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Review

The silence of the ribosomal RNA genes

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Abstract. Over the past decade emerging evidence has indicated that epigenetic factors control and regulate nuclear processes. The genes encoding ribosomal RNA (rRNA) represent an ideal model to study how epigenetics and chromatin can modulate gene expression. The reason for this is that in each cell, the rRNA genes exist in two distinct types of chromatin structure: an 'open' one corresponding to transcriptionally active genes and a 'closed' one representing the silent genes. Recent studies indicate that an epigenetic network mediates the transcriptional state of rDNA. Interplay of DNA methylation, histone modification and chromatin-remodeling activities establishes silencing at the rDNA locus in higher eukaryotes as well as at the underdominant genes in hybrid cells. The aim of this review is to summarize current knowledge about the active and silent states of rRNA genes and of nucleolar organizing regions and to analyze the mechanisms involved in the establishment and inheritance of rDNA silencing.

Key words. NOR; histone and DNA modification; NoRC; nucleolar dominance.

Introduction

Growing cells require continuous ribosome synthesis to ensure that subsequent generations contain sufficient ribosomes to support protein synthesis. The control point in the complex process of ribosome biogenesis is transcriptional regulation of ribosomal (rRNA) genes, which encode ribosomal RNA and are transcribed by the dedicated transcription machinery of RNA polymerase I (Pol I). Transcription of rDNA generates rRNA precursors (pre-rRNA, 45S in mouse, 47S in human) that are subsequently cleaved and processed into 28S, 18S and 5.8S rRNAs. These rRNAs are then packaged with ribosomal proteins to form the large and small subunits of ribosomes.

A specific set of transcription factors is dedicated to transcription of rDNA into pre-rRNA. Preinitiation complex formation requires the synergistic action of the upstream binding factor UBF and the promoter selectivity factor, termed TIF-IB in mouse and SL1 in humans [reviewed in ref. 1]. The assembly of a productive transcription initiation complex is achieved by the interaction of UBF with PAF53, the mammalian homologue of the yeast Pol I subunit A49, and by interaction of TIF-IB/SL1 with the transcription factor TIF-IA [2–4]. Although there is a certain homology between the components of the Pol I transcriptional machinery from different species, the rDNA promoter sequences from mammalian, yeast and amphibians are in some degree divergent. However, the modular architecture is in most cases evolutionarily conserved, consisting of a 150-bp-long DNA sequence containing two control elements, the upstream control element (UCE) and the core. Apparently, a structural code, in addition to primary sequences, directs specific protein/DNA interactions at the rDNA promoter and may play important functions in transcriptional control [1].

An actively cycling eukaryotic cell expends between 35 and 60% of its total nuclear transcription effort in making ribosomal rRNAs. Changes in this commitment are likely to have extensive repercussions on the cellular economy, limiting proliferation rates and perhaps even cell fate [ref. 5 and references therein]. One likely explanation for

the high levels of rRNA synthesis in the cell is the presence of large numbers of rDNA copies per genome, ranging from less than 100 to more than 10,000 [6]. Alternatively, the efficient initiation of transcription by Pol I and its associated factors can meet the demand for high levels of rRNA transcripts. Several results indicate that there is not a good correlation between the cellular growth rate and the number of rRNA genes. Two yeast strains containing different numbers of rRNA genes [140 and 42] display similar rRNA transcription levels [7]. Electron microscopy analysis has shown that similar amounts of RNA polymerase I are engaged in transcription in both strains, a strong indication that it is the initiation rate and not the number of transcribed genes that determines the level of rRNA transcription in exponentially growing yeast [7]. Consistent with this, maize inbred lines can vary almost tenfold in their rRNA gene content (2.5×10^3 and 2.4×10^4 rRNA genes in a diploid) yet have similar morphological characteristics and growth rates [8]. The same is true for aneuploid chicken cells that contain different numbers of rRNA copies and display the same levels of rDNA transcription [9].

Early electron microscopic studies revealed that not all rRNA genes are loaded with RNA polymerases, indicating that a fraction of the rRNA genes is not transcribed [10, 11]. Even when there is a high demand for rRNA synthesis, i.e. during cellular growth and proliferation, a fraction of rRNA genes remains transcriptionally silent. Consistent with this, in vivo crosslinking with psoralen, an intercalating drug that can introduce crosslinks into DNA sites that are not protected by nucleosomes, showed that two classes of rRNA genes co-exist in each cell: one accessible and the other inaccessible to psoralen [12, 13]. Further analysis indicated that the fraction of genes accessible to psoralen displays a chromatin structure free of regularly spaced nucleosomes, which represents the active genes. In contrast, the fraction of genes inaccessible to psoralen exhibits a chromatin structure with regularly spaced nucleosomes that corresponds to the silent genes [13]. The relative amount of these two chromatin structures is similar both in growing and resting cells as well as during interphase and metaphase, indicating that these two chromatin states are stably propagated throughout the cell cycle and maintained independently of transcriptional activity [13].

Research over the past decade has revealed that the chromatin structure is far more than a static carrier of the genetic information encoded in DNA as it actively and dynamically mediates regulation of processes requiring protein access to DNA, i.e. transcription, recombination and replication. Emerging evidence indicates that epigenetic factors control and regulate most nuclear processes. These factors alter chromatin structure by covalent DNA modifications, covalent histone modifications and nucleosome reorganization. These changes seem to be interpreted by proteins that recognize a particular modification and facilitate the appropriate downstream biological effect [14]. The co-existence in the same cell of two distinct rDNA chromatin structures makes the rRNA genes an ideal model to study how chromatin can modulate gene expression.

The aim of this review is to summarize current knowledge about the active and silent states of rRNA genes and to analyze the mechanisms involved in the establishment and inheritance of rDNA silencing.

Active and silent nucleolar organizing regions

rDNA transcription generates pre-RNAs that are processed, modified and assembled with ribosomal proteins, which then form the ribosome subunits. The biogenesis of ribosomes involves a large number of complexes at each step and the consequence of this activity is the formation of a distinct sub-nuclear structure, the nucleolus [15]. The nucleolus is the result of the fusion of several nucleolar organizing regions (NORs), located on the short arm of acrocentric chromosomes. For example, in humans, the 400 copies of rRNA genes are distributed in a non-uniform manner on acrocentric chromosomes 13, 14, 15, 21 and 22 [16]. Nucleolus formation represents the prototype of dynamic nuclear organization involving chromosome territories, providing a striking link between specialized transcription and nuclear compartmentalization. In vertebrates, at the onset of mitosis, the nucleolus disintegrates and rDNA transcription ceases [ref. 17 and references therein]. Mitotic silencing of rDNA transcription occurs from prophase to telophase and is accompanied by an inhibitory phosphorylation of the transcription factor SL1 directed by cdk1-cyclin B, a strong indication that regulation occurs at the level of transcription initiation [18, 19]. On metaphase chromosomes, NORs can be identified as secondary constrictions and, due to the abundance of argyophilic proteins, they can be visualized by silver staining [20]. Because of these characteristics, these structures are often termed AgNORs. However, not all NORs form secondary constrictions or can be silver stained during metaphase. The number of silver-positive NORs varies between four and ten [21] and in dividing HeLa cells, usually six out of ten NORs can be silver stained [22]. Several results suggest that AgNORs contain the active rRNA genes. During metaphase, NORs that are transcriptionally active in the previous interphase form secondary constrictions and they can be silver stained [23]. Moreover, despite mitotic compaction of the genome, AgNORs display a chromatin structure that is ten-fold less condensed than the rest of the chromosomes [24, 25]. This ‘defect’ of mitotic rDNA condensation and the consequent retention of an ‘open’ chromatin structure has been proposed to be an advantage

for early transcription of rDNA at the exit from mitosis. This idea implies that during mitosis, in the absence of transcription, specific proteins are associated with NORs depending on their function during interphase. In support of this, components of the Pol I transcription machinery, such as RNA polymerase I, the upstream binding factor UBF and the TATA-binding protein-containing factor SL1, are still bound at the acrocentric chromosomes and are exclusively associated with AgNORs [25, 26–29]. These results strongly support the idea that the transcriptional apparatus derived from maternal cell nucleoli is maintained in an assembled state on the active NORs. If AgNORs correspond to the active rDNA arrays, one can imagine that the remaining NORs should correspond to the silent rDNA clusters. Recent studies have revealed that acrocentric chromosomes including rDNA repeats but negative to silver staining contain methylated CpG sequences, suggesting that these NORs include transcriptionally silent rRNA genes [30].

Nucleoli reformation begins in late anaphase or early telophase when RNA Pol I transcription is reinitiated [25, 26, 31, 32]. Nucleolar assembly is generally accepted to be mainly a two-step process [33, 34]. The first step involves activation of the transcription machinery that depends on the decrease of cdk1-cyclin B activity [18, 35, 36]. The second step corresponds to recruitment of processing factors in the nucleolus via the formation of prenuclear bodies (PNBs) distributed through the telophase nucleus. The PNBs are preassembled complexes composed of nucleolar proteins and small nucleolar RNAs involved in rRNA processing and they can be considered as prepackaged nucleolar complexes whose primary function is nucleotide modification and processing of rRNA. As cells enter into G1, rRNA gene transcription resumes and PNBs migrate towards the NORs where they fuse or, as recently suggested, they transfer material to form the dense fibrillarin components of the newly built nucleolus [37–39]. All these events result in the formation of multiple small nucleoli. Several results indicate that nucleoli formation is intimately linked with rDNA transcription. Inhibition of Pol I transcription abrogates formation of nucleoli in G1 nuclei of daughter cells. Under these same conditions, the PNBs, whose formation is not affected, fill the nuclei [40, 41], suggesting that targeting of PNB to nucleoli and recruitment of the rRNA processing machinery depend on rDNA transcription. On the other hand, processing factors (nucleolin and fibrillarin) together with pre-rRNA synthesized at the G2/M phase of the previous cell cycle have recently been demonstrated to be recruited to active NORs early in telophase. Unlike the fusion of PNBs to the nucleoli, this early recruitment of processing factors and pre-rRNAs is independent of RNA Pol I transcription, suggesting that nucleoli formation at the end of mitosis occurs before or at the onset of rDNA transcription [42].

As cells progress through the cell cycle, the multiple small nucleoli that form around the active NORs fuse into one or a few large nucleoli, a process commonly referred to as nucleolar fusion [43]. The mechanisms involved in the process of NOR trans-association are still unclear. Given the strong correlation between nucleoli formation and rDNA transcriptional activity, it would be reasonable to think that silent NORs are excluded from nucleoli. However, this does not seem to be the case. Recent results from different laboratories indicated that nucleoli are also composed of silent NORs [44–46]. Immuno-FISH analysis on mouse>human cell hybrids, where human rRNA genes are transcriptionally silent, showed that human acrocentric chromosomes associate with hybrid cell nucleoli, indicating that a NOR can associate with a nucleolus irrespective of its transcriptional activity [45]. Consistent with this, mouse CpG-methylated rRNA genes, corresponding to the silent rDNA copies, and the nucleolar remodeling complex NoRC, which is associated exclusively with silent rRNA genes, are found localized within nucleoli [44, 46, 47]. Apparently, the presence of silent NORs in the nucleolus seems to contradict the dependency of nucleoli formation on rDNA transcriptional activity. One explanation, as suggested by McStay and collaborators [45], could be that nucleoli initially form only around transcriptionally active NORs. Subsequently, there is a dynamic reorganization of acrocentric chromosomes within the interphase nucleus that is independent of the transcriptional activity status. Why silent NORs are included in the nucleoli remains unclear. It is reasonable to think that the heterochromatic structure of silent rRNA genes mediates the integrity of the nucleolus. In yeast, the mechanism responsible for rDNA silencing involves the Sir2 protein, a member of the silent-information regulatory (Sir) proteins [48]. Sir2 binds to Net1, which specifically associates with rDNA and recruits Sir2 to the nucleolus [49]. In *net1Δ* cells, Nop1, the yeast homologue of vertebrate fibrillarin, redistributes over the entire nucleus, suggesting that the integrity of the nucleolus as a compartment has been lost. Consistent with these results, the nucleoli of more complex organisms maintain a tight association with the heterochromatic regions from several chromosomes, including those that are devoid of an NOR and would not therefore be expected to be involved in nucleogenesis [50]. It stands to reason that interactions between heterochromatin-binding proteins are likely to contribute to clustering of NORs and, consequently, to construction of the nucleolar compartment. Candidate domains are the centromeres and the heterochromatic sequences both proximal and distal to the NOR on the p arms of human acrocentric chromosomes. Human centromeres cluster around nucleoli [51–53] and have been reported to be integral components of purified nucleoli [37]. The heterochromatic sequences on the p arm comprise arrays of tandemly repeated satellite DNA,

including β satellite and satellite 1, 2 and 3 [54–58]. Pericentromeric heterochromatic regions of HSA1, 9 and Y contain some of the satellites sequences present on the p arm and, although they are non-acrocentric chromosomes, appear to associate with nucleoli [49–54, 59], suggesting a role for these sequences in NOR assembly. If these genomic regions play a role in nucleoli formation, it will be important to analyze whether NOR trans-association is mediated by the DNA sequence itself or by a specific chromatin structure. Moreover, whether this process is restricted to silent NORs or whether it is commonly used by all NORs remains to be determined. Alternatively, one can imagine that a protein(s) specifically associated with silent rRNA genes can mediate or facilitate assembly of silent NORs into nucleoli. To date, the only protein complex known to be associated specifically with silent rRNA genes is the nucleolar remodeling complex NoRC [60]. NoRC has recently been demonstrated to be the key determinant in the establishment of rDNA silencing in human and mouse cells [60–62]. Recent data from our laboratory indicate that TIP5, the largest subunit of the NoRC complex, associates with RNAs [R. Santoro et al., unpublished data]. Given that nucleoli form at the onset of rRNA transcription, one can imagine that assembly of silent NORs into nucleoli can be achieved by NoRC through rRNA association. Testing this hypothesis will be a priority for future studies.

Epigenetic regulation of active and silent rRNA genes

In higher eukaryotes, the relative amount of active and silent rRNA genes is maintained independently of transcriptional activity [13], suggesting that these chromatin states must be maintained throughout the cell cycle and propagated from one cell generation to the next. It is therefore likely that epigenetic mechanisms are involved to mark the transcriptional state of any given rRNA gene and to ensure the inheritance of the chromatin structure to the daughter cells. Methylation at cytosine bases located 5′ to a guanosine in a CpG dinucleotide is an epigenetic mark known to be involved in gene transcriptional repression [reviewed in ref. 63]. Establishment of the DNA methylation pattern is mediated by the maintenance DNA methyltransferase DNMT1 and by de novo DNA methyltransferases DNMT3b and 3a. DNA methylation is the unique epigenetic modification that is not erased by the passage of the replication fork, thus representing a strong memory mark for the inheritance of silent chromatin states. Yet, until recently, the role of cytosine methylation in rRNA gene regulation was unclear [64]. Methylated ribosomal genes are efficiently transcribed when injected into oocyte nuclei or when transcribed in vitro [65, 66], suggesting that DNA methylation is not sufficient for transcriptional repression. On the other hand, loss of rDNA methylation within the enhancer region accompanies the onset of rDNA transcription during embryonic development of *Xenopus laevis*, indicating an inverse correlation between methylation of the enhancer repeats and transcriptional activity [67]. Moreover, analysis of CpG methylation content of ribosomal genes that are accessible (active) and inaccessible (inactive) to psoralen crosslinking indicated that only silent rDNA copies are DNA methylated [68]. Recent results favor a direct role for DNA methylation in rDNA transcriptional repression [47]. Treatment of mouse cells with 5-azacytidine, an inhibitor of cytosine methylation, stimulates pre-rRNA synthesis by 40–50%, suggesting that lack of DNA methylation alleviates transcriptional repression of the corresponding fraction of silent rRNA genes (40–50%). Moreover, transcription of in-vitro-methylated transfected rDNA minigenes is severely impaired, indicating a direct link between the methylation status of rDNA and transcriptional activity. Most important, methylation-dependent transcriptional silencing can be reproduced in vitro but only when methylated rDNA templates are assembled into chromatin. Conversely, transcription on naked rDNA templates is not affected, providing an explanation why some early studies failed to establish a correlation between CpG methylation and rDNA transcription [65, 66]. All these results provide a link between DNA methylation and chromatin in the regulation of gene expression. The involvement of DNA methylation in the regulation of rDNA transcription is also supported by recent data showing that rDNA promoter sequences from tumor samples are hypomethylated when compared with matching normal tissues. This result is consistent with the relatively high level of rRNA synthesis in rapidly proliferating tumors [69].

Mouse and human rDNA promoter sequences diverge in their content of CpG dinucleotides. The human rDNA promoter harbors a CpG island whereas the mouse promoter does not, suggesting that the mechanisms of methylation-mediated silencing in mice may be distinct from that in humans. Consistent with this, methylation of a single CpG within the UCE of the mouse rDNA promoter impairs binding of the transcription factor UBF to rDNA chromatin, thereby preventing initiation complex formation [47]. On the other hand, there is an inverse correlation of transcriptional activity with methylation density of the human rDNA promoter. Recent results indicate that the methyl-binding protein MBD2 associates with silent human rRNA genes and specifically represses transcription of methylated genes [69].

In the last decade, much effort has been invested in the analysis of the involvement of posttranslational modifications of histone proteins in the organization of chromosomal domains and gene regulation [70]. The recent development of antibodies recognizing specifically modified histones has enabled the use of chromatin immunoprecip-

itation (ChIP) methods to analyze the link between chromatin structure and gene expression with respect to histone modifications. For example, histone acetylation has been found associated with an open chromatin configuration such as that found at transcriptionally active promoters. In contrast, methylation of lysine 9 of histone H3 (H3-K9) is a marker of condensed inactive chromatin of the sort associated with the inactive X chromosome and pericentromeric heterochromatin. A modified ChIP assay, developed in our laboratory, provided a means to discriminate between unmethylated and methylated DNA immunoprecipitated from formaldehyde-crosslinked chromatin. The rationale of this method is that after digestion of immunoprecipitated DNA with the methylation-sensitive enzyme *Hpa*II, only CpG-methylated DNA (i.e. resistant to cleavage) can be amplified by PCR, whereas lack of DNA methylation will not yield PCR products [61]. Using this method, we demonstrated that active rRNA genes display euchromatic structures, free of methylated CpG sequences and associated with hyperacetylated histone H4, histone H3 methylated at lysine 4, RNA polymerase I and the transcription factor UBF. Conversely, silent rRNA genes are characterized by heterochromatic features, including methylated CpG sequences, hypoacetylated histone H4, histone H3 methylated at K9, histone H4 methylated at K20 and the heterochromatin protein HP1 [61], a finding that links the 'histone code' to the 'cytosine methylation code' [70–72]. A growing body of evidence indicates that there is an interplay between cytosine methylation, histone modifications and chromatin-remodeling activities. DNA methyltransferases and DNA-binding proteins that specifically recognize methylated cytosine residues have been shown to interact with histone deacetylases and histone methyltransferases, providing a pathway by which DNA methylation can induce histone modifications [73–78]. The finding that DNA methylation in *Neurospora crassa* is profoundly altered by mutations that disrupt histone methylation suggests that alternative mechanisms of epigenetic modification are possible [79]. The role of nucleosome dynamics in the establishment of defined transcriptional states is demonstrated by the fact that mutations in the ATPase domain of putative chromatin-remodeling factors in *Arabidopsis*, *ddm1*, and in mouse, *Lsh*, induce reduction of cytosine methylation levels and alteration of histone modification patterns [80–83]. With regard to rRNA genes, the mutual dependence between these chromatin modifier activities is shown by the involvement of the nucleolar-remodeling complex NoRC in the establishment of rDNA silencing. NoRC is an ATP-dependent chromatin-remodeling complex that has been identified in mouse and human cells. This complex consists of two subunits, i.e., SNF2h, the mammalian homologue of *Drosophila* ISWI, and TIP5, a >200-kDa protein that shares several domains with the largest subunits of the human ATP-dependent chromatin-remodeling complexes ACF, WCRF, CHRAC and WICH [84–88]. NoRC associates with the rDNA promoter region of silent genes and represses rDNA transcription through recruitment of histone-modifying and DNA-methylating activities, thereby establishing and/or maintaining a repressive higher-order chromatin structure [60–62]. These results suggest that NoRC serves as a scaffold that coordinates the activities of several macromolecular complexes that modify histones, methylate DNA and establish a 'closed' chromatin state.

A common view of the connection between the different chromatin modifier complexes is that they act in a synergistic way, to ensure the propagation of the chromatin state [reviewed in ref. 72]. On the other hand, a sequential order of events may be crucial for establishing a certain chromatin structure. In dissecting the pathway that is conducive to rDNA silencing, we have recently shown that a temporal order of epigenetic events controls DNA methylation and leads to transcriptional repression [62]. Silencing of rDNA is initiated by recruitment of the NoRC to the rDNA promoter through interaction with the transcription terminator factor TTF-I bound to the promoter-proximal terminator T_0 (fig. 1). In a subsequent step, NoRC interacts with the histone deacetylase HDAC1 and with a yet unknown histone H3-K9 methyltransferase, leading to deacetylation and methylation of nucleosomes at the rDNA promoter. Our data show that these histone modifications are not sufficient for transcriptional repression, indicating that these heterochromatic marks per se do not prevent the access of transcription factors to rDNA chromatin. Importantly, inhibition of histone deacetylation impairs NoRC-mediated CpG methylation at the rDNA promoter, while inhibition of DNA methylation does not affect NoRC-mediated histone modifications. Moreover, NoRC ATP-dependent chromatin remodeling activity is required for CpG methylation at the rDNA promoter, thereby impairing binding of UBF to rDNA and preventing the formation of preinitiation complexes. It is reasonable to imagine that, after establishment of transcriptional repression, i.e. CpG methylation of the rDNA promoter, further steps of heterochromatin formation and subsequent spreading over the rRNA genes are required to set up heterochromatic structures. Supporting this idea, methyl-binding proteins and the heterochromatin protein HP1 bind to rRNA genes [60, 69]. Both proteins interact with histone modifier complexes, i.e. histone deacetylases and H3-K9 and H4-K20 methylases [74, 75, 77, 89, 90], suggesting their involvement in establishing heterochromatin at the rDNA locus. MBD2 has been recently found to associate with human rRNA genes [69]. The methyl-binding proteins show different binding ability with respect to the CpG methylation density. MBD1, MBD2 and MBD4 require multiple methylated CpGs whereas MeCP2 can bind to a

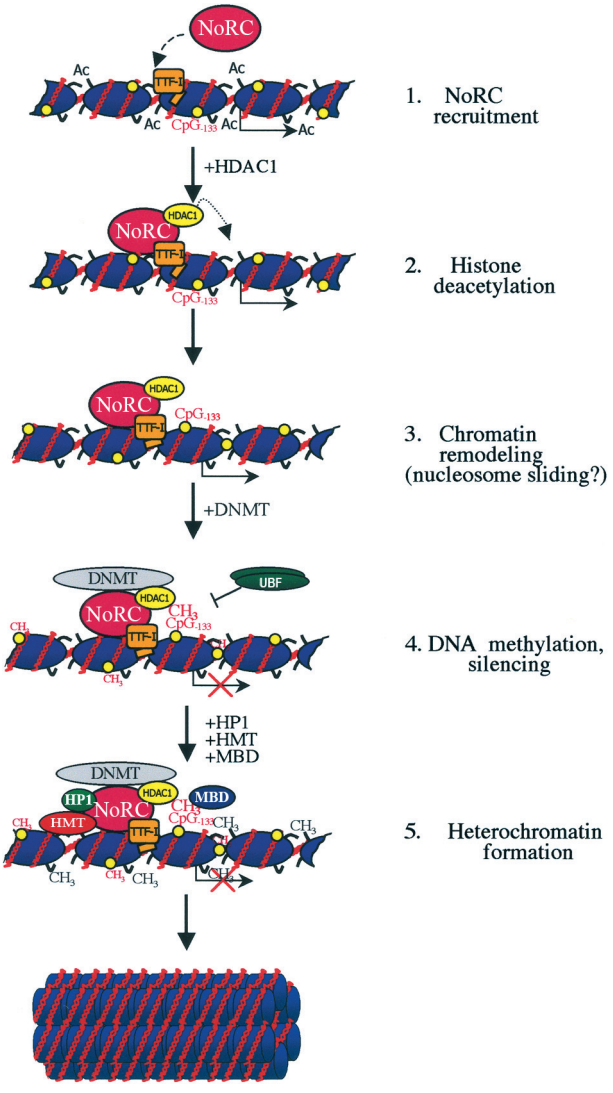

Figure 1. Model representing the order of events leading to mouse rDNA silencing. (1) Transcription termination factor TTF-1 is bound to the T_0 element within the rDNA promoter of transcribed genes and the tails of histone H4 are acetylated. In this first step, NoRC is recruited by TTF-I to the rDNA promoter. (2, 3) NoRC recruits the histone deacetylase HDAC1, mediates deacetylation of histone H4 tails and remodels rDNA chromatin. Deacetylation of histone H4 per se is not sufficient to silence rDNA but it may act as a 'flag' or signal for SNF2h-mediated nucleosome remodeling. Alternatively, nucleosomal remodeling and histone deacetylation may proceed in parallel. (4) NoRC recruits a DNA methyltransferase (DNMT) which methylates the CpG residues within the rDNA promoter (yellow circles). Importantly, histone deacetylation and nucleosome remodeling are required for rDNA methylation. The action of SNF2h may be required beforehand to open the chromatin, thereby relieving either a steric constraint or exposing the CpGs for methylation. (5) Methylation of CpG_{-133} impairs binding of UBF to the upstream control element (UCE) within the rDNA promoter. As a consequence of this, transcription initiation complex formation is impaired and transcription is repressed. The association of HP1, a methyl-binding protein (MBD) and a yet unknown histone methyltransferase with silent rRNA genes suggest that further steps of heterochromatin formation are required to silence permanently the rDNA locus. This heterochromatin structure will give rise to a silent NOR, i.e. devoid of secondary constrictions and negative to silver staining.

single symmetrically methylated CpG [ref. 91 and references therein]. In contrast to the human rDNA promoter, the mouse rDNA promoter does not harbor a CpG island, suggesting that a different MBD protein, e.g. MeCP2, could be involved in rDNA heterochromatin formation. The sum of these epigenetic modifications can give rise to a closed and compacted chromatin structure, resulting in the absence of secondary constrictions and lack of silver staining of silent NORs during metaphase.

Inheritance of active and silent rRNA genes

One question that arises is how the epigenetic and transcriptional state of each individual rRNA gene is inherited during cell division. Recent results showed that in mouse cells, the epigenetic state of a given silent rRNA gene is propagated to the daughter cells [92]. Moreover, studies in a HeLa cell line showed that RNA polymerase I, UBF and SL1 are always associated with the same NORs [22]. In contrast to higher eukaryotes, studies in yeast indicated that all rRNA genes have the same probability to be active or silent, suggesting that the chromatin state is not propagated. In yeast *Saccharomyces cerevisiae*, the replication machinery entering upstream to a transcriptionally active ribosomal rRNA gene generates two newly replicated coding regions regularly packaged into nucleosomes, indicating that the active chromatin structure cannot be directly inherited at the replication fork [93]. The absence of epigenetic memory can be explained by the fact that yeast lacks DNA methylation and can modulate the number of actively transcribed genes depending on the growth conditions [94, 95]. Supporting the idea that yeast and higher eukaryotes do not share the same mechanisms to silence rRNA genes, electron microscopy analysis showed that yeast active and silent rRNA gene copies are randomly distributed. Conversely, in higher eukaryotes, the rDNA copies are clustered and distributed on active and silent NORs, indicating that there are regulatory mechanisms that act on a scale much larger than a single rRNA gene [96].

The concept of replication of genomic material implies not only high fidelity in the duplication of DNA sequences but also maintenance and propagation of the chromatin state. In the last decade, much effort has been put into unraveling the mechanisms involved in the re-establishment of the epigenetic information through multiple rounds of cell division [reviewed in ref. 97]. In eukaryotic organisms, chromosomal DNA replication initiates at multiple sites on the chromosomes at different times following a temporal replication program [98]. The predicted S phase length, based on estimated number of origins, average fork speed and the assumption that all origins fire simultaneously, would be dramatically shorter than the observed S phase length [99]. The pres-

ence of a temporal-order replication program in all eukaryotic cells argues that such a program does have functional importance. In higher eukaryotes there is some correlation between replication timing and transcriptional activity. Although this is not a universal rule, there is a tendency for active genes to be replicated early whereas silent and heterochromatic domains are more likely replicated late. For example, the β-globin locus, which replicates early in cells expressing the globin genes (erythroid cells), becomes late replicating in non-erythroid cells [100]. Similarly, at the onset of X inactivation, when noncoding Xist RNA coats the future inactive X, the first epigenetic change that occurs is a shift to late replication of this X chromosome [101]. The 'window of opportunity' model provides one of the most interesting suggestions for explaining the need for replication timing [reviewed in ref. 102]. According to this model, an active gene that replicates in early S phase is exposed to factors that are required for the formation of active transcription complexes, whereas a silent gene replicating in late S phase experiences a different nuclear environment, which is more conducive for the generation of repressive structures. In support of this, reporter genes microinjected into nuclei of cells in early S phase are packaged into chromatin containing deacetylated histones and they are better templates for transcription. The opposite was true when the reporter gene was transfected during late S phase [103]. However, this model has its own limitations, since it cannot explain how chromatin of transcriptionally active genes replicating in late S phase and heterochromatin replicating in early S phase can be inherited [104, 105].

rRNA genes are replicated both in early and in late S phase [106]. Similar to the two X chromosomes in female cells, active and silent rRNA genes differ with respect to their replication timing, active rRNA genes being replicated early and the silent ones replicated late in S phase [92]. Importantly, the NoRC complex is exclusively associated with late-replicating silent rRNA genes but not with early replicating active copies, suggesting that inheritance of silencing at nascent rDNA is mediated by NoRC. Supporting this, NoRC has been shown to interact with the maintenance DNA methyltransferase DNMT1 [60]. DNMT1 is recruited to replication foci via its interaction with the proliferating cell nuclear antigen PCNA, a protein involved in DNA repair and replication processes [107]. Remarkably, DNMT1 interacts with the histone deacetylase HDAC2 at late replication foci, suggesting that DNA methylation acts as a marker for subsequent histone modifications [76].

NoRC is recruited to the rDNA promoter through interaction with the transcription terminator factor TTF-I bound to the promoter-proximal terminator T_0 [62]. Given that TTF-I associates with both active and silent rRNA genes, one can reasonably assume that this interaction is not the only one to mediate specific recruitment of NoRC to silent genes. TTF-I has been reported to trigger an ATP-dependent nucleosome-remodeling activity that is required to transcribe in vitro rDNA templates assembled into chromatin [108]. A prediction would be that, similar to the situation at silent genes, TTF-I targets a chromatin modifier complex to establish an rDNA chromatin architecture that is conducive to transcription. If this is true, how can TTF-I distinguish which complex to recruit to the rDNA promoter? One explanation may be that the selectivity of TTF-I in recruiting the 'right complex to the right place' could be dictated by other proteins or by pre-existing epigenetic modifications at the rDNA promoter. Alternatively, targeting of these complexes could occur during DNA replication (fig. 2). In this scenario, NoRC will be available only during the late S phase and will therefore be recruited to nascent silent genes. Conversely, the yet unknown 'active' chromatin-remodeling complex will be present during the early S phase and thus TTF-I will target it to the active newly replicating genes. As third hypothesis, one may suggest that ongoing transcription on active rRNA genes will deny access to the silencing machinery. Future experiments will address this issue.

Dominant and underdominant rRNA genes

Nucleolar dominance is a phenomenon in plant and animal hybrids whereby rRNA genes inherited by one parent are transcribed and those from the other parent are silent. The reason for nucleolar dominance is still unclear. Early studies of nucleolar dominance indicated that when the NOR of species A is dominant over the NOR of species B and B is stronger than C, then A is invariably dominant over C [96]. This suggests that NOR transcriptional activity has a defined hierarchy. One can imagine that the number of rRNA copies dictates such a hierarchy. In this case, the species containing the highest number of rDNA copies will compete better for the available Pol I transcription factors and, consequently, will be dominant over the species with the lower amount of rDNA repeats. However, there is no good correlation between the number of rRNA genes and nucleolar dominance. In allohexaploid bread wheat, the most active NOR has only half as many rRNA genes as the second most active NOR [109], indicating that it is not the number of rRNA genes of one species that determines the dominance. Early studies in *Xenopus* led to an alternative hypothesis for the mechanisms controlling nuclear dominance: the enhancer imbalance hypothesis [110]. *X. laevis* and *X. borealis* have different types and number of repetitive elements in their intergenic spacer (i.e. more enhancer repeats in *X. laevis*). In *Xenopus* hybrids, transcription of rRNA genes from *X. laevis* is dominant over *X. borealis* rRNA gene transcrip-

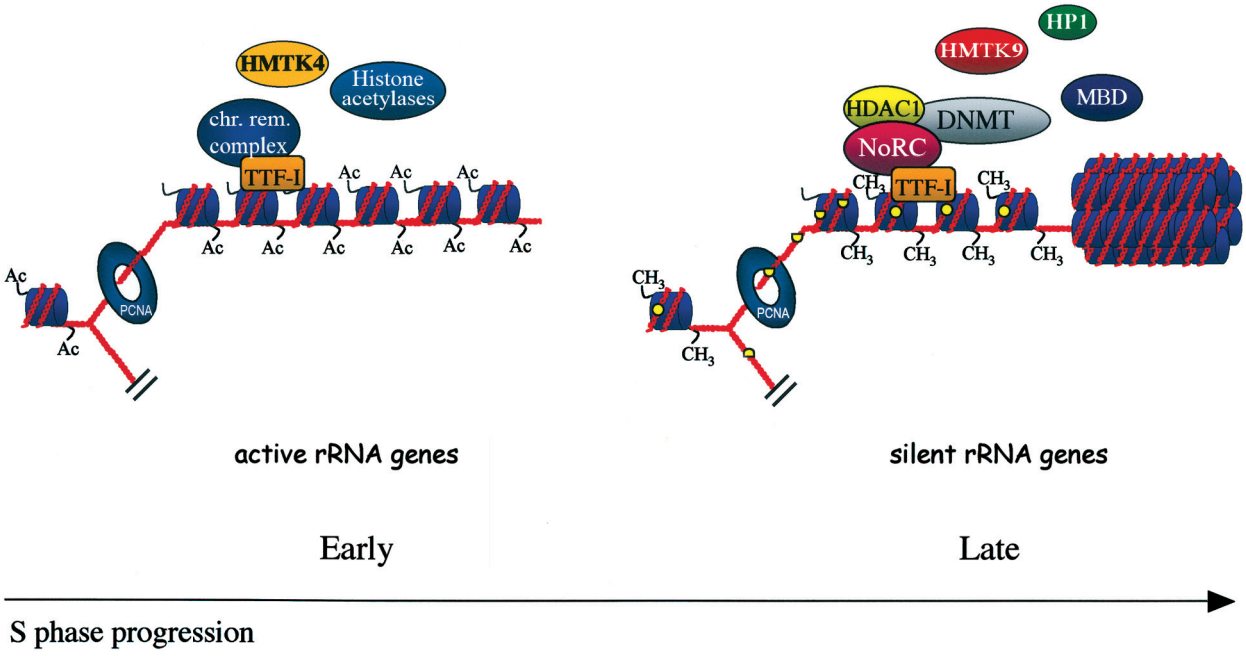

Figure 2. Establishment of active and silent rDNA chromatin states during DNA replication. Active and silent rDNA repeats replicate in a biphasic manner, active genes replicating early and silent ones replicating late in S phase [92]. According to the 'window of opportunity' model, a gene that replicates in early S phase is exposed to factors that are required for the formation of active transcription complexes, whereas a silent gene replicating in late S phase experiences a different nuclear environment, which is more conducive for the generation of repressive structures. In this scenario, TTF-I, which associates with both active and silent rRNA genes, may recruit the 'right complex to the right place.' According to this model, the yet unknown 'active' chromatin-remodeling complex (chr. rem. complex) will be present during early S phase and thus TTF-I will target it to the active newly replicating genes. Conversely, NoRC and all the components that mediate heterochromatin formation and silencing will be available only during late S phase and will therefore be recruited by TTF-I to nascent silent genes. Yellow circles represent methylated CpG residues, hemimethylated sites are depicted as hemicircles. HMTK9 and HMTK4, K9 and K4 histone methyltransferases; MBD, methyl-binding proteins.

tion. Co-injection of *X. laevis* and *X. borealis* minigenes into oocytes results in a preferential transcription of *X. laevis* minigenes [111], analogous to the situation in hybrids. Studies with recombinant constructs, in which the promoter and intergenic spacer sequences are swapped, demonstrated that the intergenic spacer of *X. laevis* is responsible for the differential transcription activity [112]. These results suggested that the mechanism to discriminate rRNA genes in a hybrid cell is based on the affinities of DNA-binding transcription factors. In other words, the dominant species containing stronger enhancer might sequester a transcription factor(s) and, as a consequence, may deny access of this factor(s) to the underdominant rRNA genes [110, 112]. If this is true, a dominant NOR with relatively few genes should still be dominant over an underdominant NOR with a greater number of genes. However, in *Arabidopsis suecica*-like hybrids, dominance is reversed as the genome ratios are changed from 1:3 to 2:2 or 3:1 (*A. thaliana*:*A. arenosa*), a fact not predicted by the enhancer imbalance hypothesis. Moreover, in *Arabidopsis* and *Brassica*, no difference has been detected in the ability of dominant and underdominant plant rRNA genes to compete for transcription factors, either in vitro or in vivo [113, 114], indicating that transcription factor competition is not sufficient to determine nucleolar dominance.

Mouse-human hybrid cells represent a particular class of nucleolar dominance regulation. In some of these hybrid cell lines, mouse rRNA genes are transcribed whereas in other lines only human rRNA genes are active [ref. 96 and references therein], indicating lack of hierarchy in NOR activity between these two species. Importantly, these two species cannot interbreed. Studies with cell-free extracts have shown that a human rRNA gene can be reprogrammed to transcribe in a mouse extract if human SL1 transcription factor is added to the reaction [115]. Similarly, a mouse rRNA gene can be transcribed in a human cell-free extract if the reaction is supplemented with mouse SL1. These results strongly suggest that mouse and human DNA control elements within the rDNA promoter region contain fundamental divergences and, as a consequence, they cannot be recognized by the transcriptional machinery of the other species. Loss or inactivation of genes for mouse or human SL1 could explain how in some hybrid cell lines mouse NORs are dominant whereas in other lines human rRNA genes are transcriptionally active. However, there is no experimental evidence concerning this point. The species-specific tran-

scription factor hypothesis seems to be a likely explanation for nucleolar dominance in hybrids between species with divergences in the rDNA-promoter-controlling elements. However, this model cannot explain nucleolar dominance in hybrids between species that can interbreed. For example, underdominant genes from *Brassica* and *Arabidopsis* can be transcribed when transfected into protoplasts of the dominant species, indicating the absence of species specificity in rDNA transcription [113, 114]. Although very different, the species-specific transcription factor hypothesis and the enhancer imbalance hypothesis are not in conflict. One way to interpret the data discussed above is that the NOR activity from species that cannot interbreed (mouse-human) is regulated by mechanisms distinct from those used by species that are sufficiently closely related to be transcribed by the Pol I transcriptional machinery of the other species (*Xenopus, Arabidopsis*).

In addition to these hypotheses, several results indicated that chromosomal domains flanking the NORs could affect nucleolar dominance. In *Brassica*, underdominant rRNA minigenes are fully transcribed when transfected into a hybrid cell where the chromosomal underdominant genes are silent [114], suggesting a possible role for the NOR chromosomal environment in the control of rDNA transcriptional activity. Additional evidences came from cytogenetic analysis of hybrids between *Drosophila melanogaster* and *Drosophila simulans*. D. *melanogaster* has a NOR on the X and Y chromosomes, D. *simulans* has a NOR on the X chromosome. In hybrid XX females, the *melanogaster* NOR is dominant and in hybrid XY males, the *melanogaster* Y-associated NOR is dominant over the *simulans* X-associated NOR [116]. Rearrangements in the heterochromatin flanking the *melanogaster* Y-associated NOR do not affect transcriptional activity at this NOR but they do prevent silencing at the *simulans* X-associated NOR in trans, underscoring the importance of chromosomal context and loci in nucleolar dominance. Consistent with this, translocation of an NOR to another chromosome can change its transcriptional state [117]. Interestingly, translocation experiments performed in Tricale (the hybrid of wheat and rye) indicate that sequences on the long arm of rye chromosome 1R and chromosome 2R are both needed to silence the rye NOR in the hybrid cells [118]. How can the chromosomal environment influence NOR activity in the nucleolar dominance process? One possible explanation for these results is that chromosomal domains flanking NORs can regulate rDNA transcriptional activity by determining replication timing and/or epigenetic modifications.

There is substantial evidence that epigenetic mechanisms are involved in the enforcement and inheritance of nucleolar dominance. For example, treatment with chemical inhibitors of DNA methylation and/or histone deacetylation alleviates silencing at the underdominant rRNA genes [119–121]. Recent results from Pikaard's laboratory indicate that a concerted DNA methylation/histone methylation switch regulates nucleolar dominance in plants [122]. A key component of this process is HTD1, a plant-specific histone deacetylase that localizes to the nucleolus and is required for H3 lysine 9 deacetylation and subsequent H3 lysine 9 methylation. The analogy with the above-described epigenetic mechanisms to establish rRNA gene silencing in mouse and human cells [47, 60, 62] suggests that nucleolar dominance may be regulated by similar mechanisms. Of interest will be to determine whether an NoRC-like factor is involved in the epigenetic silencing at the underdominant genes.

Although epigenetics may explain the establishment and maintenance of silencing at the underdominant genes, it is unlikely to be involved in the process of choosing the rRNA genes of one progenitor to be silenced. One possible scenario is that epigenetic silencing occurs after the choice of the NOR to be underdominant. One could imagine that in the absence of the Pol transcription machinery, the silent underdominant genes are more exposed to the action of the DNA methyltransferases and histone deacetylases/methylases. Whether targeting of these chromatin modifier complexes is mediated by a specific factor remains to be determined. Conversely, genes that have been chosen to be transcriptionally active will be refractory to epigenetic modifications conducive to silencing. In support of this, the transcription factor UBF has been found associated with sequences along the entire repeat of active rRNA genes [123, 124]. Although the reason for this is not yet clear, recent results indicate that UBF promotes recruitment of SL1 and the Pol I subunits and induces large-scale chromatin decondensation when targeted to a large array of heterologous UBF-binding sequences integrated into ectopic sites on human chromosomes or to silent lac operator arrays [124, 125]. This suggests that transcriptionally active genes, covered and loaded with UBF and polymerases, target positive epigenetic regulators and, consequently, are refractory to silencing modifier complexes. Intriguingly, the histone variant H3.3, which is assembled into DNA in a replication-independent manner, has been found to be deposited at the active rDNA arrays [126]. Although H3.3 and H3 variants differ in only four amino acids, the modifications commonly associated with transcribed genes, e.g. di- and trimethylation of lysine 4 and acetylation of lysine 9, 14, 18 and 23, have been found to be enriched in the H3.3 variants. In contrast, dimethylation of lysine 9, a modification associated with repressed genes, is enriched in histone H3 [127]. Thus, enrichment of the H3.3 histone at dominant rRNA genes may be involved in the establishment of euchromatic structures at the active NOR and may contribute to the protection of active rDNA repeats against epigenetic modifications conducive to silencing.

Conclusions

Although our knowledge of the mechanisms involved in the establishment of rDNA silencing has improved in recent years, why a fraction of rRNA genes is epigenetically silenced and retained through evolution remains to be determined. Dosage compensation may be a reasonable explanation but it fails to explain why in nucleolar dominance one set of genes should be selectively silenced [96]. As discussed above, one possibility is that rDNA silencing serves a structural role by mediating NOR trans-association and nucleoli integrity. Although there is no good correlation between cellular growth rate and number of rRNA genes, there are several indications that the control of the number of silent genes could have its own function. Analysis of CpG methylation content in germ cells and liver of male rats has revealed age-dependent hypermethylation in the 5′ region of the rRNA gene repeats [128]. Importantly, the augmented transcription of rRNA genes observed in cancer cells is inversely correlated with the CpG methylation content at the rDNA promoter [69] and aberrant rDNA methylation has also been found associated with developmental disorders, such as the α-thalassemia X-linked mental retardation syndrome [129]. These results clearly show that loss of rDNA silencing may affect cell metabolism by enhancing rRNA transcription. However, whether loss of silencing is the cause or the effect in these pathologies remains undetermined. Moreover, we are still ignorant about the chromatin modifier complexes involved in the formation of transcriptionally active rDNA chromatin. Understanding how chromatin can modulate transcription during the cell cycle or during external stimuli as well as how RNA polymerase I and the transcription factors can overcome the chromatin barrier at initiation and during elongation will shed new light on the basic mechanisms of rDNA transcriptional regulation.

A growing body of evidence indicates that disruption of the balance of the epigenetic networks can cause several major pathologies, i.e. cancer, syndromes involving chromosomal instabilities and mental retardation. Thus, dissecting the cause-and-effect relationship between the epigenetic marks and determining how all the chromatin modifier complexes can co-ordinate with each other has great potential for the development of therapies based on the use of inhibitors for enzymes controlling epigenetic modifications. The co-existence of two distinct epigenetically modified rDNA chromatin structures represents an excellent model to study the basic chromatin-mediated mechanisms that cells use to regulate gene expression.

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