New Insights into Nucleolar Architecture and Activity

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The nucleolus is the most obvious and clearly differentiated nuclear subcompartment. It is where ribosome biogenesis takes place and has been the subject of research over many decades. In recent years progress in our understanding of ribosome biogenesis has been rapid and is accelerating. This review discusses current understanding of how the biochemical processes of ribosome biosynthesis relate to an observable nucleolar structure. Emerging evidence is also described that points to other, unconventional roles for the nucleolus, particularly in the biogenesis of other RNA-containing cellular machinery, and in stress sensing and the control of cellular activity. Striking recent observations show that the nucleolus and its components are highly dynamic, and that the steady state structure observed by microscopical methods must be interpreted as the product of these dynamic processes. We still do not have detailed enough information to understand fully the organization and regulation of the various processes taking place in the nucleolus. However, the present power of light and electron microscopy (EM) techniques means that a description of nucleolar processes at the molecular level is now achievable, and the time is ripe for such an effort.

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I. Introduction

The eukaryotic nucleus is structurally and functionally compartmentalized; it contains a number of membraneless domains or subcompartments that are revealed by the localization of specific nucleic acid sequences or proteins by hybridization probes, antibodies, or green fluorescent protein (GFP)-tagging (Misteli, 2005; Spector, 2001). It has been proposed that this organization increases the efficiency of cellular processes by bringing together, in space and time, factors involved in RNA synthesis and ribonucleoprotein (RNP) maturation, as well as DNA replication and repair. Increasing the effective concentration of the required macromolecules in a given nuclear subcompartment increases the probability of the interactions that are needed for corresponding biochemical processes to take place. Surprisingly, the importance of nuclear compartmentalization really only began to be appreciated during the 1990s, when cell biologists studying the nucleus started to make extensive use of the tools of nonisotopic immunocytochemistry and in situ hybridization.

The most prominent of these nuclear domains is the nucleolus, whose key function is the synthesis and processing of ribosomal RNAs (rRNAs) and their assembly into ribosomal subunits (r-subunits) (Busch and Smetana, 1970; Hadjiolov, 1985). However, it has also become clear that even typical nucleolar components may be located in other places than the nucleolus; their location can be altered by cell cycle stage, modulation of cell growth or cell differentiation (Bicknell et al., 2005; Silva et al., 2004). The same is true for numerous proteins or macromolecular complexes previously considered as nonnucleolar (Desterro et al., 2005; Mekhail et al., 2005; Politz et al., 2005). These observations underscore the importance of cell biology, in particular using microscopical approaches, because these relationships are very difficult to analyze by molecular biology and biochemistry alone (Sleeman, 2004). For this reason cell biology has become one of the pillars of the rediscovered discipline of systems biology (Gorski and Misteli, 2005; Lazebnik, 2002; von Bertalanffy, 1950).

A different location of a macromolecule is likely to imply a different function. Thus it has been established that nucleoli have nonstandard functions besides being ribosome factories (Olson et al., 2002; Pederson, 1998a,b). Among these are involvement in mRNA export or degradation, biosynthesis of the signal recognition particle (SRP), biogenesis of some snRNAs and tRNAs, the sequestration of regulatory proteins, control of the cell cycle, aging, and stress sensing (Gerbi et al., 2003; Handwerger and Gall, 2006; Mekhail et al., 2005; Olson and Dundr, 2005; Olson et al., 2002; Pederson 1998a,b; Raška et al., 2006; White, 2005).

Exciting progress is evident both in the understanding of ribosome biogenesis and in the expanding list of nonribosomal nucleolar functions (Raška et al., 2006). A major source of this enormous progress is intensive proteomic research on nucleoli in yeast. The yeast model is also amenable to powerful
genetic analyses and improved biochemical approaches. These tools have enabled the characterization of an unexpectedly large number of nucleolar proteins and RNAs, as well as the isolation of various ribosomal substructures (Fatica and Tollervey, 2002; Tschochner and Hurt, 2003). Thus, despite 35 years or more of intensive research into the nucleolus, most of what is known today about the molecular details of the many steps of pre-rRNA processing and modification, ribosome biogenesis, and export of r-subunits has emerged only in the past few years (Tschochner and Hurt, 2003).

Understanding of ribosome biosynthesis in higher eukaryotes is advancing rapidly as well, also mainly through proteomic research (Andersen et al., 2002, 2005; Couté et al., 2006; Pendle et al., 2005; Scherl et al., 2002), although the pace of advancement is slower than with yeast because of the greater technical difficulties and the lack of such powerful genetic resources as are available in yeast. At the same time, it has become clear that the process of ribosome biogenesis, both in yeast and in higher eukaryotes, is much more complex than originally foreseen (Tschochner and Hurt, 2003).

In this review, progress in the understanding of how the processes of the ribosome biosynthetic pathway as well as non-ribosomal functions are integrated within nucleolar architecture will be discussed. The primary focus will be on nucleoli of mammalian cells. However we will also describe results from yeast and other species where relevant, and in particular where equivalent data has not yet been determined for mammalian cells. Ribosomal proteins (r-proteins) and rRNA sequences are highly conserved across the eukaryotes so that such inferences across species and kingdoms are often very illuminating. However, these inferences should also be treated with a degree of caution, because there are also fundamental differences. For example, the nucleolus in the yeast Saccharomyces cerevisiae does not disassemble in mitosis. In metazoans and higher plants, rRNA synthesis ceases in prophase and the nucleolus disassembles. rRNA synthesis then resumes in telophase, accompanied by the reassembly of the nucleolus (DiMario, 2004; Hernandez-Verdun, 2006; Olson and Dundr, 2005). Furthermore, some findings with important relevance for medicine are specific to mammals or even only to humans (Kamath et al., 2005; Kopp and Huang, 2005; Maggi and Weber, 2005; Raška et al., 2006; Yao and Yang, 2005).

II. Historical Background

A detailed description of nucleolar research in the classical era is given in the extensive volume by Busch and Smetana (1970). The nucleolus was first described in 1781 by Fontana, who noted its occurrence in the slime of an eel and reported it in a simple remark in his work on the venom of vipers
The name “nucleolus” was coined by Valentin (1839), who noticed that most cells had a secondary nucleus or a “nucleus within a nucleus.” Some 100 years after Fontana’s discovery, when Montgomery published an extensive review on the subject (Montgomery, 1898), the nucleolus became one of the first intracellular structures to be described in detail. In this review the variability of both size and number of nucleoli and their disappearance and reappearance at mitosis was described. These remarkable conclusions still hold true today. Some of the most important scientific contributions came as late as the early 20th century, when Heitz related the formation of nucleoli with chromosome location and McClintock studied X-ray–induced chromosomal rearrangements. Both scientists built on the cytogenetic methods introduced by Zacharias (1883), who first combined the histological concept of chromatin with the chemical substance nuclein (Dahm, 2005), a term introduced by Miescher and still preserved in today’s name of deoxyribonucleic acid. Heitz (1931) observed a direct correlation between the number and lengths of secondary constrictions, that is, regions of mitotic chromosomes with a thin appearance, where DNA could not be detected by the Feulgen reaction, and the number and size of nucleoli. McClintock (1934) suggested that the chromatin at the secondary constriction was the nucleolar organizing element (now termed nucleolar organizing region, NOR), because this region alone, without the original secondary constriction, was able to give rise to a separate nucleolus. These data definitively established the nucleolus as a genetically determined element.

Brachet (1940) and Caspersson and Schultz (1940) demonstrated that nucleoli are enriched in RNA. Due to its higher content of RNPs, higher density, and greater refractive index with respect to the surrounding nucleoplasm, the nucleolus had been clearly seen in nuclei in cytological specimens, first by straightforward phase contrast microscopy and later on by electron microscopy (EM). As it was the most prominent structure in the nuclei of most eukaryotic cells, the nucleolus attracted the interest of many investigators and was intensively studied by many research groups. This interest culminated in the early 1960s when the primary function of the nucleolus as a factory in which rRNA is synthesized and ribosomes are assembled was established (Birnstiel et al., 1963; Brown and Gurdon, 1964; McConkey and Hopkins, 1964; Perry, 1965; Ritossa and Spiegelmann, 1965; Scherrer et al., 1963).

During this period, the introduction of the first isolation procedures (Monty et al., 1956; Vincent, 1952) and importantly the standardization of such methods (Busch et al., 1963; Maggio et al., 1963) made biochemical investigations of the nucleolus possible. These investigators isolated nucleoli from starfish oocytes and from rat liver nuclei in a procedure that used sonication to break nucleolus-associated chromatin, releasing the nucleoli.

With the advent of EM, it became possible to define nucleolar structure in much more detail than had been possible with light microscopy (LM).
These high-resolution studies showed that the nucleolus lacks a membrane, but contains dense granular material and fibrillar structures (Bernhard and Granboulan, 1958; Swift, 1958). Important findings about the distribution of DNA and RNA in nucleoli resulted from combining EM with autoradiography. Granboulan and Granboulan (1965) were the first to show that labeled RNA was located in the intranucleolar fibrillar areas of cultured cells after the incorporation of tritiated uridine. The rRNA was also shown to move subsequently toward the granular region (Granboulan and Granboulan, 1965; Unuma et al., 1968; von Gaudecker, 1967). In situ hybridization was also first used in the 1960s to locate the r-genes (Pardue and Gall, 1969). Extra-chromosomal nucleoli containing amplified copies of the r-genes were shown to exist in specialized cells such as developing amphibian and insect oocytes (Brown and Dawid, 1968; Gall, 1968). An important development in the understanding of the nucleolus was the spectacular EM visualization of transcriptionally active r-genes in the form of “Christmas trees” (CTs) (Miller and Beatty, 1969).

The introduction of the new nucleic acid technology in the latter part of the 1970s brought new breakthroughs in nucleolar research. At the same time, however, this started a steady decline of interest in the nucleolus compared to other nuclear structures and nonribosomal processes. This was because the new technology made it possible to study individual genes in a way that had previously been difficult or impossible. Paradoxically, it actually became easier in some ways to study the expression of a unique human gene by means of gel techniques than to study 400 human r-genes found in clusters on five pairs of chromosomes (Raška et al., 2006).

A new wave of interest in the nucleolus began in the last decade of the 20th century. This new interest mainly resulted from enormous progress in the technology for cell biology, particularly developments in microscopy. It became possible to observe living cells expressing GFP-tagged (nucleolar) proteins in real time by digital microscopy, either using confocal microscopes with photomultipliers or fluorescence microscopes equipped with sensitive charge-coupled device (CCD) cameras. In a breakthrough in the cell biology of the nucleolus, microscopy of an r-protein tagged with GFP was used to establish the flux of r-subunits from nucleoli to nucleoplasm and cytoplasm (Hurt et al., 1999). Very soon, these qualitative live-cell observations were quantified by computer modeling of the kinetics of the processes. In this way, much has been learned about the function, mutual interactions, and unexpectedly high dynamics of nuclear and nucleolar proteins using such techniques as fluorescence recovery after photobleaching (FRAP), inverse FRAP, fluorescence loss in photobleaching (FLIP), and fluorescence resonance energy transfer (FRET) (Cheutin et al., 2004; Dundr et al., 2002; Handwerger and Gall, 2006; Misteli, 2001a; Phair and Misteli, 2000).
III. Ribosomal Genes

A. Synthesis and Maturation of Ribosomal RNA, and Biogenesis of Ribosomes

1. Organization of r-genes

The nucleolus is formed around genetic loci on the chromosomes called nucleolar organizing regions (NORs), which consist of tandemly repeated genes for rRNA (Fig. 1). Several NORs can exist within a species, but each organizer usually resides on a separate chromosome. Human cells, for example, have NORs on the short arms of the five acrocentric chromosomes (13, 14, 15, 21, and 22). They contain about 400 r-genes (Hadjiolov, 1985). In the budding yeast, S. cerevisiae, the NOR is found on the long arm of chromosome XII and contains about 100–200 tandem repeats in a single cluster (Planta, 1997).

In most species, including mammals, each r-gene repeat consists of 18S, 5.8S, and 28S rRNA coding sequences, transcribed internal and external spacers (ITS and ETS) and a non-transcribed intergenic spacer (Fatica and Tollervey, 2002; Hadjiolov, 1985) (Fig. 1). According to some data, the length and structure of the nontranscribed spacer may vary considerably even within the same NOR (Caburet et al., 2005; Lebovsky and Bensimon, 2005). In the yeast S. cerevisiae, each of the tandemly repeated r-genes also carries a 5S gene within the “non-transcribed” spacer region (Neigeborn and Warner, 1990).

![Diagram of human ribosomal genes and their transcripts. Reproduced from Raška et al. (2004) with permission of Elsevier.](image-url)
Expression of rDNA genes is closely associated with the fibrillar regions of the nucleolus (Biggiogera et al., 2001; Goessens, 1984). These regions are thus considered to be where the initiation of pol I-dependent transcription takes place. The organization of the transcription sites is still not understood in detail (Ploton et al., 2004; Raška et al., 2004), but current evidence clearly indicates that they are located in the dense fibrillar component and its boundary with the fibrillar centers (see Section IV.A.3).

Evidence shows that in mammalian cells in culture about half of the r-gene repeats are transcriptionally silent. This can be demonstrated by psoralen cross-linking (Sogo et al., 1984), by the observation that approximately 50% of r-gene repeats are methylated (Santoro and Grummt, 2001) and by the fact that not all NORs are associated with transcription factors during metaphase. On the other hand, in a few human cell lines, the majority (possibly all) of the genes are active (McStay, personal communication). In the HT1080 human cell line, it has been demonstrated that all NORs probably have associated transcription factors and all repeats are likely to be psoralen-accessible and therefore active (McStay, personal communication). Interestingly, this cell line contains a constitutively active oncogenic ras allele leading to permanent extracellular signal-regulated kinase (ERK) activation (Plattner et al., 1999). ERK is a known activator of pol I transcription through phosphorylation of the transcription initiation factor TIF-IA (Zhao et al., 2003) and the upstream binding factor (UBF) (Stefanovsky et al., 2001, 2006).

In “normal” human cells the chromosomal distribution of active versus inactive genes is an interesting question. It seems there are two classes of NORs. Some NORs are fully repressed and not associated with any transcription factors. The remaining NORs apparently contain a mosaic of active and inactive genes (McStay, personal communication; Raška et al., 2004).

In many species, including mammals, NORs can cluster together and participate in the formation of a given nucleolus. This occurs, for example, in early embryogenesis or at the end of mitosis when the disassembled nucleoli are reassembled. During mitosis, some NORs, probably those that were transcriptionally active in the previous interphase, form prominent secondary constrictions, which appear as gaps when stained with Giemsa or specific fluorochromes. The chromatin in these regions is 10-fold less condensed than the rest of the chromosome (Heliot et al., 1997; Sumner, 1982). Previously inactive r-genes apparently give rise to a compacted form of the NOR locus and no secondary constrictions are seen in the metaphase chromosomes (Mais et al., 2005).

Mitotic NORs, as well as interphase nucleoli, can be selectively stained with silver at the light and electron microscopic levels. This is due to the high concentration of so-called Ag-NOR proteins, generally referred to as argyrophilic proteins. The major Ag-NOR proteins detected during active transcription in interphase are nucleolin and B23, the two major nucleolar proteins. Ag-NOR
staining of mitotic NORs seems to be due to the largest pol I subunit and UBF (Heliot et al., 1997; Roussel and Hernandez-Verdun, 1994; Roussel et al., 1996). Recent data suggests that extensive binding of UBF is responsible for the morphology of NORs in the secondary constrictions of mammalian mitotic chromosomes (Mais et al., 2005). This may explain why silver staining is equivalent to labeling of the pol I machinery as a protein marker of secondary constrictions.

2. Pol I Transcriptional Machinery

Transcription of the nuclear genome in animals and fungi is divided among three RNA polymerases: pol I, pol II, and pol III. Plants also have a fourth RNA polymerase, which is involved in transcriptional silencing mechanisms (Herr et al., 2005; Kanno et al., 2005). The coordinated activity of RNA polymerases I, II, and III is required for the ribosome biogenesis. The important step of ribosome biosynthesis, nucleolar transcription of rDNA, is carried out by pol I. Besides pol I and the r-genes, a number of pol I-associated factors and rDNA-specific transacting factors are required for efficient rDNA transcription. In yeast, two basal factors, upstream activating factor (UAF), associated with the TATA-box binding protein (TBP), and core factor (CF), respectively, bind the upstream and core promoter elements (UE and CE, respectively). These factors are involved in the earliest steps of the pol I activation, in formation of the preinitiation complex (PIC) (Moss, 2004; Nomura et al., 2004).

Similarly, basal transcription factors have been identified in human cells: the promoter selectivity factor (SL1; also known in mouse as transcription initiation factor TIF-IB); transcription initiation factor IA (TIF-IA/Rrn3); and UBF. SL1 is composed of TBP and at least three TBP-associated factors (TAFs) (Comai et al., 1992). Before transcriptional initiation, PIC, which includes these three factors, is assembled on the promoter region of the DNA (Comai, 2004; Grummt, 2003; Moss, 2004; Moss and Stefanovsky, 2002; Prieto and McStay, 2005; Russell and Zomerdijk, 2006). UBF binds to the rDNA promoter via its high-mobility-group (HMG) boxes whereas the SL1 complex has an important role in controlling the promoter specificity through the TAFs (Heix et al., 1997). Following this, PICs recruit an initiation-competent subfraction of pol I to the rDNA promoter. This is achieved by UBF interaction with the PAF53 subunit of pol I, by association of TIF-IA with the initiation-competent subpopulation of pol I, and by interaction of SL1 with TIF-IA and UBF (Grummt, 2003; Mais et al., 2005; Prieto and McStay, 2005). UBF has been shown by both biochemical and microscopy approaches to be located not only at the promoter, but also extensively over the r-gene cluster. It is responsible not only for the specialized decondensed state of r-chromatin in interphase, but also for the secondary constrictions
seen in mitosis (see Section III.A.1). However, it has been proposed that SL1 may serve as a molecular scaffold on the rDNA, stabilizing the assembly and binding of other PIC factors (Friedrich et al., 2005; Russell and Zomerdijk, 2006).

Several reports have shown that topoisomerase I (topo I) is another key regulator of nucleolar transcription (Christensen et al., 2002; Leppard and Champoux, 2005; Scheer and Rose, 1984). Topo I is associated with pol I throughout the entire cell cycle, presumably being necessary for the organization of the nucleolus and reinitiation of rDNA transcription after mitosis (Christensen et al., 2002). However, inhibitors of the rRNA transcription machinery—camptothecin, an inhibitor of topo I, or the pol I inhibitor actinomycin D—cause topo I to dissociate from pol I and to leave the nucleolus, independently of the status of rRNA transcription (Christensen et al., 2004).

Several reports show actin and a nuclear isoform of myosin I (NMI) to be physically associated with both the pol I holoenzyme and the r-genes, suggesting that these cytoskeletal proteins are part of the nucleolar transcription machinery (Fomproix and Percipalle, 2004; Miralles and Visa, 2006; Pederson and Aebi, 2005; Philimonenko et al., 2004). Some authors have concluded that NMI is important for the initiation of transcription and that actin is involved both in the initiation and elongation (Percipalle and Visa, 2006; Philimonenko et al., 2004). de Lanerolle et al. (2005) have proposed a speculative model in which the NMI acts as an actin-dependent motor that powers the passage of the transcription machinery along the DNA template.

3. Maturation of Precursor rRNA

The product of pol I transcription is a long precursor rRNA (pre-rRNA), encompassing the coding regions, ITS and ETS of the r-genes (Fig. 1). This precursor is modified by approximately 200 base methylations and pseudouridylations before the three mature rRNAs are produced by cleavage. Each modification is made at a specific position in the pre-rRNA. These positions, and the cleavage sites, are specified by “guide RNAs,” which bind to the pre-rRNA through base-pairing of about 15 nt. This brings an RNA-modifying enzyme to the appropriate position or promotes cleavage of the pre-rRNA. All of these guide RNAs are members of a large class of RNAs called small nucleolar RNAs (snoRNAs) (Eliceiri, 1999; Kiss, 2002; Lestrade and Weber, 2006; Mattick and Makunin, 2005). snoRNAs are approximately 60- to 150-nt long and exist as small nucleolar RNP particles (snoRNPs). All snoRNPs can be classified on the basis of the conserved motifs of their component snoRNAs as either C/D box (containing RUGAUGA and CUGA elements) or H/ACA box (containing ANANNA and ACA elements) (Henras et al., 2004). Most box C/D snoRNPs guide methylation of ribose in
pre-rRNA whereas most box H/ACA snRNPs direct the isomerization of specific uridines to pseudouridines (Borovjagin and Gerbi, 2005; Kiss, 2002; Mattick and Makunin, 2005).

After the base modifications, the precursor rRNA is cleaved by endo- and exonucleases in the two ITS and the 5′ and 3′ ETS sequences to generate the mature 18S rRNA, for the small r-subunit, and 5.8S and 28S rRNA, for the large r-subunit. Very early in the transcription process, proteins and snoRNAs associate with the nascent pre-rRNA. In yeast it is clear that the pre-rRNA may be cleaved co-transcriptionally, before transcription of the entire gene is complete (Gallagher et al., 2004; Granneman and Baserga, 2005; Henras et al., 2005). However, the extent of co-transcriptional processing of the pre-rRNA precursor is not yet clear in higher eukaryotes (see also Section III.B).

In the yeast *S. cerevisiae* and a few other simpler eukaryotes, the other ribosomal RNA, the 5S rRNA, is contained within the basic rDNA unit, as it is in prokaryotes, and is transcribed in the nucleolus by pol III. In vertebrates and plants, the 5S rRNA is transcribed in the nucleoplasm outside the nucleolus and is then transported to the nucleolus. It is not known why the 5S rRNA should be transcribed separately (Dechampesme et al., 1999; Raué, 2004).

An essential step of ribosomal biogenesis is the assembly of 28S, 18S, and 5.8S rRNAs with 5S RNA and with the r-proteins (Dechampesme et al., 1999; Gerbi and Borovjagin, 2004). The genes encoding the r-proteins are transcribed by pol II. It is interesting that many snoRNAs are encoded in introns of r-protein genes in the sense orientation and are produced by exonucleolytic processing of the debranched intron after splicing. The association of rRNAs with r-proteins results in the formation of RNP precursors, which mature into the 40S and 60S subunits of the ribosome and are exported through the nuclear pore into the cytoplasm (Fatica and Tollervey, 2002; Rudra and Warner, 2004; Tschochner and Hurt, 2003).

4. Biogenesis of Ribosomes

Most of our understanding of the individual steps of ribosome biogenesis, including nuclear export, has been obtained from the model yeast, *S. cerevisiae* (Johnson et al., 2001; Tschochner and Hurt, 2003). Proteomic methods backed by genetic and biochemical analyses, as well as EM of isolated preribosomes, have shown that the composition of the preribosome subunits is highly dynamic and involves over 150 proteins (Bernstein et al., 2006; Dragon et al., 2002; Fatica and Tollervey, 2002; Fromont-Racine et al., 2003; Granneman et al., 2005; Nagahama et al., 2004; Nissan et al., 2004; Tschochner and Hurt, 2003). These proteins have various activities, and include nucleases, RNA helicases, GTPases, AAA-ATPases (ATPase associated with various cellular activities) and many other late-acting factors responsible for ribosome export. The systematic analysis of yeast r-proteins has revealed that most
of them carry out essential roles in ribosome biogenesis, making them indispensable for growth. Different r-proteins control distinct steps of nuclear and cytoplasmic pre-rRNA processing and, thus, ensure that only properly assembled ribosomes become engaged in translation. Comparative analysis of dynamic and steady-state maturation assays reveals that several r-proteins are required for efficient nuclear export of pre-18S rRNA, suggesting that they form an interaction platform with the export machinery. In contrast, the presence of most other r-proteins is required before nuclear export is initiated (Ferreira-Cerca et al., 2005). This last study draws a correlation between the in vitro assembly, structural localization, and in vivo function of r-proteins.

The current model for ribosomal export, which is apparently conserved from yeast to higher eukaryotes, is that karyopherin/exportin CRM1 associates with the pre-60S particles in the nucleus through binding to a non-ribosomal protein, Nmd3p, which in turn is linked to the r-protein Rpl10p. Although the name suggests its involvement in nonsense-mediated decay of mRNA, Nmd3p in fact plays a role as an adaptor molecule in the export of both small and large r-subunit and provides the nuclear export signal (Hedges et al., 2005; Trotta et al., 2003). This allows for the formation of an export complex in a Ran-GTP–dependent manner and unidirectional trafficking of nascent 60S subunits to the cytoplasm (Gadal et al., 2001; Ho et al., 2000; Thomas and Kutay, 2003; Trotta et al., 2003). It is proposed that Rpl10p binds to the 60S subunit only in the cytoplasm, and Nmd3p is then released through the action of two other cytoplasmic factors, the Rpl10p chaperone and a GTPase (West et al., 2005).

Although nuclear export of the pre-40S particles is also affected if CRM1 function is inhibited by leptomycin B (Moy and Silver, 1999; Thomas and Kutay, 2003), the link between the karyopherin and these preribosomal particles remains unclear. By tracking the nuclear exit of this precursor, the r-protein Rps15 has been identified as a determinant of preribosomal nuclear export in human cells (Leger-Silvestre et al., 2004; Rouquette et al., 2005).

B. Visualization of Ribosomal Genes by Miller Spreads

The only method so far available for the detailed visualization of active rDNA genes involves the disintegration of nucleoli by a hypotonic spreading method described almost 40 years ago (Miller and Beatty, 1969). This spreading technique enables the r-genes to be visualized by EM, and shows the gene axis with many engaged pol I molecules, increasing lengths of the attached nascent transcripts with the globular RNP particles (knobs) at the 5’-ends, and the non-transcribed spacer regions between the repeated genes. These spectacular preparations of genes from nucleoli are known as
FIG. 2  Electron micrographs of nucleoli together with corresponding spread preparations of nucleolar chromatin in the form of CTs. Thin-sectioned nucleoli (A, D, F) and corresponding CTs (B, C, E, G) from yeast, Xenopus oocyte, and mouse cell, respectively. Thin-sectioned nucleoli exhibit a tripartite organization with FC, DFC, and GC. Note that the yeast CTs in (B) exhibit various degrees of pol I loading and that the yeast CT in (C) instead corresponds to an r-gene regularly loaded with pol I. The arrows in (C) show large terminal knobs. These large knobs do not always remain associated with the transcripts at the 3′ end of the gene (the relevant part of the 3′ end is designated with an arrowhead); the disappearance of the knobs reflects a co-transcriptional release of the 40S r-subunit (Osheim et al., 2004). In (E), a group Xenopus CTs, normally loaded with pol I, is shown. The arrowheads in (G) designate terminal knobs on nascent pre-rRNAs from mouse. The FC, DFC, and GC are designated by F, D, and G.
Christmas trees (CTs) (Fig. 2). The terminal knobs that can be seen on the nascent rRNA transcripts correspond to rRNA processing complexes. These complexes contain U3 snoRNP, which base-pairs with sequences near the 5’ end of the pre-rRNA (Mougey et al., 1993; Scheer and Benavente, 1990; Sharma and Tollervey, 1999). Chooi and Leiby (1981) showed in CTs from mammalian cells that r-proteins associate in a specific sequence with nascent pre-rRNA; proteins of the small r-subunit are associated with the 18S sequence of the pre-rRNA, whereas those from the large r-subunit are associated with the 28S sequence of the pre-rRNA.

It is emphasized that CTs are visualized in a standard way only using certain cell types, typically yeast cells and maturing amphibian or insect oocytes (Dawid and Brown, 1968; Gall, 1968). In maturing oocytes of amphibians (and insects), amplification of r-genes takes place. These cells may possess thousands of fully active r-genes and the CTs are “easily” visualized in spread preparations. In amphibian oocytes, r-genes have the capacity to form extrachromosomal rDNA copies that replicate independently and give rise to many extrachromosomal nucleoli (Dawid and Brown, 1968; Gall, 1968). Due to the presence of interfering chromatin structures in mammalian somatic cells, a complete r-gene repeat has never been visualized and the few pictures obtained of r-genes show either CTs with maximal pol I loading or inactive r-genes covered by nucleosomes. These results were interpreted to support the binary model of r-genes (Reeder, 1999) in which each gene is a “binary unit” that is either on or off and, if on, is heavily loaded with transcribing pol I. The recent findings by French et al. (2003) in yeast challenge the binary model. These authors demonstrate the presence of active and inactive genes that are found intermingled and randomly distributed within spreads of tandem repeats of r-genes. In addition, the individual active genes are characterized by different levels of pol I loading. CTs correspond to fully extended genes, and their length usually greatly exceeds the diameter of nucleoli. This indicates that the genes and their nascent transcripts are considerably shortened and compacted in their native state.

CT spreads have been used to study early rRNA processing in yeast, because they are easy identified by their high transcriptional activity (French
et al., 2003). The terminal knobs are dynamic entities. Initially, they are about 15 nm in size. Later during transcription these knobs change into a larger knob (~40 nm, Fig. 2), which corresponds to the so-called small subunit (SSU) processome required for pre-18S rRNA processing (Dragon et al., 2002). The large knobs are cleaved co-transcriptionally from the nascent transcript at variable frequency, releasing the pre-40S ribosome (Gallagher et al., 2004; Granneman and Baserga, 2005; Osheim et al., 2004). The frequency of co-transcriptional cleavage decreases as cells grow to higher density (Osheim et al., 2004). Interestingly, EM of transcriptionally active Dictyostelium rRNA genes has revealed that r-RNA processing also occurs during transcription: co-transcriptional cleavage has been observed in the ITS, indicating that both this step, and the earlier cleavage releasing the 5′ ETS, can take place on nascent transcripts (Grainger and Maizels, 1980). However, co-transcriptional processing of rRNA has not been shown to occur in other higher eukaryotes.

The factor responsible for SSU processome assembly and compaction has been recently identified (Hoang et al., 2005). Analysis of CTs has also helped to identify the small subunit protein Rpa12p of pol I with its termination factor in yeast (Prescott et al., 2004). High-resolution pictures of pre-60S yeast r-particles have been obtained (Nissan et al., 2004).

IV. Nucleolar Structure and Function

A. Nucleolar Structure

1. Nucleolar Structural Components

In ultrathin EM sections prepared by standard procedures, most nucleoli display a concentric arrangement of three types of components, defining a tripartite organization (Fig. 2):

1. The innermost component structures called the fibrillar centers (FCs), are lightly stained regions in which a fine fibrillar substructure can often be seen (Recher et al., 1969). Based on their EM appearance and the presence of pol I and UBF in both FC and mitotic NORs, FCs are considered to be the interphase “counterparts” of mitotic NORs (Goessens, 1984). However, in our view it is clear that FCs do not encompass active rRNA genes (Gonzalez-Melendi et al., 2001; Koberna et al., 2002; Raška, 2003; Raška et al., 2006; see later). Importantly, the rRNA gene arrays of individual NORs must unravel and give rise to several FCs, because the number of FCs is much higher than the number of NORs.
2. FCs are mostly surrounded by the dense fibrillar component (DFC). The DFC has the appearance of densely packed fibrils. In mammalian nucleoli, the DFC is densely stained in conventional electron micrographs, whereas in plants the DFC staining is considerably less dense. In some cases, the DFC occupies large volumes of the nucleolus, occasionally interspersed with small FCs (so-called “reticulated” nucleoli or nucleoli with “nucleolonema”) (Busch and Smetana, 1970; Sato et al., 2005).

3. The peripheral nucleolar region forms a considerable part of the nucleolar volume. It has a grainy appearance, consisting of RNP granules 15 to 20 nm in size. Consequently it is called the granular component (GC) (Busch and Smetana, 1970; Leger-Silvestre and Gas, 2004; Mosgöller, 2004).

Besides these three principal nucleolar components, strands of more or less condensed chromatin may penetrate into the nucleolus from the surrounding perinucleolar condensed chromatin. In this way, one or more FCs in each nucleolus can be in direct contact with chromatin fibers. In some places these are seen in EM thin sections as nucleolar interstices that interrupt the layer of the DFC. Furthermore, lightly stained regions of 0.1 to 1.0 μm in diameter or larger are also seen in micrographs of nucleoli. They are referred to as nucleolar vacuoles, but it is not clear whether they represent a functional nucleolar compartment (Goessens, 1984; Hadjiolov, 1985; Schwarzacher and Wachtler, 1993). Large central nucleolar vacuoles are particularly common in plant nucleoli.

In the “typical” nucleolus of a higher animal cell, the GC occupies ∼75% of the volume of the nucleolus, with the DFC accounting for only ∼17% and the FCs only ∼2%. A typical higher plant nucleolus has a much higher proportion of DFC (up to 70%), with FCs nearer 1% (Jordan and McGovern, 1981; Shaw and Jordan, 1995). However, the DFC in those nucleoli where it accounts for such a high proportion often does not appear very electron dense in conventional thin section. It can be difficult to distinguish the components of many types of nucleoli and it appears that there is often a gradual transition between the DFC and the GC. In reality, far from being uniform structures fitting into one general or ideal scheme, nucleoli show great variability of structure throughout the plant and animal kingdoms (Shaw and Jordan, 1995).

Studies with dextrans of different molecular weights show that nucleoli are permeable to macromolecules up to 2000 kDa (Handwerger et al., 2005). These findings suggest a simple “sponge” model of nucleoli, in which the interior of each subcompartment is penetrated by “nucleoplasm” forming a network of nucleolar channels of different sizes providing easy access to macromolecules from the nucleoplasm (Handwerger et al., 2005). This model is consistent with the highly dynamic nature of nucleolar compartments (see also Section IV.B).
2. Nucleolar Organization

The previously described tripartite nucleolar arrangement has recently been challenged with a concept of bipartite nucleoli in which only the amniotes, including mammals, possess the three structural components whereas the anamniote nucleoli have no FC (Thiry and Lafontaine, 2005). This idea is based on the hypothesis that, during evolution, a third nucleolar compartment emerged at the transition between the anamniotes and the amniotes, following a substantial increase in size of the rDNA intergenic region. This hypothesis cannot be true as currently formulated, because, for instance, amphibian (anamniote) nucleoli do possess FCs (Handwerger et al., 2005; Mais and Sheer, 2001; Fig. 2), as, also, do plants (Shaw and Jordan, 1995). Also, FCs were originally defined by their morphology and cytochemistry (Goessens, 1973; Recher et al., 1969). The immunolocalization results, on which the bipartite hypothesis is extensively based (Thiry and Lafontaine, 2005), were not obtained until 15 years later (Scheer and Rose, 1984). Interestingly, using cryoelectron microscopy of rapidly frozen, vitrified sections (CEMOVIS) (Al-Amoudi et al., 2004), in which cellular structures are preserved and observed as closely as possible to their native state, the three standard nucleolar components are almost indistinguishable; the GC is the only nucleolar structural constituent visible in such preparation, and FC and DFC cannot be distinguished (Bouchet-Marquis et al., 2006). But this results from the lack of amplitude contrast in the unstained native cryo-sections, as in freeze-substituted nucleoli, which should correspond well to the native cryo-preparations, the tripartite organization of nucleoli is seen (von Schack and Fakan, 1993).

The overall structure of a nucleolus is largely determined by its activity in ribosome biogenesis. For example, dormant human lymphocytes from peripheral blood, which have low rates of ribosome biogenesis, frequently contain a single small nucleolus, termed a ring-shaped nucleolus, containing just one FC (Raška et al., 1983; Smetana et al., 1967, 1968). Upon cell activation, the size, shape and internal organization of the nucleolus are changed. Thus actively growing and cycling mammalian somatic cells, which require a high level of ribosome production, usually have one or more large nucleoli, each containing many FCs (Koberna et al., 2002; Ochs and Smetana, 1989; Raška et al., 1983). It should be noted, however, that the higher nucleolar activity in rat neurons is accompanied by the occurrence of a giant FC (Lafarga et al., 1994; Navascues et al., 2004).

Any simple structural model of the nucleolus, such as the tripartite model, must be an oversimplification. Nucleoli are host to a number of different biochemical processes taking place at any moment in a growing cell. This activity produces many protein and nucleoprotein complexes of different sizes, including pre-ribosomal particles. This simple fact necessarily makes
any simple structural model of the nucleolus, such as the tripartite model, an oversimplification. Furthermore, GCs have been considered to represent pre-ribosomes at various stages of maturation (Busch and Smetana, 1970) and are thus necessarily heterogeneous. However, many granules do not contain RNA (Politz et al., 2005) so the GC contains not only ribosomal particles but also RNA-free zones that are likely to serve other functions. Similarly, fibrillarin, a specific marker of the DFC, is located close to r-genes during transcription, probably associated with nascent transcripts, but is not located as close to the r-genes during their replication (Pliss et al., 2005). This suggests the DFC is functionally heterogeneous. The subnuclear Cajal bodies are known to share a number of common protein and RNP factors with nucleoli (Gerbi et al., 2003) and are often associated with, or even engulfed within, the nucleolus (Ochs et al., 1994). Finally, a number of studies describe nonstandard functions for the nucleolus (see Section IV.D) and show that “non-nucleolar” proteins can frequently be sequestered in nucleolar subdomains (Hoang et al., 2005; Mekhail et al., 2005; Tsai and McKay, 2005). Clearly any simple model of the nucleolus is insufficient to account for these observations.

3. Nucleolar Structure and Christmas Trees

Nucleolar morphology has been correlated with various steps of the ribosome biogenesis through mapping of various ribosomal and nucleolar components (Raška et al., 2006). However, the active rRNA genes in the form of CTs have not yet been truly seen in thin-sectioned nucleoli. In fact, an intense debate about their location has raged since 1984.

Pioneering ultrastructural autoradiographic images showed first incorporated sites of tritiated uridine in the DFC (Granboulan and Granboulan, 1965). This result indicated that the DFC is the site of nucleolar transcription. However, 20 years after these autoradiographic data were published, pol I was exclusively localized to FCs by a pre-embedding immunogold approach (Scheer and Rose, 1984), challenging numerous autoradiographic studies situating nucleolar transcription in the DFC (Fakan and Bernhard, 1971; Fakan and Nobis, 1978). An avalanche of “rectified” high-resolution autoradiographic studies situating active r-genes in the FCs ensued (Thiry and Goessens, 1991). Later on, it became apparent that the pre-embedding pol I mapping result (Raška, 2003, 2004) could be biased because the approach revealed pol I in FCs, but would not detect it in the DFC (Raška et al., 1995). It should be also noted that in immunocytochemical labeling, one cannot say whether the immunogold signal corresponds to active or inactive pol I. In fact, there is one active pol I molecule for about 10 inactive ones within nucleoli of mammalian cells (Dundr et al., 2002). In our opinion the pol I signal in FCs corresponds to nontranscribing pol I molecules (Raška et al., 2006).
The best current approach for identifying CTs is to map nonisotopically incorporated modified nucleotides such as bromo-uridine (BrU) into nascent rRNA transcripts by immunocytochemistry. This method allows sensitive detection with a resolution far greater than autoradiographic methods. Current data using mapping of incorporated BrU into nascent transcripts shows that CTs, at least in mammalian cells as well as onion and pea root cells, are found in the DFC, including the border region between the DFC and FC (Casafont et al., 2006; Cmarko et al., 2000; Gonzalez-Melendi et al., 2001; Melcak et al., 1996; Raška et al., 2004; Shaw and Brown, 2004) (Figs. 3 and 4). Thus, unequivocal evidence exists for CTs within the DFC, which necessarily must include the DFC/FC borders. There is no convincing evidence that CTs are found entirely within FCs. Furthermore, because there is a consensus that pre-rRNA processing and pre-ribosome assembly are organized vectorially away from the r-genes, location of CTs purely at the DFC/FC border would seem to be sterically impossible. Ultimately this question will be subsumed by high-resolution structural data showing the molecular organization of the different complexes and their higher order topology; already the argument about DFC versus FC seems somewhat archaic (Raška et al., 2006).

Nevertheless, the view that active r-genes are present in the FCs is still maintained by several groups (Derenzini et al., 2005; Ploton et al., 2004; Thiry et al., 2000). The findings of Mais and Scheer (2001), who mapped active r-genes in FCs of extranucleolar nucleoli from intact Xenopus laevis oocytes as well as from isolated germinal vesicles by LM immunocytochemistry, seem to support this view. The latter authors failed, however, to show significant transcription signal at the ultrastructural level. It should be noted that LM may lack the necessary resolution to locate nucleolar transcription sites, as has been shown for human cells (Malinsky et al., 2002; Raška, 2003). Thus, it will be necessary to confirm the LM results of Mais and Scheer (2001) at the EM level. The oocyte nucleoli should provide excellent sensitivity for this approach, because they possess on average 1500 fully active genes per nucleolus. This corresponds to approximately 15,000 nascent rRNA chains, which provide more than 10^6 potential bromine epitope targets per nucleolus resulting from BrU incorporation. In addition, isolated Xenopus germinal vesicles represent an ideal subject for nascent/newborn rRNA localization approaches using halogenated uridine triphosphates.

Once transcription has started, it is assumed that the maturation of the pre-ribosomes takes place in a vectorial arrangement (Jordan, 1984; Mosgöller, 2004; Raška et al., 2004, 2006). Pre-rRNA initially accumulates in DFC and the first processing steps take place in this region. The later steps of ribosome biogenesis take place in the GC and subsequently almost mature r-subunits move out of the nucleolus and reach the cytoplasm (Granboulan and Granboulan, 1965; Lazdins et al., 1997; Olson and Dundr, 2005; Shaw et al., 1995). The vectorial maturation of pre-rRNA is correlated with the vectorial distribution of the nucleolar machinery successively involved in
the different processing steps (Beven et al., 1996; Biggiogera et al., 1989; Hernandez-Verdun, 2006). Actin and myosin I have been detected within nucleoli (Miralles and Visa, 2006; Philimonenko et al., 2004), but apparently do not play a role in the movement of pre-rRNP complexes.

4. Nucleolar Mitotic Disassembly and Assembly

In higher eukaryotes, as the cells proceed through the phases of mitosis, the nucleoli disperse and disappear and pol I transcription gradually decreases until it is completely silenced (DiMario, 2004; Hernandez-Verdun, 2005, 2006; Leung and Lamond, 2003; Olson and Dundr, 2005). While transcription factors such as UBF and SL1 as well as topo I and several pol I subunits remain associated with the mitotic NORs (Chen et al., 2005; Leung et al., 2004; Roussel et al., 1996), the remaining pol I subunits, as well as the majority of rRNA processing and ribosome assembly components, move from the DFC and GC out of the nucleolus. Thus by the end of prophase, the nucleolus is no longer visible (Gautier et al., 1992; Olson and Dundr, 2005). Some factors, including several snoRNAs, move toward the nuclear envelope and then associate either with the condensing chromosomes forming a sheath surrounding the chromosomes called the perichromosomal region (Gautier et al., 1992; van Hooser et al., 2005; Yasuda and Maul, 1990), or are found in the cytoplasm and appear to become associated with the spindle apparatus (Gassmann et al., 2005; Hernandez-Verdun and Gautier, 1994). The perichromosomal region has been observed in a variety of plant and animal cells (Hernandez-Verdun and Gautier, 1994) and is suggested to have a role in the equal distribution of proteins to daughter cells required for early nuclear and nucleolar assembly following mitosis (van Hooser et al., 2005).

At anaphase, the nucleolar proteins associated with the perichromosomal region become diffuse within the cytoplasm and can associate to a large extent with cytoplasmic particles called nucleolus-derived foci (NDF). These are large bodies (1–3 μm in diameter) that can number as many as 100 per cell. These NDF contain not only a number of processing components but also partially processed pre-rRNA molecules (Dundr and Olson, 1998). As the cells proceed through telophase the number of NDFs decreases and another kind of particle, the prenucleolar bodies (PNB), appear in the cell. PNBs are discrete fibrogranular structures seen in both animals and plants. They are considered to contain prepackaged nucleolar complexes (Angelier et al., 2005; Jimenez-Garcia et al., 1994; Savino et al., 2001). The PNBs represent an intermediate step in the recruitment pathway of nucleolar processing factors. Why this step is necessary, and why the various factors are not recruited directly on to newly transcribed rRNAs is currently not clear. It has been proposed that the PNBs could serve as assembly platform of processing complexes at this period of the cell cycle (Hernandez-Verdun, 2006). Finally, the redistribution of existing nucleolar material, including NORs, takes place
between the two daughter cells (Chen et al., 2005; Fuchs and Loidl, 2004; Leung et al., 2004; Olson and Dundr, 2005).

The mechanism that governs the disassembly of nucleoli in prophase is linked to the repression of rDNA synthesis that occurs during mitosis. This mitotic silencing is regulated by the cyclin-dependent kinase (Cdk)/cyclin B kinase, which phosphorylates many specific substrates (Chou et al., 2006). It has been shown that phosphorylation of the TAF110 subunit of SL1 results in the suppression of pol I activity, and also prevents protein-protein interaction between the SL1 complex and UBF, thus disrupting the PIC (Heix et al., 1998; Kuhn et al., 1998; Sirri et al., 2000, 2002). In addition to the transcription factors, nucleolin and B23 are also physiological substrates for Cdk/cyclin B (Peter et al., 1990). Their mitotic phosphorylation status probably contributes to the dispersion of the nucleolus during prophase. Interestingly, an important role for B23 in maintenance of nucleolar structure was shown (Gonda et al., 2006; Louvet et al., 2006; see also Section IV.B).

At the end of mitosis, during telophase, reactivation of the ribosome assembly machinery takes place. The cellular phosphatase Cdc14p reverses Cdk/cyclin B-mediated phosphorylation to restore SL1 activity, which apparently induces the first events of nucleologenesis (Sirri et al., 2002; Trautmann and McCollum, 2005). Moreover, even after pol I activity begins to be restored at the end of mitosis, UBF, which has also been inactivated by phosphorylation, must be reactivated (Klein and Grummt, 1999; Olson and Dundr, 2005). This suggests that reactivation of the ribosome synthesis machinery occurs in multiple steps, probably allowing separate regulation of different stages.

In yeast, the nucleolus does not disassemble (Strunnikov, 2005) but stays a morphological entity. In budding yeast, in particular, no significant mitotic change in pol I transcription has been observed (Fuchs and Loidl, 2004; Spellman et al., 1998). However, the rDNA structure does undergo significant changes in mitosis (Granot and Snyder, 1991; Guacci et al., 1994). It has been shown that the essential structural maintenance of chromosomes complex, condensin, plays a key role in organizing the mitotic structure of the rDNA chromatin (Bhalla et al., 2002; Freeman et al., 2000; Hirano, 2005). A mechanism, which uses the Cdc 14 early-anaphase release (FEAR)/mitotic exit network (MEN)/Cdc14 pathways, seems to have adapted the function of condensin for the specific task of segregating nucleoli without disassembly (D’Amours and Amon, 2004; see also Section IV.D.3).

B. Nucleolar Plasticity and Dynamics

Groundbreaking photobleaching experiments have led to a complete reassessment of the extent of both nuclear and nucleolar protein dynamics (Handwerger and Gall, 2006; Misteli, 2001a; Olson and Dundr, 2005; Phair
and Misteli, 2000) and similar results show the rapid diffusion of RNA (Politz et al., 2003). Thus, it has been demonstrated that proteins diffuse freely through the nuclear space, including the nucleolus, and the mean residence time of most nucleolar proteins in nucleoli can be calculated to be only a few tens of seconds (Misteli, 2001a). The nucleolus exists as a discrete structure because certain proteins, some of which remain to be characterized, bind to the rDNA, forming a more or less stable core on which the complex set of nucleolar interactions and dynamic processes is built. The surrounding nucleoplasmic components continually exchange with this nucleolar “supercomplex,” and the steady-state composition of the nucleolus is to a great extent the result of the fact that the nucleolar residence time of nonnucleolar proteins, which do not find interacting partners in nucleoli, is one or more orders of magnitude shorter than that of nucleolar proteins. Thus, the nucleolus represents a steady-state structure with its components in dynamic equilibrium with the surrounding nucleoplasm (Raška et al., 2006).

In a recent study, drug-induced inhibition of rRNA processing events was shown to cause dispersion of rDNA clusters and disassociation of the late rRNA processing proteins, which accumulated in distinct masses away from the transcription sites (Louvet et al., 2005). Removal of the drug led to nucleolar reformation and restarted rRNA processing. Dynamical behavior in response to cellular stress has also been shown by the observation of nucleoli after transcriptional arrest by pol I inhibitor treatments, which leads to the segregation of nucleolar components and the formation of structures termed nucleolar caps, surrounding the central nucleolar body (Shav-Tal et al., 2005). In this study, photobleaching experiments showed that nucleolar caps are dynamic structures, and they were shown to require energy for their formation.

It should be noted that nucleolar proteins are not recruited to nucleoli through a common nucleolar targeting signal, but through functional interactions with other macromolecules already present in nucleoli; for both nucleoli and other nuclear subcompartments, the term retention signal is more accurate than targeting signal (Misteli, 2005). A GTP-driven cycle has been identified as the first possible mechanism for protein retention in the nucleolus (Misteli, 2005; Tsai and McKay, 2005). Nucleostemin (see Section IV.D.1) has GTP-binding sites, is preferentially present in stem cells and is down-regulated during differentiation (Tsai and McKay, 2005). The GTP-bound form of nucleostemin is localized to the nucleolus. Exchange of GTP in the nucleolus reverses the nucleolar binding and nucleostemin is released to the nucleoplasm. The involvement of a GTP switch indicates that nucleolar localization can be regulated and may be responsive to extracellular stimuli through signaling pathways.

What, then, is responsible for the maintenance of the typical steady-state nucleolar structure? The classical explanation (Scheer and Hock, 1999) is that pol I driven transcription, with r-genues serving as nucleation sites, organizes
and maintains nucleoli. However, Gonda et al. (2003) have shown in Xenopus that the maintenance of nucleolar morphology can be uncoupled from pol I-driven transcription by the action of specific intrinsic proteins. These RNA binding proteins, FRGY2a and FRGY2b, reversibly disassemble the nucleolus in vitro and in vivo, although pol I transcription continues. This provides the first evidence that the maintenance of nucleolar morphology can be uncoupled from pol I-driven transcription by specific intrinsic proteins. The abundant nucleolar phosphoprotein B23 is an interacting partner of the proteins (Gonda et al., 2006). Therefore, although transcriptional activity of pol I is necessary, it is not sufficient for the maintenance of nucleolar structure, which must depend on the complex intermolecular interaction of nucleolar components. The role of these proteins in nucleolar disassembly may be
through their non–sequence-specific rRNA binding, which may prevent the interaction of rRNA with other RNAs or proteins.

Findings of Louvet et al. (2006) also show that phosphorylation status of protein B23 is important in nucleolar organization. This phosphorylation is dependent on casein kinase 2 and mutation of the major phosphorylation site on B23 induces the reorganization of nucleolar components, which separate from each other. It has been proposed that casein kinase 2 phosphorylation could have a global role in nuclear activity (Louvet et al., 2006).

In an important study, Dundr et al. (2002) tagged subunits of pol I and PIC with green fluorescent protein (GFP) and determined the kinetics of assembly and elongation of the pol I transcription complexes by FRAP and related techniques. They concluded that the pol I complex is disassembled after the termination of transcription and a new complex is reassembled from subunits on r-genes for a new round of transcription. However, this “hit-and-run” mechanism for pol I has been questioned in yeast (Schneider and Nomura, 2004). Biochemical studies show that the pol I complex remains stable after its dissociation from the DNA, the two largest subunits within the pol I complex remaining associated through many rounds of transcription (Schneider and Nomura, 2004). These results support the idea that preassembled, ready-to-use multiprotein pol I transcription factories are present in nucleoli. It also appears that in human cells, separate subunits of pol I move coordinately,
either as part of a stable complex or through the actions of common receptors 
(Andersen et al., 2005). Because pol I subunits and the mechanism of transcrip-
tion in yeast and mammals are similar, it would be worthwhile confirming 
the dynamics of pol I subunit exchange in mammalian cells by more direct 
means like those used by Schneider and Nomura (2004).

The cell needs precisely equimolar amounts of rRNA and r-proteins for 
ribosome assembly (Rudra and Warner, 2004). Research suggests that this 
balance may be achieved posttranscriptionally or posttranslationally rather 
than at the level of transcriptional regulation (Andersen et al., 2005; 
Lamond, personal communication). Mass spectrometric proteomic analysis 
showed that, after proteasome activity is blocked with any of three different 
inhibitors, there is a large nuclear accumulation of r-proteins, indicating that 
normally a large turnover of r-proteins by the proteasome occurs. The rapid 
synthesis and turnover of r-proteins has been confirmed by analysis of 
individual GFP-tagged r-proteins in stable cell lines. Pulse-labeling tech-
niques, and the complementary imaging approach of whole cell photobleach-
ing in stable cell lines expressing GFP-tagged nucleolar proteins, show that 
the r-proteins are synthesized and accumulated in nucleoli faster than any of 
the other nucleolar proteins. A proposed working model is that r-proteins are 
made in excess and rapidly imported into the nucleus and nucleolus. Then 
either they associate with rRNA, assemble into an r-subunit and are exported 
to the cytoplasm, or they get ubiquitinylated and degraded in the nucleus 
(Andersen et al., 2005; Lamond, personal communication). This model received 
support from Stavreva et al. (2006) who showed that ubiquitin and the 
proteasome play a role in ribosome biogenesis.

Thus, current understanding of the functional organization of the nucleo-
lus is that it is a self-organizing, dynamic system (Misteli, 2001b). Nucleolar 
components are continuously exchanged with the nucleoplasm, their detailed 
dynamics generating the observed stable overall nucleolar configuration 
(Fig. 5). In turn, the fine control and tuning of gene expression is ultimately 
responsible for the generation and maintenance of this highly dynamic, but 
permanent, subcompartment. Conversely, the nucleolus is an open system, 
and even though it is a steady-state structure, it is far from equilibrium. Its 
morphology is directly linked to its functional status and can quickly and 
even dramatically react to external stimuli (Louvet et al., 2005; Politz et al., 
2003; Rasˇka et al., 2004; Shav-Tal et al., 2005) (Fig. 6).

C. Nucleolar Proteomics: Spotlight on the Naming of 
the Parts

High throughput characterization of proteins has now been applied to several 
species, and is beginning to show in much more specific detail the range 
of other activities that are located in the nucleolus. For example, three
separate mass spectrometric analyses of purified human cell culture nucleoli have now been published (Andersen et al., 2002, 2005; Scherl et al., 2002), and one of purified plant (Arabidopsis) cell culture nucleoli (Pendle et al., 2005). In both species, many proteins were identified whose biochemical activity is known but which were not previously thought to be located in the nucleolus. It could be argued that many of the proteins identified were contaminants—such a large and poorly defined structure as the nucleolus is likely to be impossible to fully separate from other nuclear and cellular components. Nevertheless, the proteomic analysis has been confirmed by systematic protein expression studies using GFP-tagged proteins. For example, Pendle et al. (2005) showed that the vast majority (87%) of the proteins identified by proteomic analysis were nucleolar-located by structural criteria as well. Many of the unexpected human proteins have also subsequently been confirmed as being in the nucleolus by structural methods such as GFP tagging and immunofluorescence (Fox et al., 2002a). It is also notable that

FIG. 5 Dormant and phytohemagglutinin-stimulated human lymphocytes. (A) and (B) show light micrographs, (C) and (D) show equivalent thin section EMs. (A) Human peripheral blood lymphocyte stained for RNA containing structures with toluidine blue (Smetana et al., 1966), contains a small nucleolus (arrow) with RNA enriched structures only at its periphery. (B) After 48 h stimulation, lymphocytes contain large nucleoli (arrow). (C) Prior to stimulation, one large FC is seen in the nucleolus of the dormant cell. (D) After 48 h stimulation several tiny FCs are scattered in the large nucleolus of the stimulated cell. Bars: 5 μm (A, B) and 0.5 μm (C, D). Reproduced from Raška et al. (2004) with permission of Elsevier.
FIG. 6 Schematic of nuclear or nucleolar rearrangement. The degree of transience in a structure and thus the mean residence time for a molecule depends not only on the interacting structural motifs but also on the strength of interactions of the remaining part of the molecule through its other structural motifs. The diagram illustrates that it is possible for a given component to be localized into two different structures through two different interaction motifs. (A) shows a schematic arrangement of components in two spatially separated compartments or subcompartments. (B) shows the
a large proportion of the nucleolar proteins identified in these proteomic analyses is currently completely uncharacterized, further supporting the view that we have as yet a very incomplete picture of the biochemistry that is taking place in the nucleolus. See (http://www.lamondlab.com/nopdb/; http://bioinf.scri.sari.ac.uk/cgi-bin/atnopdb/home) for current databases of nucleolar proteins identified.

A second source of nucleolar protein identification has been screens in which cDNAs have been randomly fused to GFP and the subcellular localization of the fusion proteins determined. This approach has turned up an eclectic mix of proteins localized in the nucleolus, some expected as nucleolar constituents, others not. In pilot screens aimed at systematically localizing proteins in mammalian cells, in yeast (Huh et al., 2003), and in plant cells (Koroleva et al., 2005), 2–3% of GFP fusions were highly enriched within the nucleolus. As expected, these include many RNA processing functions as well as protein kinases and phosphatases that may regulate various aspects of rRNA metabolism (Koroleva et al., 2005). Perhaps more surprising was the finding that certain transcription factor-like proteins were preferentially located in the nucleolus, whereas related family members were found mainly in the nucleoplasm. See (http://gfp-cdna.embl.de/) for a database of these proteins.

In their nucleolar analysis, Andersen et al. (2005) made use of an isotopic labeling method in mass spectrometry termed SILAC (stable isotope labeling by amino acids in cell culture) to analyze the dynamics of the nucleolar proteins. By growing cells in isotopically substituted amino acids, cultures were differentially labeled, and each culture was subjected to a different treatment by a metabolic inhibitor. The cultures were then pooled and nucleoli were purified and analyzed by mass spectrometry. In this way the same peptides were produced from each set of nucleoli in the same way, but their source could be identified by their differing masses, and the ratios of dynamic redistribution of one component caused by its displacement by a new (e.g., newly synthesized) factor with a higher affinity. (C) shows an example of a change (e.g., phosphorylation/dephosphorylation) in a structural motif, which results in a dynamic relocalization similar to that seen in (B). Alternatively, this component itself can be modified and redistributed as shown in (D). We emphasize that the interactions within a given complex may be regulated by factors that are spatially separated, and “remote” regulation is therefore possible in this scheme. Such remote regulation may be missing in in vitro systems. This underlines the importance of in situ studies of the cell. Importantly, the dynamics of transient interactions, producing shorter or longer residence times of components in a given (nucleolar) compartment, cannot be shown in this simplified drawing. The scheme illustrated here is applicable to both ribosomal and “non-canonical” functions of nucleoli even though this simple scheme does not explicitly encompass the complex intermolecular interaction of nucleolar components as shown for instance in Gonda et al. (2003, 2006). (From the work of Ivo Melčák and Ivan Raška; Copyright permission of Vesmir spol. s r. o.).
their abundance in the nucleoli of the differently treated cell cultures could be calculated. This adds valuable quantitative data to the mass spectroscopic analysis. This and similar approaches provide very powerful methods for examining the behavior and dynamics of defined sets of proteins by proteomics.

Leung et al. (2003) carried out a bioinformatics analysis of the proteomic analyses of nucleoli available, and also compared the spectrum of proteins identified with those that had been identified as nucleolar by previous studies. They found 121 proteins identified in the literature up to 2002; this was increased to about 400 proteins by the first two human nucleolar proteomics analyses (Andersen et al., 2002; Scherl et al., 2002). Leung et al. (2003) also analyzed the amino acid composition and motif content of the identified proteins and found a greater incidence in specific motifs, such as FGGR and RGGF, which are consensus sequences for asymmetric arginine dimethylation, compared to the entire genome. More generally, bioinformatic analyses of the nucleolar proteome are beginning to suggest a chimeric origin for eukaryotic genomes, with proteins from the nucleolus showing a putative archaeabacterial ancestry, in contrast to the presumed eubacterial and cyanobacterial origin of mitochondria and chloroplasts (Staub et al., 2004).

D. Non-Conventional Roles of the Nucleolus

Besides the functions directly related to ribosome biogenesis, it has been shown that the nucleolus is directly involved in or implicated in a number of other cellular processes (Olson et al., 2002; Pederson, 1998a). In many cases the discovery of these additional functions has been the result of studies localizing factors involved in diverse processes, and often these initial studies have been confirmed by more detailed biochemical analysis. However, for many of these “unconventional” nuclear activities the experimental data is currently restricted to a single species, and it is not possible to say as yet whether these unconventional roles are general functions of the nucleolus or adaptations in individual species or groups of species.

In addition to the functions discussed later, there is a fairly large and growing literature showing a link between the nucleolus and viral infections (Hiscox, 2002, 2003 for reviews), including DNA, RNA, and retroviruses. Viral proteins colocalize with nucleolar proteins such as nucleolin, B23, and fibrillarin, and can cause their redistribution during infection. These proteins may be used by the viral replication cycle, which itself may be located in the nucleolus. Apart from using host proteins for replication, viruses may also target the nucleus and nucleolus to disrupt host functions and to inhibit antiviral responses (Hiscox, 2003). A detailed discussion of this important topic is beyond the scope of this review.
1. Nucleolus and Cancer

Nuclear morphology has long been one of the key factors used in tumor grading. In principle, morphometric parameters of nucleoli, such as number, size (area), and distance from nuclear membrane, can be determined in toluidine blue-stained specimens (Busch and Smetana, 1970; Nafe and Schlote, 2004; Smetana et al., 1966). Furthermore, following the early observations of Montgomery (1895) that rapidly growing cells typically exhibited larger and more numerous nucleoli, cancer biologists quickly made the conceptual connection between AgNOR staining and cell proliferation (Derenzini et al., 1990). AgNOR staining appeared to be of diagnostic and prognostic value in different types of human tumor, including pharyngeal carcinoma, multiple myeloma, breast cancer, prostate carcinoma, uterine leiomyoma, thyroid neoplasm, as well as in leukemia patients (Camargo et al., 2005; Madej et al., 2006; Romao-Correia et al., 2005; Smetana et al., 2005, 2006; reviewed in Maggi and Weber, 2005).

Additional prognostic information is based on the occurrence of the perinucleolar compartment (PNC), a structure that has been closely linked to malignant transformation by both in vitro and in vivo experiments (Kamath et al., 2005; Kopp and Huang, 2005). PNCs have been detected in paraffin-embedded breast tissue samples and are positively correlated with the disease progression. These findings suggest that formation of PNC is indicative of the degree of aggressive behavior of breast cancer cells and show the potential for observation of the PNC to monitor the clinical progression of this type of cancer (Kamath et al., 2005; Kopp and Huang, 2005).

Much of the work from the past decade has focused on the ability of newly discovered nucleolar oncogenes to promote and suppress tumorigenesis via nucleolus-based mechanisms. Several papers have shown that the c-Myc protein, the product of the c-myc proto-oncogene, is localized in the nucleolus, where it controls the synthesis of rRNA by pol I, in addition to its role in controlling pol II and pol III transcription (Oskarsson and Trumpp, 2005). Grandori et al. (2005) demonstrated, in human cells, that c-Myc associates with the TBP and TAF components of SL1. Furthermore, the association of TBP with the promoter increases in the presence of high levels of c-Myc and rapidly decreases upon c-Myc down-regulation. This suggests that c-Myc positively regulates the efficiency of pol I recruitment to target promoters. Grewal et al. (2005) have proposed that c-Myc regulates rRNA transcription during larval development of flies. It has also been proposed that the nucleolus sequesters c-Myc in quiescent cells, while providing a pool of c-Myc that is readily available to reach its targets in the nucleus (Sanders and Gruppuso, 2005). Finally it has been shown that c-Myc is able to regulate the activity of all three polymerases in mammalian cells, and thus to coordinate overall ribosome synthesis and cell growth (Arabi et al., 2005). These observations
indicate a key role for c-Myc in tumor-promoting pathways through the regulation of ribosome biogenesis.

Nucleostemin is a protein that is predominantly localized to the nucleolus, and undergoes dynamic shuttling between the nucleolus and the nucleoplasm using a GTP-based mechanism (Tsai and McKay, 2005). It has no known involvement in ribosome biosynthesis, but rather appears to play a role in controlling cell growth and proliferation. Nucleostemin is preferentially expressed in neural stem cells, embryonic stem cells, and several cancer cell lines where it functions in a p53-dependent manner (Tsai and McKay, 2002). It is concentrated in rRNA-deficient domains within the granular component of the nucleolus (Politz et al., 2005). This suggests that the nucleolar granular component is not only involved in ribosome synthesis but also contains zones related to other functions (Politz et al., 2005).

2. P53 and Stress Sensing

pRb (retinoblastoma protein) and p53 play important roles both in the control of cell cycle progression and in ensuring sufficient cell growth for the generation of two normal daughter cells. Both of these oncosuppressor proteins are concentrated in the nucleolus. Interestingly, in human breast cancer, nucleolar size and activity are closely related to these proteins (Trere et al., 2004). The pRb tumor suppressor protein has a crucial role in regulating the G1- to S-phase transition, and its phosphorylation by cyclin-dependent kinases is an established and important mechanism in controlling pRb activity.

The p53 transcription factor is the major mediator of cellular stress responses in mammalian cells. Elevated levels of p53 inhibit cell growth or may lead to apoptotic cell death (Ryan et al., 2001). More than 50% of human cancers have impaired p53 function, making the pathways of p53 regulation and action the subject of intense research (Lane, 1992; Levine, 1997). Normally p53 is maintained at a very low level. When nucleoplasmic levels of p53 rise, this leads to the activation or repression of a large number of genes, which either allows the cell to recover from the insult or to be removed by apoptosis (Oren, 2003). This makes p53 the principal guardian of the genome preventing the initiation of cancer and tumor progression (Olson, 2004).

Although there are certainly many levels of posttranslational regulation of p53, including localization (Gostissa et al., 2003), one of the major controls is in the degradative pathway involving murine double minute clone 2 (MDM2). MDM2 can function as an E3 ubiquitin ligase, ubiquitinating p53 and targeting it for degradation by the proteasome. MDM2 also ubiquitinates itself, and the gene for MDM2 is a target of p53 transcriptional activation (Ryan et al., 2001). Thus p53 is at the center of a complex feedback network, and
the level of the protein depends on the activity of the MDM2 pathway. A major regulator of MDM2 is ARF (so called because it is encoded in an alternative reading frame of p16INK4a, an inhibitor of CDK4/6, which regulates the retinoblastoma protein). In fact the gene encoding ARF/p16INK4a is the second most frequently inactivated gene in human cancer (Ruas and Peters, 1998). ARF is localized to the nucleolus, and can relocalize to nuclear bodies, where it is associated with MDM2 and p53, suggesting a nucleolar link to cancer and cell stress response (David-Pfeuty and Nouvian-Dooghe, 2002; Kashuba et al., 2003; Zhang and Xiong, 1999).

Both MDM2 and MDM2-p53 have been shown to be bound to r-protein L5 in RNP complexes (Marechal et al., 1994), and the complexes were also shown to contain 5S and 5.8S rRNAs, suggesting either that MDM2 and p53 have a role in ribosome biogenesis, or that ribosomes are involved in nuclear export of MDM2 and p53 (Tao and Levine, 1999). Thus it was proposed that ARF binds to MDM2, sequestering it in the nucleolus and preventing its export to the cytoplasm. However, recently a more direct functional link between the nucleolus and p53 regulation has been suggested (Olson, 2004; Rubbi and Milner, 2003). These authors noted that most stress treatments that activated p53 also cause nucleolar breakdown or disruption. The two exceptions were leptomycin B (LMB), which inhibits nuclear export, and MG132, which inhibits proteasome activity. They proposed that, although the downstream p53 control involved nuclear export and proteasomal degradation in agreement with previous hypotheses, and with the activity of LMB and MG132, the upstream “stress sensor” was the nucleolus itself in all cases. To show that p53 was not itself the cause of nucleolar disruption, Rubbi and Milner (2003) demonstrated that representative stress treatments also caused nucleolar disruption in p53 null cells. Next they showed that UV irradiation at doses that would normally trigger a p53 response, would not do so if the irradiation was targeted to affect the nucleoplasm but not the nucleolus. Finally they showed that direct disruption of the nucleolus by injecting an antibody against UBF did cause a p53 response, although other antibodies had no effect on p53.

Thus it is possible that a functional and structurally intact nucleolus may be necessary to maintain p53 levels low. The mechanism for this is not yet clear. It is possible that the nucleolus and ribosome biogenesis is required for export of p53-MDM2. Alternatively ARF may be normally sequestered in the nucleolus and released on nucleolar breakdown; the released ARF would then inhibit MDM2 and allow levels of p53 to rise. However, this is unlikely to be the whole story. For example, ARF is not present in all cell types, even in mammals, and stresses such as DNA damage can cause nucleolar segregation by p53-independent mechanisms (Al-Baker et al., 2004). Bernardi et al. (2004) have shown that PML (promyelocytic leukemia tumor suppressor protein), which regulates posttranslational modifications such as acetylation
and phosphorylation, sequesters MDM2 to the nucleolus after DNA damage. This nucleolar localization is independent of ARF, but may be mediated by association with r-protein L11, given that L11 knockdown impairs PML localization to the nucleolus after DNA damage.

ARF can regulate the cell cycle in the absence of p53. B23 (also called Nucleophosmin, NPM, a prominent nucleolar phosphoprotein with ribonuclease activity and necessary for ribosome assembly) (Savkur and Olson, 1998) was identified as a binding partner for ARF (Brady et al., 2004); hyperproliferative signals were shown to up-regulate ARF, which resulted in nucleolar retention of B23/NPM and concomitant cell cycle arrest. Itahana et al. (2003) also showed that ARF interacts with NPM/B23, promoting its polyubiquitination and degradation; overexpression of B23/NPM induced cell cycle arrest in normal fibroblasts, whereas knockdown inhibited pre-rRNA processing and induced apoptosis. On the other hand, overexpression of ARF inhibited pre-rRNA processing and ribosome biogenesis (Sugimoto et al., 2003). B23/NPM and MDM2 compete for the same interaction site in ARF, and knockdown of B23/NPM enhanced MDM2-ARF association and decreased the localization of ARF to the nucleolus, whereas overexpression of B23/NPM antagonized ARF function and increased its nucleolar localization (Korgaonkar et al., 2005). B23/NPM also acts directly as a repressor of p53, binding to the N terminus of p53 and setting a threshold for p53 response to irradiation (Maiguel et al., 2004). Is B23/NPM sequestered to the nucleolus by ARF, or is ARF kept there by B23/NPM interaction, or are both required for ribosome biogenesis and kept in the nucleolus by other interactions? As yet there is no clear answer; what is clear is that there is a complex network of direct interactions and regulation between these proteins, ribosome biogenesis, and nucleolar organization. A complete understanding of this regulation will require a thorough analysis of the interactions, the stoichiometry, and the dynamics of the various components.

3. Cell Cycle Regulation

Nucleolar breakdown and division are closely coordinated with mitosis but do not necessarily occur at the same time in all species. In fungi, many of the nucleolar components are retained until late mitosis and are separated by a mechanism that may involve the telophase spindle: for example, the BIMG protein phosphatase, a nucleolar resident involved in the metaphase anaphase transition, is retained in the nucleolus throughout mitosis and can be observed streaming from the mother nucleolar mass into the daughters during telophase in the fungus Aspergillus nidulans (Fox et al., 2002b). Two pathways are involved in nucleolar segregation in budding yeast, the FEAR (cdc14 early anaphase release) pathway that initiates breakdown and the MEN (mitotic exit) pathway that maintains it (Shaw and Doonan, 2005).
Cdc14p is a dual specificity protein phosphatase that acts as the effector of the FEAR signaling transduction pathway. Cdc14p is anchored in the nucleolus by the Net1p protein as part of a nucleolar complex, which has been termed RENT (regulator of nucleolar silencing and telophase exit) (Shou et al., 2002) and is involved in the inactivation of the mitotic CDK protein kinase to enable mitotic exit. Net1p may also anchor other nucleolar proteins including Pol I and it stimulates rRNA synthesis both *in vitro* and *in vivo*. Condensin, the protein that reorganizes chromosomes into their highly compact mitotic structure, becomes highly enriched on the rDNA during mitosis (Bhalla et al., 2002; Freeman et al., 2000). Cdc14p also targets condensin to rDNA during anaphase, promoting its compaction. Why compaction of the rDNA is temporally separated from that of the rest of the genome is unclear, but may be related to late mitotic retention of the nucleolus. Machin et al. (2005) showed that resolution of the sister rDNAs is independent of spindle function but occurs prior to cdc14p-mediated compaction of the rDNA. These findings suggest that the physical retention of the Cdc14 phosphatase within the nucleolus until after the metaphase/anaphase transition may provide a structural mechanism to impose order on the later mitotic events in yeast.

Does FEAR-mediated nucleolar separation occur in higher eukaryotes? Although a recent report indicates that Cdc14-like proteins are present in the nucleolus in Xenopus and play an, as yet undefined, role in cell division (Kaiser et al., 2004), it seems likely that the mechanism of nucleolar disassembly and rDNA separation is considerably different. In Xenopus oocytes two proteins have been identified that can induce nucleolar disassembly independently of transcriptional inhibition (Gonda et al., 2003; see Section IV.B).

### 4. Signal Recognition Particle

The best characterized of the other nucleolar activities is in the assembly of the SRP. The SRP is a complex of an RNA with several proteins that targets translation of certain proteins to the endoplasmic reticulum (ER) by initially blocking their translation and then releasing the translational arrest on binding to an SRP receptor in the ER (Walter and Johnson, 1994). Its function is thus closely linked with that of the ribosome as part of the general translation machinery. At least part of the assembly of the SRP has been shown to occur in the nucleolus. Jacobson and Pederson (1998) showed that when SRP RNA was injected into mammalian culture cells it rapidly localized to the nucleolus, then gradually relocated to the cytoplasm, suggesting that the SRP was being assembled in the nucleolus and then exported. *In situ* hybridization and biochemical fractionation confirmed this localization (Chen et al., 1998; Politz et al., 2000). Similar results have been obtained for Xenopus oocytes (Sommerville et al., 2005). Many, but not all, of the protein components of the mature cytoplasmic SRP (SRP19, SRP68, and...
SRP72 proteins but not SRP54 protein) have been localized to the nucleolus in vertebrates (Politz et al., 2000) and in yeast (Grosshans et al., 2001). These results suggest that assembly of the SRP has a nucleolar phase, but must be completed in the cytoplasm. This parallels the behavior of the r-subunits (see Section III.A.4). It has also been shown in both mammalian cells (Alavian et al., 2004) and yeast (Ciufo and Brown, 2000; Grosshans et al., 2001) that export of the SRP requires CRM1, again in common with the export of small and large r-subunits (see Section III.A.4).

5. tRNA and RNAse P

Nucleolar-localized tRNA was detected in early studies of RNA biosynthesis (Halkka and Halkka, 1968; Sirlin et al., 1966). More recently, precursors to tRNAs have been detected in the nucleolus by in situ hybridization (Bertrand et al., 1998). Pre-tRNAs are trimmed at 5' and 3' ends and undergo base modifications such as pseudouridylation and 3' end procession (Hopper and Phizicky, 2003). Both protein and RNA components of RNAse P, which catalyzes 5' trimming of pre-tRNAs, have been localized to the nucleolus (Jacobson et al., 1997; Jarrous et al., 1999), as has cbf5p/Dyskerin, which catalyzes these and other pseudouridylations. It has been shown that most tRNAs in S. cerevisiae are exported from the nucleus as aminoacyl tRNAs, and that, in mutants defective in aminoacylation of tRNA, tRNAs accumulate in the nucleolus (Steiner-Mosonyi and Mangroo, 2004), suggesting that the machinery for aminoacylation is also present in the nucleolus. It has also been shown that charging of tRNA with amino acids occurs in the nucleus of Xenopus oocytes, again suggesting a proofreading function (Lund and Dahlberg, 1998). Fluorescence in situ hybridization studies have shown that a high proportion of tRNA genes are located in or at the periphery of the nucleolus in S. cerevisiae and that this colocalization is dependent on transcriptional status, being reduced or abolished in mutants of pol III polymerase which transcribes tRNA or in which the tRNA promoter is nonfunctional (Thompson et al., 2003). In S. cerevisiae pol II transcribed genes are silenced by proximity to tRNA genes. This silencing may be due to the clustering of the dispersed tRNA genes to the nucleolus; it is hypothesized that sequestration of a pol II transcribed gene into the nucleolus by virtue of its location near a tRNA gene takes it into a nuclear region lacking the relevant polymerase subunits (Wang et al., 2005).

Thus both SRP and tRNAs may pass through the nucleolus as they are transcribed, matured, or assembled, although the genes for SRP RNA and tRNAs are not close to the rRNA genes in the genome. Other than a general relation in function, it is not known why these components should be associated with the nucleolus. Perhaps a clue comes from some parallels with 5S r-RNA (Pederson and Politz, 2000), which is transcribed from a
number of extranucleolar tandem repeat loci in most eukaryotes and then imported into the nucleolus to participate in ribosome assembly. However, in several eukaryotes (e.g., *S. cerevisiae*, *Mucor racemosus*, *Dictyostelium discoideum*, *Torulopsis utilis*) and many prokaryotes (e.g., *Escherichia coli*) the 5S genes are contained within the rDNA repeat (Hadjiolov, 1985), and so must be transcribed in the nucleolus or the equivalent region of the prokaryotic nucleoid. However, in other simple eukaryotes (e.g., *Schizosaccharomyces pombe*, *Neurospora crassa*, *Acanthamoeba castellanii*) the 5S repeats are separate from the rest of the rRNA genes. It is assumed that the 5S genes became separated from the other rRNA genes early during eukaryotic evolution, for unknown reasons. But it seems that the complex assembly of ribosomes still requires the addition of 5S RNA, probably with cognate proteins attached, at a specific stage of ribosome biogenesis, which in turn requires the 5S RNA to be imported into the nucleolus rather than being added later in the nucleus or cytoplasm (Pederson and Politz, 2000). Furthermore there are reports of active 5S loci being associated with the nucleolus in a number of plant species, where the genes are separated in the genome from the rRNA as in most higher eukaryotes (Highett *et al.*, 1993; Montijn *et al.*, 1999).

Following this line of reasoning, there is now evidence that the informational components of the eukaryotic genome, such as the transcription and translation machinery, had their origins in an archaeal prokaryotic ancestral genome (Staub *et al.*, 2004). In the case of at least two present-day archaea (*Methanobacterium thermoautotrophicum*, *Methanothermus fervidus*) both the SRP RNA and two tRNA genes are located within an rRNA repeat (Pederson and Politz, 2000). It has been proposed that a nucleolar phase may have been retained as part of a mechanism of prechecking for correct functionality before export of these factors, which if they function incorrectly in the cytoplasm have the potential to cause widespread damage (Pederson and Politz, 2000). On the other hand it may simply be that the assembly processes are just too complex to be substantially reorganized.

6. Small Nuclear and Nucleolar RNAs

Small nuclear RNAs (snRNAs) are involved in splicing and form the cores of the spliceosomal complexes. Small nucleolar RNAs are involved in ribosome biogenesis, as discussed previously. The snRNAs and snoRNAs themselves contain modified bases, and these modifications are guided by small RNAs found specifically in Cajal bodies (CBs) called scaRNAs, suggesting that this part of snRNA maturation takes place in the CBs (Darzacq *et al.*, 2002; Jady *et al.*, 2003; Liu *et al.*, 2006). In plants it has been shown that most snoRNAs are transcribed as polycistronic precursors (Leader *et al.*, 1997). In situ studies have shown that these precursors are present in both CBs and in nucleolus,
suggesting that processing occurs in both locations (Shaw et al., 1998). CBs have been shown to have a role in the recycling/assembly of the U4/U6/U5 tri-snRNP, as RNAi knockdown of specific factors necessary for tri-snRNP assembly causes U4/U6 di-snRNP to accumulate in CBs (Schaffert et al., 2004). FRET microscopy has further shown the assembly of the U4/U6 di-snRNP occurring in CBs (Stanek and Neugebauer, 2004). A role for CBs in the maturation of U2 snRNP has also been shown (Nesic et al., 2004).

Thus it is clear that snoRNAs and their cognate proteins are, as expected, found in the nucleolus. Little is known about the mechanisms that mediate these changes in location, but Boulon et al. (2004) have shown that two factors previously known to mediate snRNA export, PHAX and CRM1, are involved in transport of U3 and other snoRNAs to the nucleolus in human cells. It is proposed that U3 precursors bind PHAX, which targets the complex to the CBs, and that subsequently CRM1 is bound to further target the U3 complexes to the nucleolus. Similar targeting to specific nuclear compartments is observed in yeast U3 maturation (Verheggen et al., 2002), which involves transport to the yeast nucleolar body, the yeast counterpart of the CB, and then out to the surrounding nucleolus. It has also been shown in yeast that Srp40p/Nopp140 is required to retain the snoRNA U14 in the nucleolar body and Nsr1/nucleolin is required to transport U14 out to the nucleolus (Verheggen et al., 2001).

Good evidence supports that U4/U6.U5 and U2 snRNAs are also in the nucleolus, and undergo part of their maturation or assembly processing there, and that the individual snRNAs are transported independently to the nucleolus (Gerbi and Lange, 2002; Lange and Gerbi, 2000; Yu et al., 2001). A nucleolar phase for snRNPs is strongly supported by the proteomic analyses of the nucleoli in both human and plant cells (Andersen et al., 2002, 2005; Pendle et al., 2005; Scherl et al., 2002), which showed many snRNA-associated proteins, particularly the Sm class of proteins. Live cell studies in human cells microinjecting plasmids encoded GFP-tagged Sm proteins have shown that the fluorescent proteins, and by implication the snRNPs, are seen first in CBs and the nucleolus, before being later localized to nuclear speckles. Taken together these studies point to a role for the nucleolus in the maturation of snRNAs and the assembly of snRNPs.

7. mRNA

The nucleolus has been implicated in mRNA export or surveillance, or even in translation in a number of experiments going right back to the 1960s, when its conventional role in rDNA transcription and ribosome biogenesis was being established. Harris (1967) investigated heterokaryons between human HeLa and chicken erythrocyte cells (Pederson, 1998a). The erythrocyte nucleus is condensed, lacks a nucleolus, and is transcriptionally silent, but
in the heterokaryons it becomes reactivated, forms a nucleolus, and eventually chicken proteins are produced in the cytoplasm of the heterokaryon. Harris showed that no proteins of chicken origin were produced until the chicken nucleus had reformed a nucleolus, suggesting that lack of a functional nucleolus prevented export of mRNAs from the chicken nucleus. Although the idea was not taken seriously at the time, various pieces of supporting evidence have accumulated. For example, spliced c-myc RNA was localized to the nucleolus in mammalian cells (Bond and Wold, 1993). The nucleolus in mRNA transport-defective yeast mutants has been shown to be disrupted and fragmented (Kadowaki et al., 1994; Schneiter et al., 1995). Furthermore, heat shock (Tani et al., 1995) or mutation of nucleolar proteins such as pol I or Mtr3p, also implicated in mRNA transport (Kadowaki et al., 1995), result in accumulation of polyA+ RNA in the nucleolus in yeast. Ideue et al. (2004) have obtained evidence in S. pombe that a subset of poly A+ mRNA associates transiently with the nucleolus during export; in transport defective mutants, an intron-containing transcript accumulated in the nucleolus, whereas transcripts from the intronless cDNA did not accumulate. Using a GFP-based reporter system in living yeast cells, Brodsky and Silver (2000) showed that mRNA processing factors were required for nuclear export of mRNAs, demonstrating a clear coupling between mRNA processing and export. Moreover mRNAs containing a particular 3’ untranslated region sequence from the ASH1 transcript accumulated in the nucleolus.

In the proteomic analysis of Arabidopsis nucleoli, Pendle et al. (2005) found many of the proteins of the mRNA exon junction complex, and confirmed their nucleolar location. The exon junction complex is a complex that binds to spliced transcripts 20–24 nucleotides upstream of splice sites, and contains splicing proteins, such as RNPS1 and SRM160, RNA export/localization factors, such as Aly/REF, UAP56 and TAP/P15 (although TAP is not present in plants), Magoh and Y14, and factors involved in nonsense-mediated decay, such as Upf1, Upf2, and Upf3. It also contains eIF4AIII, related to a transcription initiation factor (Tange et al., 2004). Many of the proteins were highly concentrated in the nucleolus as judged by GFP fusion localization. This suggests that the nucleolus is involved in some way in mRNA export or in surveillance and nonsense-mediated decay mechanisms.

ADAR1 and ADAR2 are RNA editing enzymes. They deaminate adenosine to inosine in long double-stranded RNA molecules and in specific mRNAs. ADAR2 is localized in the nucleus and nucleolus, whereas ADAR1 is distributed throughout the cytoplasm, nucleus, and nucleolus (Desterro et al., 2003; Nie et al., 2004; Sallacz and Jantsch, 2005), but as with all nucleolar proteins both enzymes are in dynamic flux. Within the nucleolus ADAR2 is concentrated in a novel compartment that is clearly different from the FCs, DFC, or GC. One of the few characterized substrates
for these enzymes in human cells is the glutamate receptor GluR-B. On expression of this substrate RNA, both ADAR1 and ADAR2 leave the nucleolus and associate with nucleoplasmic sites containing GluR-B precursor RNA. ADAR1 editing activity is regulated by SUMOylation; on modification of ADAR1 by SUMO-1 conjugation, editing activity is reduced (Desterro et al., 2005; Vitali et al., 2005). However, localization of the protein does not appear to be affected by SUMOylation.

8. Translation

Currently, the only known mechanism for detecting premature termination codons (PTCs) in mRNAs involves the EJCs in a pioneer round of translation. If a ribosome encounters a stop codon while there still remain EJCs downstream (i.e., toward the 3' end), this means that the stop codon must be a PTC; the transcript is then targeted for NMD (Maquat, 1995). Interestingly, mRNA seems to become immune to NMD once it is clearly cytoplasmic, again suggesting the involvement of the nucleus in the surveillance process (Stephenson and Maquat, 1996). Thus detection of PTCs in the nucleus is likely to imply at least verification of the open reading frames in some way occurring within the nucleus. The nucleolar proteomic studies have identified a number of translation factors, both initiation factors and elongation factors, in the human (http://www.lamondlab.com/nopdb/) and plant nucleolus (http://bioinf.scri.sari.ac.uk/cgi-bin/atnopdb/home). This therefore leads directly to the question whether such nuclear or nucleolar translation can be directly demonstrated.

In fact, evidence was published for protein translation in pea nucleoli during the 1960s (Birnstiel and Hyde, 1963; Birnstiel et al., 1961). After the definitive demonstration that translation occurred in the cytoplasm, these results were assumed to be due to cytoplasmic contamination and were for the most part forgotten. However, this topic has again been the subject of recent investigation, this time using modern cell biological methods. Cook and colleagues supplied human HeLa cells with a labeled aminoacyl tRNA and then after a short incubation detected where the labeled nascent proteins were located by fluorescence microscopy (Iborra et al., 2001). This clearly showed 9–15% of the labeling within the nucleus. Nathanson and colleagues (2003) have repeated the experiments, and in their experiments nuclear translation accounted for at most 1% compared to the 9–15% detected by Iborra et al. (2001). They concluded that any labeled protein detected in the nucleus did in fact originate from cytoplasmic contamination. Thus the issue of nuclear translation is still highly controversial (Iborra et al., 2004), and will require further studies to arrive at a generally accepted conclusion.
9. Telomerase

The standard mechanism for chromosome replication in eukaryotes cannot extend to the very ends of the DNA strands, and therefore in the absence of an alternative mechanism the chromosomes would become shorter in successive generations, eventually losing genes essential to viability. The solution to this problem is to have multiple tandem repeats of a short sequence at the ends of each chromosome (TTAGGG in humans), which are extended by a separate pathway. This extension is catalyzed by a reverse transcriptase (TERT), which copies the repeated sequence from an associated RNA (TERC) (Cech, 2004). Most somatic cells do not have active telomerase, which is proposed to limit the number of rounds of cell division that they can support, and has been suggested to be a factor in aging and carcinogenesis (Maser and DePinho, 2002). Inappropriate activation of telomerase or presence of the specific telomere binding proteins could have catastrophic consequences. For example, double strand breaks in the DNA could give rise to sites for telomerase elongation, and thus cause chromosome fragmentation. Immortalized cell lines, such as those derived from cancers, often have reactivated telomerase (Counter et al., 1992). Thus telomere biology has assumed considerable importance in both cancer and aging research (Blasco et al., 1999; Khakhar et al., 2003; Maser and DePinho, 2002).

There is evidence for the localization of telomerase in the nucleolus, either as part of its assembly pathway or sequestered there as part of the regulation of its activity (Teixeira et al., 2002). Telomerase RNA contains a box H/ACA domain (Mitchell et al., 1999a), in common with one of the classes of snoRNAs, which may localize the telomerase RNA to the nucleolus (Lukowiak et al., 2001; Teixeira et al., 2002). The H/ACA domain in both snoRNAs and telomerase RNA interacts with the pseudouridyl synthase, dyskerin/cbf5p (Mitchell et al., 1999b). However, Fu and Collins (2003) have shown that snoRNAs and telomerase RNA have distinct biogenesis pathways, despite their common H/ACA motif, and, in common with scaRNAs, telomerase RNA has a CB localization signal (Jady et al., 2004). The reverse transcriptase catalytic subunit of human telomerase, hTERT, also has a nucleolar localization domain (Etheridge et al., 2002), and thus the RNA and reverse transcriptase components are both localized to the nucleolus and are presumed to be assembled there (Mitchell and Collins, 2000). Microinjected Xenopus telomerase is also localized to the nucleolus by the H/ACA box, and Pendle et al. (2005) found a dyskerin homologue in the Arabidopsis nucleolar proteome (Narayanan et al., 1999). Plasmodium falciparum has a very large telomerase reverse transcriptase, pfTERT, which is localized to a discrete nuclear compartment associated with the nucleolus (Figueiredo et al., 2005).

Strong evidence for the functional relevance of telomerase localization comes from studies of human cell lines (Wong et al., 2002). A functional
GFP fusion with hTERT was used to monitor the location of active telomerase. In primary cell lines the hTERT was localized in the nucleolus, but specifically redistributed in late S-phase, when telomere replication takes place. In contrast, in tumor and transformed cell lines, hTERT was excluded from the nucleoli, whereas ionizing radiation caused reassociation of telomerase with the nucleolus in both types of cell, presumably as a result of DNA damage (Wong et al., 2002). The prominent nucleolar phosphoprotein nucleolin, which probably has RNA chaperone and helicase functions, interacts with hTERT, and the interaction is also dependent on the telomerase RNA (Khurts et al., 2004). This interaction is likely to be involved in the dynamic localization of telomerase.

The telomere repeat binding factor TRF2, which recognizes the telomere repeats at chromosome ends, is also localized in the nucleolus in a cell cycle–specific manner, perhaps being sequestered there to regulate its activity (Zhang et al., 2004). Again, it may be argued that proteins such as TRF2, if inappropriately expressed throughout the nucleus, might bind to replication intermediates or double strand breaks and thus cause new telomeric structures to be nucleated, with disastrous consequences. Thus sequestration in a separate compartment may be a safety mechanism.

V. Concluding Remarks and Future Directions

Ribosome biogenesis represents a huge investment of resources and energy for a cell. For example, in cultured human HeLa cell, 14,000 ribosomal subunits leave the nucleoli per minute (Görlich and Mattaj, 1996). Similarly, in S. cerevisiae it has been calculated that a rapidly growing cell must produce about 2000 ribosomes a minute (Warner, 1999), with each nuclear pore importing about 1000 r-proteins and exporting 25 ribosomes each minute. This in turn implies that approximately 60% of the total RNA transcription in these cells must be rRNA. Thus, ribosome supply is one of the principal limitations to cell growth, given a favorable environment. This justifies the enormous investment in the machinery to make ribosomes; in effect the nucleolus can be considered as one of the most powerful engines of cell growth. But, as with any powerful engine, adequate regulation and control is fundamental to its safe use. This may be one explanation for the links that are emerging between the nucleolus on the one hand and the cell cycle and stress responses on the other.

But why is the nucleolus apparently involved in so many other activities unconnected with ribosome biogenesis? One common theme is an involvement in maturation of diverse RNAs. It is possible that these processes use common or closely related RNA-binding proteins. The concentration of such
proteins into the dynamic nuclear domain represented by the nucleolus may then have the effect of also localizing RNA maturation processes other than ribosome biogenesis. This may or may not have an adaptive advantage in increasing efficiency, depending on whether the other RNA processes ever become limiting to growth. An alternate explanation that has been suggested for the nucleolar localization of apparently unconnected factors is that the nucleolus is a subnuclear compartment that has a markedly different composition and structure from the rest of the nucleus, and that it may be used for sequestration of various factors away from the nucleoplasm, and their regulated release when required.

A full understanding of nucleolar organization is likely to require more understanding of the evolutionary history of the eukaryotic nucleus and nucleolus. The biogenesis of ribosomes and of other RNA-containing cellular machines and its regulation is enormously complex. It is possible that the spatial organization of these processes was present very early in eukaryotic evolution, and that later evolutionary changes simply could not unravel the preexisting complex network of spatial and functional interactions. Organization may be retained that lacks any simple present-day rationale.

Whatever the reasons, it is clear that the nucleolus harbors hundreds of different biochemical processes taking place at any moment in a growing cell. This activity produces many protein and nucleoprotein complexes of different sizes, including preribosomal particles. This simple fact necessarily makes any simple structural model of the nucleolus, such as the tripartite model, an oversimplification. Our knowledge of nucleolar ribosome biogenesis as well as many new nucleolar nonribosomal processes has expanded dramatically in the past few years, but we are still lacking detailed in situ structural information about the cascade of steps in ribosome biogenesis, as well as about the unconventional nucleolar functions. Even a question as elementary as the location of the active r-genes has been debated for more than 20 years (Ploton et al., 2004; Raška et al., 2004). However, rather than trying to force observations into a particular simplistic preconceived model, we should concentrate on obtaining an ultrastructural description of the nucleolus at the molecular level.

Are current methods and technology sufficient to describe the functional organization of nucleoli in molecular detail? We are confident that in many cases the answer is yes, either through straightforward high-resolution microscopy or by immunolabeling techniques or both in combination. Raška et al. (1989), for instance, have showed that that there is a subtle structural difference between the replication domains labeled with antibodies to proliferating cell nuclear antigen and the surrounding unlabeled chromatin; this difference is only revealed by immunogold labeling. Huge advances in the resolution of LM are now being made and these will help to bridge the gap between LM and EM (Gustafsson, 2005; Gustafsson et al., 1999;
Hofmann et al., 2005). Recently, the conventional optical diffraction limitations of fluorescence microscopy have been overcome using stimulated emission depletion microscopy, and by this technique objects separated by less than about 40 nm can be resolved (Hofmann et al., 2005; Simpson, 2006; Willig et al., 2006).

Another powerful approach is provided by correlative LM and EM, including tomographic EM and ultrastructural identification of the nucleolar distribution of proteins tagged with GFP or other markers (Grabenbauer et al., 2005; Koster and Klumperman, 2003; Raška, 2003). A first step is to identify CTs clearly in situ; a start has been made in this (Gonzalez-Melendi, 2001; Raška, 2003), and modern developments of LM and EM make this goal eminently achievable. We are confident that this will open the way to describe the nucleolus at the same level of molecular detail as has already been achieved with the Golgi and endocytic pathways (Raška et al., 2006).

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