Chapter 2

Establishment and Maintenance of Open Ribosomal RNA Gene Chromatin States in Eukaryotes

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Abstract

In growing eukaryotic cells, nuclear ribosomal (r)RNA synthesis by RNA polymerase (RNAP) I accounts for the vast majority of cellular transcription. This high output is achieved by the presence of multiple copies of rRNA genes in eukaryotic genomes transcribed at a high rate. In contrast to most of the other transcribed genomic loci, actively transcribed rRNA genes are largely devoid of nucleosomes adapting a characteristic "open" chromatin state, whereas a significant fraction of rRNA genes resides in a transcriptionally inactive nucleosomal "closed" chromatin state. Here, we review our current knowledge about the nature of open rRNA gene chromatin and discuss how this state may be established.

Key words Nucleolus, Nucleolar organizer region (NOR), Ribosomal DNA, Ribosomal RNA genes, RNA polymerase I, Transcription, Chromatin, Nucleosome, Preinitiation complex (PIC), High mobility group (HMG) box proteins, Upstream binding factor (UBF), Hmo1, Psoralen cross-linking, Chromatin immunoprecipitation (ChIP), Chromatin endogenous cleavage (ChEC), Electron microscopy (EM)

1 Introduction

This short review aims at summarizing research which has contributed to our current understanding of characteristic chromatin states at eukaryotic rRNA gene loci sharing many conserved features from yeast to human. This subject has also (partly) be covered by other reviews in the past, which are recommended for further reading [1–5]. The regulation of RNAP I transcription by epigenetic mechanisms (including posttranslational covalent modifications of histones and other components of the RNAP I transcription machinery, as well as DNA-methylation) has been reviewed in great detail in the past [6–9] and will not be subject of this article.

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In the nucleus of eukaryotic cells, the genetic information encoded in the DNA is assembled in the structure of chromatin. The basic unit of chromatin is called the nucleosome and consists of approximately 146 bp of DNA wrapped around a histone octamer (reviewed in [10–12]). The tight wrapping of the nucleic acid around the protein core renders the DNA in part inaccessible which plays an important role for the regulation of essential nuclear processes like replication, DNA-repair, and transcription. To deal with nucleosomal DNA, eukaryotic cells developed mechanisms altering chromatin structure at distinct loci in specific situations (reviewed in [13, 14]). Accordingly, characteristic changes in chromatin structure and posttranslational covalent modification state of chromatin components correlate with transitions in the transcriptional status of individual genes. Understanding the relationship between chromatin structure and transcription will be essential to fully comprehend the complex process of eukaryotic gene expression.

2 Visualization of rRNA Transcription

2.1 Actively Transcribed rRNA Genes Are Prominent Structures in Nuclear Chromatin Spreads

In eukaryotes, there are at least three different nuclear RNAPs, numbered I–III, each of which has a distinct set of target genes (reviewed in [15], see also short reviews by Merkl et al. and Pilsl et al., this issue). Whereas RNAP II transcribes all protein coding genes, RNAP III is dedicated to the synthesis of small noncoding RNAs including the 5S rRNA and tRNAs. In all organisms—with only one known exception [16]—RNAP I has only one acknowledged genomic target locus from which it synthesizes a large precursor transcript encompassing the sequences of three out of four rRNAs (hereafter called rRNA gene). Remarkably, rRNAs produced by RNAP I account for more than 60% of cellular RNA synthesis in exponentially growing *S. cerevisiae* cells (hereafter called yeast, reviewed in [17]). This is achieved by a strong promoter with a stably bound preinitiation complex (PIC) (reviewed in [18]), as well as the multimerization of the genomic templates (reviewed in [19]). Thus, rRNA genes exist as clusters of repeated transcription units, the so-called nucleolar organizer regions (NORs), at one or more genomic locations depending on the organism. Actively transcribed NORs are part of the nucleolus, the dominant nuclear substructure of early ribosome biogenesis, whereas inactive NORs do not associate with nucleoli (reviewed in [20]).

Even slightly before the different nuclear RNAPs were biochemically defined [21, 22], it was possible to visualize the enzymes transcribing their target loci in chromatin spreads by electron microscopy (EM) [23–25]. The first transcription units that were unambiguously identified by this method were the actively

transcribed extrachromosomal ribosomal RNA genes isolated from amphibian oocytes [25]. These transcription units showed a characteristic "Christmas-tree" like appearance with tightly packed elongating RNAPs forming the stem, and protein-coated nascent rRNAs extending from the polymerases forming the branches of the trees. Depending on the preparation, Christmas trees can be decorated with "terminal balls" representing preribosomal assembly intermediates [26]. Ever since, rRNA gene Christmas-trees have been observed in chromatin spreads from different cell types of many organisms (reviewed in [27, 28]). These studies yielded important insights in aspects of RNAP I transcription at the single molecule level. More recently, the spreading technique combined with cryo-EM tomography allowed to obtain more detailed structural information about yeast RNAP I transcribing its native template [29]. In fact, shortly after the first Christmas trees had been described, the conserved repeated "beads-on-the-string" nature of nontranscribed eukaryotic chromatin came into the focus of the researchers [30, 31]. The beads-on-the-string seen in EM were biochemically defined as complexes of DNA and histone octamers, the fundamental units of chromatin named nucleosomes [32–34].

2.2 rRNA Genes Transcribed by RNAP I Are Nucleosome Depleted

The above analyses of chromatin spreads provided first insights into how different RNAPs deal with the chromatin template (reviewed in [27]). Thus, nonribosomal chromatin moderately transcribed by (presumably) RNAP II remained at least partially covered with nucleosomes [35–37]. This indicated that nucleosomes may either persist or are reestablished upon RNAP II transcription. In contrast, there was evidence that RNAP I and RNAP III transcription occurred exclusively on nucleosome depleted templates even in situations when transcription rate was reduced (reviewed in [27, 38–40]). Complementing EM analyses, endonucleases were used as molecular probes to investigate chromatin structure (reviewed in [41–43]). Endonucleases can only poorly access DNA assembled into a nucleosome. Therefore, endonuclease cleavage at a genomic region of interest can be used to deduce information about local nucleosome occupancy. In such assays, DNA in actively transcribed rRNA gene chromatin was more accessible than DNA in nontranscribed rRNA gene chromatin, supporting the view of nucleosome depletion at RNAP I transcribed regions [44, 45]. This notion was corroborated by in vivo and in vitro cross-linking of chromosomal DNA with psoralen (reviewed in [46] and references therein). Psoralen is a parent compound of naturally occurring substances which can intercalate in DNA (reviewed in [47] and references therein). Upon exposure to long-wave ultraviolet (UVA) radiation, psoralen incorporation leads to DNA-interstrand cross-links. As observed for nucleases, nucleosome formation prevents psoralen intercalation into nucleosomal DNA tightly interacting with the histone octamer

[48, 49]. Therefore, psoralen cross-links are restricted to accessible nucleosome-free DNA regions. After DNA isolation from psoralen cross-linked chromatin, nucleosomes leave a characteristic footprint of approximately 146 bp of non–cross-linked DNA surrounded by cross-linked linker DNA. This can be analyzed by EM of the cross-linked DNA under denaturing conditions, in which the non–cross-linked DNA regions are visualized as single stranded DNA bubbles. Consistent with nucleosome depletion, DNA isolated from psoralen treated actively transcribed rRNA gene chromatin was heavily cross-linked and largely devoid of single-stranded DNA bubbles [50].

2.3 rRNA Genes Coexist in At Least Two Different Chromatin States

Psoralen cross-linking alters the mobility of deproteinized DNA fragments in native agarose gel electrophoresis [50]. A high-degree of psoralen incorporation in nucleosome-depleted DNA leads to a strong retardation of the corresponding fragment, whereas lower psoralen incorporation in nucleosomal DNA yields faster migrating fragments. In combination with Southern blot analysis this technique can be used to monitor nucleosome occupancy at specific restriction fragments obtained from psoralen cross-linked chromosomal DNA [50–52]. DNA-fragments deriving from psoralen cross-linked transcribed regions of rRNA genes from a human cell line and yeast cells migrated as two major bands with low and high mobility in native agarose gel electrophoresis, indicating that these genes adapt at least two different chromatin states [51, 52]. Nascent RNA was exclusively cross-linked to the fragment of low mobility, suggesting that nucleosome occupancy at actively transcribed rRNA genes is strongly decreased [51, 52]. This led to the model that rRNA genes may coexist at least in a nucleosome depleted, "open" chromatin state and a transcriptionally inactive nucleosomal "closed" chromatin state. It should be noted, that psoralen cross-linking measures nucleosome occupancy at selected loci, but does not allow straightforward conclusions about the transcriptional state of a gene (reviewed in [2, 3] and references therein). Therefore, open rRNA genes are not necessarily actively transcribed, although actively transcribed rRNA genes appear to be always in an open chromatin state. To date, no other chromosomal locus with a psoralen accessibility similar to open rRNA genes has been identified. Strikingly, psoralen cross-linking of heavily transcribed RNAP II-dependent genes did not yield fragments with the low mobility expected for fully cross-linked DNA [53]. This may corroborate the observations in EM that RNAP II transcribed gene regions retain a significant number of nucleosomal particles [35, 37]. Thus, the open rRNA gene chromatin state is probably unique regarding the extent of nucleosome depletion and the size of the nucleosome depleted region.

With the advent of chromatin immunoprecipitation (ChIP) the nucleosome-depleted nature of actively transcribed rRNA genes was challenged (reviewed in [2, 3]). In ChIP experiments, histone

molecules coprecipitated rRNA gene fragments from extracts obtained from yeast cells carrying a reduced number of rRNA gene copies [54]. Because under these conditions, the majority of rRNA genes were transcribed [55], it was concluded that RNAP I transcribes a dynamic, nucleosomal chromatin template. A subsequent study based on chromatin endogenous cleavage (ChEC) experiments in yeast supported rather the model of robust histone/nucleosome depletion at actively transcribed rRNA genes [56]. ChEC is performed in yeast strains in which a protein of interest is expressed in fusion with Micrococcal nuclease (MNase) from *Staphylococcus aureus* [57]. MNase is a secreted DNA and RNA endo- and exonuclease which has long been used to study chromatin structure (reviewed in [41, 43], see chapter of Teubl et al. in this issue). MNase activity strictly depends on calcium. Thus, due to the low intracellular calcium concentrations recombinantly expressed MNase fusion proteins are not active. After isolation of crude nuclei from cells expressing MNase-fusion proteins and addition of calcium, the tethered nuclease will cut neighboring DNA. Specific cleavage events in genomic DNA can be monitored by Southern blot analysis, primer extension or high-throughput sequencing to reveal which DNA regions were in the proximity of the MNase fusion proteins [58, 59]. ChEC experiments can also be performed in combination with psoralen cross-linking to determine if a factor of interest preferentially associates with the open or the closed rRNA gene chromatin state [56, 60]. Using this method, it could be shown that RNAP I subunits fused to MNase specifically degraded the highly psoralen cross-linked fragments derived from open rRNA gene chromatin in yeast. In contrast, histone-MNase fusion proteins preferentially degraded the poorly psoralen cross-linked fragments derived from closed rRNA gene chromatin. These results supported the hypothesis that RNAP I transcribes a histone/nucleosome depleted chromatin template [56]. The apparent contradiction of these results to the conclusions derived from ChIP experiments [54] may be explained by the notion that the term histone depletion does not exclude that a few histone molecules occasionally associate with open rRNA gene chromatin. Additionally, even in yeast cells with a lower rRNA gene copy number, a small subpopulation of rRNA genes resides in the closed nontranscribed nucleosomal chromatin state [55, 61]. This nucleosomal subpopulation might then be detected in ChIP analyses, which—unlike the combination of ChEC with psoralen cross-linking analyses—does not distinguish between DNA fragments derived from open or closed rRNA gene chromatin. In higher eukaryotes ChEC combined with psoralen cross-linking analyses have not been conducted so far. Nevertheless, endonuclease accessibility of human rRNA gene loci combined with high throughput sequencing (HTS) suggested that nucleosome occupancy is reduced at rRNA genes when compared to intergenic regions [62]. More recently, a

deconvolution histone ChIP-HTS analysis combined with genetic manipulation of RNAP I transcription in mice strongly supported that histone depletion at active rRNA genes is conserved from yeast to mammals [63, 64].

2.4 HMG-Box Proteins Are Architectural Components of Open rRNA Gene Chromatin States

The high mobility group (HMG) box is a structural motif first described in eukaryotic proteins with a high electrophoretic mobility (reviewed in [65]). HMG box proteins are involved in the regulation of many important DNA-dependent processes in the nucleus presumably due to their acknowledged function as architectural chromatin components. Along these lines, HMG-box proteins are conserved constituents of open rRNA gene chromatin (reviewed in [2, 63, 66, 67]). In vertebrates, the upstream binding factor (UBF) was initially identified to be required for proper recruitment of the basal RNAP I PIC at the rRNA gene promoter [68–70]. UBF has a characteristic arrangement of up to six tandemly repeated HMG-boxes (reviewed in [71]). Upon dimerization UBF may wrap DNA in one single loop in a structure called the "enhancesome" in vitro [72]. It was suggested that this structure establishes a characteristic architecture at the rRNA gene promoter (reviewed in [71]). In vivo, UBF molecules spread along the entire rRNA gene region transcribed by RNAP I [62, 63, 66, 73, 74]. Additionally, UBF is preferentially recruited to repetitive enhancer DNA elements preceding the rRNA gene promoter in higher eukaryotes [62, 63, 69, 73, 74]. UBF was shown to localize to NORs harboring active rRNA genes [75]. During the cell division cycle, UBF stays associated with NORs which have been transcriptionally active even upon RNAP I transcription shutdown at mitosis [76–78]. In this condition, UBF likely maintains NORs bearing active rRNA gene clusters in a hypocondensed, open chromatin state leading to "secondary constrictions" visualized by light microscopy in plant mitotic chromosomes early in the twentieth century [79–81]. Yeast contains a bona fide UBF homologue Hmo1 [82, 83]. As UBF, Hmo1 is a chromatin component of actively transcribed rRNA gene regions but has only one HMG-box and—in contrast to UBF—no reported role in RNAP I PIC formation [56, 84–86]. Both murine UBF and yeast Hmo1 are required to maintain the nucleosome depleted open rRNA gene chromatin state in the absence of RNAP I transcription [61, 63]. In agreement, both UBF and Hmo1 can destabilize nucleosomal templates in vitro [87, 88]. Furthermore, Hmo1 mediated DNA compaction, bridging and looping observed in vitro was suggested to be implicated in the maintenance of the nucleosome depleted rRNA gene chromatin state in vivo [89]. UBF and Hmo1 bind to various DNA sequences in vitro [90–92] and genome wide ChIP analyses suggested that both proteins additionally associate with nucleosome depleted promoter regions of highly transcribed RNAP II dependent genes [84–86, 93]. This indicates that UBF

and Hmo1 may generally recognize structures associated with highly transcribed DNA templates. This hypothesis was substantiated in in vitro studies in which Hmo1 bound preferentially to DNA templates thought to mimic transcription intermediates [91]. Along these lines it has also been suggested that Hmo1 protects negatively supercoiled DNA at gene boundaries in vivo [94], indicating that (r)DNA topology may be important for Hmo1 recruitment. Whereas there is evidence that recruitment of Hmo1 to rRNA gene sequences is dependent on prior RNAP I transcription [61, 86], RNAP I transcription may not be required to recruit UBF to the rRNA gene promoter or enhancer elements [95]. Thus, integration of rRNA gene enhancer repeats from *Xenopus laevis* at different loci in human chromosomes led to the formation of "pseudo-NORs" resulting in secondary constrictions in mitotic chromosomes (reviewed in [67, 96]). These structures were bound by human UBF, other components of the basal RNAP I transcription machinery and ribosome biogenesis factors [95, 97] but appear to be transcriptional inactive. Although, psoralen accessibility at pseudo-NOR sequences has not been tested to date, it is assumed that they reside in an open chromatin structure. Taken together, both Hmo1 and UBF are architectural proteins of open rRNA gene chromatin and involved in the maintenance of the nucleosome depleted state [56, 61, 63, 73]. Additionally, UBF binding at the rRNA gene promoter (and likely at enhancer regions) is likely related to its role in RNAP I PIC formation (reviewed in [18, 66]). As an essential PIC component, UBF—but not Hmo1—is required for the establishment of the open rRNA gene chromatin state in vivo [56, 63, 73].

2.5 Molecular Requirements to Establish the Open rRNA Gene Chromatin State

As indicated above, there is a tight correlation between RNAP I transcription and the establishment of the open chromatin state. In fact, studies in yeast indicated that the equilibrium of open and closed chromatin states observed in asynchronously dividing cells may be explained as the result of RNAP I transcription dependent opening and replication dependent closing of rRNA genes [61]. In each synthesis phase of the cell division cycle, replication leads to nucleosome deposition and chromatin closing at rRNA genes on both sister chromatids [98]. After rRNA genes have been replicated, opening of rRNA genes correlates with the onset of RNAP I transcription. In higher eukaryotes, cell division cycle dependent rRNA gene chromatin transitions were likely dismissed in early psoralen cross-linking studies in which interphase cells were compared with metaphase cells [51]. However at least in one study, time course experiments during the cell division cycle in a human cell line revealed very similar rRNA gene chromatin state transitions to those observed in yeast [99]. In agreement with a requirement for RNAP I transcription for establishment of the open rRNA gene chromatin state in replicating yeast cells, all rRNA genes adapt the

closed chromatin state when RNAP I transcription is impaired [61]. In addition, there was good correlation between downregulation of RNAP I transcription and closing of rRNA gene chromatin in various physiological relevant situations. Thus, rRNA gene chromatin closing occurs when cells grow to stationary phase [51, 52, 100, 101], upon UV-damage [102, 103], or when cells differentiate [104]. On the other hand, upregulation of RNAP I transcription upon transfer of stationary yeast cells in fresh growth media correlates with rapid opening of rRNA genes [100, 101]. Furthermore, the observed opening of rRNA genes when replication-mediated chromatin closing was prevented strictly depends on ongoing RNAP I transcription [61].

It remains unclear if RNAP I transcription alone suffices to establish open, nucleosome-depleted rRNA gene chromatin. Thus, additional factors may assist RNAP I to convert genes from the closed to the open chromatin state. The "Facilitates Chromatin Transcription" (FACT) complex, known to support RNAP II transcription, was shown to associate with rRNA genes in yeast and human, copurified with human RNAP I and enhanced RNAP I nonspecific transcription from chromatin templates in vitro [39, 105]. In yeast, the chromatin remodeling factors Ino80, Isw1, and Isw2, as well as the Swi/Snf complex associate with rRNA genes in vivo and ex vivo and may modulate rRNA gene chromatin structure [106–108]. Furthermore, bona fide RNAP II transcription (elongation) factors TFIIH, Paf1, Spt4/5, Spt6, and THO were reported to support RNAP I transcription [109–114]. In addition, early ribosome biogenesis factors might be components of rRNA gene chromatin in yeast and higher eukaryotes perhaps supporting efficient RNAP I transcription [97, 115]. Finally, a constantly growing number of factors which are involved in (epigenetic) posttranslational covalent modifications of chromatin components (including proteins and nucleic acids), as well as noncoding RNAs may influence RNAP I transcription and the establishment of rRNA gene chromatin states (reviewed in [6–9]). Since most of the above factors have acknowledged roles in various nuclear processes, it is, however, often difficult to distinguish between direct and indirect effects on RNAP I transcription and rRNA gene chromatin structure.

3 Conclusions

Our current knowledge of molecular mechanisms required for establishment of the open rRNA gene chromatin state is mostly based on in vivo observations or ex vivo analyses of relatively crude cellular fractions. In the future, analyses with defined in vitro reconstituted chromatin will probably allow to stringently test current hypotheses and to describe molecular mechanisms which are

responsible for chromatin opening (see [116] and chapter by Merkl et al., this issue). One promising approach may involve the investigation of isolated native rRNA gene chromatin templates. Chromosomal rRNA gene domains in their native chromatin context can be purified from yeast, and initial analyses indicate that important features of the in vivo chromatin structure are maintained upon isolation [107]. The purified chromatin can be subjected to functional biochemical assays, or single molecule structural analyses [107, 117]. The reconstitution of transcription using highly purified components of the RNAP I transcription machinery and the native chromatin template in vitro might closely reflect the in vivo situation. Together, these studies may help to derive a detailed understanding about the interplay between chromatin structure and transcription on a molecular level. Given the tight connection between RNAP I transcription and early steps in ribosome biogenesis [115], it should be attempted to include selected ribosome biogenesis factors in the purified system. Perhaps, this may lead us soon to the first Christmas trees "grown" in vitro.

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