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# Conventional and Nonconventional Roles of the Nucleolus

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As the most prominent of subnuclear structures, the nucleolus has a well-established role in ribosomal subunit assembly. Additional nucleolar functions, not related to ribosome biogenesis, have been discovered within the last decade. Built around multiple copies of the genes for preribosomal RNA (rDNA), nucleolar structure is largely dependent on the process of ribosome assembly. The nucleolus is disassembled during mitosis at which time preribosomal RNA transcription and processing are suppressed; it is reassembled at the end of mitosis in part from components preserved from the previous cell cycle. Expression of preribosomal RNA (pre-rRNA) is regulated by the silencing of individual rDNA genes via alterations in chromatin structure or by controlling RNA polymerase I initiation complex formation. Preribosomal RNA processing and posttranscriptional modifications are guided by a multitude of small nucleolar RNAs. Nearly completed ribosomal subunits are exported to the cytoplasm by an established nuclear export system with the aid of specialized adapter molecules. Some preribosomal and nucleolar components are transiently localized in Cajal bodies, presumably for modification or assembly. The nonconventional functions of nucleolus include roles in viral infections, nuclear export, sequestration of regulatory molecules, modification of small RNAs, RNP assembly, and control of aging, although some of these functions are not well established. Additional progress in defining the mechanisms of each step in ribosome biogenesis as well as clarification of the precise role of the nucleolus in nonconventional activities is expected in the next decade.

**KEY WORDS:** Nucleolus, Nucleolar proteins, Ribosome biogenesis, Preribosomal RNA, Small nucleolar RNA, RNA polymerase I, Cajal body, Signal recognition particle, Aging mechanisms. © 2002, Elsevier Science (USA).

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## I. Introduction<sup>1</sup>

### A. Historical Perspective

Although the nucleolus is the most prominent of all subnuclear bodies, the recognition of its importance has occurred slowly and gradually. Over the past half-century there has been steady progress in understanding ribosome assembly by a relatively small group of investigators, with nucleolar research never achieving the “star” status of other biological problems. In recent years, interest in the nucleolus has been reinvigorated, not only because major progress has been made in elucidating its important primary function, but because intriguing new roles have also been discovered. As we rapidly move toward a much clearer picture of the mechanisms of ribosome biogenesis, there is still much uncertainty about the significance of the nontraditional activities of the nucleolus.

The initial identification of a nucleolus inside a nucleus has been attributed to Fontana (1781); however, the first actual description of a nucleolus was probably that of Wagner (1835), who termed it “Keimfleck” or “macula germinatova” in the oocyte nuclei of several species. The name “nucleolus” was coined by Valentin (1839), who noticed that most cells had a secondary nucleus or a “nucleus within a nucleus.” The early history of the nucleolus has been critically reviewed by Franke (1988). These early observations of the nucleolus as a subcellular structure remained strictly descriptive until the early 1960s, when its key function as a factory for ribosome assembly became established (Hadjiolov, 1985; Scheer and Hock, 1999; Olson *et al.*, 2000). Research on the nucleolus until about 1985 has been thoroughly covered in two extensive volumes (Busch and Smetana, 1970; Hadjiolov, 1985). More recently, Thiry and Goessens (1996) reviewed many of the ultrastructural aspects of the nucleolus. The major focus of the current review will be on research conducted since 1990.

<sup>1</sup>Abbreviations used: ARF, alternate reading frame or ADP-ribosylation factor; BLM, Bloom syndrome gene product; CB, Cajal body or coiled body; cdc, cell division cycle; CK2, casein kinase 2; DFC, dense fibrillar component; ERCs, extrachromosomal rDNA circles; ETS, external transcribed spacer; FC, fibrillar center; FRAP, fluorescence recovery after photobleaching; GC, granular component; GFP, green fluorescent protein; HDAGs, hepatitis delta antigens; IGS, intergenic spacers; ITS, internal transcribed spacer; NB, nucleolar body; NDF, nucleolus-derived foci; NES, nuclear export signal; NLS, nuclear localization signal; NOR, nucleolar organizer region; NOS, nucleolar localization signal; NTS, nontranscribed spacer; PNB, prenuclear body; pol, polymerase; PR, perichromosomal region; PT, proximal terminator; Rb, retinoblastoma protein; RBD, RNA binding domain; rRNA, ribosomal RNA; RNP, ribonucleoprotein particle; rDNA, ribosomal DNA (genes for pre-rRNA); SL, selectivity factor; snoRNA, small nucleolar RNA; SMN, survival motor neuron; SRP, signal recognition particle; SV40, simian virus 40; tRNA, transfer RNA; UBF, upstream binding factor; UPE, upstream promoter element; WRN, Werner syndrome gene product.

## B. Importance of the Nucleolus

### 1. The Nucleolus as a Subcellular Compartment in Eukaryotic Cells

The major feature that distinguishes eukaryotes from prokaryotes is the presence of a physical barrier between the nucleus and cytoplasm. This compartmentalization confers selective advantage on eukaryotic organisms in several ways (Alberts *et al.*, 1994). First, DNA in the nucleus is protected from mechanical forces generated by cytoskeletal elements in the cytoplasm. Second, the compartmentalization allows transcription and translation to be separated. This, in turn, facilitates the production of messenger RNAs from complex precursor RNAs, which can be spliced and processed in the nucleus before they are exposed to the cytoplasmic translational apparatus. However, recent evidence for translation inside the nucleus (Iborra *et al.*, 2001) may weaken the case for the importance of nuclear and cytoplasmic partitions. Third, the nuclear envelope provides a means of regulating transcriptional events by controlling the import of regulatory factors into the nucleus (Carmo-Fonseca *et al.*, 2000). Thus, the nuclear compartment provides the eukaryotic cell with the ability to deal with large genomes and complex regulatory systems that would be unmanageable in an unpartitioned cell.

The nucleus has its own subcompartments, which are not separated from the remainder of the nucleoplasm by membranes, as are the organelles of cells. Instead, they are organized around chromosome territories or other macromolecular complexes. These subnuclear compartments are often called “nuclear bodies” (Brasch and Ochs, 1992; Matera, 1999), of which the most prominent and most thoroughly studied is the nucleolus.

### 2. Advantages of an Organized System of Ribosome Assembly

In active cells, a large proportion of the cell's energy is devoted to production of proteins, which are synthesized on ribosomes. Each cell contains about 10 million ribosomes (Alberts *et al.*, 1994). In growing and dividing cells, this same number of ribosomes must be produced in every cell generation. To respond to this need, one might expect the system for ribosome production to be organized in a highly efficient manner. Using an industrial analogy, an organism with ribosome production structured in multiple assembly lines distributed among a few compact factories might have a competitive advantage over one with this machinery generally dispersed throughout the nucleus. In a centralized system of production, each assembly line would have ready access to a supply of parts (ribosomal proteins and RNAs) brought to a single location as well as an abundance of tools (enzymes and assembly factors) that could be shared among the assembly lines. After one assembly unit finishes a product, a nearby unit could utilize the tools without delay. In fact, the analogy would seem to apply to nucleoli, which are

organized around the tandemly repeated genes for pre-rRNA present in the nucleolar organizer regions (NORs) of chromosomes. Each of these genes could be viewed as the beginning of one of the many assembly lines in a nucleolus. Since cells normally contain only one to four nucleoli per cell, ribosome production would also be highly centralized and might meet the standard of an efficient manufacturing process. However, as discussed below, under certain conditions, ribosome biogenesis is able to proceed in the absence of nucleolar structure. Thus, the importance of specific nucleolar architecture for ribosome productions is not entirely clear. The apparent permissiveness in structure in relationship to its primary function hints that the characteristic nucleolar organization could be more important for its nonconventional functions.

### **3. New Roles for the Nucleolus**

Although our understanding of the process of ribosome biogenesis has been markedly advanced during the past 40 years, new and surprising roles for this puzzling subnuclear body have been uncovered during the past decade. These include nuclear export, sequestration of regulatory molecules, modification of small RNAs, RNP assembly, and control of aging. The new roles are not nearly as well established as the old ones. Nevertheless, these unexpected functions have caught the attention of numerous researchers from disparate fields and have breathed new life into an old area of investigation.

## **II. Nucleolar Structure and Assembly**

### **A. Interphase Nucleolus**

#### **1. Foundation of the Nucleolus: Tandemly Repeated Genes for Preribosomal RNA**

All eukaryotic species have multiple copies of genes for preribosomal RNA (rDNA). The number of copies varies from under 50 to several thousand per haploid; see Hadjiolov (1985) for a compilation of gene multiplicity data for a large number of species. In some organisms, especially amphibians, the transcription units may be visualized by electron microscopy after dispersal of the nucleoli. In these nucleolar spreads, the growing RNA chains look like the branches of a Christmas tree, where the gene is the trunk. During mitosis the genes for pre-rRNA are usually localized to secondary constrictions of chromosomes. They have the ability to initiate the formation of nucleoli during interphase; hence, they are called nucleolar organizer regions or NORs. In certain cells, especially oocytes of some species, rDNA is also present as amplified extrachromosomal DNA, resulting

in as many as one million copies of the rDNA genes and thousands of nucleoli per cell (Bird, 1980). In somatic cells the NORs are found on multiple chromosomes; for example, in humans there are five chromosomes that contain NORs. Theoretically, in a diploid cell one nucleolus could arise from each NOR to produce 10 nucleoli; however, the nucleoli have a tendency to fuse during interphase (Anastassova-Kristeva, 1977; Wachtler *et al.*, 1984). Consequently, the number of nucleoli decreases as the cell progresses through interphase, with only one to four nucleoli typically found in mammalian cells.

## 2. Dynamic Aspects of Nucleolar Structure

**a. Nucleolar Subcomponents** Although the tandemly repeated genes for pre-rRNA are necessary for the formation of nucleoli, they are not sufficient for producing a true nucleolar structure. This occurs when the genes are transcribed, generating two structures that are found in all nucleoli, the dense fibrillar component (DFC) and the granular component (GC) (Scheer and Hock, 1999). The former component contains newly synthesized preribosomal RNA (pre-rRNA) and a collection of proteins, while the latter comprises nearly completed preribosomal particles (Fig. 1). A third component, the fibrillar center (FC), is usually seen in the nucleoli of most metazoans, but it is generally not found in lower eukaryotes. In three dimensions, the FC is more or less a spherical structure, which is surrounded by the growing pre-rRNP particles of the DFC (Fig. 2). The zone of transcription of the pre-rRNA genes seems to be at or near the boundary between these two structures (Scheer and Hock, 1999; Mosgoeller *et al.*, 1998; De Cárcer and Medina, 2000). The growing preribosomal particles move from the DFC to the GC for final stages of assembly and processing.

The above observations reinforce the idea that transcription and ribosome assembly maintain the normal structure of the interphase nucleolus; i.e., the nucleolus seems to be “an organelle formed by the act of building a ribosome” (Mélese and Xue, 1995). This is further illustrated by the very striking effect of RNA pol I transcription inhibitors (Hadjiolov, 1985), which cause the FC, DFC, and GC to segregate into distinct regions within the nucleolus. Nucleolar structure is not only dependent on transcription by RNA pol I, but it is also sensitive to RNA polymerase II (RNA pol II) inhibition, i.e., upon treatment with 5,6-dichloro-1 $\beta$ -D-ribofuranosylbenzimidazole (DRB), the transcribing genes for pre-rRNA disperse into “nucleolar necklaces” (Le Panse *et al.*, 1999). These necklaces appear to comprise separated FCs connected by strings of DFCs. Since DRB is a protein kinase inhibitor, it is unclear whether nucleolar necklace formation is due to a shortage of RNA pol II gene products or if nucleolar protein phosphorylation is altered.

**b. Location of Transcription** The actual location of transcription in the nucleolus has been a subject of much debate for the past two to three decades. One model proposes that the site of transcription is either in the DFC itself or at the border

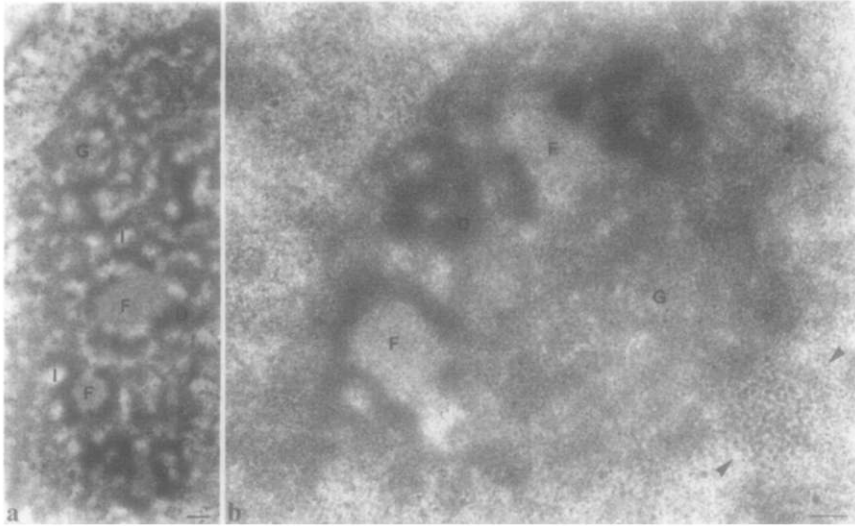


FIG. 1 Ultrastructures of mammalian cell nucleoli. Examples of HeLa cell nucleoli with two different kinds of subnucleolar organization of components are shown. (a) A reticulated type of nucleolus, which is often found in transcriptionally active cells, is shown. These nucleoli have relatively small fibrillar centers (F) surrounded by strands of dense fibrillar component (D). The nucleolar interstices (I) appear as regions of lower electron density and contain a few strands of intranucleolar chromatin. The small punctate granular components (G) are scattered throughout the nucleolus. A more common form of structural organization is seen in (b) in which the nucleoli have large and prominent fibrillar centers surrounded by dense fibrillar components. Relatively large areas are covered by the granular components, which are more clearly separated from the dense fibrillar components than in reticulated nucleoli. In (b) a Cajal body (arrowheads) appears to be attached to the nucleolus through clumps of perinucleolar chromatin, which were detected by immunogold staining using an antibody against single-stranded DNA. Notice the difference in the sizes of the intermediates of ribosome subunit assembly (15–20 nm), which make up the nucleolar granular components, compared with the coiled fibrillary strands (40–60 nm) of the Cajal body. From Olson *et al.* (2000). Scale bars = 200 nm.

between the FC and the DFC (Hozak, 1995; see Fig. 2). A second model suggests that transcription takes place inside the FC; this was proposed after finding RNA polymerase I predominantly localized in the FC (Scheer and Rose, 1984). The arguments in favor of the latter model are discussed extensively by Scheer *et al.* (1993). Recent data further support this model by showing that early incorporation of BrUTP occurs within the FCs, where it colocalizes with RNA polymerase I (Thiry *et al.*, 2000). Because of the semantic nature of arguments concerning this issue, it is likely that the location of the site of rDNA transcription will remain controversial for several years to come.

**c. Dependence of Nucleolar Structure on Transcription** In budding yeast, there is also evidence for dependence of normal nucleolar structure on RNA pol I

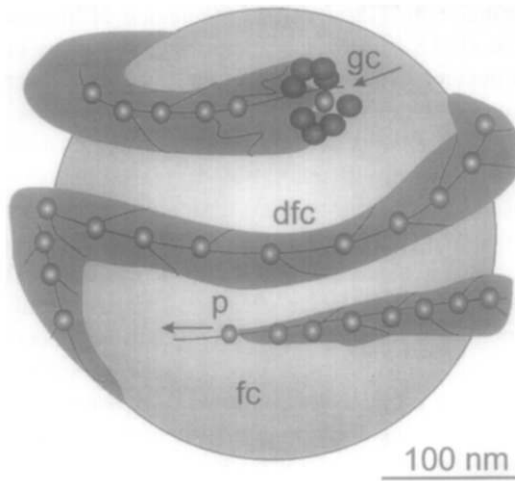


FIG. 2 A model of the nucleolus in HeLa cells, drawn roughly to scale. Each fibrillar center (fc) is covered by approximately six ribosomal transcription units, but only one is shown. The template slides (arrows) through fixed polymerases (ovals) fixed in the dense fibrillar component (dfc) on the surface of the fibrillar center, as transcripts are extruded. Supercoils are removed by topoisomerases (not shown). On termination, polymerases are recycled through the store in the fibrillar center. As the promoter advances, polymerases (p) initiate and then extrude nascent rRNA, while at the 3' end nascent rRNA in the dense fibrillar component condenses into the granular component (spheres, gc). Scale bar = 100 nm. From Hozak (1995).

transcription (Oakes *et al.*, 1998). The initial studies used a mutant strain of *Saccharomyces cerevisiae* in which RNA pol I is inactive and rDNA transcription is driven from a plasmid by RNA pol II. In this strain, the crescent-shaped nucleolus associated with the nuclear envelope is absent and several granules, termed mininucleoli, replace the nucleoli. Subsequent studies used a series of mutants in which the rDNA is placed either in a plasmid or in the chromosome and transcription can be performed with either RNA pol I or RNA pol II (Wai *et al.*, 2000). When rDNA is transcribed from a multicopy plasmid by RNA pol II, a condensed nucleolus forms, but it does not associate with the nuclear envelope. The latter interaction requires transcription from tandemly repeated rDNA genes. However, none of the mutants used in this study was able to form a typical crescent-shaped nucleolus, suggesting that normal nucleolar structure requires a specific rDNA organization as well as RNA pol I transcription.

**d. Ribosome Biogenesis Outside of the Nucleolus** As shown by Karpen *et al.* (1988), ribosome biogenesis can also take place in euchromatic regions outside of the NORs in higher eukaryotes. Single pre-rRNA genes inserted into non-NOR chromosomal sites of *Drosophila* nuclei were capable of transcribing pre-rRNA



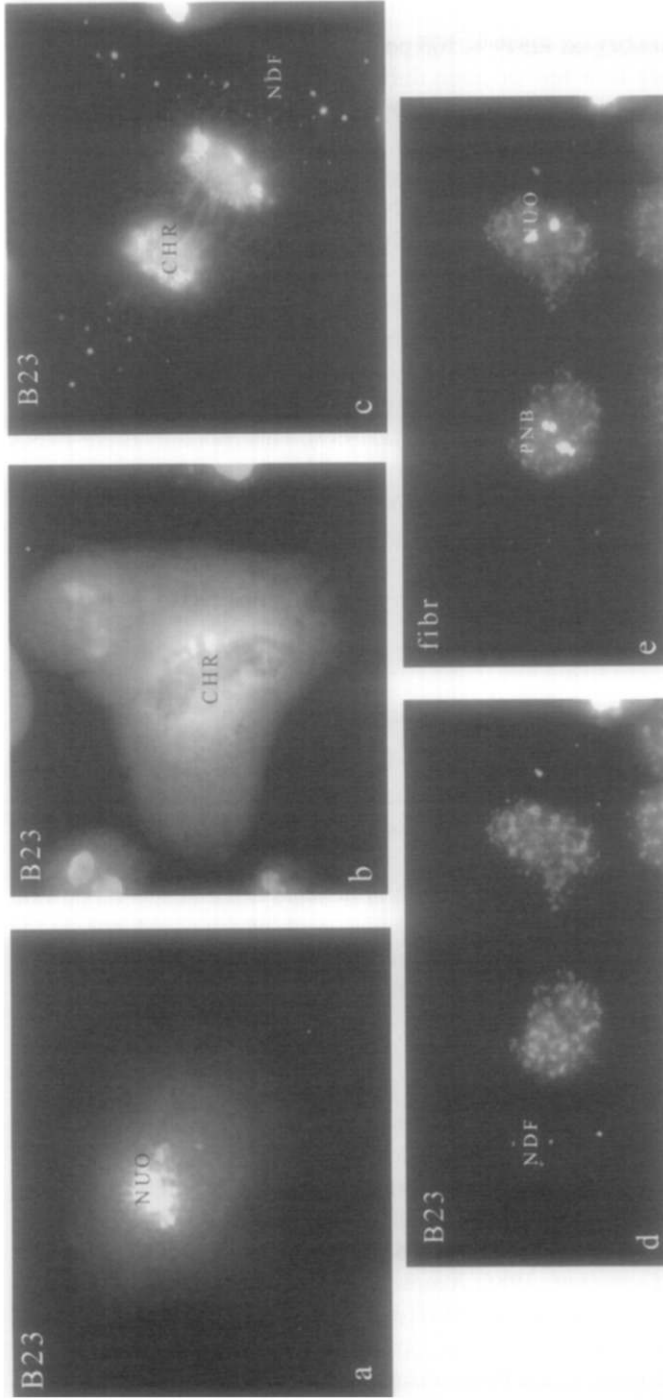
and forming “mininucleoli.” Thus, neither the tandem arrangement of rDNA nor the location of the rDNA in heterochromatic regions seems to be required for transcription and nucleolar formation in higher eukaryotes. Nierras *et al.* (1997) developed a strain of yeast in which the tandem array of rDNA genes is deleted, but instead carries many copies of a plasmid that contains a single rDNA gene. Although the transcriptional efficiency was decreased in this strain, pre-rRNA processing was normal and the strain grew reasonably well. Immunofluorescence microscopy revealed that yeast fibrillarin (Nop1p) was not present in a single nucleolar structure, but dispersed throughout the nucleus in this strain. These data confirm that a well-defined nucleolar structure is not essential for preribosome production. Although a nucleolus organized around the tandem array of rDNA may have some advantage to the organism, it seems to be less important for ribosome biogenesis than previously believed. The dynamic aspects of nucleolar structure reinforce a growing realization that there is considerable flexibility in the functional organization of the cell nucleus in general (Wolffe and Hansen, 2001) and that rigid compartmentalization is probably not essential for ribosome assembly.

## B. Nucleolus during Mitosis

### 1. Relocation of Nucleolar Components

One of the most remarkable features of the cell cycle is the disassembly of the nucleolus during mitosis and its subsequent reassembly as the daughter cells reenter interphase. The structural disassembly of the nucleolus is preceded by the arrest of ribosomal RNA gene transcription (Scheer and Hock, 1999) and accompanied by the suppression of preribosomal RNA (pre-rRNA) processing (Dundr and Olson, 1998). Where do all of the nucleolar components go after the nucleolus loses its normal interphase structure? Not only do specific classes of components have different locations during mitosis, but these locations also change during progression through mitosis. Of the nucleolar constituents studied thus far, the RNA Pol I transcriptional apparatus appears to be the least mobile; it remains anchored to the chromosomal NORs throughout the mitotic cycle (Scheer and Hock, 1999). However, most of the other nucleolar components separate from the transcription machinery during this period.

Aside from the transcription machinery, the mitotic behavior of the pre-rRNA processing components has been studied extensively. In prophase these components are located in the cytoplasm and associated with the perichromosomal regions (PRs). In metaphase they distribute among the cytoplasm, PRs, and the spindle apparatus. However, in anaphase this material also appears in large cytoplasmic particles called nucleolus-derived foci (NDF) (Dundr and Olson, 1998) especially in actively growing cell types (Fig. 3). The NDF are large bodies (1–3  $\mu\text{m}$ ), which



**FIG. 3** Locations of nucleolar proteins during mitosis in CMT3 cells. The cells were subjected to immunofluorescence microscopy using antibodies to protein B23 and fibrillar. In (a) B23 distributes among the nucleoplasm and nucleolar remnants (NDO) as the nucleolus disperses in prophase/prometaphase. In metaphase (b), B23 is found in the cytoplasm, the chromosome periphery, and possibly the mitotic spindle. In anaphase (c), B23 decorates the perichromosomal region and is also present in numerous nucleolus-derived foci (NDF). In telophase (d), protein B23 is seen in NDF and in numerous PNBs, with little or no signal in nucleoli. When the labeling for B23 and fibrillar is compared in the same cells (d, e), there is a stronger signal for fibrillar in nucleoli (e) but reduced labeling in PNBs, compared with the B23 signal in the same locations (d). CHR, chromosomes.

reach a maximum number (as many as 100 per cell) during anaphase, after which their number declines to a few or none during telophase (Dundr *et al.*, 1997; Zatssepina *et al.*, 1997; Dundr and Olson, 1998). The decrease in the number of NDF seems to coincide with the appearance of prenucleolar bodies (PNBs) and reforming nucleoli. The NDF contain the proteins fibrillarin, protein B23/No38, nucleolin, p52, and the hPop1 subunit of RNase P as well as several snoRNAs (U3, U8, and U14). They also contain partially processed pre-rRNA, apparently a mixture of high-molecular-weight (45 S and 46 S) pre-rRNA (Dundr and Olson, 1998). The latter work and a more recent study (Doussset *et al.*, 2000) confirm the results of a much earlier investigation, which indicated that pre-rRNA is preserved during mitosis (Fan and Penman, 1971). Similar complexes containing processing intermediates and nucleolar proteins have been observed in immunoprecipitates from metaphase cells (Pinol-Roma, 1999). Thus, not only is pre-rRNA transcription shut down during mitosis, but pre-rRNA processing is also suppressed during the same period in the cell cycle. A major role of the NDF might be the temporary storage of partially processed pre-rRNAs and associated pre-rRNA processing components. However, the molecular composition of the NDF, their mechanism of assembly, and precise role in nucleolar reassembly remain unclear.

## 2. Postmitotic Reassembly of the Nucleolus

A proposed model for reassembly of the nucleolus at the end of mitosis is illustrated diagrammatically in Fig. 4. Many of the pre-rRNA processing components make their way back into the nucleus during telophase; there are two possible routes to that destination. First, some of this material is undoubtedly carried into nuclei by mitotic chromosomes as the perichromosomal layer. A second portion might be imported into nuclei from a cytoplasmic pool, which could include the NDF. At least one component, protein B23, seems to be transferred from NDF into telophase nuclei (Dundr *et al.*, 2000). After dissociating from the NDF this material might be transported through the nuclear envelope (Fig. 4) by the established nuclear import system. Inside the telophase nucleus, the processing components along with partially processed pre-rRNA are incorporated into prenucleolar bodies (PNBs) (Medina *et al.*, 1995; Verheggen *et al.*, 1998; Dundr *et al.*, 2000). The PNBs are morphologically distinct particles, which appear in newly formed daughter nuclei in telophase concomitantly with nucleolar reassembly. The PNBs contain many constituents of the mature interphase nucleolus, but in contrast to true nucleoli, they do not contain rDNA and they do not synthesize pre-rRNA (Lepoint and Goessens, 1978; Ochs *et al.*, 1985; Azum-Gelade *et al.*, 1994; Jimenez-Garcia *et al.*, 1994). When RNA pol I transcription is reinitiated at the chromosomal NORs in telophase, material from the PNBs is apparently transferred to nucleoli; i.e., the nucleolar size increases as the PNBs disappear (Jimenez-Garcia *et al.*, 1994; Gautier *et al.*, 1994). Recent studies using time-lapse fluorescence microscopy

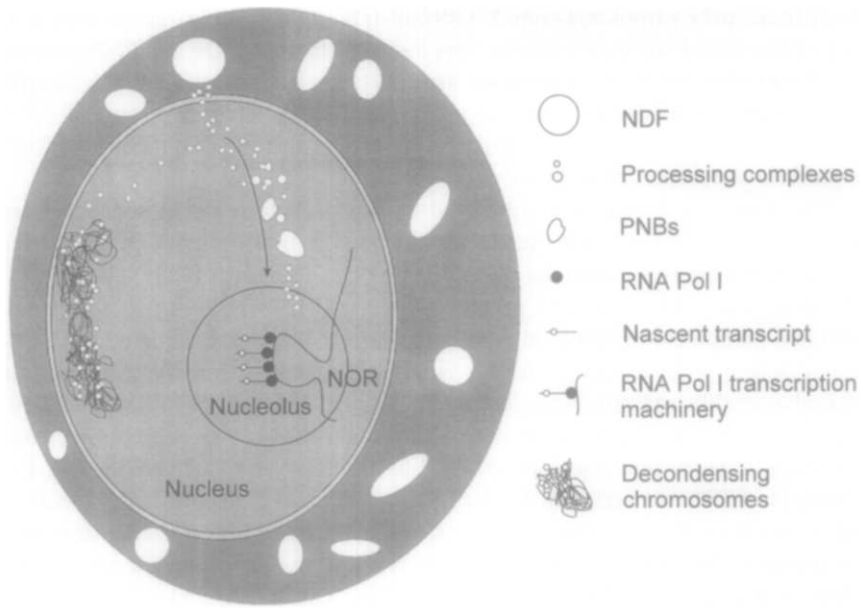


FIG. 4 Proposed model for formation of a nucleolus in telophase. Nucleoli disintegrate early in mitosis, and nucleolar components distribute to various parts of the cell. The transcriptional apparatus remains attached to the nucleolar organizer regions (NORs) on chromosomes whereas the processing complexes adhere to the chromosome periphery or are dispersed in the cell. Large particles called nucleolus-derived foci (NDF) are assembled from the processing complexes in anaphase. The diagram suggests that in telophase, prenucleolar bodies (PNBs) are formed from the processing complexes, which could be derived from two sources. The first is from the periphery of chromosomes, which are in the process of decondensing. The dispersing chromosomes are positioned around the inside surface of the nuclear envelope. The second source is the NDF, which dissociate into small particles that are eventually imported into nuclei. The material appears to flow in streams of small particles from disintegrating PNBs into newly forming nucleoli as transcription is reactivated. From Dundr *et al.* (2000).

in living cells showed that fibrillarin (Dundr *et al.*, 2000) and Nop52 (Savino *et al.*, 2001) are transferred from PNBs to growing nucleoli during telophase. Incorporation of protein constituents of PNBs into nascent nucleoli seems to be a stepwise process, with the proteins required for early stages of pre-rRNA processing (e.g., fibrillarin) relocating from the PNBs to the nucleolus before late ones (e.g., B23). There is also evidence for the presence of pre-rRNA sequences in telophase nucleoli before RNA pol I transcription begins (Verheggen *et al.*, 1998).

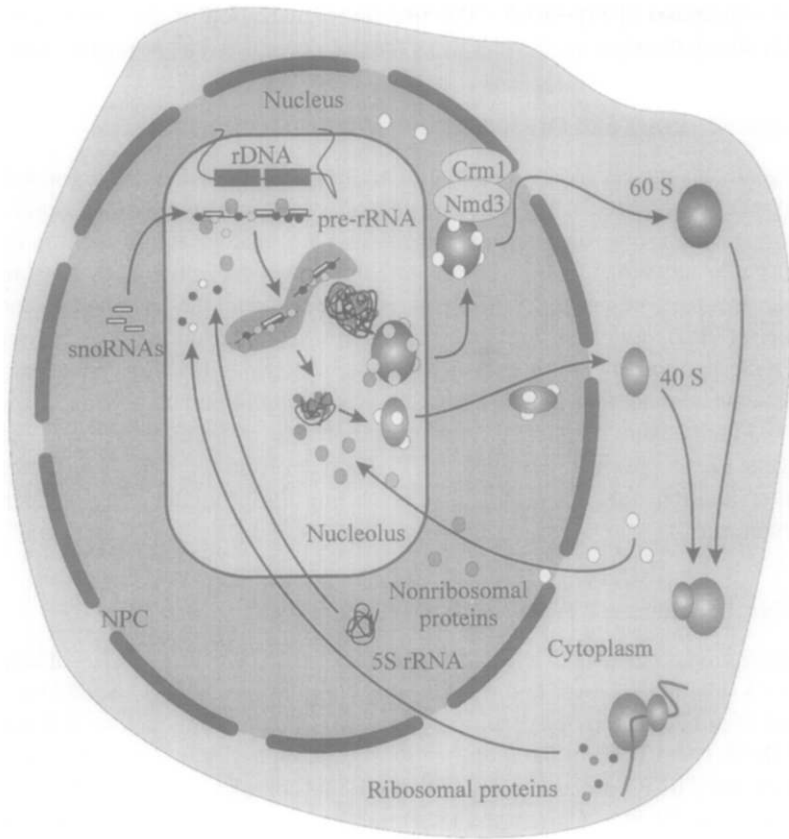
Experiments using fluorescence recovery after photobleaching (FRAP) indicate that components of the NDF and PNBs exchange at nearly identical rates, suggesting that they have similar structures (Dundr *et al.*, 2000). The rates of exchange

were found to be surprisingly rapid, with half-times of recovery from photobleaching of less than 1 sec for fibrillarin. This indicates that the NDF and PNBs are not fixed particles, but their components are constantly changing. Thus, the transfer of material from PNBs into the growing nucleoli seems to occur through dissociation of individual components from the PNBs, possibly utilizing simple shifts in equilibria and diffusion to carry out the process.

The studies by Dundr *et al.* (2000) also showed that pre-rRNA sequences are present in the PNBs; since these particles are not transcriptionally active, the RNA must be derived from the maternal cell. Thus, it seems likely that processing complexes associated with partially processed pre-rRNA enter telophase nuclei and participate in rebuilding the nucleolus. In the model depicted in Fig. 4, transcription is an essential step in building nucleolar structure, whereby the stored assembly and processing components are transferred from PNBs to elongating pre-rRNA chains in the developing nucleoli. However, there are cases in which transcription does not seem to be necessary for nucleologenesis. For example, rDNA transcription is not required for the formation of nucleoli in early *Xenopus* development (Verheggen *et al.*, 1998) or for DFC-like structures to appear in *Xenopus* anucleolate mutants (Hadjiolov, 1985). Recent studies also show that nucleolin, fibrillarin, and pre-rRNAs can localize to newly forming nucleoli in the absence of pre-rRNA transcription (Dousset *et al.*, 2000). The latter work suggests that the early stages of nucleolar assembly occur before transcription has been activated. In summary, the concept that the nucleolus is "formed by the act of building a ribosome" (Mélese and Xue, 1995) seems to be essentially correct, but this general rule may not apply to every physiological condition.

### III. The Ribosome Assembly Process

As illustrated in Fig. 5, the main business of the nucleolus may be distilled into a few operations: transcription and processing of pre-rRNA into 18 S, 5.8 S, and 28 S rRNAs, modification of the RNAs, incorporation of 5 S rRNA, attachment of ribosomal proteins, and eventual export of the small and large subunits to the cytoplasm. At first glance, this would seem to be a relatively simple process; however, during the past 10 years the number of proteins and RNAs implicated in ribosome biogenesis has grown enormously. The process is much better understood in yeast than in higher eukaryotes, but many unanswered questions remain for all organisms, especially with regard to the timing and mechanisms of individual steps. Space does not permit a detailed discussion of all aspects of the process; instead, we will highlight some of the more interesting and recently discovered features of each step.



**FIG. 5** Major steps in eukaryotic ribosome biogenesis. During and after transcription, nonribosomal proteins and small nucleolar RNAs (snoRNAs) associate with the preribosomal RNA (pre-rRNA) transcript. Methylation and pseudouridylation of the nascent pre-rRNA are guided by the snoRNAs. 5 S rRNA, a component of the 60 S subunit, is added to the maturing complex. The pre-rRNA undergoes a series of cleavages ultimately resulting in 18 S, 5.8 S, and 28 S rRNAs. The complex is split into the two precursor particles for the small (40 S) and large (60 S) ribosomal subunits. Ribosomal proteins are added to the precursor complexes at various stages of assembly. The nearly mature subunits are transported out of the nucleus through the nuclear pore complex (NPC); the 60 S precursor utilizes the Nmd3 protein as an adaptor for binding to the Crm1 nuclear export factor. The small and large subunits are eventually incorporated into ribosomes in the cytoplasm.

### A. Transcription

Because this topic has been reviewed extensively (Paule and White, 2000; Jacob and Ghosh, 1999; Reeder, 1999; Grummt, 1999), only a few key aspects will be covered. As indicated below, several essential features of the transcription system

affect expression of pre-rRNA: structure and organization of the rDNA repeats, rDNA chromatin structure, assembly of the pol I complex, and regulatory factors.

### 1. Gene Structure and Organization

The genes for pre-rRNA are arranged in multiple copies, which are separated by intergenic spacers (IGS). The IGSs are generally longer than individual genes and the organization of promoters, enhancers, and terminators within them is complex (Paule and White, 2000). The essential promoter for initiation of basal transcription is the core promoter; however, another segment, the upstream promoter element (UPE), aids in assembly of the complex formed on the core promoter. The IGSs also contain multiple spacer promoters intermingled with enhancers; the latter sequences act to augment formation of stable initiation complexes (Reeder, 1999). Finally, there is a main terminator complex downstream from the 3'-end of the gene plus a proximal terminator (PT) upstream from the UPE; the PT seems to have multiple functions, including protecting the promoter from wandering polymerases.

### 2. RNA Polymerase I Transcriptional Machinery

Transcription is carried out by RNA polymerase I (pol I), which consists of at least 11 or 14 subunits in mouse and yeast, respectively (Reeder, 1999). In addition, initiation of pol I transcription requires at least two DNA-binding factors, designated TIF-IB/SL1 and UBF in the mouse system (Grummt, 1999). Two other factors, TIF-IA and TIF-IC, are associated with pol I; this complex is recruited to the promoter by TIF-IB/SL1. Current thinking suggests that UBF binds and bends DNA in a way that positions the core promoter and the upstream promoter element so that TIF-IB/SL1 interacts with both parts of the promoter. Thus, four basal factors are required for the optimal interactions needed for assembly of the initiation complex (Fig. 6). Although this complex can be formed from individual components *in vitro*, recent evidence suggests that parts of it might be preassembled in Cajal bodies (Gall, 2001; see below). Subsequent to initiation, transcription elongation is aided by factor TFIIS, which induces cleavage of the 3' ends of halted complexes (Schnapp *et al.*, 1996). Efficient elongation also requires topoisomerase I or II activity (Schultz *et al.*, 1992). Finally, termination is achieved with the aid of a factor called TTFI in mouse and Reb 1 in yeast (Reeder, 1999).

### 3. Regulation of rDNA Transcription

Pre-rRNA expression is regulated by several different mechanisms including (1) complete repression of transcription, (2) maintaining the activity of a subset of genes, and (3) fine-tuning the activity of the transcriptional machinery. Several major points of transcriptional regulation are summarized in Fig. 6.

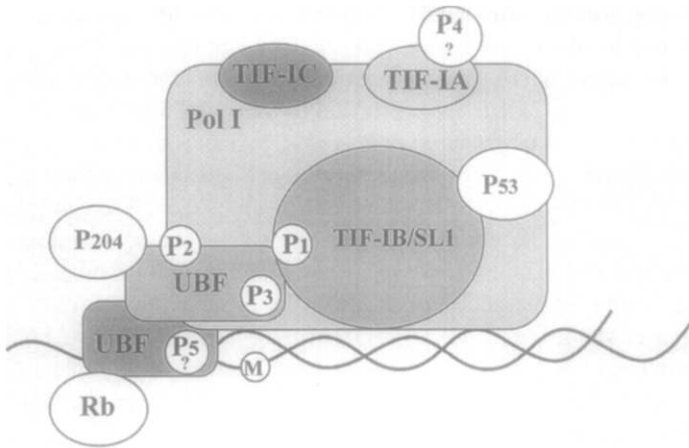


FIG. 6 Regulation of preribosomal RNA transcription in eukaryotes. The RNA polymerase I (Pol I) initiation complex contains transcription initiation factors, TIF-IA, TIF-IB/SL1, TIF-IC, and UBF. The DNA template is indicated by the two curved lines. Gene silencing may be achieved by methylation of DNA (M), which prevents initiation complex formation. In mitosis, transcription initiation is inhibited by phosphorylation of TIF-IB/SL1 (P1) and UBF (P2). Another phosphorylation site (P3) in UBF is involved in its reactivation at the end of mitosis. Phosphorylation of TIF-IA (P4) is believed to control transcriptional activity in the transition between growing and stationary phase cells. An additional phosphorylation site in UBF (P5) may play a role in the general regulation of its activity. UBF activity is suppressed by the retinoblastoma protein (Rb) and by the interferon-inducible nucleolar protein p204. Pol I transcription is also inhibited by the interaction of the tumor suppressor p53 with TIF-IB/SL1.

**a. Control during Mitosis** Among the RNA polymerase systems, the pol I machinery is unique in that it remains associated with the nucleolar organizers during mitosis in an inactive form. What causes the inactivation and how is postmitotic reactivation accomplished? At the beginning of mitosis, the promoter-binding factor, SL1, is inactivated by phosphorylation by a *cdc2/cyclin B*-dependent protein kinase (Grummt, 1999). Two subunits from the SL1 complex, TBP and hTAF<sub>110</sub>, become phosphorylated *in vitro*, but only the latter protein controls its activity. Phosphorylation of SL1 impairs its interaction with the upstream binding factor UBF, which prevents formation of a preinitiation complex. Presumably, dephosphorylation of SL1 reverses its inactivation. However, even after RNA pol I initiation competence is restored at the end of mitosis, another level of suppression must be overcome: UBF is rendered inactive also by phosphorylation (Klein and Grummt, 1999). UBF is reactivated later in G<sub>1</sub> phase, apparently by both dephosphorylation and new phosphorylation at a different site with *cdk4-cyclin D1*- and *cdk2-cyclin E*-dependent kinases (Voit *et al.*, 1999).

**b. Silencing a Subset of Genes** The multiplicity of genes coding for essentially the same gene product provide eukaryotic organisms the opportunity to regulate



expression by limiting the number of genes available for transcription. This is accomplished by utilizing a feature that is unique among transcribed genes in eukaryotes; the active rDNA genes do not appear to contain nucleosomes, at least in their typical form (extensively reviewed by Lucchini and Sogo, 1998). In the active genes, the DNA compaction ratio is close to one, compared with almost 6 in nucleosomal genes. Using psoralen crosslinking, Sogo and associates have shown that there are two classes of rRNA genes, one in which the DNA readily crosslinks with psoralen and another that does not. The active genes, including the transcribed regions and flanking sequences, contain DNA that becomes crosslinked, indicating a reduced protection of the DNA by proteins. In contrast, the rest of the nontranscribed spacer as well as the inactive genes seem to have conventional nucleosomal structures. It is not known whether histones are completely removed from the active rDNA chromatin, or are simply moved out of the way to give a nonnucleosomal appearance.

It has been suggested that transcription factors or the transcriptional process itself cause disruption of the nucleosomal structure. Support for this view comes from the fact that a nucleolar-specific chromatin-remodeling complex has been isolated and found to be associated with the pol I transcriptional machinery (Strohner *et al.*, 2001). How are individual ribosomal genes selected to be active or inactive? Santoro and Grummt (2001) suggest that a very simple mechanism leads to rDNA silencing. They showed that methylation of a single CpG dinucleotide in the upstream control element of the rDNA promoter (133 bp upstream from the initiation site) abrogates rDNA transcription. This methylation event inhibits binding of the transcriptional factor UBF to nucleosomal rDNA, which in turn prevents initiation complex formation. Thus, in the absence of transcription, the default structure of rDNA is the relatively standard nucleosomal form. In other words, transcription must be allowed to occur to generate the unique chromatin structure found in the active genes for pre-rRNA.

**c. Adjusting Transcription** In the previous two instances, the genes for pre-rRNA are either on or off; i.e., there is essentially a digital control mechanism in operation. This raises the question of whether nature has developed a method for continuous adjustment or analogue control of rRNA production? As it turns out, there are several ways to adjust the rate of rRNA transcription in response to changing physiological conditions (Hannan *et al.*, 1998b; Kuhn, 1998). However, a limited number of factors in the pol I transcription machinery are utilized for transduction of cellular signals. One of these is factor TIF-IA in mouse (Kuhn, 1998), which copurifies with pol I and is essential for *in vitro* transcription. TIF-IA appears to be the same as factor C\* (Brun *et al.*, 1994) or TFIC (Mahajan and Thompson, 1990). When cells are in logarithmic growth phase, pre-rRNA transcription is at a maximal rate; this decreases to near zero when cells enter stationary phase. The rate of transcription is reflected in the activities of cell-free extracts prepared from either growing or stationary phase cells. Addition of TIF-IA purified from growing

cells to stationary phase extracts restores the transcriptional activity of the latter extracts to nearly that of the growing cell extracts (Schnapp *et al.*, 1993). Although it seems well established that TIF-IA plays a major role in controlling transcriptional activity, the mechanism by which it does this is not well understood. There is evidence that phosphorylation events affect the activity of the factor, but a cause and effect relationship has not been established.

As already indicated above, a key player in the regulation of RNA pol I transcription is UBF, which affects rRNA production by at least three different mechanisms (Hannan *et al.*, 1998b). The first is by controlling the amount of UBF available to the pol I transcription apparatus; there is evidence that UBF is a limiting factor in certain instances. For example, overexpression of UBF1 in cardiac myocytes leads to an increase in the rate of rDNA transcription as much as 4.5-fold (Hannan *et al.*, 1996). Stimulation of pre-rRNA transcription by insulin (Hannan *et al.*, 1998a) is also accompanied by increases in UBF. Second, the level of phosphorylation of UBF correlates with its activity (Hannan *et al.*, 1998b). Although UBF is phosphorylated by casein kinase II (Voit *et al.*, 1992) it is not clear whether this or other enzymes are responsible for the increased activity of the factor. Cyclin-dependent kinases also seem to be involved with reactivation of UBF at the end of mitosis (Voit *et al.*, 1999; see above). Finally, UBF is inactivated by the retinoblastoma protein (Rb), with the result that UBF-dependent transcription is inhibited (Hannan *et al.*, 1998b, 2000; Klein and Grummt, 1999). Rb interacts with UBF to prevent formation of a UBF-SL1 complex; this interaction is also governed by phosphorylation by a complicated, poorly understood mechanism. It is interesting that suppression of rDNA transcription is accomplished by sequestration of UBF in another system, namely the interferon-inducible nucleolar protein p204, which also binds UBF (Liu *et al.*, 1999). In contrast, pol I transcription is inhibited by the tumor suppressor p53 by its interaction with SL1; this also prevents formation of the UBF-SL1 complex (Zhai and Comai, 2000).

## B. Assembly of Preribosomal RNP Complexes

As revealed by X-ray crystallographic analyses, the ribosome is a highly compact structure in which the rRNAs contribute most of the mass (Ramakrishnan and Moore, 2001). Given the length of the pre-rRNA transcript and the large number of opportunities for the formation of base pairs at improper locations, the task of producing correctly folded rRNAs in the ribosomal subunits is a formidable one. Thus, it is easy to understand the necessity for the association of proteins and snoRNAs with the transcript early in its life cycle, before mismatches occur. For example, it is suggested that binding of U3 snoRNA to the 5' end of 18 S rRNA prevents the premature formation of a central pseudoknot and that an RNA helicase participates in this activity (Colley *et al.*, 2000). In fact, at least 17 different helicases (see Table I) have been implicated in yeast ribosome assembly,

TABLE 1

## Nucleolar Proteins

Class/name of component	Proposed function/source/comments	References
<b>Ribonucleases</b>		
<b>Endonucleases</b>		
RNaseP, RNaseMRP	Cleavage of rRNA precursors in yeast and humans	van Hoof <i>et al.</i> , 2000
PR1	Cleaves single-stranded regions of RNA in mouse	Eichler and Craig, 1994
Rnt1p	RNase III family, involved in processing the 3' end of 25 S rRNA, cleaves pre-U3 in yeast	Nagel and Ares, 2000;
B23	Cleaves specific sites in ITS2 of vertebrates	Herrera <i>et al.</i> , 1995; Savkur and Olsson, 1998
<b>Exonucleases</b>		
AtRtp41	5' → 3' exonuclease, 5.8 S rRNA processing in yeast and plants	Chekanova <i>et al.</i> , 2000
Xm1p/Ski1p, Xm2p/Rat1p	5' → 3' exonuclease, required for the final steps in the formation of 25 S rRNA	Geerlings <i>et al.</i> , 2000
Mtr3p, Rrp41p/Ski6p, Rrp42p, Rrp43p, Rrp45p, Rrp46p, Rrp44p/Dis3p, Rrp4p	3' → 5' exonucleases, exosome components, processing of 7 S pre-rRNA to the mature 5.8 S rRNA in yeast	Chekanova <i>et al.</i> , 2000; Brouwer <i>et al.</i> , 2001
Rrp6p/PM-Sc1 100p, Rrp40p	Exosome components, processing of rRNA and snoRNAs in yeast and humans	Chekanova <i>et al.</i> , 2000; Brouwer <i>et al.</i> , 2001
Rex1p, Rex2p, Rex3p	Members of RNase D family, required for 5.8 S rRNA trimming in yeast	van Hoof <i>et al.</i> , 2000
<b>Topoisomerases</b>		
Topoisomerase I (topo I)	Catalyzes single-strand breaks independent of ATP hydrolysis, human	Hyttel <i>et al.</i> , 2000
Topoisomerase II (topo II)	Changes DNA topology by breaking double-stranded DNA; requires ATP hydrolysis, in mammalian cells; two isoforms, $\alpha$ , $\beta$	Tsutsui <i>et al.</i> , 2001; Mo and Beck, 1999
Topoisomerase III	Involved in rDNA metabolism, belongs to topoisomerase IA subfamily	Mohaghegh and Hickson, 2001

Molecular chaperones				Szebeni and Olson, 1999; Davis <i>et al.</i> , 1998;
Protein chaperones		Chaperones found in nucleoli of vertebrates		Yárgas-Roig <i>et al.</i> , 1998; Knowlton, 1999; Corporeau <i>et al.</i> , 2000
B23, HDJ-2, Hsp70, Hsp72, Hsc70				Isaac <i>et al.</i> , 1998
Nopp140/p130		Associates with NAP57 in yeast and vertebrates, putative chaperone of snoRNPs		
RNA chaperones		Ribosome maturation in <i>S. pombe</i>		Lalev and Nazar, 2001
RAC				
Proteins implicated in pre-rRNA processing				
Proteins associated with H/ACA snoRNAs		Box H/ACA snoRNA core proteins; Cbf5p is a putative pseudouridine synthase		Kiss, 2001
Nop10p, Gar1p, Nhp2p, Cbf5p		Human homologues of yeast Cbf5p and Gar1p		Dez <i>et al.</i> , 2001; Yang <i>et al.</i> , 2000
NAP57/Human dyskerin, hGar1p		Homologous to Nhp2 in yeast		Maiorano <i>et al.</i> , 1999
p17nhp2		Protein component of snoRNP, has role in pre-rRNA processing in yeast		Kressler <i>et al.</i> , 1999
Sbp1p		Maturation of diverse RNAs, putative RNA helicase		Kressler <i>et al.</i> , 1999
Sen1p				
Proteins associated with box C/D snoRNAs		Box C/D snoRNA core proteins		Kiss, 2001
Nop1p, Nop5p/58p, Nop56p, Snu13p				

(continues)

TABLE 1 (continued)

Class/home of component	Proposed function/source/comments	References
Sof1p, Imp3p, Imp4p, Mpp10p, Sof1p, Lcp5p	Specific components of the U3 snoRNP, pre-18 S rRNA processing in yeast	Zanchin and Goldfarb, 1999; Lee and Baserga, 1999; Kressler <i>et al.</i> , 1999
U3-55k/hU3-55k	Interacts with U3 RNA in <i>Xenopus</i>	Lukowiak <i>et al.</i> , 2000
NAP65	Rat homologue of yeast Nop5p/58p	Yang <i>et al.</i> , 2000
Fibrillarin/U3-RNP	Vertebrate homologue of yeast Nop1p	Barneche <i>et al.</i> , 2000
AtFib1, AtFib2	Functional homologue of Nop1p in plants	Barneche <i>et al.</i> , 2000
193-aa protein/FibM	Archaeal fibrillarin homologue, similar to Prp31, Nop56/Sik1p, Nop5p/Nop58	Hickey <i>et al.</i> , 2000
p62	Interact with U16 snoRNA, fibrillarin, and p68 in <i>Xenopus</i>	Filippini <i>et al.</i> , 2000
65-kDa and 68-kDa proteins	C/D-stem motif binding activity in mouse and <i>Xenopus</i>	Weinstein and Steitz, 1999
Other proteins implicated in ribosome biogenesis		
Gar2	Required 18 S rRNA and ribosomal subunit production	Sicard <i>et al.</i> , 1998
Rrp8p	Functionally linked to Gar1p and involved in pre-rRNA cleavage at site A2 in yeast	Bousquet-Antonelli <i>et al.</i> , 2000
Nop2p, Nop4p/Nop77p, Nop8p, Nop56p/Sik1, Nop5p/Nop58	Involved in 60 S subunit assembly	Zanchin and Goldfarb, 1999
Nop7p, Nip7p, Brx1p, Nog1p, Has1p, Erb1p, Rpf1p, Ebp2p, Rpl1p, Ytm1p, Spb1p, Nop15p, Nop16p, R1p7p, Drs1p	Proteins associated with Nop7p, Nug1p in yeast, late stages of 60 S subunit assembly	Zanchin and Goldfarb 1999; Harnpicharnchai <i>et al.</i> , 2001; Bassler <i>et al.</i> , 2001
Rrp5p	Involved in 18 S and 5.8 S rRNA synthesis in yeast	Eppens <i>et al.</i> , 1999
Nucleolin/Nsr1p	Factor for assembly and maturation of pre-RNP particles in vertebrates	Ginisty <i>et al.</i> , 1999

rRNA-modifying enzymes and associated proteins			
p120/No1	Has rRNA 2'-hydroxymethyltransferase activity		MacCallum and Hall, 2000
cbf5p	Putative rRNA pseudouridine synthase in yeast		Pogacic <i>et al.</i> , 2000
NAP57/human dyskerin	Rat and human homologues of Cbf5p		Pogacic <i>et al.</i> , 2000
Nop60Bp	Putative pseudouridine synthase in <i>Drosophila</i> related to NAP57		Phillips <i>et al.</i> , 1998
Treacle	Mutated in Trecher Collins syndrome in humans, structurally related to Nopp 140		Isaac <i>et al.</i> , 2000
Fibrillarin	Putative RNA O-methylase in vertebrates, homologue of yeast nop1p		Wang <i>et al.</i> , 2000
Helicases			
Multifunctional DNA and RNA helicases			
Nucleolin	Multifunctional phosphoprotein shows DNA and RNA helicase activity in mammals		Tuteja and Tuteja, 1998
Sen1p	Related to DEAD box RNA helicase and type I DNA helicase in yeast		Rasmussen and Culbertson, 1998
DNA helicases			
RecQ4, RecQ5, RecQL/RecQ1	RecQ DNA helicases in humans		Yankiwski <i>et al.</i> , 2000
WRN/hWRNp, BLM	RecQ DNA helicases, altered in Werner and Bloom's syndromes		Mohaghegh and Hickson, 2001
xBLM, DmBLM, Sgs1p, Rqh1p	RecQ DNA helicases, identical to human BLM in <i>Xenopus</i> , <i>Drosophila</i> , and yeast		Mohaghegh and Hickson, 2001
NDH II/DNA helicase II	Member of DEXH DNA helicase family		Zhang <i>et al.</i> , 1999
PDH65 (DNA helicase 65)	Involved in rDNA transcription and pre-rRNA processing in pea		Tuteja <i>et al.</i> , 2001
p50, p55	Coordination of snoRNA processing and snoRNP assembly in mouse		Newman <i>et al.</i> , 2000

(continues)

TABLE 1 (continued)

Class/name of component	Proposed function/source/comments	References
RNA helicases		
Protein NOH61, Has1p	Members of DEAD Box RNA helicase family in humans and yeast	Zirwes <i>et al.</i> , 2000; Bassler <i>et al.</i> , 2001
Dhr1, Dhr2	DEAH Box RNA helicases, required for 18 S rRNA synthesis in yeast	Colley <i>et al.</i> , 2000
p68	DEAD Box RNA helicase, activation of rDNA transcription, interacts with fibrillarlin	Nicol <i>et al.</i> , 2000
II/Gu (RHI/Gu)	RNA helicase, member of the DEAD Box superfamily in human, HeLa	Garcia <i>et al.</i> , 2000
Fa1p, Rrp3p, Rok1p, Dbp4p, Dbp8p	RNA helicases, required for 40 S subunit synthesis in yeast	De la Cruz <i>et al.</i> , 1999
Dbp3p, Dbp6p, Dbp7p, Dbp9p, Dbp10p, Dob1p/Mtr4p, Drs1p, Mak5p, Nop56p/Sik1, Spb4p, hNOP56	RNA helicases required for 60 S subunit synthesis in yeast	De la Cruz <i>et al.</i> , 1999; Zanchin and Goldfarb, 1999
	Putative human RNA helicase, involved in 60 S subunit synthesis	Gao <i>et al.</i> , 2000; Sun <i>et al.</i> , 2001
Nucleolar proteins of undefined function		
Cgr1p	Colocalizes with Nop1p in yeast	Sun <i>et al.</i> , 2001
C7 protein	Mouse homologue of <i>Drosophila</i> late puff product L82 and isoform of human OXR1	Fischer <i>et al.</i> , 2001
pp135	Constituent of fibrillar components of nucleoli	Vandelaer and Thiry, 1998
Spb1p	Associated with Nop1p and Nop58p, binds 5'-adenosyl methionine	Pintard <i>et al.</i> , 2000

further supporting the need for directed formation of proper RNA conformation (Lafontaine and Tollervey, 2001).

Processing and RNA remodeling components begin associating with pre-rRNA during transcription as indicated by the presence of electron-dense “terminal balls” near the leading ends of nascent transcripts (Mougey *et al.*, 1993). The terminal balls contain U3 small nucleolar RNA (snoRNA) and a number of unidentified proteins; these structures might be equivalent to the “processomes,” which are complexes of processing proteins and snoRNAs in yeast (Maxwell and Fournier, 1995). Thus, it is clear that many of the components of the processing machinery are added before the pre-rRNA transcripts are completed, but the exact timing of addition of individual proteins and snoRNAs is yet to be determined.

Do the components find their way to the growing pre-rRNP particles by a directed or by a stochastic process? Several recent reports suggest the latter pathway. Using FRAP to measure exchange of proteins in nuclear compartments, Phair and Misteli (2000) showed that although some proteins may “reside” in nuclear substructures such as the nucleolus, they are highly mobile and exchange rapidly with the nucleoplasm. The high mobility of the snoRNP protein fibrillarin as well as its targeting to transcription centers was shown by Snaar *et al.* (2000). Finally, studies by Chen and Huang (2001) indicate that the processing and assembly components nucleolin, fibrillarin, RNase MRP subunits, Rpp29, and B23 all have high mobilities and that they exchange rapidly between the nucleolus and the nucleoplasm. However, their mobilities are higher in the nucleoplasm than in the nucleolus, suggesting that engagement in ribosome biogenesis slows them down. Taken together, these studies suggest that inside the nucleus, pre-rRNA processing components reach their destinations through diffusion. Furthermore, their nucleolar localization is due to the specific functional roles they perform rather than by general nucleolar targeting.

### C. Pre-rRNA Processing

The pathways of pre-rRNA processing are generally well defined in vertebrates and in yeast (Sollner-Webb *et al.*, 1996; Eichler and Craig, 1994; Lafontaine and Tollervey, 2001). Although the final RNA products destined for ribosomal subunits are relatively simple, the route to their production is surprisingly complex, as illustrated for the yeast system in Fig. 7. Processing proceeds in orderly, temporally regulated events; in general, the 5' half of the transcript is processed before the 3' half. The order of processing events is similar in yeast and vertebrates, but with important differences in details. Pre-rRNA processing does not rely strictly on site-specific nucleases to directly generate the ribosomal RNA products. Instead, there are initial cleavages in the spacer regions of pre-rRNA by endonucleases followed by trimming in both directions by exonucleases to yield the final three species of ribosomal RNA. The nucleases known to participate in this process are



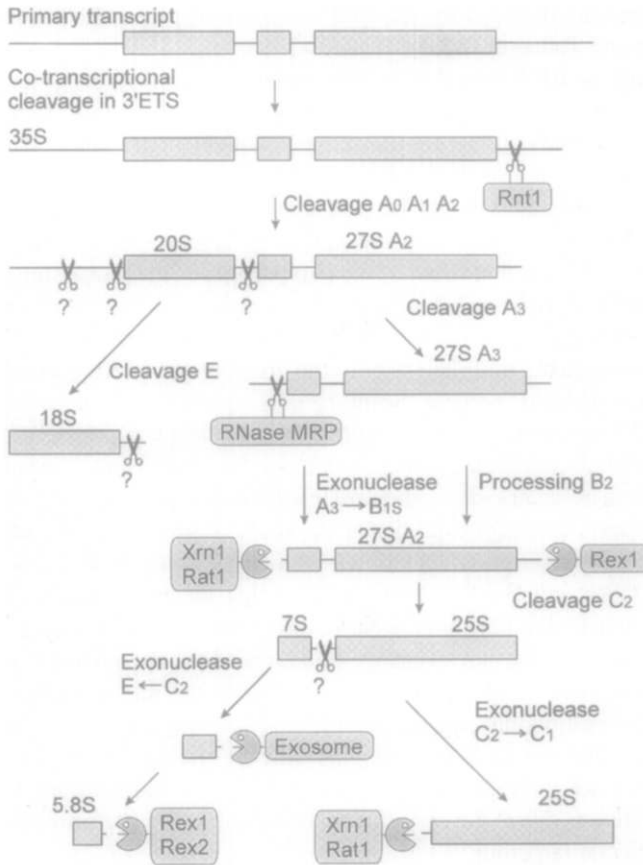


FIG. 7 Features of preribosomal RNA processing in eukaryotes. The pathway is presented is for the best characterized eukaryote, the budding yeast *Saccharomyces cerevisiae*. The mature ribosomal RNAs are generated by sequential endonuclease cleavage, with some of the mature rRNA termini generated by exonuclease digestion. Scissors with question marks indicate that the endonuclease responsible is unknown. In yeast, an alternative pathway generates a minor 5' extended form of the 5.8 S rRNA; for simplicity this has been omitted from the figure. Adapted from Lafontaine and Tollervy (2001).

listed in Table I. Many of the 3' → 5' exonucleases are contained in particles called "exosomes" (Allmann *et al.*, 2000). At least two of the endonucleases, RNase P and MRP RNase, contain small RNAs as essential components (Tollervy and Kiss, 1997; Jarrous *et al.*, 1999). There is evidence that the first processing event in rodent nucleoli occurs before the pre-rRNA transcript is completed (Lazdins *et al.*, 1997), although another study using primate cells indicates that at least some of the transcripts achieve full length before any processing takes place (Dundr and Olson, 1998).

### 1. Role of Small Nucleolar RNAs

Since the specificity for cleavage of pre-rRNA does not appear to reside in the nucleolar ribonucleases themselves, how does the processing machinery generate very precise ends on the final rRNA products? Our understanding of the mechanism of pre-rRNA processing has been greatly facilitated by the discovery of an unusually large number of snoRNAs (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997; Weinstein and Steitz, 1999; Elicieri, 1999), which play major roles in directing the cleavage reactions. More than 150 snoRNAs have been identified in various eukaryotic species and it is estimated that there may be as many as 200 in vertebrates (Elicieri, 1999). A comprehensive database of yeast snoRNAs has been compiled (Samarsky and Fournier, 1999).

The snoRNAs can be divided into two major classes, box C/D and box H/ACA, as shown in Fig. 8. The functions of the snoRNAs in pre-rRNA processing are rapidly becoming elucidated, as illustrated below by a few examples. Both classes of snoRNAs are involved in cleavage reactions (Elicieri, 1999). The most highly characterized C/D box snoRNA, U3, is essential for cleavage at both ends of the 18 S rRNA and at the 5' end of 5.8 S rRNA (Kass *et al.*, 1990; Savino and Gerbi, 1990). Interestingly, U3 snoRNA appears to act as a bridge to draw together the

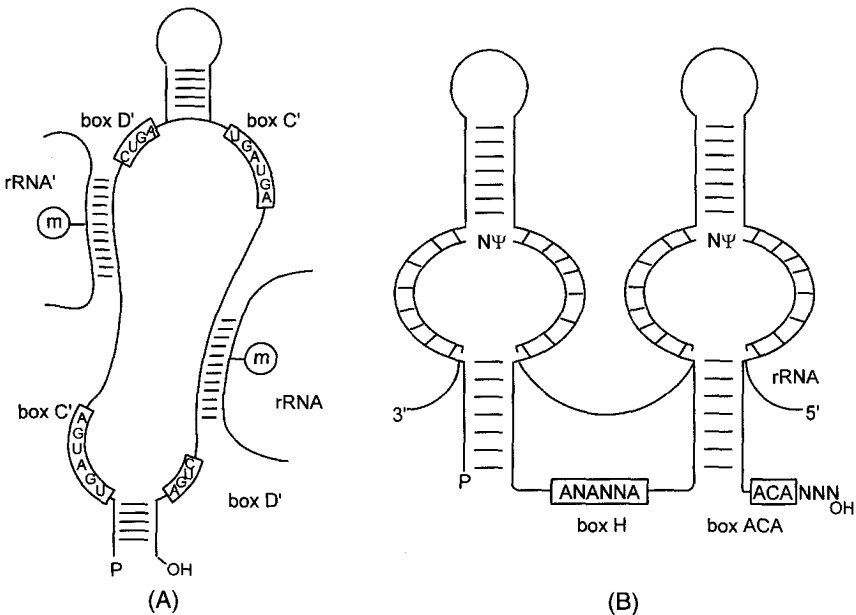


FIG. 8 Structural and function of (A) 2'-O-methylation and (B) pseudouridylation guide snoRNAs. The consensus sequences of boxes C, C', D, D', H, and ACA are indicated (R is a purine and N stands for any nucleotide). From Kiss (2001).

5' and 3' ends of the 18 S rRNA coding sequence to coordinate the cleavage of sites in the vicinity of these regions (Borovjagin and Gerbi, 2001). In the case of H/ACA box snoRNAs, E1, E2, and E3 are each involved with different stages of 18 S rRNA formation (Mishra and Elicieri, 1997). The exact manner in which the snoRNAs participate in the cleavage reactions is not well understood. Although base pairing between the snoRNA and the pre-rRNA is required for processing to proceed, it is not clear whether the snoRNAs serve as guides to direct the nucleases to the cleavage sites or if they act as RNA chaperones to produce folding patterns recognized by the nucleases (Peculis and Greer, 1998; Borovjagin and Gerbi, 1999).

## 2. Preribosomal RNA Modification

The posttranscriptional modifications of pre-rRNA fall into three categories: (1) ribose methylation, (2) base methylation, and (3) pseudouridylation. As discussed below, progress has been made on the locations of these modifications and how they are placed at those sites, but their real function remains largely an open question.

**a. Methylation** Ribose methylation at the 2'-O position occurs on about 100 and 55 sites in vertebrates and yeast pre-rRNA, respectively, which are found in the conserved regions of mature rRNA (Maden and Hughes, 1997). The modification takes place during or immediately after synthesis. How does the ribosome assembly machinery know where to place the methyl groups on a very long RNA transcript? The answer is again found in the snoRNAs, in this case the ones belonging to the C/D box class. Segments of the pre-rRNA targeted for methylation form duplexes with the D and D' boxes of the snoRNA; these guide the methylase to the sites to be modified (Weinstein and Steitz, 1999). Initial clues about the identity of the methylase enzyme came from mutational studies suggesting that the yeast fibrillar homologue Nop1p was responsible for the activity (Tollervey *et al.*, 1993). More recently, X-ray crystallographic studies showed that the archaeobacterial homologue of fibrillar contains a domain with a three-dimensional structure very similar to those found in known methyltransferases (Wang *et al.*, 2000). Thus, it is highly probable that specific methylation is directed through the association of fibrillar with various C/D box snoRNAs. Although earlier studies suggested that ribose methylation is important for correct pre-rRNA processing in mammalian cells, this does not seem to be the case in yeast (Maden and Hughes, 1997). Even more elusive is the role that methylation might play in the function of the ribosome. Finally, very little is known about RNA base methylation, which does not seem to be directed by guide snoRNAs.

**b. Pseudouridylation** The other major pre-rRNA modification is pseudouridylation, in which the attachment of the base to the sugar is flipped from the nitrogen

at position 1 to the carbon at position 5. There are nearly as many pseudouridines present in pre-rRNA as there are methyl groups. As with methylation the modified residues are located in the conserved regions of the mature rRNAs (Ofengand and Bakin, 1997). The conversion of the uridine to pseudouridine at specific sites is directed by sequence-specific base pairing by the H/ACA class of snoRNAs. Members of the latter class of RNA have one or two functional antisense regions capable of forming duplexes with pre-rRNA (Weinstein and Steitz, 1999). There are several candidates for eukaryotic pseudouridine synthases, including Cbf5p in yeast (Watkins *et al.*, 1998) and NAP57 in rat (Meier and Blobel, 1994); the actual demonstration of the activity in these or other proteins will be an important step in establishing their true identity. As with O-methylation, the function of the pseudouridine residues in rRNA remains poorly understood.

## D. Preribosomal Particle Maturation

### 1. Addition of Ribosomal Proteins

Although the past decade has brought us much progress in our understanding of pre-rRNA processing and modification, research on the addition of ribosomal proteins and 5 S rRNA to and final maturation of the pre-rRNP particles has been largely dormant until recently. Most of what is known about these events in vertebrates is derived from work done in the 1970s and early 1980s using biochemical methods (summarized by Hadjiolov, 1985). Although there was early evidence for the attachment of certain ribosomal proteins during transcription (Chooi and Leiby, 1981), this has not been confirmed in later studies. The general consensus is that about half of the large subunit proteins and one-third of the small subunit proteins are added to the growing complex very early. The remaining proteins are attached sequentially as the particles pass through the nucleolus and nucleoplasm, with some ribosomal proteins possibly added in the cytoplasm.

The sequence of incorporation of yeast ribosomal proteins has been compiled by Kressler *et al.* (1999). This information is also based largely on data collected in a few studies more than 20 years ago. Of the 80-odd ribosomal proteins, approximately 24 associate with a 90 S particle, which contains the full-length pre-rRNA transcript. Another dozen are incorporated after the 90 S particle splits into 66 S and 43 S particles. It is not clear whether the remaining ribosomal proteins are added in the nucleoplasm or after export of the subunits to the cytoplasm (see below).

### 2. Incorporation of L1/L5 and 5 S rRNA

At least one ribosomal protein, L1 in yeast or L5 in vertebrates, is found in an RNP complex before it is assembled into the pre-rRNP particle, in this case with

5 S rRNA. In vertebrates, 5 S rRNA is synthesized in the nucleoplasm and forms a 7 S RNP particle, which is then exported to the cytoplasm (Steitz *et al.*, 1988). Before reentry into the nucleus the 5 S rRNA combines with ribosomal protein L5. Recent studies indicate that the association of rpL5 with 5 S rRNA occurs during translation and suggest that the 5 S rRNA plays the role of a chaperone in the folding of L5 (Lin *et al.*, 2001). In yeast, L1 is not only essential for the stable assembly of 60 S subunits, but it is also required to maintain the stability of newly synthesized 5 S rRNA (Deshmukh *et al.*, 1993). Dechampesme *et al.* (1999) showed that assembly of the L1–5 S rRNA complex into the maturing pre-rRNP particles was essential for cleavage in the yeast ITS2 and for accumulation of 60 S subunits. The complexity of this one portion of ribosome assembly illustrates the mutual dependence of ribosome components on each other as well as the apparent necessity to maintain the correct sequence of events in ribosome biogenesis.

### 3. Late Stages of Maturation

New approaches and advances in technology should facilitate more rapid progress in the problem of preribosome maturation. Two recent studies have utilized a tandem affinity purification method for isolation of pre-rRNP particles at intermediate to late stages of assembly; the method relies on the expression of epitope-tagged yeast nucleolar proteins that are present in specific classes of particles. The proteins in these complexes were identified by a combination of high-performance liquid chromatography (HPLC), sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and mass spectrometry. In one of these, Harnpicharnchai *et al.* (2001) used tagged Nop7p to purify 66 S preribosomes containing 27 SB, 25.5 S, and 7 S pre-rRNAs; these RNAs represent two consecutive steps in pre-rRNA processing. At least 70 different proteins copurified with Nop7p; 45 of these were ribosomal proteins, including 31 of the 46 proteins in the 60 S subunit. The remaining proteins identified were nonribosomal proteins, some of which have been shown to play specific roles in ribosome biogenesis and others with unknown functions. In another study, Bassler *et al.* (2001) used tagged Nug1p to precipitate precursors to the yeast 60 S subunit. This particle contained substantial amounts of mature 25 S rRNA, its late precursors, and mature 5.8 S rRNA. It also contained 30 large ribosomal subunit proteins, a number similar to that found by Harnpicharnchai *et al.* (2001) as well as 22 nonribosomal proteins. With a few exceptions there was remarkable agreement in the complement of ribosomal proteins identified in the two studies. More striking was the observation that 8 of the nonribosomal proteins were previously shown to purify with the nuclear pore complex. The authors suggest that the Nug1p-containing particle is almost ready for export to the cytoplasm and may be at a slightly more advanced stage of maturity than the Nop7p particle.

The absence of some large subunit proteins in late stage pre-rRNP particles examined in the above studies suggests that certain ribosomal proteins are added

in the cytoplasm. Additional evidence for this is provided by the observation that yeast L7 is one of the last proteins incorporated and that it cycles on and off the large subunit in the cytoplasm (Dick *et al.*, 1997). The latter protein is also essential for joining the large and small subunits and the authors suggest that its presence or absence could be a mechanism for regulating translation. Obviously, the cytoplasmic phase of ribosome assembly needs clarification. Furthermore, because translation has been shown to occur in the nuclei of cells of vertebrates (Iborra *et al.*, 2001) it seems likely that all ribosomal proteins that are essential for protein synthesis are present in the subunits in the nuclei. Is it possible that some proteins in cytoplasmic ribosomes are not essential for translation, but that they carry out other, as yet undefined functions? Or are some fully assembled ribosomal subunits imported back into the nucleus to participate in protein synthesis?

## E. Export of Ribosomal Subunits

### 1. General Features of Export

Nuclear export of ribosomal subunits is a facilitated, energy-dependent, unidirectional, and spatially ordered process. The latter point is reinforced by electron microscopic studies that showed ribosomes or their subunits lining up on tracks in the nucleoplasm, presumably as they are exported from the nucleolus to the cytoplasm in *Schizosaccharomyces pombe* (Léger-Silvestre *et al.*, 1997). The coupling of the later steps in ribosome maturation is suggested by the finding that three nucleolar proteins, Noc1p, Noc2p, and Noc3p, are required for both maturation and transport (Milkereit *et al.*, 2001). These proteins are conserved in evolution from yeast to mammals. The authors propose that the three Noc proteins are essential for intranuclear movement of ribosomal precursor particles, which in turn is necessary for certain steps in pre-rRNA processing. In other words, ribosome biogenesis seems to be a vectorial process, where transport is essential for assembly and maturation. Specific movement within the nucleus seems to be crucial for positioning the subunits for transport into the cytoplasm.

Recent studies on the mechanisms of nuclear export of ribosomal subunits have utilized novel assay systems. One of these takes advantage of the fact that one stage of pre-rRNA processing occurs in the cytoplasm in yeast (Moy and Silver, 1999). Using this method, it was shown that export of small ribosomal subunits depends on the Ran-GTPase system and on a subset of proteins from the nuclear pore complex. The importance of Ran in export of ribosomal subunits has been confirmed by a system using GFP-tagged ribosomal protein L25 (Hurt *et al.*, 1999). A similar assay system utilized GFP-tagged ribosomal protein L11b in yeast (Stage-Zimmerman *et al.*, 2000). In the latter study, it was found that L11b accumulated in the nucleus when 60 S ribosomal subunit assembly was impaired and also when several nucleoporins and transport factors were mutated.

## 2. Adaptation of the General Nuclear Export System

Although the conventional nuclear export system is generally utilized for moving ribosomal subunits into the cytoplasm, there are somewhat different pathways for the small and large subunits, which also differ from the routes taken by other macromolecules. A distinct pathway for ribosome export was first suggested by experiments showing that saturation of the export system with 40 S ribosomal subunits does not affect the export of tRNA (Pokrywka and Goldfarb, 1995). It is now apparent that special molecules are needed to adapt the macromolecular complex to the export system. A major factor for export of leucine-rich nuclear export signal (NES)-containing proteins is Crm1p or exportin 1 (Stade *et al.*, 1997). Ho *et al.* (2000a) found that nuclear export of the 60 S subunit in yeast utilizes the latter factor, but another protein called Nmd3p is required as an adaptor between Crm1p and the ribosomal subunit (illustrated in Fig. 5). The Nmd3p protein contains the NES needed to interact with Crm1p. Because Nmd3p binds the 60 S, but not the 40 S subunits (Ho *et al.*, 2000b), it will be interesting to see if a separate adaptor protein is required for nuclear export of 40 S subunits.

## F. The Relationship between the Cajal Body and the Nucleolus

### 1. Exchange of Material

The Cajal body (CB), formerly known as the coiled body, was originally called the nucleolar accessory body, implying that the CB and the nucleolus are in some way related (Matera, 1999). In fact, CBs are often juxtaposed with nucleoli as illustrated in Fig. 1. This relationship is also supported by the fact that several proteins; e.g., fibrillarin and Nopp140, are found in both the nucleolus and the CB. Because the latter protein has been shown to move between the two structures, it could be a functional link between the two subnuclear bodies (Isaac *et al.*, 1998). Furthermore, coilin, the usual marker for CBs, was also shown by the latter group to be an interacting partner of Nopp140. An essential, mutually beneficial interaction between the two bodies is suggested by studies showing that the expression of mutant forms of coilin can disrupt nucleolar structure and, conversely, mutants of the Nopp140 affect CB structure (Matera, 1999).

Mutational analyses have revealed that some truncated coilin mutants localize to the nucleolus; these investigations have led to the identification of a cryptic nucleolar localization motif within the protein (Hebert and Matera, 2000). However, the cellular signals that naturally cover/uncover the coilin nucleolar localization signal have yet to be discovered. This raises the possibility that coilin itself might traffic through the nucleolus, possibly as a carrier of components to be modified or processed.

The actual transfer of material from the CB to the nucleolus is supported by studies demonstrating that intergenic spacer regions from snoRNA precursors are present in CBs and in nucleoli. The movement of the snoRNAs from CBs to nucleoli is dependent on the presence of the box C/D motif, but this motif is not necessary for localization in CBs (Narayanan *et al.*, 1999a). Splicing factors for mRNA also follow a similar pathway. Recently it was shown that fluorescently labeled Sm proteins (components of snRNPs) first accumulate in CBs, then in nucleoli, and finally in speckles (Sleeman and Lamond, 1999). The observation that CBs move from the nucleolar periphery into the nucleolus (Boudonck *et al.*, 1999) opens the possibility that the transfer of the material is accomplished by direct contact between the CB and the nucleolus. This is reinforced by studies showing that CBs exhibit a highly dynamic behavior within the cell; the movements include translocations through the nucleoplasm, joining of bodies to form higher structures, and separation of smaller bodies from larger CBs (Platani *et al.*, 2000). The joining and separation of the CBs coupled with their interactions with the nucleolus and other nucleoplasmic structures further support the proposed transport function of this organelle.

What is actually happening to the small RNAs in the CBs remains an unresolved issue. Are the RNAs being modified by methylation or pseudouridylation in the CBs? Or are the CBs needed for proper assembly of snoRNP particles? It should also be pointed out that some studies indicate that intact CBs are not essential for nucleolar functions (Almeida *et al.*, 1998) and some cells do not have any CBs. Therefore, much work needs to be done to establish the true relationship between the Cajal body and the nucleolus.

## **2. Assembly of the Transcriptional Apparatus**

An intriguing model has been proposed by Gall (2001), which suggests that transcription complexes for each of the three nuclear RNA polymerases are assembled in the CBs. Experimental data show that RNA polymerase II actually passes through the CBs; however, evidence for the presence of RNA polymerase I in CBs is largely based on antibody staining. The model suggests that complexes called “transcriptosomes” are assembled from the polymerases, transcription factors, and RNA processing components in the CBs. Separate transcriptosomes are then transported to other parts of the nucleus, including nucleoli in the case of the RNA pol I complexes.

## **3. A Cajal Body Homologue in Yeast**

Although CBs have not been found in lower eukaryotes, a probable equivalent structure has been observed within yeast nucleoli themselves (Verheggen *et al.*, 2001). The authors named this structure the nucleolar body (NB). The NBs do not contain coilin, but the human survival of motor neuron (SMN) protein, a marker for



gems or CBs, localizes to the NBs. The box C/D snoRNAs move through the NBs as they do through the CBs, and this traffic is dependent on proteins Srp40 and Nsr1p, which are yeast homologues of mammalian Nopp 140 and nucleolin, respectively. Thus, in the case of yeast, a highly specialized subnucleolar structure seems to carry out the functions of CBs found in vertebrates. The NB might truly be the “nucleolar accessory body” that has not left the nucleolus. This raises interesting evolutionary questions: were CB functions originally contained in the nucleoli of primitive eukaryotes? If so, was it necessary to move the CB out of the nucleolus to accommodate additional nucleoplasmic functions needed by higher eukaryotes? Is the yeast NB located in the nucleolus instead of the nucleoplasm because yeast has no carrier molecule (e.g., coilin) to transport components between the nucleolus and the CB homologue?

#### **IV. Nucleolar Proteins**

Technology for rapid identification of proteins has advanced to the point where databases of all proteins present in nucleoli from one or more cell types will soon be available. Two examples of progress in this direction were presented above, where nearly all of the proteins in pre-rRNP particles were rapidly identified by mass spectrometry (Harnpicharnchai *et al.*, 2001; Bassler *et al.*, 2001). A nucleolar proteome project, in which the full complement of nucleolar proteins from HeLa cells has been analyzed by mass spectrometry, is now complete (Andersen *et al.*, 2002); the availability of these data will greatly facilitate progress in our understanding of the protein composition of the nucleolus. However, functional analyses as well as development of nucleolar protein interaction maps will be needed for a thorough understanding of the workings of the nucleolus.

Numerous proteins known or presumed to be involved in pre-rRNA processing and ribosome assembly have been identified (Table I). A few of the well-characterized ones are described in more detail below.

##### **A. Nucleolin**

###### **1. Activities Related to Ribosome Biogenesis**

One of the most extensively studied nucleolar proteins is the abundant phosphoprotein nucleolin. Because the characteristics of this protein have been reviewed extensively (Ginisty *et al.*, 1999; Tuteja and Tuteja, 1998; Srivastava and Pollard, 1999), only a few interesting features will be covered. Initially called protein C23 (Orrick *et al.*, 1973), nucleolin or similar versions have been found in a wide range of eukaryotes from yeast to humans. The relationships of the unusual structural

features of the protein to its functions have long been a source of curiosity. The sequence begins with alternating basic and highly acidic segments in its N-terminal one-third, followed by four RNA-binding domains (RBDs) and ends with a short sequence that is rich in glycine and dimethylated arginine (RGG motif). Some of these motifs are found in other nucleolar proteins; e.g., highly acidic segments in protein B23 and the RGG motifs in fibrillarin (Lischwe *et al.*, 1985). Although many different activities have been ascribed to nucleolin, its primary function seems to be in facilitating the early stages of pre-rRNA processing, possibly by first interacting with the 5' region of pre-rRNA and then recruiting processing components.

Our understanding of the participation of nucleolin in ribosome assembly has evolved over more than a quarter of a century. This role was first suggested when it was shown to be present in pre-rRNP particles (Olson *et al.*, 1974). Later studies indicated that nucleolin is directly associated with pre-rRNA (Herrera and Olson, 1986) and that the segments resembling RNA binding domains are capable of binding RNA (Bugler *et al.*, 1987). Nucleolin has a relatively high affinity for two sequences upstream from the first processing site in pre-rRNA (Ghisolfi-Nieto *et al.*, 1996). Interaction of nucleolin with these sites may be essential for the primary processing event (Ginisty *et al.*, 1998) and for assembly of the primary processing complex (Ginisty *et al.*, 2000). As determined by nuclear magnetic resonance (NMR), the molecular basis of the sequence recognition is that the first two RNA binding domains bind on opposite sides of an RNA stem-loop, forming a clamp that brings the 5' and 3' ends of the recognition sequence together and stabilizes the stem-loop (Allain *et al.*, 2000a,b; Bouvet *et al.*, 2001). This is consistent with earlier observations showing that nucleolin is capable of promoting secondary structure in complex RNAs (Sipos and Olson 1991). Thus, nucleolin seems to be part of a complex associated with nascent pre-rRNA and involved in the early stages of pre-rRNA processing. The ability of nucleolin to bind several ribosomal proteins through its RGG (or GAR) domain (Bouvet *et al.*, 1998) suggests that it plays a broader role in the beginning phases of pre-rRNP particle assembly.

## 2. Other Activities

Other activities of nucleolin are unrelated to ribosome biogenesis and extend well beyond the confines of the nucleolus. Some of the proposed functions stem from its ability to alter nucleic acid secondary structure. For example, nucleolin is one of the polypeptide components of the B cell-specific transcription factor and switch region binding protein, LR1 (Hanakahi *et al.*, 1997). The other polypeptide is a specific isoform of hnRNP D; this combination of polypeptides results in a sequence-specific duplex DNA binding protein (Dempsey *et al.*, 1998). The promotion of switch recombination appears to be related to the nucleic acid annealing activity in nucleolin, which resides in the C-terminal region (Hanakahi *et al.*, 2000).

The RGG domain is essential for this activity and is responsible for the ability of nucleolin to dissociate from single-stranded DNA. A different B cell-specific DNA recombination complex designated SWAP contains nucleolin, protein B23, poly(ADP-ribose) polymerase, and SWAP-70 (Borggreve *et al.*, 1998). However, little is known about the role of nucleolin in this complex.

## B. Nucleolar Protein B23

Protein B23 is another well-characterized, abundant nucleolar phosphoprotein (Olson, 1990) with multiple activities, but whose actual functions in ribosome biogenesis are less well defined than other proteins. Because of the recent interest generated by this protein as well as the absence of single review on it, we are covering protein B23 in more detail than other nucleolar proteins.

### 1. General Properties

**a. Initial Characterization** Protein B23 was initially identified by Orrick *et al.* (1973) in a comparison of nucleolar proteins from normal rat liver versus Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis. Various laboratories have given the protein different names: numatrin because of its presence in the nuclear matrix (Fields *et al.*, 1986), nucleophosmin (abbreviated NPM; Chan *et al.*, 1989), and NO38 in *Xenopus laevis* (Schmidt-Zachmann *et al.*, 1987).

**b. Behavior under Varying Physiological Conditions** The amount of protein is increased in hypertrophic rat liver (Ballal *et al.*, 1974), and its level is six-fold higher in HeLa cells than in normal human liver cells (Busch *et al.*, 1984). Conversely, protein B23 levels are reduced in cells undergoing apoptosis (Patterson *et al.*, 1995; Tawfic *et al.*, 1995). As might be expected, conditions that increase the stability of protein B23 seem to have antiapoptotic effects (Chou and Yung, 2001). However, the opposite result was obtained by Martelli *et al.* (2000) who found that protein B23 is relatively stable during apoptosis.

Increases in protein B23 levels are associated with receptor-mediated induction of mitogenesis in normal cells and with neoplastic growth in various cell types (Feuerstein and Mond, 1987; Feuerstein *et al.*, 1988). A chromosomal translocation results in the fusion of the amino-terminus of protein B23 to the catalytic domain of anaplastic lymphoma kinase; this produces a protein in which the tyrosine kinase is permanently activated leading to development of non-Hodgkin's lymphoma (Morris *et al.*, 1994). Antibodies to protein B23 are also identified in the sera from patients with autoimmune diseases, including systemic rheumatic disease (Pfeifle *et al.*, 1986), systematic lupus erythematosus, chronic graft-versus-host disease (Wesierska-Gadek *et al.*, 1992), and hepatocellular carcinoma (Imai *et al.*, 1992).

**c. Subcellular Locations** Protein B23 is primarily located in the nucleolar granular component, which contains maturing preribosomal RNP particles, as demonstrated by Spector *et al.* (1984). It is also present to some extent in the dense fibrillar component, but it is clearly absent from the fibrillar centers (Biggiogera *et al.*, 1991). Consistent with its location in the granular component, it has been shown to be associated with the maturing preribosomal RNP particles (Prestayko *et al.*, 1974; Olson *et al.*, 1986; Yung *et al.*, 1986; Zirwes *et al.*, 1997a; Pinol-Roma, 1999). Its presence in the nucleolus is dependent on active rDNA transcription as indicated by its translocation to the nucleoplasm after treatment of cells with serum starvation (Chan *et al.*, 1985; Yung *et al.*, 1990), inhibitors of pre-rRNA synthesis such as actinomycin D (Yung *et al.*, 1985a), or agents that block pre-rRNA processing, including toyocamycin (Yung *et al.*, 1985b; Finch *et al.*, 1997). In contrast, inhibitors of protein synthesis have no effect on the translocation process (Chan *et al.*, 1985; Yung *et al.*, 1985b), suggesting that protein B23 is interacting with pre-rRNA rather than other proteins. Although primarily located in nucleoli, protein B23 is also known to shuttle between the nucleus and cytoplasm (Borer *et al.*, 1989).

During mitosis, protein B23 disperses to various subcellular locations. Ochs *et al.* (1983) showed that during metaphase and anaphase, it is present at the chromosome periphery and in the interzone of the mitotic spindle. Protein B23 also appears in nucleolus-derived foci during anaphase and in prenucleolar bodies in telophase (see section above for more details) (Dundr *et al.*, 1997; Dundr and Olson, 1998; Dundr *et al.*, 2000). In addition, it is associated with centrosomes in early prometaphase and the mitotic apparatus in metaphase (Zatsepina *et al.*, 1999). Protein B23 reenters the nucleolus in the late stages of telophase or early G<sub>1</sub> phase.

**d. Features of the Primary Structure** The cDNA and amino acid sequences of protein B23 have been deciphered in chicken (Borer *et al.*, 1989), human (Chan *et al.*, 1989), mouse (Schmidt-Zachmann *et al.*, 1988), *Xenopus laevis* (Schmidt-Zachmann *et al.*, 1987), and rat (Chang *et al.*, 1988). As discussed below, protein B23 is expressed as at least two isoforms. The sequence of protein B23 is highly conserved among higher eukaryotes. For example, there is only one amino acid difference between the rat and mouse proteins and a 90% identity between human and rat sequences. The amino-terminal 119 residues of mammalian protein B23 show about 60% homology to *Xenopus laevis* nucleoplasmin, which suggests that these proteins arise from a single multigene family (Schmidt-Zachmann *et al.*, 1987; Schmidt-Zachmann and Franke, 1988). Other proteins in the nucleoplasmin/nucleophosmin family include mouse Npm3 (MacArthur and Shackelford, 1997) and *Xenopus* NO29 (Zirwes *et al.*, 1997b). The latter is an acidic protein that is present in preribosomal particles from *Xenopus* oocytes.

Figure 9 illustrates the arrangement of unique segments in the linear sequence. The N-terminal residue is modified, but the nature of the posttranslational

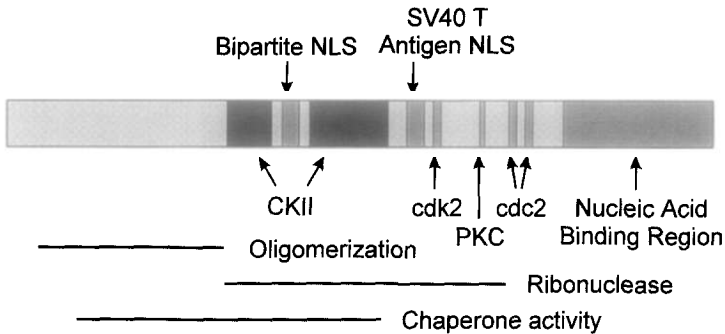


FIG. 9 Structural features of protein B23.1 polypeptide. Two highly acidic segments (black) in the center of the polypeptide chain contain phosphorylation sites for casein kinase II (CKII). There is a bipartite nuclear localization signal (NLS) in the region between the two acidic segments and an SV40 T antigen type NLS downstream from the second acidic segment. Sites for cdk2 and cdc2 kinases as well as protein kinase C (PKC) are indicated. The nucleic acid binding activity is located in the C-terminal end. The general regions of the polypeptide that are essential for oligomerization and ribonuclease and chaperone activity are indicated by lines under the diagram.

modification has not been determined. There are two highly acidic regions in the center of the molecule. Nuclear localization signal (NLS) sequences are found on either side of the second acidic region. The first one is a bipartite, nucleoplasmin-like NLS whereas the second NLS resembles the SV40 large T type (Peculis and Gall, 1992). The C-terminal 37 residue tail that is unique to B23.1 is rich in aromatic and basic amino acid residues and is essential for its nucleic acid binding activity (Wang *et al.*, 1994; see below). Studies by Umekawa *et al.* (1993) suggest that the secondary structure of protein B23 is composed predominantly of  $\beta$ -sheet (39.4%) and  $\beta$ -turns (27.1%) and to a small extent  $\alpha$ -helix (9.8%) and random coils (23.7%). No other information on the three-dimensional structure of the molecule is currently available.

**e. Mammalian Protein B23 Genes** Chang *et al.* (1990) studied the gene encoding the rat protein B23 in detail. The entire gene for B23 spans 11 kb and includes 12 exons and 11 introns and codes for two forms of the protein, B23.1 and B23.2, in the rat. Equivalent forms in humans have been deduced from the genomic sequence (Chan *et al.*, 1997). The B23.1 mRNA arises from exons 1 through 9 and 11 and 12, whereas the B23.2 mRNA is encoded by exons 1 through 10. Thus, the two mRNAs are a result of alternative splicing from a single gene where the 5' untranslated region and the first 9 exons are identical, but their 3' ends are different.

As a consequence of this genomic arrangement, the two proteins differ only at the C-terminal ends. The first 255 amino acids are identical in both forms, with 37 and 2 residue extensions beyond the point of identity in B23.1 and B23.2,

TABLE II  
Interactions and Activities of Nucleolar Protein B23/NPM

Interacting partner or activity	Detection method	References
Casein kinase II	Yeast two-hybrid system	Grein <i>et al.</i> , 1999
HIV-1 Rev protein	Affinity chromatography	Fankhauser <i>et al.</i> , 1991
Nucleolin/C23	Yeast two-hybrid system, coimmunoprecipitation	Li <i>et al.</i> , 1996
Nucleolar antigen p120	Affinity chromatography	Valdez <i>et al.</i> , 1994
Interferon regulatory factor-1	Affinity chromatography, immunoprecipitation	Kondo <i>et al.</i> , 1997
Centrosome/mitotic spindle	Immunofluorescence	Zatsepina <i>et al.</i> , 1999
Control of centrosome duplication	Immunofluorescence, transfection analyses	Okuda <i>et al.</i> , 2000
ATP binding activity	Nitrocellulose binding assay	Chang <i>et al.</i> , 1998
Nucleic acid binding activity	Filter binding assays	Dumbar <i>et al.</i> , 1989
Nuclear localization signal-containing peptides	Equilibrium dialysis	Szebeni <i>et al.</i> , 1995
Stimulation of nuclear import	<i>In vitro</i> nuclear import assays	Szebeni <i>et al.</i> , 1997
Ribonuclease activity	In-gel assays	Herrera <i>et al.</i> , 1995
Molecular chaperone activity	Aggregation, thermal denaturation assays	Szebeni <i>et al.</i> , 1999
Stimulation of DNA polymerase $\alpha$	Enzyme assays	Takemura <i>et al.</i> , 1994
Promotion of single-strand DNA annealing	DNA transfer assay	Borggreffe <i>et al.</i> , 1998
Histone chaperone activity	Nucleosome formation	Okuwaki <i>et al.</i> , 2001a
Stimulation of adenovirus replication	DNA replication assays	Okuwaki <i>et al.</i> , 2001b
Modulation of hepatitis delta RNA replication	Transient transfection assays	Huang <i>et al.</i> , 2001

respectively. The organization of the C-terminal segments is identical in both the rat and human proteins (Chan *et al.*, 1997).

## 2. Activities

Protein B23 is a multifunctional protein with several measurable activities and interactions with other macromolecules. These are summarized in Table II.

**a. Nucleic Acid Binding** The location of B23 in the dense fibrillar and granular regions suggests that protein B23 interacts with RNA. The possible interaction with RNA is supported by the observations that the protein has a relatively high affinity for single-stranded nucleic acids and it is able to perturb nucleic acid structure, destabilizing the RNA helix and disrupting base stacking (Dumbar *et al.*, 1989).

However, there is currently no evidence that protein B23 directly interacts with RNA in the nucleolus *in vivo*.

Feuerstein *et al.* (1990) showed that the protein binds either single-stranded or double-stranded DNA. The possibility that protein B23 interacts with DNA as a factor in DNA replication was considered by Takemura *et al.* (1994). Their studies revealed that protein B23.1 stimulates the activity of DNA polymerase  $\alpha$ , but not DNA polymerase  $\beta$  or  $\gamma$ , whereas B23.2 has no effect. The C-terminal end of B23.1 is essential for the stimulation (Umekawa *et al.*, 2001), which is consistent with this segment of the molecule containing the nucleic acid binding activity (Wang *et al.*, 1994; Hingorani *et al.*, 2000). Protein B23 also copurifies with DNA polymerase  $\alpha$  primase, supporting the idea that protein B23 is part of an enzyme complex required for DNA replication (Feuerstein *et al.*, 1990). Finally, protein B23 is part of a B cell-specific DNA recombination complex and its nucleic acid binding activity is undoubtedly important in the ability of protein B23 to promote DNA reannealing (Borggreffe *et al.*, 1998).

**b. Ribonuclease Activity** Studies by Herrera *et al.* (1995) showed that protein B23 possesses endoribonuclease activity and is classified as a phosphodiesterase. The action of B23 ribonuclease on different regions of pre-rRNA was studied using transcripts synthesized *in vitro* from cloned rDNA segments. Although no specific cleavages were detected in transcripts containing sequences from the 5' external transcribed spacer or the first internal transcribed spacer, the enzyme preferentially cleaves a few sites in the second internal transcribed spacer (ITS2) 250 nt downstream from the 3' end of the 5.8 S pre-rRNA (Savkur and Olson, 1998). The ability of the enzyme to preferentially cleave in the ITS2 region of pre-rRNA along with its subnucleolar localization suggests that protein B23 could participate in late stages of ribosome biogenesis.

**c. Molecular Chaperone Activity** The density of the nucleolus indicates that it contains very high concentrations of protein, especially, during periods of active ribosome assembly. A major consequence of this "macromolecular crowding" could be the aggregation and immobilization of proteins in the process of being incorporated into ribosomes. This tendency to aggregate might be relieved by the action of molecular chaperones (Ellis, 1997) and it seems likely that specialized molecular chaperones exist in the nucleolus to aid in the ribosome assembly process.

The suggestion that protein B23 is a molecular chaperone came from studies on its interaction with the HIV-1 Rev protein. Rev localizes predominantly to the nucleolus of HIV-1-infected cells (Kubota *et al.*, 1989) and is associated with protein B23 (Fankhauser *et al.*, 1991). Protein B23 has also been shown to stimulate the import of Rev into the nucleus (Szebeni *et al.*, 1997). Because the Rev protein has a tendency to aggregate under normal physiological conditions (Wingfield *et al.*, 1991), protein B23 could act as a molecular chaperone by preventing aggregation,

thereby improving the solubility of Rev. Protein B23 also interacts with other nucleolar proteins, including nucleolin (Li *et al.*, 1996), nucleolar protein p120 (Valdez *et al.*, 1994), and protein kinase CKII (Grein *et al.*, 1999).

Based on the above findings, protein B23 was tested for chaperone activity using several protein substrates typically used in chaperone assays (Szebeni and Olson, 1999). It was found to prevent aggregation of the Rev protein in a concentration-dependent manner. B23 also suppresses the aggregation of other proteins during thermal denaturation and preserves enzyme activities under high temperature conditions. In addition, B23 promotes the renaturation and restoration of activities of enzymes that had been previously denatured with guanidine-HCl. Finally, protein B23 preferentially binds denatured substrates and hydrophobic regions when complexed with denatured proteins. All of these characteristics strongly suggest that protein B23 is a molecular chaperone. Association of other proteins with protein B23 could serve as a transitional phase in assembly of proteins into the ribosome; i.e., protein B23 or other specialized chaperones could escort ribosomal proteins to the proper "station on the assembly line."

A histone chaperone activity has also been proposed for protein B23. This is based on the observation the protein B23 binds to histones, preferentially to histone H3, and mediates the formation of nucleosomes (Okuwaki *et al.*, 2001a). Furthermore, protein B23 serves as a template activation factor to stimulate adenovirus replication (Okuwaki *et al.*, 2001b). The authors suggest that the stimulation is also due to a histone chaperone activity and that the protein B23 assists in the remodeling of chromatin.

### 3. Structure–Function Relationships

Protein B23 has a tendency to oligomerize and probably exists as a hexamer of approximately 230 kDa (Yung and Chan, 1987; Schmidt-Zachmann *et al.*, 1987). Oligomerization is promoted by moderate concentrations of salt or low concentrations of divalent cations (Herrera *et al.*, 1996). The presence of a hexameric form in living cells was confirmed using crosslinking by nitrogen mustard (Chan, 1989). Deletion analysis by Liu and Chan (1991) suggested that oligomerization requires both the N- and the C-terminal domains of the protein. Also, intermolecular disulfide bond formation from cysteine 22 seems to enhance the stability of the oligomers, although this is not essential for their formation (Fields *et al.*, 1986; Zirwes *et al.*, 1997a). More recent studies indicate that the amino-terminal half of the molecule is both necessary and sufficient for oligomer formation (Hingorani *et al.*, 2000).

The crystal structure for the N-terminal domain of *Xenopus* nucleoplasmin reveals that the protein forms a pentamer and two pentamers associate to form a dodecamer (Dutta *et al.*, 2001). This region of the molecule consists almost entirely of  $\beta$ -sheets. Because the N-terminal regions of nucleoplasmin and protein B23 have a high degree of sequence homology it is tempting to speculate



that the B23 oligomers would have similar structures. The question of whether the oligomeric structure of B23 is a hexamer as earlier data suggest or if it is a pentamer as in nucleoplasmin should be answered by a crystallographic structure of the intact B23 or its N-terminal region.

Wang *et al.* (1994) demonstrated that isoform B23.1 binds nucleic acids whereas the minor isoform B23.2 does not, indicating that the C-terminal tail unique to B23.1 is required for this activity. In fact, a 5-kDa C-terminal fragment seems to account for nearly all of the nucleic acid binding activity. Hingorani *et al.* (2000) extended this work with a series of deletion mutants to show that basic and aromatic segments at the N- and C-terminal ends, respectively, of the nucleic acid binding region were required for activity. Although some RNA binding proteins contain aromatic residues in their binding domains (Dreyfuss *et al.*, 1993), there is little or no similarity between the 5-kDa fragment and these proteins. Hence, this region may represent a novel nucleic acid binding motif.

Hingorani *et al.* (2000) also showed that the molecular chaperone activity was contained in the N-terminal half of the molecule, with important contributions from both nonpolar and acidic regions. The central portion of the molecule is required for ribonuclease activity and possibly contains the catalytic site; this region overlaps with the chaperone-containing segment of the molecule. The chaperone activity also correlates with the ability of the protein to form oligomers. The C-terminal, nucleic acid binding region enhances the ribonuclease activity but is not essential for it. In summary, the three activities reside in mainly separate, but partially overlapping segments of the polypeptide chain of protein B23 (Fig. 9).

#### 4. Protein B23 Phosphorylation

Protein B23 has long been known to be a phosphoprotein (Olson, 1990), with several potential sites of phosphorylation. The first acidic segment in the rat protein contains a major casein kinase II (CK2) site (serine 125) identified previously by Chan *et al.* (1986). The second acidic segment has a potential CK2 site at threonine 185. In addition to the CK2 sites, there are two *cdc2* (cell division cycle 2; p34<sup>cdc2</sup>-cyclin B) phosphorylation sites on threonines 232 and 235 (Peter *et al.*, 1990). There is a site for protein kinase C on serine 225 (Beckmann *et al.*, 1992). Finally, a CDK2 protein kinase site resides on threonine 198 (Jones *et al.*, 1981; Tokuyama *et al.*, 2001).

The roles of the sites of phosphorylation are only partially understood. The phosphorylation of protein B23 by CKII correlates with the rate of synthesis of nascent pre-rRNA in the nucleolus (Olson, 1990) whereas the *cdc2* sites are phosphorylated only during metaphase (Peter *et al.*, 1990). One of the most interesting findings is that the CDK2 site controls centrosome duplication in mammalian cells (Okuda *et al.*, 2000). Binding of protein B23 to the centrosome prevents its duplication until late G<sub>1</sub> phase; phosphorylation at T199 releases protein B23 allowing centrosome duplication. Because protein B23 has been shown to be present at the

spindle poles during mitosis (Zatsepina *et al.*, 1999), the protein must reassociate with the centrosomes prior to or during metaphase.

In summary, although protein B23 is one of the most abundant proteins in the nucleolus and has several measurable activities, its presumed role in ribosome biogenesis remains elusive. In contrast, its extranucleolar functions in control of centrosome duplication and in DNA recombination seem somewhat more certain. It is also curious that no yeast homologue has been found. Does this mean that there are tasks that must be accomplished in vertebrate nucleoli that are not necessary in yeast or is there a greater need for protein B23 functions outside of the nucleolus in higher eukaryotes?

### C. Proteins Associated with Small Nucleolar RNAs

#### 1. Proteins Associated with Box C/D snoRNAs

The vast multiplicity of small nucleolar RNAs raises the question of whether the protein components of the snoRNPs equally as diverse. A partial answer is that there are sets of core proteins common to each of the two major groups of snoRNPs (Table I). In yeast, at least four different proteins are essential for the functions of the box C/D snoRNPs: Nop1p (fibrillarin in vertebrates), Nop5p/Nop58p, Nop56p, and Snu13p (Kiss, 2001).

**a. Fibrillarin** Of all snoRNA-associated proteins, fibrillarin is the most extensively studied, fibrillarin or its budding yeast equivalent Nop1p has been shown to be essential for pre-rRNA processing, pre-rRNA methylation, and ribosome assembly (Tollervey and Kiss, 1997). As indicated above it is all but certain that fibrillarin is a methylase because of a domain shared with known methyltransferases (Wang *et al.*, 2000). However, it is not clear whether this protein participates directly in other pre-rRNA processing activities or if it is required simply to maintain the integrity of the snoRNP particles. Although U3 is the snoRNA most commonly associated with fibrillarin, other snoRNAs also bind specifically to fibrillarin, including U16 in *Xenopus laevis* (Fatica *et al.*, 2000). In *Trypanosoma brucei*, at least 17 different C/D box snoRNAs were found in association with fibrillarin (Dunbar *et al.*, 2000).

Fibrillarin was initially identified because it was the sole protein recognized by the autoimmune serum from a patient with scleroderma (Ochs *et al.*, 1985). It was given the name fibrillarin because the serum labeled the fibrillar centers and the dense fibrillar components of nucleoli. The protein contains an unusual, 31-residue segment, rich in glycine and arginine at its amino-terminal end (GAR domain); all of the arginine residues are in the form of unsymmetrical dimethylarginine (DMA) (Lischwe *et al.*, 1985). A very similar protein called B-36, which also contains a GAR domain, was isolated earlier from *Physarum* (Christensen *et al.*, 1977; Christensen and Fuxa, 1988). As with the GAR domain in nucleolin, the

functional significance of this segment is not clear. It is curious that a putative methyltransferase is itself methylated, although there may be no relationship between the two kinds of methylation. When a GFP fusion of the GAR domain is expressed in mammalian cells, it localizes to nucleoli, but this segment is not essential for nucleolar localization of fibrillarin (Snaar *et al.*, 2000). Fibrillarin also contains a sequence resembling the RNA binding domains found in many RNP proteins; this domain is required for localization to both nucleoli and Cajal bodies.

**b. Other C/D Box Proteins** Although a common set of proteins is carried by all C/D box snoRNPs, there are other proteins that associate with RNPs containing specific snoRNAs. For example, the small protein Mpp10 specifically associates with U3 snoRNA in yeast and is required for three cleavage events that generate mature 18 S rRNA (Lee and Baserga, 1997). The U3 snoRNP complex contains two other proteins, Imp3p and Imp4p, which interact with Mpp10; both of these are also essential for 18 S rRNA formation (Lee and Baserga, 1999). Finally, at least two additional proteins, Sof1p (Jansen *et al.*, 1993) and Lcp5p (Wiederkehr *et al.*, 1998), are uniquely present in the U3 snoRNP. Thus, at least seven different proteins are contained in the most prominent C/D box snoRNP. One wonders whether the protein complexity will grow as the multitude of snoRNPs in this class are isolated and thoroughly examined.

## 2. Proteins Associated with Box H/ACA snoRNAs

The box H/ACA snoRNPs have a different set of common proteins. In yeast, these are Cbf5p (Nap57 in vertebrates), Gar1p, Nhp2p, and Nop10p (Kiss, 2001). Cbf5p/Nap57 most likely is the enzyme that performs the pseudouridylation, but the other three proteins are also essential for this modification. The true level of diversity of proteins found in the box H/ACA snoRNPs has yet to be determined.

## V. Nonconventional Roles of the Nucleolus

During the past decade an increasing number of unexpected proteins and RNAs have been detected within the nucleolus and, as a result, numerous new functions have been ascribed to it. For most of these components, the reason for their nucleolar location is largely speculative; while for others, there are at least plausible explanations for their presence. Figure 10 summarizes several of the nontraditional molecules found in the nucleolus and the possible reasons for their presence. Hopefully, a maturation process over the next several years will sort out the functions where nucleolar location is truly significant from those in which they are simply there by coincidence.

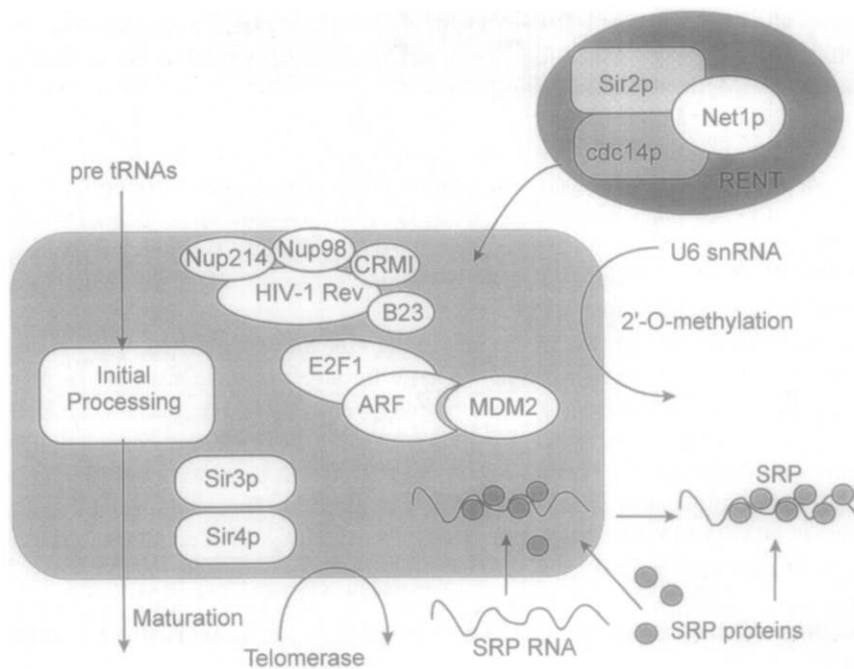


FIG. 10 Nonconventional roles of the nucleolus. Several examples of activities not related to ribosome biogenesis are indicated. These include tRNA maturation, the proposed methylation of U6 snRNA, and partial assembly of signal recognition particles (SRPs) and the telomerase complex. The yeast cell cycle is regulated through nucleolar sequestration of the cdc14p protein phosphatase in the RENT complex, which also contains the proteins Sir2p and Net1p. The HIV-1 Rev protein interacts with protein B23 in the nucleolus and also recruits the nuclear export factor hCRM1 and nucleoporins Nup98 and Nup214 to the nucleolus. The activity of tumor-suppressor protein p53 is regulated by sequestration of components that control its degradation (MDM2 and E2F1), which bind to the ARF protein in the nucleolus. Finally, the silencing proteins Sir3p and Sir4p relocate from telomeres to nucleoli during aging in yeast, presumably in response to accumulation of extrachromosomal circles of rDNA.

## A. The Role of the Nucleolus in Subnuclear Storage and Nuclear Export

### 1. Viral Proteins and RNAs

**a. HIV-1 Components** One of the first nucleolar proteins unrelated to ribosome biogenesis to attract attention was the HIV-1 Rev protein (Kjems and Askjaer, 2000). The Rev protein forms a specific complex in the nucleolus with protein B23 (Fankhauser *et al.*, 1991). However, because ongoing transcription is required for its nucleolar localization, Rev probably also interacts with pre-rRNA (Dundr *et al.*, 1995). As indicated above, protein B23 is proposed to serve as a

molecular chaperone for Rev (Szebeni *et al.*, 1999). Although a reengineered Rev protein is capable of regulating HIV-1 mRNA splicing without ever visiting the nucleolus (McDonald *et al.*, 1992), the nucleolar location could be important for optimal Rev function, i.e., for storage or maintaining a threshold level of Rev, preventing its nonproductive shuttling or protecting it from degradation (Dundr *et al.*, 1995). More recently, it was shown that Rev is capable of recruiting nucleoporins Nup98 and Nup214 as well as the nuclear export factor hCRM1 to the nucleolus (Zolotukhin and Felber, 1999). The latter study suggests that a Rev–hCRM1–nucleoporin complex assembles in the nucleolus along with Nup98 and Nup214, which aid in moving the complex to the nuclear pore complex.

While nucleolar location of the Rev protein is well established, there is also some evidence that the HIV-1 mRNA passes through the nucleolus at some point in its life cycle. A hammerhead ribozyme that specifically cleaves the HIV-1 mRNA was engineered into U16 snoRNA, which resulted in the ribozyme accumulating in nucleoli (Michienzi *et al.*, 2000). In HIV-1-infected cells expressing this ribozyme, there was significant suppression of HIV-1 replication. These findings revive the idea of HIV-1 mRNA trafficking through the nucleolus, which could also serve as a staging area for nuclear export of the message.

**b. Other Viral Proteins** Several other viral proteins are located in the nucleolus, at least during part of their life cycles. One of these is the coronavirus nucleoprotein (N), which is involved in several aspects of viral replication and is localized to both cytoplasmic and nucleolar compartments. Hiscox *et al.* (2001) propose two possible roles for the nucleolar localization of the N protein. While in the nucleolus, its interaction with ribosomal proteins could cause dissociation of the coronavirus core and release of the genomic RNA. Alternatively, the N protein could subvert host cell translation by disrupting formation of new ribosomes or interfering with the cell cycle. The N protein might then up-regulate translation of the viral RNA by binding to its 5' end, which might recruit existing ribosomes.

The adenovirus protein V is not only localized to the nucleolus during infection, but it also causes two prominent nucleolar proteins, nucleolin and protein B23, to relocate to the cytoplasm (Matthews, 2001). It is known that adenovirus infection inhibits both synthesis and processing of pre-rRNA; however protein V by itself does not have this inhibitory effect. The disruption of the cellular balance by adenoviruses may occur by either of two ways. First, it may interfere with the synthesis and processing of rRNA resulting in disruption of cellular processes (Matthews, 2001). Second, the virus could enhance its own replication by relocating nucleolin, which, in turn, could interfere with replication through its ability to repress transcription (Yang *et al.*, 1994).

Two hepatitis delta virus antigens (HDAGs) localize to nucleoli of human hepatocyte-derived cells (Huang *et al.*, 2001). These form a complex, which contains both proteins B23 and nucleolin. Expression of the HDAGs in the human cell lines not only up-regulates the level of B23 mRNA, but interaction of the antigens

with protein B23 also enhances the hepatitis delta virus replication. These studies further illustrate the multifunctional nature of protein B23 (see above). Whether the modulation of viral replication is due to its ability to bind nucleic acids or its molecular chaperone activity or both remains to be determined.

Less is known about the function of a number of other viral proteins that localize to the nucleolus. These include the UL3 protein of herpes simplex virus type 2 (Yamada *et al.*, 1999) and human papillomavirus type 16 E7 protein (Zatsepina *et al.*, 1997). It is interesting that the latter protein interacts with the retinoblastoma-1 protein, which is capable of regulating rDNA transcription (see above).

## 2. Regulation of Gene Expression by Spatial Confinement

**a. p53 Regulation** The activity of tumor-suppressor protein p53 is partially regulated by a nucleolar sequestration mechanism (Zhang and Xiong, 2001). Cellular levels of p53 are controlled by a balance between synthesis and degradation, with the latter performed in the cytoplasm and dependent on the oncoprotein MDM2. MDM2 has intrinsic E3 ligase activity that conjugates ubiquitin to p53; the ubiquitination seems to trigger the export of p53 to the cytoplasm where it is degraded by proteasomes. Another protein, p19<sup>ARF</sup> (p14<sup>ARF</sup> in humans; hereafter designated as ARF), forms a complex with MDM2, which attenuates p53 degradation by inhibiting its ubiquitination. Interestingly, ARF seems to be exclusively nucleolar and MDM2 increasingly becomes localized to nucleoli when coexpressed with ARF. As with several other nucleolar proteins, highly basic regions of ARF are important for nucleolar localization (Rizos *et al.*, 2000). The protein is found in the granular component of the nucleolus and its presence there is dependent on continuing transcriptional activity (Lindström *et al.*, 2000). Thus, a mechanism of controlling p53 levels seems to be through the nucleolar tethering of part of its degradative system. However, more recent studies using human cells indicate that certain versions of ARF can form a complex with MDM2 outside of the nucleolus (Llanos *et al.*, 2001). This work suggests that nucleolar localization is not absolutely essential for ARF activity toward MDM2, at least in the human system. Although the actions of ARF could be carried out strictly in the nucleoplasm, the authors suggest that the nucleolar location might be a means of stabilizing or storing ARF, or even providing a backup system for controlling p53 levels.

The above studies describe a regulatory mechanism involving inhibition of p53 degradation. By contrast, some cells lack p53 and in these, tumor suppression is achieved in part by targeting transcription factors for degradation. Martelli *et al.* (2001) found that ARF mediates tumor suppression by targeting some members of the E2F (E2F1, -2, and -3) family of transcription factors that promote cell cycle progression. As with p53, proteolysis of E2F1 is dependent on the presence of a functional proteasome. Curiously, coexpression of ARF and E2F1 results in the relocalization of E2F1 to the nucleolus, but how this event triggers degradation

has not been determined. In any event, ARF acts in a multifunctional capacity in promoting tumor suppression in response to oncogenic signals. How ARF can protect p53 from degradation in one instance and target other transcription factors for degradation in another remains an unresolved issue. Furthermore, the real significance of nucleolar localization in this process is still open to question.

**b. Cell Cycle Regulation in Yeast** Nucleolar anchoring is involved in control of cell division in the yeast *S. cerevisiae*. The exit from mitosis in this organism is controlled by the protein phosphatase Cdc14p, which both promotes the degradation of a cyclin subunit and the accumulation of a protein kinase inhibitor. Cdc14p is regulated by sequestration in the nucleolus for most of the cell cycle, but it is released from that site during anaphase (Visintin *et al.*, 1999). Furthermore, the protein responsible for its nucleolar anchoring has been identified as Cfi1p, a protein related to Reg1p, a regulatory subunit for protein phosphatase 1. Concurrent research (Shou *et al.*, 1999) showed that Cdc14p is part of a larger nucleolar complex, which not only contains Cfi1p (called Net1p by this group), but also Sir2p and possibly Nan1p. This is termed the RENT complex, an acronym for “regulator of nucleolar silencing and telophase exit.” Sir2p (silent information regulator) is a regulator of the silencing of rDNA repeats as well as a subtelomeric repressor molecule (Cockell and Gasser, 1999). Its location in the nucleolus is also dependent on Net1p. The nucleolus may serve as a general “sequestration center” for temporary inactivation of regulatory molecules such as Cdc14p. Because the nucleolus completely disintegrates during mitosis in higher organisms (see above), this mechanism for controlling exit from mitosis probably applies only to yeast and other lower eukaryotes.

**c. Activities of Sir2p** One of the aforementioned proteins, Sir2p, has some unusual activities, including a histone deacetylase activity (reviewed by Shore, 2000). The latter activity is not surprising since histones in the deacetylated state would be expected to be associated with silenced chromatin. What is surprising is the enzymatic mechanism by which this protein acts. Sir2p has been shown to be an NAD-dependent histone deacetylase (Tanner *et al.*, 2000). Although initially it was thought that NAD is simply a regulator of this enzyme, it has been shown to have a more direct role in the catalytic mechanism; the acetyl groups removed from the lysine residues of the histones are transferred to NAD<sup>+</sup> to form the product 1-*O*-acetyl-ADP-ribose. The generation of the latter molecule raises the possibility that it could serve as another signaling molecule.

Most studies on Sir2p have utilized the yeast system, but Luo *et al.* (2001) and Vaziri *et al.* (2001) have isolated mammalian equivalents (Sir2 $\alpha$  and hSir2 in mouse and human, respectively), which also localize to nucleoli. Past work on yeast Sir2p has focused on histone deacetylation; the more recent studies show that Sir2 $\alpha$  and hSir2 are also capable of deacetylating p53. The mammalian Sir2 proteins interact with and deacetylate p53 and suppress p53-mediated functions

including transcriptional activation and induction of apoptosis. These findings not only represent an expanded role for Sir2p, but they also add another level to the already complex regulation of p53 functions. Again, the significance of the nucleolar location of the mammalian Sir2 proteins is uncertain.

## B. Other Nucleolar Activities Not Related to Ribosome Biogenesis

### 1. Signal Recognition Particle Assembly

A handful of cellular processes not involved in conventional nucleolar functions seem to take place in the nucleolus. The first of these is the maturation of the signal recognition particle (SRP), which is a complex of a small RNA and several proteins that targets nascent secretory proteins to the endoplasmic reticulum. Jacobson and Pederson (1998) found that when SRP RNA was injected into the nuclei of mammalian cells, it initially and rapidly localized in nucleoli, after which there was a gradual decline in nucleolar signal and an increase in its presence in the cytoplasm. Later, three out of four of the SRP proteins of the S domain (SRP19, SRP68, and SRP72) but not SRP54 were found in the nucleolus (Politz *et al.*, 2000). The latter studies strongly suggest that some stage of assembly of the SRP is taking place in nucleoli of vertebrates.

Grosshans *et al.* (2001) carried out more extensive studies on the assembly of the yeast SRP, which is a ribonucleoprotein complex consisting of an RNA molecule (scR1) and six proteins. Interestingly, four of the six proteins are found in the nucleolus (Srp14p, Srp21p, Srp68p, and Srp72p); these are the SRP core proteins. The Sec65p protein is both nucleolar and nucleoplasmic, but Srp54p is exclusively cytoplasmic. The SRP core proteins are imported into the nucleolus using the ribosomal protein import receptors Pse1p and Kap123p/Yrb4p and it is proposed that the core proteins act as RNA chaperones to aid in the correct folding and stabilization of scR1. The SRP RNA is only transiently located in the nucleolus as an intermediate product in combination with the core proteins to give the pre-SRP particle. The latter particle is then exported to the cytoplasm via the nuclear export receptor, Xpo1p, and a subset of nucleoporins. As with the mammalian SRP the final assembly is performed in the cytoplasm. Thus, although it is clear that assembly of the SRP occurs in the nucleolus, it is not obvious what nucleolar components participate in this process.

### 2. Nucleolar Linkage to RNA pol III Synthesized Transcripts

**a. tRNA Processing** In addition to producing and processing ribosomal RNA transcripts, the nucleolus is involved in the some stages of the life cycles of certain tRNAs. tRNA processing is both spatially and temporally regulated, and involves a



series of reactions. These include the removal of the 5' leader from pre-tRNAs, followed by 3' end cleavage, splicing, and multiple nucleotide modifications (Wolin and Matera, 1999; Carmo-Fonseca *et al.*, 2000). In an early stage of tRNA maturation, the La protein binds to the 3' end of the newly synthesized transcript and this serves as the substrate for the ribonucleoprotein enzyme RNase P, which removes the 5' leader sequence. Using probes against pre-tRNA introns, Bertrand *et al.* (1998) showed that the precursors for tRNA<sup>Leu3</sup> and tRNA<sup>Trp</sup>, as well as the dimeric transcript of tRNA<sup>Arg</sup>/tRNA<sup>Asp</sup>, were present in the nucleolus. The RNA component of RNase P is also localized primarily in the nucleolus, although the protein subunits of the enzyme are found in both nucleoli and coiled bodies (Jarrous *et al.*, 1999). However, it is unlikely that all of the 5' processing of tRNAs occurs in the nucleolus and it is not clear whether any 3' tRNA processing takes place in that location (Wolin and Matera, 1999).

The involvement of the nucleolus in tRNA biosynthesis is supported by studies on tRNA gene-mediated silencing of RNA polymerase II (pol II) transcription. tRNA-class polymerases (pol III) have been shown to negatively regulate neighboring RNA pol II promoters in the budding yeast *S. cerevisiae* (Kendall *et al.*, 2000). This transcription mediated silencing (tgm) is independent of the orientation of the tRNA gene and shows no requirement for binding to either the upstream pol II factors or the pol II holoenzyme. The Cbf5 protein is a probable pseudouridine synthetase associated with snoRNAs and has been shown to be involved in ribosomal RNA maturation (see above). A mutant that affects the expression of this protein has been shown to have suppressed tgm silencing. Because the biogenesis of at least some tRNAs begins in the nucleolus, this finding raised the question of a possible link between tgm silencing and the nucleolus. It was found that the CBF5 mutant nucleoli were slightly fragmented and the pre-tRNAs were localized in the nucleoplasm instead of nucleoli. This loss could be attributed to an inadequate supply of snoRNPs playing transport or structural roles. The fragmentation of the nucleoli could be due to incomplete rRNA pseudouridylation, resulting in a disruption of nucleolar organization. The authors also raise the possibility that some tRNA transcription takes place in the nucleolus. Hence the sequestered localization of tRNA genes antagonizes pol II-mediated transcription of the nearby genes.

Other posttranscriptional modifications of small RNAs may also be performed in the nucleolus. One form of the enzyme (Mod5p-II) that catalyzes the formation of isopentenyl adenosine was shown to be present in the nucleoli of yeast (Tolerico *et al.*, 1999). However, its nucleolar localization is not essential for the production of the modified tRNA, suggesting that the presence of this enzyme in the nucleolus is one of those natural occurrences with no real functional significance.

**b. Spliceosomal RNA Biosynthesis** Evidence for nucleolar involvement in biosynthesis of spliceosomal RNAs is beginning to accumulate. Methylation and pseudouridylation of the U6 spliceosomal RNA is guided by snoRNAs (Weinstein and Steitz, 1999; Ganot *et al.*, 1999) and it is suggested that U6 snRNA might

acquire these modifications as it transiently associates with nucleoli. Lange and Gerbi (2000) showed that U6 snRNA indeed visits the nucleolus. U6 snRNA could be tethered in the nucleolus as a result of base pairing with the guide snoRNAs that participate in the modification process. Once this is completed the U6 molecule might default back to the nucleoplasm.

### 3. A Possible Role of the Nucleolus in mRNA Transport

Another potentially important nucleolar function is its proposed involvement in mRNA transport. Cell fusion experiments in the late 1960s indicated that a functional nucleolus is necessary for the formation of certain mRNAs (Pederson, 1998). Again, in the early 1990s a few reports provided evidence to support that conclusion (Schneiter *et al.*, 1995). First, poly(A) RNA accumulates in fragments of nucleoli of *S. cerevisiae* or in *S. pombe* strains defective in mRNA transport. Second, mutations in certain nucleolar proteins also induce the accumulation of poly(A) RNA in yeast nucleoli. Finally, poly(A) RNA collects in nucleoli of heat-shocked yeast cells. More recent studies have confirmed that RNAs containing a certain 3' untranslated region (*ASH1*) accumulate in the nucleolus under stress conditions that block mRNA export (Brodsky and Silver, 2000). These observations have led to the suggestion that the nucleolus plays a role in export of some mRNAs, at least in yeast and possibly in some viral systems (see above). However, because supporting data for this is sparse, it is too early to draw any firm conclusions about nucleolar involvement in mRNA transport.

### 4. Tissue-Specific Expression of Small Nucleolar RNAs

One of the more intriguing recent findings is the discovery of brain-specific snoRNAs. Cavaille *et al.* (2001) identified three C/D box snoRNAs and one H/ACA box snoRNA that are exclusively expressed in mouse or human brains. Curiously, unlike all snoRNAs isolated to date, these show no complementarity to pre-rRNA, in spite of the fact that they reside in nucleoli. Instead, one of the C/D box snoRNAs has a segment complementary to the serotonin 2C receptor mRNA, suggesting a potential role in the processing of this mRNA. Even more interesting is the observation that the H/ACA box snoRNA is encoded in an intron for the brain-specific serotonin 2C receptor gene. These findings have the potential for opening up research in an entirely new field in which small RNAs might play an unexpected role in regulating expression of tissue-specific proteins.

## C. Nucleolus in Aging and Diseases

### 1. Yeast as a Model System

In recent years, a number of studies have suggested a linkage between aging and the nucleolus, especially in yeast. The observation that stimulated the current

interest was a mutation in the *SIR4* gene, which lengthened the life span of yeast (Guarente, 1997). In the strain carrying this mutation, the silencing proteins Sir3p and Sir4p relocate from telomeres to nucleoli. The relocation is dependent on another gene, *UTH4*, which extends the yeast life span. More importantly, in aging wild-type cells the Sir complex also relocates from telomeres to nucleoli. At this point another player enters the nucleolar scene, the *SGS1* gene. Mutations in this gene cause premature aging in yeast and also induce a redistribution of Sir3p from the telomeres to the nucleolus. Mutations in *SGS1* also cause nucleolar fragmentation; this seems to be due to the accumulation of extrachromosomal rDNA circles (ERCs). The movement of proteins from telomeres to the nucleolus might be a mechanism for protection against damage caused by formation of ERCs. Mutations in the gene for another nucleolar protein, Fob1p, slow the production of the ERCs and extends the life span of yeast (Defossez *et al.*, 1999). The wild-type Fob1p protein causes unidirectional blocks in rDNA replication forks and it is proposed that these blocks result in chromosomal breaks, thereby triggering premature aging.

## 2. Aging in Mammals

Deficiencies in DNA helicases of the RecQ family are associated with cancer and premature aging (Mohaghegh and Hickson, 2001). Several of these, including the *SGS1* protein in yeast (see above) and the proteins involved in Bloom and Werner syndromes in humans, have nucleolar locations, at least during part of the cell cycle.

**a. Bloom Syndrome** BLM is the RecQ DNA helicase that is altered in Bloom syndrome, an autosomal recessive condition resulting in genomic instability and leading to increased frequencies of cancer. BLM is normally found in the nuclear domain 10 in the nucleoplasm; however, it shifts to the nucleolus during S phase where it colocalizes with the Werner syndrome protein, WRN (Yankiwski *et al.*, 2000). Consistent with its proposed role in maintaining the stability of repeated sequence elements, BLM is also present in a subset of telomeres. The DNA helicase activity of BLM is necessary to ensure that replication from telomeres can proceed smoothly subsequent to DNA damage, thereby lowering the occurrence of cancers (Neff *et al.*, 1999). This activity is also required to reduce recombinatorial events within rDNA repeats, which may lead to loss of chromosomal integrity. Although BLM colocalizes with WRN, the two proteins seem to exist in functionally distinct complexes (Brosh *et al.*, 2001). Both of these proteins exhibit the capacity to resolve triple helices found distributed within the genome, and any loss of this ability may lead to genomic instability.

**b. Werner Syndrome** The WRN helicase, associated with premature aging in Werner syndrome (Yu *et al.*, 1997), has been localized to transcriptionally active

nucleoli, suggesting a link between rDNA transcription and the functioning of the nucleoli. WRN also interacts with DNA polymerase  $\delta$  and recruits it to the nucleolus, suggesting a role of WRN in DNA replication (Szekely *et al.*, 2000). Finally, there is accelerated methylation of ribosomal RNA genes during cellular senescence of Werner syndrome fibroblasts (Machwe *et al.*, 2000). However, the latter phenomenon has not been directly linked to alterations in the WRN protein. It is interesting that the Werner's syndrome protein (WRN) is localized to nucleoli in human cells, but the equivalent protein in mouse is not (Marciniak *et al.*, 1998). This is apparently due to sequence differences between the human and mouse protein in the C-terminal region that directs it to the nucleolus (Suzuki *et al.*, 2001). There is also some controversy about whether significant amounts of the WRN protein are actually localized to the nucleolus (Shiratori *et al.*, 1999). Because there is no evidence of accumulation of ERCs or nucleolar fragmentation in aging mammalian cells, this raises the questions of whether the nucleolus has anything to do with Werner syndrome or if the aging yeast model is relevant to humans. Obviously, more definitive studies are needed to clarify the significance of the nucleolus in the premature aging syndromes.

**c. *Telomerase in the Nucleolus*** Yeast may or may not be a good model for studies on aging in humans, although there is clearly evidence for redistribution of telomeric components to nucleoli as yeast age (Guarente, 1997). In mammalian cells, there is evidence for a normal presence of telomeric components in nucleoli. A small proportion of telomerase RNA localizes to nucleoli of HeLa cells (Mitchell *et al.*, 1999a). This could be related to the fact that telomerase RNA contains a domain that resembles the H/ACA box of small nucleolar RNAs. Fluorescently labeled telomerase RNA also localizes to nucleoli when injected into *Xenopus* oocytes (Narayanan *et al.*, 1999b). It is suggested that the telomerase RNP is partly assembled in the nucleolus. Dyskerin, a putative pseudouridine synthase that is mutated in the human disease dyskeratosis congenita (DKC), also associates with snoRNAs of the H/ACA family as well as with telomerase RNA in the nucleolus (Mitchell *et al.*, 1999b). The latter finding supports the idea of nucleolar assembly and/or processing of telomerase since DKC cells have less telomerase RNA and lower levels of telomerase activity than normal cells.

## VI. Concluding Remarks

The past decade has brought remarkable progress in our understanding of nucleolar structure and dynamics and traditional nucleolar functions. At the same time, new information on the nucleolus challenges current views of its conventional roles and evidence for nontraditional roles raises new questions. For example, the traditional

view is that a well-organized nucleolus is essential for ribosome biogenesis. The observation that cells can assemble preribosomal particles outside of the nucleolus suggests the opposite. However, under certain physiological conditions a well-organized nucleolus might contribute to the survival of the organism, thereby placing evolutionary pressure for cells to develop a nucleolus. Is it possible that the nucleolar structure is required more for the newly uncovered, nonconventional rather than the conventional roles? Some of the nonconventional activities may need an immobile platform, thereby taking advantage of the nucleolus by default because of the dearth of "solid" structures in the nucleus.

Of the nonconventional roles of the nucleolus, SRP assembly and tRNA processing are the most convincingly demonstrated. Although a nucleolar phase or location is clearly present for the remaining nonconventional roles, there is a great deal of uncertainty about whether the nucleolus is really necessary to carry out these functions. The Cajal body seems to carry out activities related to nucleoplasmic as well as nucleolar functions. In yeast, the equivalent structure might be contained in the nucleolus itself as the nucleolar body. Thus, some of the nonconventional roles that have been discovered in yeast nucleoli could be due to activities of the nucleolar body; these are unlikely to be seen in nucleoli of higher eukaryotes. This raises the question of how far yeast can be carried as a model for mammalian nucleolar functions. What the newly discovered roles show is that the nucleolus is not simply an isolated structure, but that it interacts and communicates with the rest of the cell at several different levels. Although the traditional role of the nucleolus in manufacturing ribosomal subunits is secure, the unconventional nucleolar functions will have to stand the test of time.

In a few years all nucleolar components will be identified and their interacting partners defined. After that, what is the future for research on the nucleolus? Although it is dangerous to predict the future, we would expect the emphasis to go in two directions. The first is a shift back to more traditional areas of biochemistry and structural biology to study details of the mechanisms of each step in ribosome biogenesis and in other nucleolar functions. The second direction might be a break with the longstanding reductionist approach and move toward integrationism. The focus might then be on putting the pieces of the puzzle back together, including the development of functional computer models for the entire process of ribosome synthesis and its regulation. Whatever path is taken, we should not be surprised if a new generation of nucleologists uncovers another set of unusual activities in the nucleolus during the next decade.

## Acknowledgments

We thank Romie Brown and Kim Eaves for assistance in assembling the manuscript and the following individuals for stimulating discussions: J. Aris, S. Baserga, H. Busch, P. Chan, M. Dundr, E. Egyhazi, M. Fournier, I. Grummt, D. Hernandez-Verdun, P. Hozak, N. Huang, S. Huang, C. Lyon, G. Matera,

F. Medina, U. Meier, T. Misteli, W. Mosgoeller, S. Negi, T. Pederson, S. Pinol-Roma, I. Raška, M. Schmidt-Zachmann, C. Schoefer, P. Silver, R. Terns, M. Terns, J. Woolford, C. Verheggen, and O. Zatzepina. We apologize to those colleagues whose work was not cited because of space limitations.

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