Human Molecular Genetics, 2007, Vol. 16, Review Issue 1 ***R21–R27***
doi:10.1093/hmg/ddm020

Different epigenetic layers engage in complex crosstalk to define the epigenetic state of mammalian rRNA genes

Ingrid Grummt*

Division of Molecular Biology of the Cell II, German Cancer Research Center, D-69120 Heidelberg, Germany

Received January 3, 2007; Accepted February 7, 2007

Eukaryotic cells contain several hundred ribosomal RNA (rRNA) genes (rDNA), a fraction of them being silenced by epigenetic mechanisms. The presence of two epigenetically distinct states of rRNA genes provides a unique opportunity to decipher the molecular mechanisms that establish the euchromatic, i.e. transcriptionally active, and the heterochromatic, i.e. transcriptionally silent, state of rDNA. This article summarizes our knowledge of the epigenetic mechanisms that control rDNA transcription and emphasizes how DNA methyltransferases and histone-modifying enzymes work in concert with chromatin-remodeling complexes and RNA-guided mechanisms to establish a specific chromatin structure that defines the transcriptional state of rRNA genes. These studies exemplify the mutual dependence and complex crosstalk among different epigenetic players in the alteration of the chromatin structure during the process of gene activation or silencing.

INTRODUCTION

Epigenetic mechanisms, which involve DNA and histone modifications, cause the heritable silencing of genes without changing their coding sequence. Disruption of the balance of epigenetic networks can cause several major pathologies, including cancer and chromosomal instability. It is well established that epigenetic mechanisms are profoundly affected in tumor cells. Moreover, changes in DNA methylation and aberrant histone-modification profiles have been causally linked to diseases such as Rett syndrome, systemic lupus erythematosis and others (1). Morever, many studies have correlated deregulation of ribosome biosynthesis with cancer, showing that overexpression of ribosomal RNA (rRNA) could lead to excessive protein synthesis and thus can be an initiating step in malignant transformation (2). The synthesis of rRNA is regulated at multiple levels, e.g. via changes of the transcription rate and via the number of gene copies that are transcribed. These topics have been reviewed in the past and readers are referred to recent articles for further reading (3–8). Some recent reviews summarize the current knowledge of the epigenetic mechanisms that mediate silencing and epigenetic control of rDNA (9–12). This review provides an update on recent advances of the molecular mechanisms that control the chromatin structure of rRNA genes and presents a new, coherent picture of the complex crosstalk and mutual dependence of events that operate along a common mechanistic pathway to regulate nucleolar structure and function.

rRNA GENES EXIST IN TWO DISTINCT EPIGENETIC STATES

Eukaryotic rRNA genes (rDNA) are arranged in clusters of tandem repeats, known as nucleolar organizer regions (NORs), that are located on the short arms of acrocentric chromosomes. Though rDNA transcription units are virtually identical, a significant fraction is usually silenced by epigenetic mechanisms. There are two classes of NORs, active and inactive ones. Active NORs have a distinct chromatin structure that is evident as a secondary constriction in metaphase. On inactive NORs, rDNA appears to be packaged in a form that is indistinguishable from the surrounding heterochromatin. In higher eukaryotes, the relative amount of active and silent rRNA genes is maintained independently of transcriptional activity, suggesting that these chromatin states must be maintained throughout the cell cycle and propagated from one cell generation to the next. Several epigenetic characteristics dis-

*To whom correspondence should be addressed at: Molecular Biology of the Cell II, German Cancer Research Center, Im Neuenheimer Feld 581, D-69120 Heidelberg, Germany. Tel: +49 6221423423; Fax: +49 6221423467.

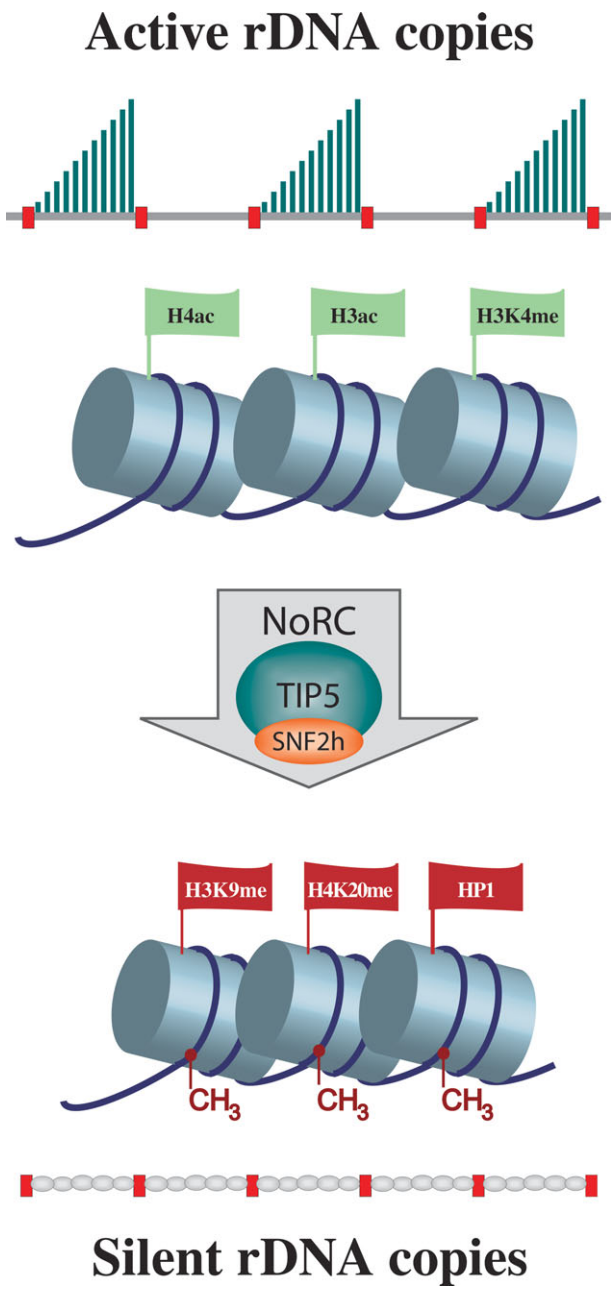

Figure 1. rRNA genes exist in two distinct epigenetic states. Eukaryotic rRNA genes are arranged in clusters of tandem repeats, known as NORs, which are located on the short arm of acrocentric chromosomes. The red boxes illustrate terminator elements that are located upstream and downstream of the transcription unit; the balls represent nucleosomes. The two classes of active and inactive NORs are epigenetically distinct. Potentially active rRNA genes exhibit an open chromatin structure, are associated with Pol I and nascent pre-rRNA (green lines) and are characterized by DNA hypomethylation, acetylation of histone H4 (Ac) and methylation of H3K4me2. Epigenetically silenced rRNA genes are demarcated by CpG hypermethylation (CH3), histone H4 hypoacetylation, methylation of H3K9 and H4K20 (Me) and association with heterochromatin protein 1 (HP1).

tinguish potentially active from inactive mammalian rRNA genes (Fig. 1). Generally, an 'open' chromatin structure that is characterized by DNA hypomethylation, acetylation of histone H4 and dimethylation of histone H3 at lysine 4 (H3K4me2) correlates with transcriptional activity, whereas CpG hypermethylation, histone H4 hypoacetylation, methylation of H3K9 and H4K20 correlate with transcriptional silencing (13–17). The coexistence of two distinct epigenetic states of rRNA genes in each cell suggests that the usage of all gene copies might be restricted to certain phases of early development, for example, during oogenesis where large quantities of ribosomes and proteins are required. In support of this, the level of global CpG methylation is significantly lower in germ line cells compared with somatic cells, indicating a cell- or tissue-specific epigenetic difference. Similar mechanisms may augment ribosome production in cancer cells, where rDNA methylation is decreased and the number of active rRNA genes is increased (18). Therefore, understanding the mechanisms that establish and maintain the active and silent state of rRNA genes will guide us in understanding the basic mechanisms the cell uses to regulate gene expression at the epigenetic level.

THE PROMOTER OF SILENT rRNA GENES IS METHYLATED AT SPECIFIC CpG RESIDUES

The major form of epigenetic information in mammalian cells is DNA methylation, the covalent addition of a methyl group to the 5′-position of cytosine within CpG dinucleotides. DNA methylation generally correlates with transcriptional silencing, and alterations in the CpG methylation pattern are hallmarks of many human diseases and cancer. In human cells, there are at least 25 CpGs residing within the bounds of the Pol I promoter. In mouse, on the other hand, methylation of a single cytosine residue (at −133) within the upstream control element (UCE) of the rDNA promoter was found to impair binding of the basal transcription factor UBF on chromatin templates (19). These results imply that DNA methylation mediates or reinforces transcriptional silencing through an effect on essential protein–DNA interactions needed for transcription initiation complex formation. Consistent with proper DNA methylation playing a role in controlled cell proliferation, comparison of the methylation profile of human hepatocellular carcinomas and normal liver showed significant hypomethylation of the rDNA promoter in tumors as compared with normal tissues (18).

Hypomethylation of rRNA genes correlates with decreased genomic stability, suggesting that silencing entails the assembly of a generally repressive chromatin structure that is less accessible to the cellular recombination machinery. In support of this, somatic knock-out of Dnmt1 in human cells led to severe rDNA demethylation, enhanced binding of the Pol I transcription machinery to rDNA and profound disorganization of the nucleolus (20). This indicates that a specific pattern of DNA methylation is not only required for the cell to decide which rDNA copy is to be transcribed, but also for the maintenance of nucleolar structure. Finally, a systematic search for alterations of DNA methylation has revealed a region of rDNA that is specifically hypomethylated with age in both spermatozoa and liver of male rats, indicating that rDNA methylation is vulnerable to age-dependent alteration of cellular functions (21).

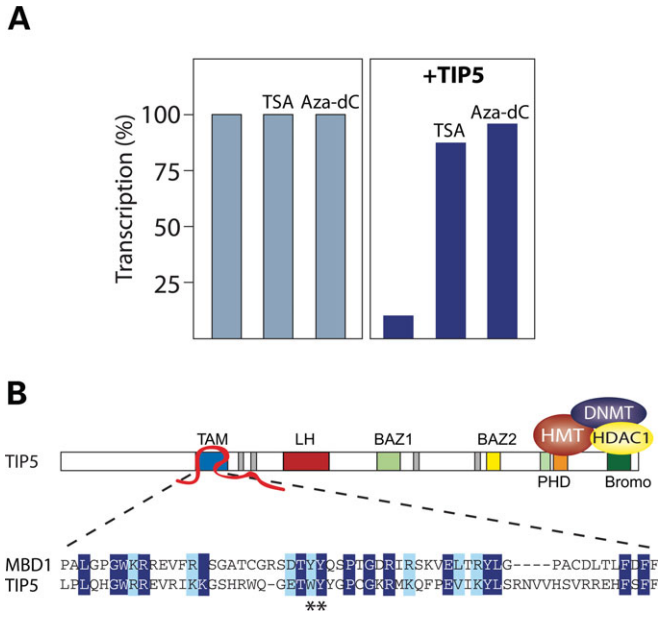

Figure 2. NoRC establishes the silent state of rDNA by recruiting histone-modifying enzymes, DNMTs and RNA. (**A**) NoRC-dependent rDNA silencing involves histone deacetylation and *de novo* DNA methylation. The bar diagram illustrates the relative level of rDNA transcription in mock-transfected cells and cells overexpressing TIP5 in the absence or presence of an inhibitor of HDAC (TSA) or DNMTs (aza-dC) activity. (**B**) Scheme illustrating the modular organization and the location of sequence motifs in TIP5 that have been associated with functions in chromatin structure and function. The C-terminal part of TIP5 contains a tandem PHD finger and bromodomain that interact with HDAC, histone methyltransferase (HMT), DNMTs and SNF2h (not shown). The TAM domain binds RNA (marked in red). An amino acid alignment of the MBD-domain of MBD1 and the TAM domain of TIP5 is shown below. Identical and homologous amino acids are indicated in dark and light blue, respectively. The asterisks indicate the position of point mutations in the RNA-binding-deficient mutant TIP5WY531/532.

NoRC, A CHROMATIN REMODELING COMPLEX THAT SILENCES rRNA GENES

In human and mouse, the transcriptionally silent state of rDNA is established by a chromatin remodeling complex, termed NoRC. NoRC is a member of ATP-dependent chromatin-remodeling machines, consisting of the ATPase SNF2h and the nucleolar protein TIP5 (22). NoRC interacts with DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and histone methyltransferases (HMTs), thereby targeting enzymatic activities to the rDNA promoter that are required for heterochromatin formation. Overexpression of NoRC represses Pol I transcription by increasing the level of specific heterochromatic marks both on the promoter of endogenous rDNA repeats and on artificial rDNA minigenes (13,16,22). Treatment of cells with methyltransferase inhibitors, such as 5-aza-2′deoxycytidine (aza-dC), or HDAC inhibitors, such as trichostatin A (TSA), prevented heterochromatin formation and transcriptional repression (16,17,23). This demonstrates that histone deacetylation, DNA methylation and transcriptional silencing are closely interconnected and suggests that in higher eukaryotes, chromatin-remodeling complexes, such as NoRC, coordinate epigenetic events that lead to transcriptional silencing (Fig. 2a).

NoRC is recruited to rDNA by interaction of its large subunit, termed TIP5, with TTF-I (transcription termination factor for Pol I) bound to a promoter-proximal terminator element, known as T_0 (24,25). TIP5 contains a number of protein domains that are important for its function. The C-terminal part of TIP5 harbors a tandem PHD finger/bromodomain, a cooperative unit that has been shown to target chromatin modifiers to gene promoters (26). The bromodomain, an approximately 100 amino acid sequence element found in many chromatin-associated proteins, plays a vital role in NoRC-mediated rDNA silencing. This domain interacts specifically with histone H4 acetylated at lysine 16 (H4K16ac), and interaction with acH4K16 is required for subsequent deacetylation of H4K5, H4K8 and H4K12. Mutational analysis revealed that the integrity of the bromodomain is essential for NoRC function, and the interaction with acetylated histone tails is a key event in NoRC-mediated rDNA silencing (27).

The TIP5 bromodomain cooperates with the adjacent PHD domain to recruit SNF2 and chromatin-modifying activities to the rDNA promoter. A fusion protein comprising the C-terminal PHD finger/bromodomain of TIP5 linked to the DNA-binding domain of TTF-I is capable of inducing rDNA silencing. This demonstrates that the bipartite PHD finger/bromodomain of TIP5 constitutes a repressive unit that is capable of establishing heterochromatic features and repressing Pol I transcription, provided that it is specifically anchored to its target gene (27). Thus, the PHD finger/bromodomain of TIP5 recruits all important enzymatic activities that lead to epigenetic changes involved in rDNA silencing, e.g. histone hypoacetylation, DNA methylation and ATP-dependent nucleosome remodeling (Fig. 2b).

An extensive analysis of the order of events that establish a local heterochromatin environment at rDNA has revealed a hierarchical order and mutual dependence of events that operate along a common mechanistic pathway to repress transcription. These studies suggest that silencing is initiated by recruitment of NoRC to rDNA by TTF-I bound to its promoter-proximal target site. Once recruited to rDNA, the PHD/bromodomain of TIP5 interacts with an SIN3-like complex, containing HDAC1, with histone methyltransferase(s), and with DNMT(s) to modify histones and to methylate rDNA (16). This study also revealed that ATP-dependent chromatin remodeling is required for DNA methylation and transcriptional silencing. Thus, NoRC acts as a scaffold that coordinates the activities of several macromolecular complexes that remodel nucleosomes, modify histones, methylate DNA and establish a 'closed' chromatin state. It is reasonable to imagine that, after the establishment of specific heterochromatic marks, further steps are required for setting up a compact heterochromatic structure that spreads over a specific rDNA cluster.

DIFFERENT NUCLEOSOME POSITIONS AT ACTIVE AND SILENT rDNA REPEATS

Active and silent rDNA copies are characterized not only by distinct epigenetic marks but also by specific nucleosome positions. Apparently, nucleosomes are positioned into

specific translational frames that characterize the 'on' or 'off' state of of rDNA. At active genes, the promoter-bound nucleosome covers nucleotides from −157 to −2, whereas at silent genes, the nucleosome is positioned 25 nucleotides further downstream. In NIH3T3 cells, the ratio of 'active' versus 'silent' nucleosome positions is approximately 1:1. This ratio changes to at least 1:5 in cells overexpressing NoRC and after differentiation of 3T3-L1 cells into adipocytes (28). TIP5Y1775F, a point mutant that does not associate with chromatin and does not trigger transcriptional silencing (27), was not capable of shifting nucleosomes from the active into the 'inactive' position. These results demonstrate that NoRC is the molecular machine that shifts the promoter-bound nucleosome downstream of the transcription start site into a translational position that is unfavorable for transcription complex formation.

It is important to note that the specific nucleosomal architecture of active genes places the DNA exit sites into close proximity, potentially allowing cooperative binding of UBF and TIF-IB/SL1 to the UCE and core promoter. On silent genes, however, a nucleosome is positioned downstream of the transcription start site, and both the UBF-binding site and the functionally important CpG residue at nucleotide −133 are placed into the nucleosomal linker region that is contacted by NoRC. In this nucleosome position, the core element is placed inside the nucleosome and the relative alignment of the DNA element with respect to the histone octamer surface has been changed. Thus, while at active genes the nucleosome juxtaposes the core and UCE sequences, both sequence elements are separated at silent genes, not allowing cooperative binding of UBF and TIF-IB/SL1. As a consequence, no transcription complexes are formed. Thus, NoRC serves at least two functions: first, as a remodeling complex that alters the position of the nucleosome at the rRNA gene promoter, and second, as a scaffold that coordinates the activities of macromolecular complexes that modify histones, methylate DNA and establish a 'closed' heterochromatic state.

NoRC CONTROLS REPLICATION TIMING OF rRNA GENES

The concept of the epigenetic code implies that the epigenetic information is memorized, that is, transmitted from one generation to the next, although the DNA must be unpackaged during replication. This raises the question of how the active and silent states of chromatin are established anew after each round of replication. In mammalian cells, replication timing is linked to transcriptional control through its liason with chromatin structure. In general, transcriptionally active euchromatin replicates early in S-phase, whereas heterochromatin replicates late in S-phase. Apparently, eu- and heterochromatic genes experience a different nuclear environment that favors the generation of an open or repressive chromatin structure, respectively. Replication timing is both the cause and consequence of chromatin structure by providing a means to inherit chromatin states that, in turn, regulate replication timing. This mode of inheritance, commonly referred to as epigenetic inheritance, is believed to be the basis for memory mechanisms that maintain cell identity and propagate stable patterns of gene expression in subsequent cell cycles. With regard to rDNA, rRNA genes replicate in a biphasic manner, active genes replicating early and silent ones replicating late in S-phase (29). NoRC is exclusively associated with late replicating rRNA genes, and moderate overexpression of TIP5 in mouse cells shifts replication timing from early to late. In addition, the number of active rDNA transcription units was decreased, the size and number of nucleoli were reduced and cell proliferation was impaired. Thus, NoRC not only mediates the inheritance of specific epigenetic marks through DNA replication but also sets the clock for silent genes replicating late.

rDNA SILENCING REQUIRES TRANSCRIPTS ORIGINATING FROM THE INTERGENIC SPACER

Recent analyses of mammalian transcriptomes have revealed that more than 50% of cellular RNA corresponds to transcripts originating from genomic regions previously classified as silent and non-functional. Although most of the identified ncRNAs have unknown functions, ncRNAs have been reported to play important roles in establishing a specific chromatin structure and gene silencing. Intriguingly, intergenic spacer (IGS) sequences that separate individual rDNA transcription units often contain one or more Pol I promoters which are similarly organized as the gene promoter. The biological function of spacer promoters and intergenic transcripts remained elusive.

A recent study has revealed that IGS transcripts play an important role in the epigenetic control of the rDNA locus (30). This study has shown that NoRC is associated with RNA originating from the IGS. RNA interaction studies revealed that 150–300 nt RNAs that are complementary to the rDNA promoter are required for high-affinity binding of TIP5. Mutational analysis of IGS transcripts in conjunction with homology-based structure prediction revealed that TIP5 recognizes the secondary structure of IGS transcripts, and binding of TIP5 to RNA changes the structure of NoRC in an induced fit mechanism (C. Mayer, unpublished data). Importantly, binding to RNA is required for the recruitment or tethering NoRC to nucleolar chromatin and heterochromatin formation. The interaction of NoRC with RNA is mediated by the TAM (TIP5/ARBP/MBD) domain of TIP5, which exhibits sequence homology to the domain (MBD) present in proteins that bind to methylated DNA. A TIP5 mutant containing two amino acid exchanges in the TAM domain was deficient in RNA binding, was less tightly associated with chromatin and did not trigger heterochromatin formation (30). Thus, the integrity of the TAM domain is required both for NoRC binding to chromatin and the establishment of heterochromatic features at the rDNA promoter.

On the basis of these results, the following picture emerges (Fig. 3). A promoter located ∼2 kb upstream of the pre-rRNA transcription start site directs the synthesis of long intergenic RNA. This IGS RNA is processed into short intermediates that are either rapidly degraded or shielded from further degradation by binding to NoRC. Once complexed with RNA, the association of NoRC with rDNA is stabilized and heterochromatin formation is reinforced. Apparently, the RNA–NoRC

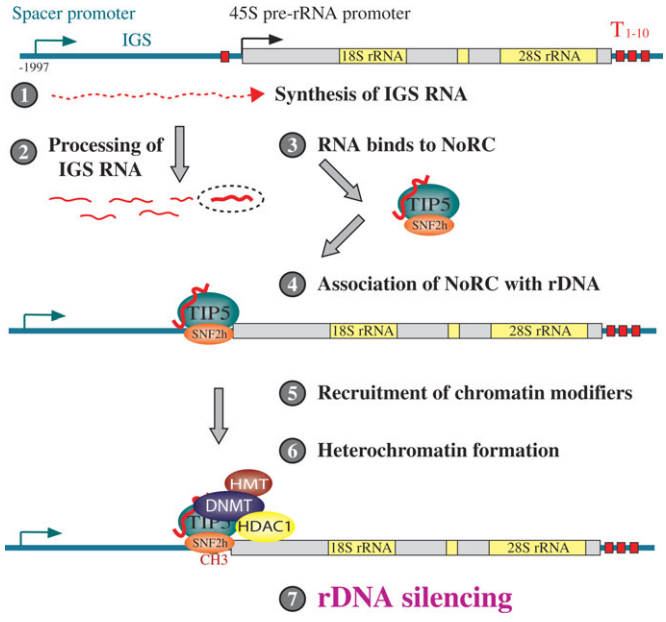

Figure 3. Model depicting the role of non-coding RNA in heterochromatin formation and gene silencing. First, intergenic spacer (IGS) transcripts are synthesized [step 1] which is then processed [step 2]. RNA that matches the rDNA promoter associates with the chromatin remodelling complex NoRC [step 3]. Once bound to RNA, NoRC is recruited to the rDNA promoter [step 4]. The large subunit of NoRC, TIP5, interacts with histone modifying enzymes, such as HDACs, HMTs, and DNMTs [step 5], leading to heterochromatic histone modifications and de novo DNA methylation [step 6]. As a consequence, transcription complex formation is impaired and rRNAgenes are silenced [step 7].

complex is utilized as a platform to recruit other RNA-binding proteins involved in silencing, such as HP1 proteins, which may facilitate further chromatin compaction. Thus, NoRC-associated IGS RNA can be added to a growing list of functional ncRNAs that originate either from processed introns or intergenic DNA regions and have distinct roles in the regulation of gene expression.

CSB AND WSTF—REMODELERS THAT ESTABLISH THE ACTIVE STATE OF rDNA?

ATP-dependent nucleosome remodeling machines are involved not only in rDNA silencing but also in transcription activation on chromatin templates. Previous *in vitro* studies have demonstrated that binding of TTF-I to the promoter-proximal terminator T_0 triggers ATP-dependent nucleosome remodeling that correlates with efficient transcription initiation on rDNA assembled into chromatin (31). The molecular mechanisms that govern activation of Pol I transcription on chromatin templates are largely unknown. As TTF-I binding and nucleosome remodeling are required both for activation and repression of Pol I transcription on chromatin templates, it is reasonable to predict that TTF-I may also recruit an 'activating' remodeling complex, distinct from NoRC, that is is capable of establishing euchromatic features at active rDNA repeats.

A possible candidate for such an activating chromatin remodeler is Cockayne syndrome protein B (CSB), a member of the SWI/SNF2-like family of DNA-dependent ATPases. CSB interacts with core histones through their N-terminal tails and is capable of disrupting protein–DNA interactions at the expense of ATP hydrolysis (32). CSB plays a key role both in transcription-coupled DNA repair and in transcription activation, and mutations in the CSB gene lead to the genetic disorder Cockayne syndrome. CSB is contained in a complex consisting of Pol I, TFIIH and TIF-IB/SL1, all of which are required for rDNA transcription. Overexpression of CSB stimulates rDNA transcription, whereas in CSB-deficient cells, rRNA synthesis is impaired (33). Consistent with CSB serving a role in the activation of Pol I transcription, depletion of CSB by siRNA reduced the association of Pol I with rDNA and inhibited pre-rRNA synthesis (our unpublished data). Like NoRC, CSB is recruited to rDNA by TTF-I bound to its cognate site adjacent to the rDNA promoter, and activation of Pol I transcription is more pronounced if both TTF-I and CSB, rather than each protein alone, are overexpressed. Activation of Pol I transcription requires the ATPase activity of CSB, indicating that the chromatin remodeling activity of CSB promotes transcription through chromatin. Thus, TTF-I and CSB synergize in transcriptional activation, a finding that underscores the functional relevance of TTF-I-mediated targeting of CSB to rDNA.

To establish the open chromatin structure at active rDNA repeats, CSB appears to synergize with WICH, a remodeling complex that consists of William syndrome transcription factor (WSTF) and the ATPase SNF2h. WICH has been shown to serve a function both in transcription and DNA replication (34). WICH is associated with the Pol I transcription machinery, and RNAi-mediated knockdown of WSTF impairs pre-rRNA synthesis (35). WSTF is enriched in isolated nucleoli, and antibodies to WSTF inhibit Pol I transcription on pre-assembled chromatin templates, but not on naked DNA, suggesting that the WSTF/SNF2h complex forms a platform for proteins that are required for transcription through chromatin. Significantly, a fraction of cellular WICH is contained in a large multiprotein complex, termed B-WICH (36). B-WICH is associated with several nucle(ol)ar proteins, including the transcription factor Myb-bp1a, the RNA helicase II/Gua, CSB and nuclear myosin 1 (NM1). Formation of B-WICH depends on active transcription, suggesting that this complex assembles at nascent 45S pre-RNA. The finding that NM1 is present in B-WICH is interesting because recent studies have revealed the indispensibility of nuclear actin and myosin in transcription by all three classes of nuclear RNA polymerases (37). Both actin and NM1 are present in isolated nucleoli (38), are associated with the Pol I transcription machinery and are required for Pol I transcription *in vivo* and *in vitro* (39). Depletion of NM1 or WSTF by siRNA inhibited pre-rRNA synthesis, suggesting that the interaction of NM1 with WSTF is required for progression of Pol I through chromatin templates (35). Thus, by analogy to rDNA silencing, the interaction of NM1 with B-WICH may recruit CSB and CSB-associated proteins to active rDNA repeats to establish a chromatin structure that facilitates transcription complex formation and elongation through chromatin.

FUTURE DIRECTIONS

Many studies have correlated epigenetic gene regulation in every aspect of tumor biology, including cell growth, differentiation, cell cycle control, DNA repair and recombination. It is less clear, however, whether changes in the epigenetic state of rRNA genes are cause or consequence of aberrant rDNA transcription in cancerous or senescent cells. The discovery and characterization of chromatin remodeling complexes, such as NoRC and CSB, that trigger the establishment and/or maintenance of specific DNA methylation patterns, histone modifications and nucleosome positions at a subset of rDNA clusters have opened new avenues for understanding the basic principles of transcriptional control at the epigenetic level. These studies have revealed that the intrinsic stability of epigenetic marks is caused by integrative cooperativity and multiple interlocking feedback mechanisms between DNMTs, histone modifying enzymes and nucleosome remodeling activities that mediate the stable commitment of specific rDNA clusters to a particular state of activity. Perturbation of this epigenetic balance is associated with alterations in rRNA synthesis and genomic instability, ultimately resulting in cellular transformation and malignant outgrowth. Though the link of epigenetic control of rDNA transcription with cell growth and proliferation is not fully understood, several scenarios can be imagined. For example, it has been postulated that silencing a fraction of rDNA repeats may enhance transcription and ribosome assembly by concentration around fewer active genes (5). It is also conceivable that silencing regulates rDNA copy usage and keeps a 'reservoir' of rRNA genes that may be used in certain developmental stages (e.g. female reproductive cells) or during carcinogenesis. Most likely, however, limiting the number of active rRNA genes might also decrease DNA damage and stabilize rDNA by repressing homologous recombination. Questions that have to be answered in the future will be to determine whether individual rRNA genes or entire NORs are subject to epigenetic control, to understand whether silencing is restricted to specific gene clusters and to examine whether heterochromatin formation spreads into neighboring sequences. Certainly, a key issue that has to be addressed concerns the role of non-coding RNA in the establishment of a specific chromatin structure and transcriptional control of rDNA. Small intergenic RNAs that are associated with the chromatin-remodeling complex NoRC and required for heterochromatin formation have joined the already known non-coding RNAs that regulate differentiation and development by a variety of mechanisms, including control of epigenetic memory, promoter selection, alternative splicing, mRNA stability and translation (40). The discovery that non-coding RNA serves a key function in nucleolar structure and epigenetic control of rRNA synthesis is likely to guide us in understanding the alterations in the Pol I transcription machinery that might precede or accompany malignant progression.

ACKNOWLEDGEMENTS

My apologies to all colleagues whose works have not been cited because of space constraints. I thank Kerstin Schmitz and Christine Mayer for critical reading and comments on this manuscript. Research in my laboratory was supported by the Deutsche Forschungsgemeinschaft (SFB/Transregio 5, SP 'Epigenetics'), the EU-Network 'Epigenome' and the Fonds der Chemischen Industrie.

Conflict of Interest statement. None declared.

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