1997). In support of this, RNAi-mediated depletion of UBF increases the level of histone H1 on rDNA and decreases the fraction of rDNA that is accessible to psoralen cross-linking (E. Sanij & R. Hannan, personal communication). These results indicate that UBF binds to rDNA on the surface of nucleosomes, similar to what has been proposed for the HMG box protein HMGB (Travers 2003). UBF binding to chromatin leads to a less compact chromatin structure that is accessible to Pol I and transcription factors, suggesting that UBF can maintain rDNA in a euchromatic, transcriptionally active state throughout the cell cycle. Because UBF does not bind to methylated rDNA repeats and silent NORs, it is not likely to be directly involved in the decision process that determines whether or not a given cluster of rRNA genes can be transcribed.

HETEROCHROMATIN IS IMPORTANT FOR NUCLEOLAR STRUCTURE

Heterochromatin plays an essential role in nuclear organization and chromosome structure. For example, centromeric heterochromatin on different chromosomes can fuse to form chromocenters during interphase (Hsu et al. 1971), indicating the involvement of centromeric heterochromatin in chromosome segregation (Bernard & Allshire 2002). Heterochromatin also plays a role in maintaining the structure of nucleoli and the integrity of rDNA repeats. Typically, nucleoli stain poorly with the fluorescent dye DAPI but are surrounded by a shell of intensely stained

heterochromatin. This so-called perinucleolar heterochromatin is composed of satellite DNA that surrounds NORs and silent rDNA clusters located on either active or silent NORs (Sullivan et al. 2001). A link between heterochromatin formation and the nucleolus is further strengthened by the observations that heterochromatin from non-NOR-bearing chromosomes associates with nucleoli (Manuelidis & Borden 1988) and that the inactive X chromosome requires transient association with nucleoli to facilitate heterochromatin formation (Zhang et al. 2007).

It is surprising that—despite its tight association with silent heterochromatin—the nucleolus is the transcriptionally most active cellular organelle. This apparent paradox suggests that heterochromatin serves an important role in nucleolar function. Indeed, there is evidence that heterochromatin prevents homologous recombination between rDNA repeats, thereby preserving nucleolar structure and rDNA stability. Loss of silencing correlates with rDNA instability, nucleolar disintegration, and cellu-

lar senescence. Among the key players that ensure rDNA stability are the NAD⁺-dependent histone deacetylase Sir2 (Silent information regulator 2) and the histone methyltransferase Su(var)3-9 (Suppressor of variegation 3-9). In *Drosophila*, inhibition of H3K9 methylation by mutation of *Su(var)3-9* destabilizes rDNA, leading to the excision of rDNA repeats and the generation of extrachromosomal rDNA circles (Peng & Karpen 2007). Likewise, mutations in yeast *Sir2* lead to increased rDNA instability and shortening of replicative life span (Sinclair & Guarente 1997). Finally, somatic knockout of DNMT1 in human cells leads to severe rDNA demethylation, which is accompanied by enhanced binding of the Pol I transcription machinery to rDNA and profound disorganization of the nucleolus (Espada et al. 2007). These results underscore the functional relevance of specific heterochromatic histone modifications and DNA methylation in deciding which rDNA copies are to be transcribed, and reveal a mechanism that determines how local chromatin structure can regulate genome stability.

SUMMARY POINTS

1. Mammalian genomes contain hundreds of rRNA gene (rDNA) repeats that are located at several nucleolar organizer regions (NORs). Active NORs form secondary constrictions on metaphase chromosomes.
2. Eukaryotic cells contain two epigenetically distinct classes of rRNA genes, one that exhibits euchromatic features and is permissive to transcription and another that has a heterochromatic conformation and is transcriptionally refractive.
3. Active and silent rDNA clusters can be distinguished by their pattern of DNA methylation, specific histone modifications, and distinct nucleosome positions.
4. The transcription termination factor TTF-I bound to the promoter-proximal terminator T₀ is the key player in establishing the epigenetically active or silent state of rDNA, recruiting either an activating [Cockayne syndrome protein B (CSB)] or a repressive (NoRC) chromatin remodeling complex.
5. CSB interacts with the histone methyltransferase G9a and HP1 γ , thereby triggering the chain of events that establish or maintain the active state of rDNA.
6. Interaction with TIP5 (TTF-I-interacting protein 5), the large subunit of the nucleolar remodeling complex NoRC, mediates heterochromatin formation and silencing by recruiting histone-modifying and DNA-methylating activities and shifting the promoter-bound nucleosome into a silent position. The fine-tuned balance between

the activating chromatin remodeler CSB and the repressive NoRC complex establishes the ratio of active to silent rDNA repeats.

7. Binding of UBF across rDNA repeats is essential for maintaining the active chromatin state throughout the cell cycle and is responsible for the formation of secondary constrictions on metaphase chromosomes.
8. Heterochromatin formation and transcriptional silencing play an important role in maintaining the structural integrity of nucleoli and genetic stability of rDNA repeats.

FUTURE ISSUES

1. Why do eukaryotes contain more rRNA genes than are needed in proliferative cells?
2. What are the mechanisms that determine CSB and NoRC recruitment and hence decide between silencing or transcriptional competence?
3. Do CSB and NoRC regulate individual rRNA genes or entire NORs?
4. Which pathways regulate the level and/or the activity of CSB and NoRC and therefore control the epigenetic state and copy usage of rDNA?
5. How is a given epigenetic state inherited, and which mechanisms are involved in spreading specific histone modifications and DNA methylation patterns across clusters of rRNA genes?
6. What is the functional impact of keeping a certain ratio of active to silent rDNA repeats throughout cell division, and how is this ratio altered during cell differentiation, senescence, and cancer?
7. How are variant and noncanonical rDNA repeats distributed among NORs, and which cell-type-specific processes select a subtype of rDNA to be transcribed?
8. How does UBF promote chromatin decondensation and the formation of secondary constriction at metaphase chromosomes?
9. Does the chromosomal context play a role in regulating the activity of individual NORs?

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Errata

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