Emerging concepts of nucleolar assembly

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Summary

The nucleolus is a large nuclear domain and the site of ribosome biogenesis. It is also at the parting of the ways of several cellular processes, including cell cycle progression, gene silencing, and ribonucleoprotein complex formation. Consequently, a functional nucleolus is crucial for cell survival. Recent investigations of nucleolar assembly during the cell cycle and during embryogenesis have provided an integrated view of the dynamics of this process. Moreover, they have generated new ideas about cell cycle control of nucleolar assembly, the dynamics of the delivery of the RNA processing machinery, the formation of prenucleolar bodies, the role of precursor ribosomal RNAs in stabilizing the nucleolar machinery and the fact that nucleolar assembly is completed by cooperative interactions between chromosome territories. This has opened a new area of research into the dynamics of nuclear organization and the integration of nuclear functions.

Key words: Nucleolus, Cell cycle control, CDK, pol I transcription, rRNA processing, Nuclear body

Introduction

In eukaryotes, the nucleus is re-assembled after each mitosis, and consequently mitotic exit is a crucial period for establishment of nuclear functions. When the chromosomes reach the mitotic poles, the nuclear envelope reforms while chromatin decondenses to form chromosome territories (for a review, see Cremer and Cremer, 2001). The nuclear machinery responsible for gene expression is then progressively imported into the nucleus or activated, and nuclear bodies are re-assembled (Spector, 2001). This period of the cell cycle allows us to investigate the initial organization of nuclear functions. Nucleolar assembly provides a good example of such dynamic organization since it is an early event that takes place in all cycling cells and requires cooperation between transcription and processing (for reviews, see Scheer et al., 1993; Scheer and Weisenberger, 1994). Furthermore, the formation of an active nucleolus is important for nuclear architecture, functional compartmentalization of the nucleus and control of cell proliferation (for reviews, see Carmo-Fonseca et al., 2000; Cockell and Gasser, 1999; Lamond and Earnshaw, 1998; Olson et al., 2000; Pederson, 1998; Strouboulis and Wolffe, 1996).

The nucleolus is the factory in which ribosome subunits are synthesized and assembled before being exported to the cytoplasm (for a review, see Hadjiolov, 1985; Mélèse and Xue, 1995; Shaw and Jordan, 1995). Ribosome biogenesis is accomplished by specific transcription and processing machineries. Consequently, the establishment of nucleolar functions at the end of mitosis depends on their activation, targeting and/or recruitment (Fig. 1). In cycling cells, nucleolar assembly is generally initiated during telophase and continues for 1-2 hours into early G1 phase (Fig. 1). In this case, mitosis follows an interphase during which nucleoli have been fully active, and nucleolar assembly benefits from machinery and complexes inherited from the previous cell cycle. Similarly, during embryogenesis, de novo nucleolar assembly integrates material of maternal origin, but it is programmed over several cell cycles, as nuclei become progressively more competent to organize and control the transcription of specific genes. In cycling cells and during embryogenesis, the assembly process has common features, although their duration is variable. In addition to the products of RNA polymerase I (pol I) transcription and processing of the precursor ribosomal RNAs (pre-rRNAs), ribosome biogenesis needs pol III transcription of 5S rRNAs as well as ribosomal protein import. The details of these other pathways involved in nucleolar assembly at the exit of mitosis are still lacking; they are therefore not discussed here.

Early work suggested that nucleolar assembly depends on the activation of the pol I transcription machinery (Benavente, 1991; Scheer and Hock, 1999; Thiry, 1996). This generates pre-rRNAs (47S in mammals), which recruit the rRNA-processing machinery. Noticeably, proteins and small nucleolar RNAs (snoRNAs) involved in rRNA processing were observed in nuclear bodies, called prenucleolar bodies (PNBs), before localizing at sites containing newly transcribed rRNAs (Jiménez-Garcia et al., 1994). From these observations, Spector and co-workers concluded that PNBs are mobile nuclear bodies that participate in the delivery of the rRNA-processing complexes to sites of ribosomal gene (rDNA) transcription (Jiménez-Garcia et al., 1994).

Here, we discuss recent findings that have illuminated the cell cycle controls on nucleolar assembly, the dynamics of delivery of the processing machinery and the role of pre-rRNAs in stabilizing nucleolar machinery. It is now clear that activation of pol I transcription at exit of mitosis is not sufficient to generate nucleolar assembly (Fig. 2). These new findings provide a more integrated view of the assembly process and its dynamics in which the localization of a component reflects its time of residency and binding affinity.

Background: pol I transcription and rRNA processing

In mammalians, the basal pol I transcription machinery is

composed of pol I, the upstream binding factor (UBF) and the selectivity factor SL1, a TBP-TAF_I (TBP-associated factor) complex (for reviews, see Comai, 1999; Moss and Stefanovsky, 1995). An important factor is the transcription termination factor (TTF-1), which mediates termination of pol I transcription and remodeling of the rDNA promoter (Längst et al., 1998). Other factors that modulate or facilitate transcription are also involved and are regulatory targets depending on different stimuli (for reviews, see Grummt, 1999; Reeder, 1999). In particular pol I transcription is closely correlated with cell growth and cell cycle, but the mechanisms controlling the correlation between the level of ribosome production and cell cycle phase remain to be clarified.

Processing of pre-rRNAs is very complex, involving cleavage, methylation and pseudouridylation (for reviews, see Smith and Steitz, 1997; Tollervey, 1996). Cleavages that remove the 5′ and 3′ external transcribed spacers and the internal transcribed spacers are controlled by several ribonucleoprotein (RNP) complexes, which act sequentially. There are early and late processing complexes, which act at early steps and late steps of rRNA processing, respectively. For example, fibrillarin participates in early rRNA processing, and Nop52 participates in late rRNA processing (Savino et al., 1999).

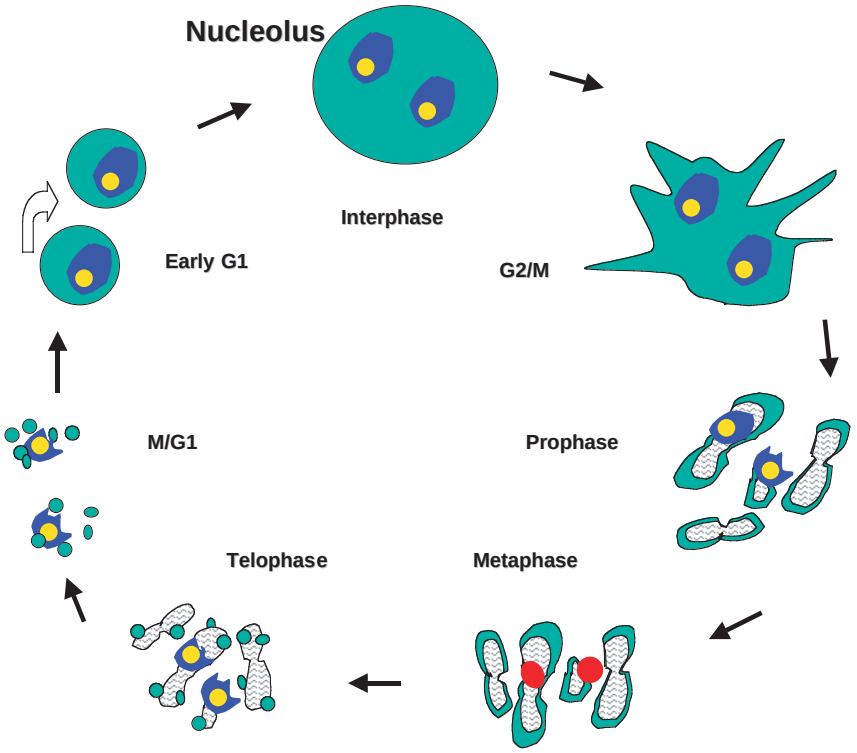

Fig. 1. Nucleolus cycle in human cells. The nucleolus during interphase is organized in three main components: the fibrillar centers in yellow containing the rDNA; the dense fibrillar component in blue corresponding to sites of transcription and early rRNA processing; and the granular component in green corresponding to late rRNA processing. At the G2/M transition, the rRNA processing machinery (green) leaves the nucleolus and during prophase becomes partially distributed over the surface of all the condensed chromosomes. The rDNA present on some chromosomes are still active (yellow and blue). At metaphase, pol I transcription is repressed (red spots for inactive rDNA). The reactivation of pol I transcription in telophase is concomitant with the gathering of the rRNA processing machinery into PNBs (green spots) at the chromosome periphery. At the end of mitosis (M/G1), nucleolar domains start to reform around the active NORs and give rise to a complete nucleolus after association of several NORs in early G1.

Cell cycle control of nucleolar assembly

In higher eukaryotic cells, entry into mitosis is accompanied by a global inhibition of transcription, in particular pol I transcription. Strikingly, the pol I transcriptional machinery – the pol I complex, UBF, SL1 and TTF-1 (Gébrane-Younès et al., 1997; Jordan et al., 1996; Roussel et al., 1996; Sirri et al., 1999) – remains associated with rDNA when pol I transcription is repressed. It assembles at sites termed nucleolar organizer regions (NORs) when interphase chromatin condenses into mitotic chromosomes. The NORs are the chromosome regions containing the repeats of rDNA genes. During mitosis, the rRNA-processing machinery becomes partially distributed over the surface of all the chromosomes. Fibrillarin, nucleolin, protein B23, PM-Scl 100, Nop52 and also snoRNAs U3 and U14, colocalize mainly at the chromosome periphery (for a review, see Hernandez-Verdun et al., 1993). The colocalization of these different factors involved in rRNA processing suggests that processing complexes are at least to some extent maintained during mitosis. They persist throughout mitosis and are found at telophase and in early G1 phase in numerous PNBs (Jiménez-Garcia et al., 1994).

How is pol I transcription repressed during mitosis? Some components of the pol I transcription machinery, such as SL1 (Heix et al., 1998) and TTF-1 (Sirri et al., 1999), are mitotically phosphorylated by CDK1–cyclin-B. CDK1–cyclin-B-mediated phosphorylation of SL1 abrogates its transcriptional activity in vitro (Heix et al., 1998), and CDK1–cyclin-B is necessary not only to establish repression but also to maintain it from prophase to telophase. Indeed, in vivo inhibition of CDK1–cyclin-B leads to dephosphorylation of the mitotically phosphorylated forms of components of the pol I transcription machinery and restores pol I transcription in mitotic cells (Sirri et al., 2000).

Interestingly, the restoration of pol I transcription by in vivo inhibition of CDK1–cyclin-B in mitotic cells leads to the accumulation of pre-rRNAs, which are not processed to form mature rRNAs. Therefore, the activation and/or relocalization of the pre-rRNA-processing machinery that normally occurs at exit from mitosis (Fig. 1) is not, or not exclusively, dependent on inhibition of CDK1–cyclin-B; pol I transcription and pre-rRNA processing might thus be regulated by distinct mechanisms. Recent results show that inhibition of CDK1–cyclin-B in mitotic cells induces the formation of PNBs, but that the presence of CDK inhibitors prevents proper relocalization of the pre-rRNA-processing machinery from these PNBs to the reforming nucleoli in early G1 phase (Sirri

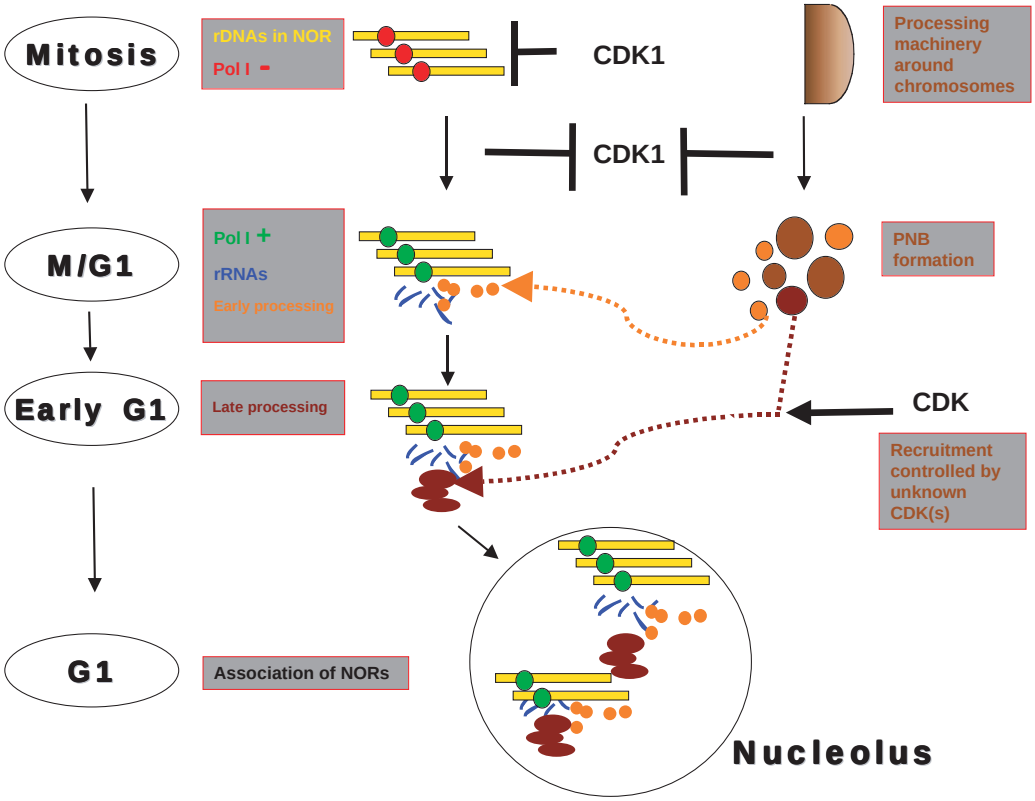

Fig. 2. Nucleolar assembly in cycling cells. During mitosis the RNA pol I machinery is associated with the rDNA in NORs (yellow bars) and the rRNA processing machinery (brown gradient) is in the cytoplasm mostly around all chromosomes. Pol I transcription is maintained repressed (red spots) during mitosis by the activity of CDK1–cyclin-B. At the end of mitosis (M/G1), the inhibition of CDK1–cyclin-B activity releases the mitotic silencing of pol I transcription (green spots) and induces the formation of PNBs corresponding to different rRNA processing complexes (orange and brown bodies). Recruitment (dashed orange arrow) of the early processing machinery is contemporary with activation of transcription (rRNA in blue), but the late processing machinery (dashed brown arrow) is recruited later by a controlled pathway (early G1). Finally in G1, association of several NORs completes nucleolar formation.

et al., 2002). An (or more than one) unidentified CDK therefore seems indispensable for proper localization of the processing machinery (Fig. 2), restoration of pre-rRNA processing and, consequently, formation of a functional nucleolus.

Dynamics of the rRNA-processing machinery

PNBs first appear as densely packed fibrils and later as mostly granular structures. This suggests that different PNBs are generated at different times or that the composition of PNBs varies (Fomproix et al., 1998; Savino et al., 2001; Verheggen et al., 2000). Because rRNA-processing complexes are first observed in PNBs and then enter the reforming nucleoli as PNBs disappear, PNBs were initially considered to be mobile structures carrying rRNA-processing complexes towards sites of pol I transcription (Bell et al., 1992; Benavente, 1991; Jiménez-Garcia et al., 1994; Ochs et al., 1985). However, examination of the dynamics of PNBs in living cells, using fluorescently tagged proteins (Dundr et al., 2000; Savino et al., 2001) and 4D time-lapse microscopy, shows that PNBs do not move from the chromosome surface to the nucleolus. Instead, oriented flow between PNBs and between PNBs and nucleoli ensures progressive delivery of the rRNA-processing machinery to the nucleoli (Savino et al., 2001). In fact, the flow could comprise large complexes, since electron microscopy has revealed particles and dense fibrils in material connecting PNBs to the nucleolus. The precise characterization of this flow will be important if we are to understand the principles behind this specific delivery. Studies of the dynamics of PNBs in living cells also indicate that the different types of PNB have different lifetimes. In addition there is an ordered recruitment of their content to the nucleolus, which indicates that this pathway is regulated.

At exit from mitosis, the processing machinery is delivered to the nucleolus through PNB formation and by directional flow. However, during interphase, analysis of the dynamics of fibrillarin by fluorescence recovery after photobleaching (FRAP) shows that fibrillarin exchanges rapidly between the nucleoplasm and nucleolus through an ATP-independent diffusion mechanism (Dundr et al., 2000; Phair and Misteli, 2000; Snaar et al., 2000). If this diffusion mechanism is also present at exit from mitosis, PNB formation could be determined by interaction(s) between the rRNA-processing complexes and partners that no longer exist after nucleolar assembly (see the discussion of the role of rRNAs below).

In addition to the PNBs, there are other bodies that contain nucleolar components. For example, several nucleolar components involved in pre-rRNA processing, including U3 snoRNA, fibrillarin, nucleolin, B23 and Nop52, can accumulate in large cytoplasmic particles termed nucleolus-

derived foci (NDF) during anaphase and telophase of various mammalian cell lines (Dundr et al., 1997; Dundr et al., 2000; Dundr and Olson, 1998). NDFs contain partially processed pre-RNAs that persist throughout mitosis (Dundr and Olson, 1998). They move quickly within the cytoplasm, and when they contact the nuclear envelope they disappear into the nucleus (Dundr et al., 2000). The dynamics of NDFs in the cytoplasm could indicate that they are associated with motors, but there is presently no direct evidence supporting this hypothesis. To explain why NDFs are not found in all cells, Dundr and Olson have suggested that, in the case of high levels of expression of components of the processing machinery, the excess material that is not retained around the chromosomes forms these cytoplasmic aggregates (Dundr et al., 1997).

The role of rRNAs in nucleolar assembly

The observation that partly processed rRNAs can be transmitted to daughter cells (Dousset et al., 2000; Dundr and Olson, 1998) in association with several rRNA processing complexes was surprising but not completely unexpected: pol I transcription is still active in prophase as the late rRNA processing complexes leave the nucleolus (Fan and Penman, 1971; Gautier et al., 1992; Prescott and Bender, 1962). These partly processed rRNAs are synthesized during prophase, stably maintained through mitosis and participate in nucleolar assembly (Dousset et al., 2000). Indeed, inhibition of pol I transcription at the time of nucleolar assembly does not impair recruitment of fibrillarin and nucleolin in the vicinity of the rDNA. The inactive nucleoli formed under these conditions contain pre-rRNAs synthesized in early prophase of the previous cell cycle, which indicates that they participate in nucleolar assembly. Therefore an active pol I machinery and elongating rRNAs are dispensable for recruitment of processing factors. However, pre-rRNAs inherited from mitosis are involved in the early stages of nucleolar assembly and present when recruitment of processing factors occurs.

These partly processed rRNAs inherited from mitosis are found in PNBs and NDFs in which processing complexes are also present (Dousset et al., 2000; Dundr and Olson, 1998). In addition, pre-RNAs and rRNA processing intermediates can be immunopurified in mitotic nucleolar processing complexes (Pinol-Roma, 1999). This could indicate that the processing complexes forming PNBs and NDFs are nucleated by these pre-rRNAs (Dousset et al., 2000; Dundr et al., 2000; Dundr and Olson, 1998; Pinol-Roma, 1999). Therefore, stable mitotic pre-rRNAs are clearly partners of the nucleolar machinery during nucleolar assembly, but their role still remains to be characterized.

During the first cell cycles of *Xenopus laevis* embryogenesis, transcription is established de novo at the mid-blastula transition (MBT) after 12 cell cycles devoid of transcription. One can therefore study assembly of the nucleolar machinery in the context of active or inactive pol I transcription. Assembly of a functional nucleolus in *Xenopus* embryos takes several cell cycles. Before any transcription, there is association of UBF with rDNA, sequential formation of PNBs and recruitment of different rRNA processing complexes. We have demonstrated that in the absence of pol I transcription, components of the rRNA-processing machinery are recruited to rDNA in association with pre-rRNAs of maternal origin (Verheggen et al., 2000; Verheggen et al., 1998). An inactive nucleolus is formed while the RNA pol I complexes accumulate in nucleoplasmic structures that exclude rDNA (Bell and Scheer, 1999; Verheggen et al., 2000).

Similarly, in mammalian embryos, functional nucleoli do not develop immediately after fertilization. Active nucleoli are assembled at species-specific stages of cleavage (Baran et al., 1996; Baran et al., 1997). In all cases, a nucleolar precursor body (NPB) is present (Fléchon and Kopecny, 1998). Nucleolar assembly occurs around these NPBs either over several cell cycles, when pol I transcription is activated early, or during one cell cycle, when pol I transcription is activated after several divisions.

Nucleolar assembly in relationship to general nuclear organization

rDNA genes are clustered in the NORs of mitotic chromosomes. In some species, in diploid cells, there is one pair of NOR-bearing chromosomes, and one nucleolus is generated around each NOR. In other species, there are several pairs of NORs, and in this case several NORs participate in the building of one nucleolus. This provides an interesting example of how several chromosome territories contribute to one functional nuclear domain. In humans, there are five pairs of NOR-bearing chromosomes, the acrocentric chromosomes 13, 14, 15, 21 and 22, some of which cooperate to form the nucleoli. In most cells, not all ten NORs are actually associated with transcription factors. For example, in HeLa cells, only six NORs are competent (i.e. associated with transcription factors) (Roussel et al., 1996). At the end of mitosis, activation of these six NORs induces six individual foci of pol I transcription close to the nuclear envelope. In living cells, these six foci move slowly within the nuclear volume without apparent coordination (Savino et al., 2001). About 1 hour after initial transcription firing, fusion of these foci is observed. Fusion is a rapid process that leads to reorganization of the nucleolus and is concomitant with general movement in the nucleus (Savino et al., 2001). These data indicate that the positioning of chromosome territories defined by NORs is significantly modified at this time.

This large-scale reorganization of the nuclear architecture cannot be due to interaction between rRNAs for several reasons. First, in living cells the nucleolar foci initially approach each other without fusing. Second, since their subsequent fusion takes place at a precise timepoint during the cell cycle, this is probably a regulated event rather than an event that depends on the amount of nucleolar activity (which varies in the different foci). Finally, even silent NORs associate with nucleoli (Sullivan et al., 2001). MacStay and co-workers have therefore proposed that clustering of NORs depends on heterochromatin adjacent to rDNA genes (Sullivan et al., 2001). An additional possibility is that heterochromatic domains of rDNA bound with Net1 and Sir2 (in yeast) maintain rDNA clustering and nucleolar integrity (for a review, see Carmo-Fonseca et al., 2000).

Conclusions and perspectives

Recent findings indicate that nucleolar assembly at exit from mitosis depends on cell cycle controls. Indeed, CDK1–cyclin-

B activity represses pol I transcription during mitosis, and its inactivation releases this silencing. The formation of PNBs is also controlled by the CDK1 pathway, whereas the recruitment of the rRNA-processing machinery appears to depend on the activity of another CDK (Fig. 2). The characterization of the CDK(s) controlling this process should be investigated in the future. Another interesting discovery is that even after assembly of the nucleolus, the nucleolus is under the control of CDK throughout interphase (Sirri et al., 2002). This is particularly relevant since some cell cycle regulators are sequestered in the nucleolus. Thus, it is important to understand how crosstalk between cell cycle regulators and the active nucleolus is achieved.

The pre-rRNAs generated by pol I transcription are localized at the sites of active rDNA gene clusters. The binding affinity of the processing proteins for these pre-rRNAs can explain the compartmentalization of the processing machinery in the functional nucleolus. During nucleolar assembly, pre-rRNAs also appear to participate in compartmentalization of the processing machinery. Mitotic pre-rRNAs are involved in the reformation of the nucleolus after mitosis, and maternal pre-rRNAs in *Xenopus* embryos are involved in the regrouping of PNBs around rDNA. In both situations, the intriguing question is how the mitotic pre-rRNAs or the maternal pre-rRNAs regroup around rDNA genes. The presence of pre-rRNAs in PNBs and NDFs could also explain the formation of temporarily organized bodies. The stability of these rRNAs could determine their lifetime. Clearly, these questions must be addressed if we are to understand the role of stable rRNAs in the formation and/or maintenance of nuclear structures.

Ribosome biogenesis involves the pol-I, pol-II and pol-III-dependent transcription pathways, the intranuclear translocation of 5S RNAs, the ordered assembly of ribosomal proteins and the export of the small and large ribosomal subunits. Presently we do not know how the coordination between these pathways is regulated and controlled. This is therefore an important goal of research in this area.

Another interesting unanswered question is how the presence of a functional nucleolus contributes to general nuclear architecture and function. The fact that a functional nucleolus is a large nuclear domain (a third of the yeast nucleus), represents the highest concentration of RNA in the nucleus, and is a site of silencing for reporter pol II genes suggests that it has a general role in nuclear function. Indeed, the nucleolus can exclude or sequester molecules that play a role outside the nucleolus. Therefore it will be important to determine whether clustering of rDNA genes has a direct effect on the organization of other genes or on the distribution of heterochromatin.

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