3.2
Eukaryotic Ribosome Synthesis

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3.2.1
Introduction

Recent proteomic developments are providing the first eukaryotic ribosomal assembly maps. In these, pre-ribosomal assembly appears to be asymmetric, at least biphasic, with the small ribosomal subunit synthesis factors binding first to the pre-rRNAs to be replaced, after the first few pre-rRNA cleavages, by proteins involved in the synthesis of the large ribosomal subunit. Pre-rRNA processing is fairly well characterized with several key-processing enzymes remaining to be identified, including most endoribonucleases. rRNA modification is also well understood and relies extensively on small nucleolar RNAs (snoRNAs) for the recognition of the sites of modification. Nucleolar routing of box C+D snoRNAs (required for sugar 2′–O methylation) involves transit through a specific nuclear locale, the human coiled/cajal body (CB) and yeast nucleolar body (NB); these are conserved sites of small ribonucleoprotein particles (RNP) biogenesis. The first proteins involved in ribosome export are being identified; however, most of these are also required for pre-rRNA processing, and presumably pre-rRNP assembly. Their precise function in RNP transport therefore awaits these effects to be uncoupled. Key factors active in ribosome synthesis are also required for the processing of many other classes of cellular RNAs, suggesting that maturation factors are recruited from a 'common pool' of proteins to specific pathways. Much remains to be done to understand how rRNP processing, modification, assembly and transport are integrated with respect to ribosome synthesis and other cellular biosynthetic pathways.

3.1.1
Prelude

Ribosome synthesis starts in the nucleolus, the site of rDNA transcription. rRNA synthesis occurs at the interface between the fibrillar center(s) (FCs) and the dense fibrillar component (DFC) with the nascent transcripts reaching out in the body of the DFC ([128]; reviewed in Ref. [104]). A dedicated polymerase, RNA Pol I (Pol I), drives the transcription of a large precursor encoding three of the four mature ribosomal RNAs (rRNAs). The fourth rRNA, 5S, is produced from a Pol III promoter. The Pol I primary transcript is modified (specific residues are selected for ribose or base modification and pseudouridines formation), processed (mature sequences are released from the precursors and the non-coding sequences discarded) and assembled with the ribosomal proteins (RPs) in pre-ribosomes (reviewed in Refs. [130, 224, 298, 299, 311, 325]). As these processes occur, the granular component (GC) of the nucleolus emerges. FC, DFC, and GC are morphologically distinct compartments present in most eukaryotes; interestingly, although controversial, recent analysis

indicate that the yeast *Saccharomyces cerevisiae* has no FC (D.L.J. Lafontaine and M. Thiry, unpublished results). The relationship between the subnucleolar structures and the different steps of ribosome synthesis is not clear at present.

The nucleolus is a highly dynamic structure, and RNA and protein components are known to exchange with the surrounding nucleoplasm with high kinetics [40, 211]. The average nucleolar residency time for human *fibrillarin* was estimated to be of only ~40 s, indicating that the remarkably stable organization of the nucleolus may in fact reflect the extremely rapid dynamic equilibrium of its constituents. It is presently unclear whether there are resident, structural, nucleolar proteins or whether the structure simply 'holds together' through multiple, weak, interactions occurring between the nascent pre-rRNAs and the numerous trans-acting factors recruited to the sites of transcription [173]. The recent proteomic characterization of this cellular compartment will probably help to address these issues ([8, 236]; reviewed in Ref. [61]).

Pre-ribosomes are released from the nucleolar structure, reach the nuclear pore complexes (NPC), presumably by diffusion, and are translocated to the cytoplasm. Both the small (40S) and large (60S) ribosomal subunits undergo final cytoplasmic maturation steps. A large number of trans-acting factors follow the pre-ribosomes to the cytoplasm and are recycled to the nucleus. Recent data suggest that the final steps of maturation may be coupled to cytoplasmic translation [240, 286].

RP genes, most often intron-containing, duplicated and expressed at distinct levels (yeast), are transcribed by Pol II. RP pre-mRNAs follow a canonical Pol II synthesis pathway (including capping, splicing, poly-adenylation, etc.; reviewed in Ref. [219]) and are routed to the cytoplasm to be translated. RPs are addressed to the nucleus and the nucleolus. Nuclear targeting operates on the NLS mode (reviewed in Refs. [163, 322, 323]); redundant importins are involved [111, 230]. Nucleolar targeting is less- well defined.

Ribosome synthesis is an extremely demanding process requiring both tremendous amounts of energy and high levels of co-regulation and integration with other cellular pathways (reviewed in Refs. [150, 214, 309]). The production of the resident ribosomal components (4 rRNAs and about 80 RPs), as well as several hundreds of RNAs and protein trans-acting factors (see below) depends on the concerted action of the three RNA polymerases, extensive RNA processing and modification reactions, RNP assembly and transport and the function of several RNPs, including the ribosome itself. With about 2000 ribosomes to be produced per minute in an actively dividing yeast cell, transcription of pre-rRNAs and RP pre-mRNAs alone represent not less than 60 and 40% of the Pol I and Pol II cellular transcription, respectively. With about 150 pores per nucleus, each pore must import close to 1000 RPs and export close to 25 ribosomal subunits per minute.

The nucleolus does not only serve the purpose of 'making of a ribosome'. In fact, it appears that most classes of cellular RNAs, including mRNAs [117, 239], tRNAs [21], snRNAs [81, 87], the SRP [42, 93, 110], RNAse P [113] and the TEL RNP [64, 192, 254] all transit through this organelle on their way to their final destinations, which can either be the nucleoplasm or the cytoplasm. Although the reason for this

trafficking is in most cases unclear at present, this presumably reflects a need to benefit from the pre-ribosomes maturation machinery. In the following, I will try to emphasize instances where common trans-acting factors are used on distinct classes of RNAs. The concept of a 'multifunctional nucleolus' has recently been elegantly reviewed [206].

Most of our current understanding of ribosome synthesis is based on research work in *S. cerevisiae*; this will be reviewed here. Other eukaryotic systems have been used successfully, including trypanosomes, Xenope, mouse and humans. Comparison between these various levels of organization is most useful and often highlights a high degree of conservation throughout the eukaryotic kingdom, e.g., most trans-acting factors identified in yeast have human counterparts.

This chapter will present an overview of eukaryotic ribosome synthesis for the non-specialists, with an emphasis on the latest developments and unresolved issues.

3.2.2 Why so many RRPs?

An excess of 200 proteins, here referred to as RRPs (ribosomal RNA processing factors) are required for ribosome synthesis and transiently associate with the pre-ribosomes. RRPs are not found in mature, cytoplasmic, particles but are recruited at various stages in the ribosomal assembly process. Recruitment presumably follows a strict temporal order. A similar number of small, stable RNAs, which localize at steady state in the nucleolus, the snoRNAs, are also involved.

Most RRPs have no known function and, in fact, apart from those few with catalytic activities or well-characterized protein domains, we clearly have no idea of what they do. Best-characterized RRPs include several endo- and exoribonucleases (Table 3.2-1), snoRNA-associated proteins, modification enzymes (ribose and base methyl-transferases, pseudouridine synthase), RNA helicases [47, 262], GTPases [86, 240, 317], AAA-ATPases [14, 77], protein kinases [295, 296], RNA binding or protein–protein interaction domain-containing proteins and proteins with striking

Table 3.2-1 Endo- and exoribonucleolytic activities involved in pre-rRNA processing.

Cleavage site	Cleavage activity	References
B_0	Rnt1p/yeast Rnase III	136
B_0-> B_2	Rex1p	187
A_0, A_1, A_2	?, ?, ?	
A_3	MRP	159
A_3- > B_{1S}	Rat1p, Xrn1p	98
B_{1L}	?	
C_2	?	
C_2- > $C_{1'}$ and $C_{1'}$- > C_1	Rat1p, Xrn1p	85
C_2- > E	Exosome Rex1p, Rex2p ?Ngl2p	176 287 65

homology to RPs [14, 59, 79, 234]. Protein–protein interaction domain include coil–coil domains, WD and HEAT repeats, crooked-neck-like tetratrico peptide repeat, etc.; distinctive RRP motifs include the Brix, GAR, G-patch, and KKD/E-domains [9, 62, 67, 83, 94, 318]. The actual catalytic activity of most RRPs remains to be demonstrated experimentally and the precise substrate of these proteins is, in most cases, not known.

Comprehensive lists of RRPs have recently been compiled by various authors with a short description of protein domains and known or presumed functions; these are freely available on-line (see useful WWW links at the end of this chapter).

3.2.3 (Pre-)ribosome Assembly, the Proteomic Era

In the early 1970s, the joint efforts of the Warner and Planta Labs defined the basics of eukaryotic ribosome assembly; this remained the core of our understanding for the next 30 years [133, 277, 282, 297, 308, 312, 313]. Metabolic labeling experiments and sucrose-gradient analysis revealed that following transcription, an early 90S pre-ribosome is formed and subsequently partitioned into a 43S and a 66S particle, precursors to the 40S and 60S subunits, respectively (see Fig. 3.2-1). The RNA content of these particles was established as 35S, 27S, and 20S pre-rRNAs for the 90S, 66S, and 43S particles, respectively. The conversion of the 43S particles to 40S subunits is closely linked to small subunit export. Few RRPs were known at that time and the protein composition of these RNP complexes was not determined.

In the absence of appropriate tools, most of the research focused on other aspects of ribosome synthesis with most of the progress being made on pre-rRNA processing and modification (see below).

There was no reason to believe *a priori* that there would be a strong bias for the association of RRPs involved in small subunit synthesis with the primary transcript. In fact, since many mutations affecting primarily 25S rRNA synthesis have negative feedback effects on early pre-rRNA cleavages (see Sect. 3.2.4 and Ref. [299]), as part of what we think is a 'quality control' mechanism (see below), the suggestion was made that early and late RRPs interact functionally; such interactions could have occurred in a single, large, 'processome'. Functional interactions between early and late RRPs are most probably prevalent but the simple view of a unique 'processome' has however been recently challenged.

The advent of efficient copurification schemes and mass-spectrometry analysis [162, 228] led several labs to isolate distinct pre-ribosomal species (currently about 12, see Table 3.2-2). Typically, these were purified from one or several epitope-tagged protein components of the rRNPs ([14, 56, 67, 91, 95, 195, 234, 318]; reviewed in Refs. [71, 310]). These preparations have achieved a much better definition in their pre-rRNA content (which parallels our current understanding of pre-rRNA processing, see Figs. 3.2-1 and 3.2-3) and the protein composition of the particles has been established accurately. In combination with high-throughput copurification and two-hybrid schemes ([74, 75, 84, 101, 244] and useful WWW links), these data

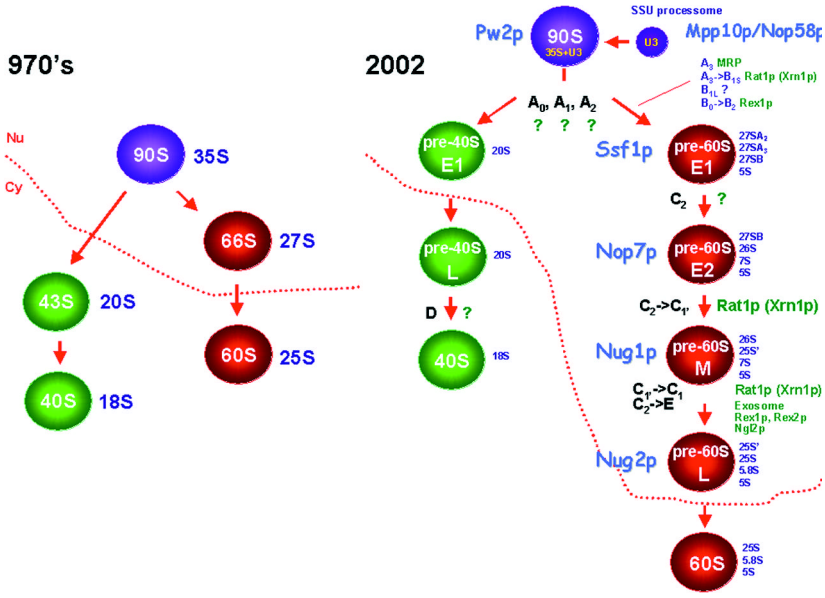


Figure 3.2-1 Ribosomal assembly pathways. See main text for a full description. Cleavage sites, processing activities and RNA content of pre-ribosomal particles are indicated, as well as the TAP-targets used for the purifications (see Table 3.2-2). Pre-ribosomes have tentatively been ordered, based on their protein and RNA content, and assigned and to the early (E), middle (M), or late (L) class. At the time of writing (Christmas 2002), several novel pre-ribosomal species are being isolated (in particular in the 40S subunit branch) and the pathway is expected to be much refined in the next few months. Largely inspired by Fatica and Tollervey [71]). Nu, nucleus; Cy, cytoplasm.

Table 3.2-2 TAP-tagged purified pre-ribosomes, as of Christmas 2002.

Pre-ribosomes	TAP targets	References
90S and U3/SSU processome	Pw2p, Rrp9p, Nop58p, YDR449c, Krr1p, Noc4p, Kre31p, Bud21p, YHR196w, YGR090w, Enp1p, YJL109c, Nop14p	91
U3/SSU processome	Mpp10p and Nop58p	56
Pre-60S E1	Ssf1p	67
Pre-60S E2	Nop7p	95
Pre-60S M	Nug1p	14
Pre-60S L	Nug2p/Nog2p	234
Seven species of early (E), medium (M) and late (L) pre-60S	Nsa3p, Nop7p, Sda1p, Rix1p, Arx1p, Kre35p, Nug1p	195

The TAP technology (Tandem Affinity Purification, 228) has been used to isolate most pre-ribosomes described to date.

provide the basis to draw the first eukaryotic (pre-)ribosomal assembly maps (see Figs. 3.2-1 and 3.2-2).

It transpires that ribosomal assembly is strongly asymmetric and at least biphasic ([56, 91]; reviewed in Ref. [71]). Early RRPs interact with nascent pre-rRNAs, mostly in association with the U3 snoRNP, now also referred to as the small subunit processome ('SSU processome'; [56], see below). Following the first three pre-rRNA cleavages, at sites A_0, A_1, and A_2 (see Figs. 3.2-1–3.2-3 and pre-rRNA processing section), this first set of factors essentially cycles-off the pre-ribosomes and is replaced by the large ribosomal subunit RRPs (Fig. 3.2-2). Pre-40S subunits are then left associated with very few factors, about a dozen of them, referred to as the SSU RRP complex [235, 335]; pre-60S subunits acquire several dozens of novel RRPs. As anticipated, there is a steady decrease in the number of these pre-60S-associated RRPs as the pre-ribosomes undergo the complex 5.8S–25S pre-rRNA processing pathway and reach the NPC. 90S and 66S particles were long known to have a higher ratio of protein to RNA content than the mature 60S subunits, as judged from buoyant densities in CsCl gradients (see, e.g., Ref. [277]). This is in contrast with 43S pre-ribosomes and 40S subunits that have about the same protein content. Late nuclear pre-60S ribosomes show the reassuring presence of known transport factors, such as the well-characterized Nmd3p/Rpl10p couple (see Sect. 3.2.7).

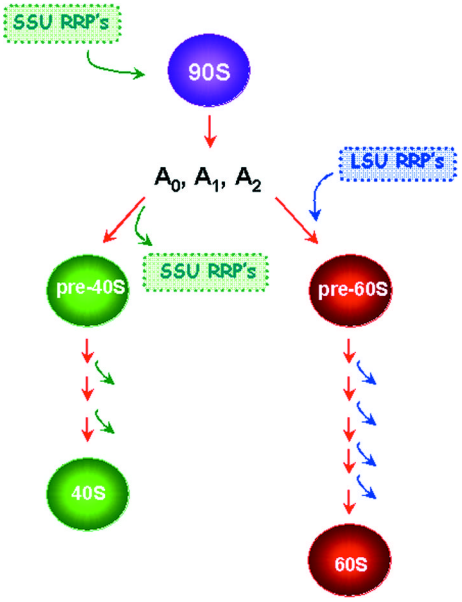


Figure 3.2-2 The 'biphasic model' for ribosomal assembly. The SSU RRPs (including the U3 snoRNP/SSU processome') associate with the primary Pol I transcript, generating the 90S pre-ribosomes. This first set of RRPs is replaced after the first three pre-rRNA processing reactions (A_0–A_2) by the LSU RRPs.

Most striking from the currently described pre-rRNPs is the conspicuous absence of most known cleavage enzymes; this could either reflect the low abundance or transient associations of these activities with the rRNP complexes.

X-ray crystallographic analysis of large ribosomal subunits revealed that while many RPs are located on the exterior of the rRNA core, several RPs show idiosyncratic extensions deeply buried into the body of the subunits in a configuration that is only compatible with concomitant foldings of the RPs and the rRNAs ([13]; reviewed in Refs. [55, 143, 220, 222]). This presumably underlies the need for close to 30 distinct remodeling activities (helicases, GTPases, and AAA-ATPases). It is remarkable that several RRPs are strikingly homologous to RPs (e.g., Imp3p/Rps9p, Rlp7p(Rix9p)/Rpl7p, Rlp24p/Rpl24p, Yh052p/Rpl1p [14, 59, 79, 234]), suggesting that they possibly 'hold in place' pre-rRNP structures during the assembly process, preventing premature, irreversible, folding steps to occur before being swapped for their homologous RPs. This strategy may even couple late pre-rRNA processing reactions to translation as eIF3j/Hcr1p is required for both 20S pre-rRNA processing and translation initiation and the RRP Efl1p is homologous to the ribosomal translocase EF-2 [240, 286].

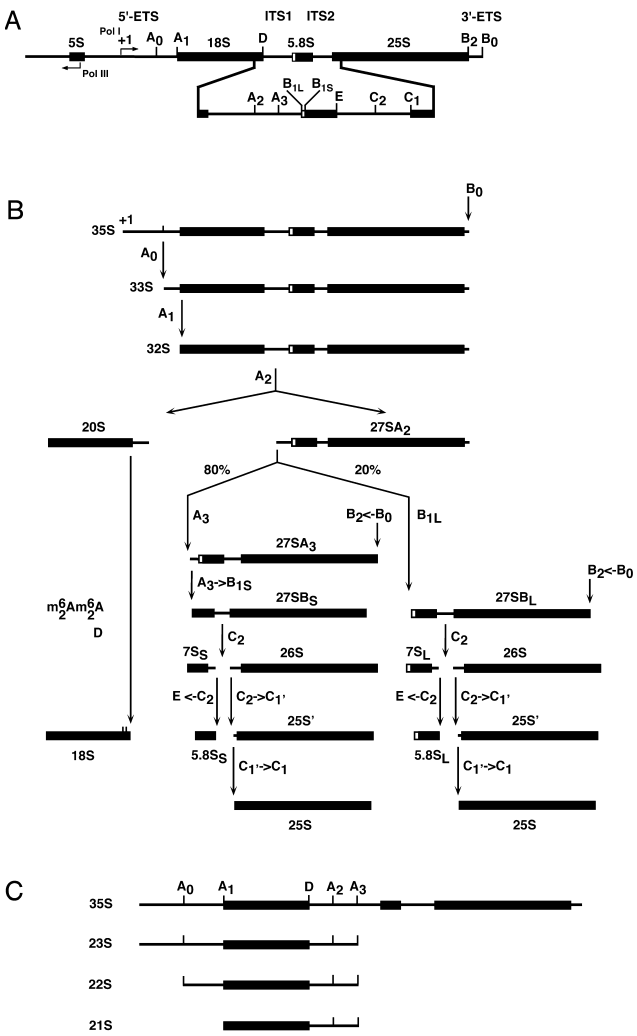
Pre-rRNP particles currently described were isolated from tagged RRPs and although clearly distinct in composition, as expected from the substantial remodeling of the pre-ribosomes that take place along the pathway, represent mixed pre-rRNP populations. It is also important to note that it is in fact mostly pre-ribosomal assembly rather than ribosomal assembly *per se* that has been addressed so far. Indeed, RPs present a particular challenge; there are usually small, highly basic and coprecipitate at high degrees with targets that are not related to ribosome synthesis. Despite these limitations, a major step has however been achieved with the isolation of particles which have a lifetime of presumably less than a minute.

Much remains to be done to understand what the RRPs exactly do, how and when they interact with the pre-ribosomes and how they 'talk' to each other.

3.2.4
Ribosomal RNA Processing, Getting there...

Pol I transcription drives the synthesis of a large pre-rRNA, 35S in yeast, containing the mature sequences for the small subunit rRNA (the 18S rRNA) and two of the large subunit rRNAs (5.8S and 25S rRNAs). Completion of transcription requires about 5 min. Mature sequences are flanked with non-coding spacers (Fig. 3.2-3A).

Figure 3.2-3 rDNA and pre-rRNA processing pathway. (A) Structure of the yeast rDNA. The 18S, 5.8S and 25S rRNAs are encoded in a single, large, Pol I transcript (35S). Mature sequences are separated by non-coding spacers, the 5′- and 3′-external transcribed spacers (ETS) and the internal transcribed spacers 1 and 2 (ITS). Processing sites (A_0 to E) are indicated. 5S is transcribed independently, in the opposite direction, by Pol III as 3′-extended precursors. The production of 5S mature 3′-ends is a multi-step process that requires Rex1p. (B) Pre-rRNA processing pathway in wild-type strains. See main text for a description of our current understanding of the processing pathway. Processing at sites C_2->E is detailed in Fig. 3.2-4.



Note that C_2 (referred to as $C_{2'}$ in Ref. [85]) was recently mapped precisely by primer extension at a position located 94 nucleotides upstream of site C_1. Previous mapping, by RNase protection, located this site slightly upstream (at position +101 with respect to C_1). Although both sites may be used in vivo, it is more probable that this difference reflects limitations inherent to the RNase mapping strategy used. In Ref. [85], the $C_{2'}$-B_2 RNA is referred to as 25.5S (C_2-B_2 is 26S here).

Note that a cryptic processing site (A_4) has recently been identified in ITS1 between A_2 and A_3 in rrp5 mutants [63]. (C) Aberrant RNA precursors commonly detected in RRP mutants. Delays in early pre-rRNA processing often results in the accumulation of the 23S, 22S or 21S RNA. These are generally not further matured.

A Pol III precursor, 7S, is processed in 3′ by the Rex1p/Rna82p exoribonuclease into 5S; the third large ribosomal subunit rRNA [213, 287]. In most eukaryotes but *S. cerevisiae*, 5S rDNA is located in extranucleolar loci as individual or repeated copies. In yeast, 35S and 7S are encoded on opposite strands within 150–200 repeated 9 kb rDNA arrays located on chromosome XII (Fig. 3.2-3A).

Mature sequences are generated from the 35S pre-rRNA following a complex multi-step processing pathway requiring both endo- and exoribonucleolytic digestions (Fig. 3.2-3B). It is thought that most cleavage sites are known and occur within about 2 min following a precise temporal order. There is a strong bias for cleavages from the 5′- to the 3′-end of the primary transcript and the synthesis of the small and large ribosomal subunits is relatively independent.

The 35S pre-rRNA is successively cleaved in the 5′ external-transcribed spacer (5′-ETS) at sites A_0 and A_1 and in the internal-transcribed spacer 1 (ITS1) at site A_2 (Fig. 3.2-3B). Endonucleolytic digestions at sites A_0 and A_1 produce the 33S and 32S pre-rRNAs, respectively. Precursors to the small and large subunit rRNAs (the 20S and $27SA_2$ pre-rRNAs, respectively) are generated by endonucleolytic cleavage of the 32S pre-rRNA at site A_2. The precise mechanism of cleavage at sites A_0–A_2 is not known; however, these reactions are tightly coupled and involve the box C+D snoRNA U3/the 'SSU processome' (see below). The 20S pre-rRNA is then exported to the cytoplasm where endonucleolytic digestion, by an unknown RRP, at site D provides the 18S rRNA [276, 282]. A complex of late small subunit RRPs has recently been described in association with the dimethyl-transferase Dim1p ([295] and see below); the endonucleolytic activity may lie in one of these.

The $27SA_2$ pre-rRNA is cleaved at site A_3 to generate the $27SA_3$ RNA. This cleavage is carried out by the endoribonucleolytic RNP complex RNase MRP. RNase MRP is highly reminiscent to another snoRNP, the ubiquitous RNase P that is involved in the 5′-end formation of tRNAs (reviewed in Refs. [183, 329]). The homology extends both to the structure of their respective RNA as well as to their protein composition (eight of the nine protein subunits are shared between the two enzymes). Snm1p is specific to RNAse MRP; Rpr2p is unique to RNase P [35, 238].

In the absence of cleavage at site A_2, pre-rRNA processing can proceed through the next ITS1 cleavage at site A_3. This can be seen as a 'rescue' pathway for such an essential activity as ribosome synthesis [183].

There are two alternative pathways of synthesis of 5.8S–25S rRNAs [98]. In the major pathway, which represents ~80% of the total processing, the $27SA_3$ pre-rRNA is trimmed to site B_{1S} (the 5′-end of the most abundant form of 5.8S, the $5.8S_S$ rRNA) by the combined action of two 5′–3′ exoribonuclases, Rat1p and Xrn1p. Rat1p is encoded by an essential gene and mostly located to the nucleus; Xrn1p is not essential and mostly localizes to the cytoplasm [114]. These two exoribonucleases often show partially overlapping functions (see, e.g., Refs. [65, 85, 98, 210]).

$27SB_S$ is cleaved by an unknown endonuclease, roughly in the middle of ITS2, at site C_2. Cleavage at C_2 provides the $7S_S$ and 26S pre-rRNAs. Processing of the 3′-end of 5.8S and the 5′-end of 25S requires a complex succession of, mostly, exoribonucleolytic digestions. During these, consecutive substrates are literally 'handed over' from one ribonucleolytic activity to the next.

The 7S is digested to site E by the successive action of the exosome complex [4, 176, 177], the Rex1p exoribonuclease and Ngl2p, a putative endonuclease [65, 287].

The exosome is a remarkable complex of 11 3′–5′ exoribonucleolytic activities involved in the synthesis and degradation of most classes of cellular RNAs ([6, 99, 109, 176]; reviewed in Refs. [178, 289]). A nuclear form of the exosome is specialized in the synthesis and turnover of large RNAs, including rRNAs and pre-mRNAs as well as most classes of small stable RNAs (snoRNAs, snRNAs, tRNAs, pre-mRNAs, SRP, RNase P, etc.); a cytoplasmic form is devoted to mRNA degradation. Rrp6p (*E. coli* RNase D), a non-essential subunit of the exosome, is specific to the nuclear form of the complex [6, 29]. Nuclear and cytoplasmic exosomes also differ by their use of specific cofactors (see, e.g., Refs. [260, 290]). The related DExH putative RNA helicase Dob1p/Mtr4p (nuclear) and Ski2p (cytoplasmic) is an example [48, 109].

7S precursors are first trimmed from site C_2, located at position +134 with respect to the 3′-end of 5.8S, to position +30 [4] (Fig. 3.2-4). This requires all the subunits of the exosome and the nuclear cofactor Dob1p/Mtr4p. 5.8S+30 pre-rRNA is then digested to position +8 by Rrp6p. 5.8S+8, also referred to as 6S, is consequently trimmed to 5.8S+5 by the multiple exoribonuclease activities of Rex1p, Rex2p and the exosome complex (notably the Rrp40p and Rrp45p subunits) [4, 287]. 5.8S+5 is finally matured to 5.8S by Ngl2p [65] (Fig. 3.2-4).

While the relationship between the subnucleolar compartments and the various ribosome synthesis steps is far from being clear, it is probable that the DFC is the site of early pre-rRNA processing, modification and assembly reactions with later processing cleavages and assembly steps occurring in the GC. SnoRNP core proteins

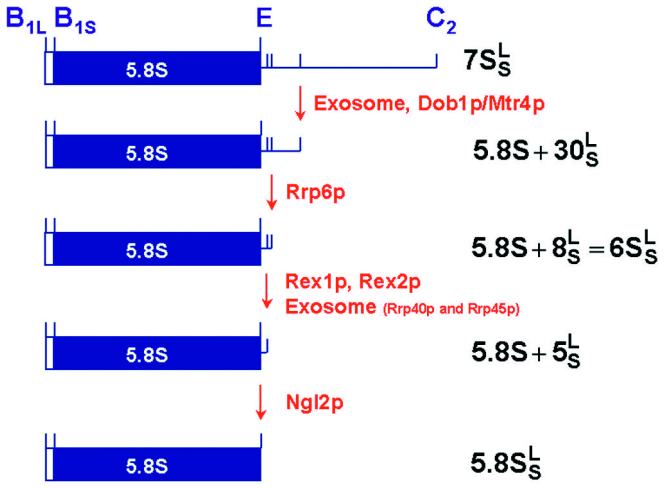


Figure 3.2-4 Multiple steps of ribonucleolytic 'hand-over' are required to synthesize the 5.8S rRNA. Successive pre-rRNA species and trans-acting factors involved are indicated. See main text for a complete description.

involved in 2′–O methylation, pseudouridines formation and early pre-rRNA processing cleavage at sites A_0–A_2 (see below) localize to the DFC [97, 156, 197]. The MRP, involved in cleavage at site A_3, is detected in the GC [225]; this is also where Rlp7p, which is required for cleavage at site C_2, has been localized [79].

Following cleavage at site A_2, the maturation of the small and large rRNAs is relatively independent. However, mutations affecting primarily the synthesis of 5.8S and 25S rRNAs frequently have negative feedback effects on early cleavages at sites A_0–A_2. The mechanism underlying these observations is not known but believed to be part of a 'quality control' mechanism (it would not appear very useful to further initiate the production of pre-ribosomes that will fail to mature properly), which presumably reflects the existence of functional interactions between early and late RRPs.

3′-end formation of other classes of RNAs, such as the snoRNAs and snRNAs, seem to follow a similar strategy of 'exoribonucleolytic hand over' [4, 288]. It is unclear, at present, whether so many distinct nucleolytic activities, with partially overlapping specificity, are required to achieve what would appear to be a fairly straightforward processing. This presumably provides potential for further 'rescue pathways' and quality controls.

The 26S pre-rRNA is trimmed to site C_1 by Rat1p and Xrn1p. This is also probably a multi-step process. Consistently, primer extension through ITS2 from an oligonucleotide specific to the 5′-end of 25S rRNA reveals strong stops at positions +9 and +18 (respective to 25S rRNA 5′-end). The species extending to site +9 (25S′) is lost in some RRP mutants [79]. In the mature subunits, 5.8S and 25S rRNAs are base-paired but the precise timing of this association in the pre-ribosomes is not known.

The major site of Pol I transcription termination (site T_2) is located at position +210 (respective to the 3′-end of 25S rRNA). Precursors extending to this site are however not detected in wild-type cells as primary transcripts are cleaved co-transcriptionally at sites +14/+49 (B_0) on both sides of an AAGN-closed stem-loop structure by the endonuclease Rnt1p [37, 136, 326]. Rnt1p is homologous to bacterial RNase III which similarly cleaves its substrates on both sides of extended stem-loop structures (reviewed in Ref. [121]). Final trimming to site B_2 (the 3′-end of 25S) is carried out by Rex1p/Rna82p [287]. An oligonucleotide specific to sequences located downstream to B_2 detects $27SA_2$ but not 27SB on Northern blots, demonstrating that processing at sites B_1 and B_2 is tightly coupled and presumably concurrent [136].

The minor pathway (used in ~20% of the cases) produce pre-rRNAs and 5.8S rRNA that are extended in 5′ by 7–8 nucleotides. This starts with cleavage of the $27SA_2$ pre-rRNA at site B_{1L} by an unknown enzyme, a presumed endoribonuclease. The resulting $27SB_L$ is then processed into 25S and $5.8S_L$ rRNAs following a pathway that is, as far as we know, essentially identical to the one described above for $27SB_S$.

It is not precisely known when the 5S RNP (5S rRNA associated with RPL5, see [52]) joins pre-60S ribosomes but its recruitment is required for efficient 27SB processing and is therefore presumably concomitant with processing at site C,

thus ensuring that all newly formed 60S subunits contain stoichiometric amounts of the three rRNAs [50, 294].

Alterations in the kinetics of cleavage are seen in many RRP mutants. These usually lead to the accumulation of aberrant precursors that are not faithfully processed to mature rRNAs but rather degraded, notably by the action of the exosome complex [4, 5]. The most often encountered abnormal species, the 23S (extending from sites +1 to A_3), 22S (from sites A_0–A_3) and 21S (A_1–A_3) RNAs, result from alterations in the kinetics of early pre-rRNA processing reactions (Fig. 3.2-3C). Analysis of these species has allowed the description of the processing in the ITS1 and led to the identification of the cleavage site A_3 [98, 154, 155, 184, 248, 268]. Alterations in the order of cleavage at later processing sites are now also known to occur and give rise to the accumulation of a full range of abnormal RNAs; e.g., A_2–C_2, A_2–E, etc. [67, 135].

Over the years, extensive mutagenesis experiments have been performed on rDNA to isolate sequences relevant in cis to pre-rRNA processing reactions. While it is far beyond the scope of this chapter to review this body of data (see Ref. [299]), it should be noted that these experiments have often highlighted how processing reactions distant in the primary rRNA sequence are in fact tightly linked; indeed, mutations in the 5′-ETS, ITS2, or 3′-ETS regions can each inhibit processing in ITS1 (see, e.g., Refs. [7, 20, 292, 293]).

While we now have a fairly complete picture of pre-rRNA processing, much remains to be done to understand the precise kinetics of the processing as well as the extensive connections between early and late cleavage events. Many processing enzymes also remain to be identified, in particular most endoribonucleolytic activities. It is possible that some endoribonucleases have already been assigned to the RRPs and await further attention; the absence of specific motifs in their sequence complicates their identification. The development of *in vitro* reconstitution assays should be most useful in this respect.

It is notable that most known cleavage factors (the exosome, the exoribonucleases Rat1p and Xrn1p, the endoribonuclease Rnt1p) involved in pre-rRNA processing are required for the synthesis and/or degradation of other classes of cellular RNAs (mRNAs, snRNAs, snoRNAs, tRNAs, SRP, RNase P, etc.). All seem to indicate that general maturation factors are recruited from a 'common pool' of proteins to specific cellular pathways. This is also illustrated by the over-increasing sets of proteonic data supporting the existence of extensive integration between ribosome synthesis and other biosynthetic pathways.

3.2.5
Ribosomal RNA Modification: A Solved Issue?

Ribosomal RNAs are extensively modified with a large majority of the modifications clustering at the most functionally relevant and conserved sites of the ribosome (tRNA- and mRNA-binding sites, peptidyl transferase center, intersubunit bridges, entry of the exit tunnel, etc.; see Chapters 6 and 8 for a functional description of the ribosome). This has recently been highlighted on three-dimensional

maps based on crystallographic analysis of archaeal and bacterial ribosomal subunits (see Ref. [49] and useful WWW links). The atomic resolution structure of the ribosome established it as a ribozyme; the peptidyl transferase center is surrounded by an RNA cage leaving little, if any, chance to the RPs to be involved in the peptidyl-transfer reaction *per se* (reviewed in Refs. [55, 143, 181] and Sect. 8.3). rRNA spacers are consistently devoid of modification.

The most frequent RNA modifications are 2′–O methylation of ribose moieties (Nm) and uridine isomerization (pseudouridines, ψ) (~50 of each in yeast; twice this amount in humans) (Figs. 3.2-5b and d). The sites of these modifications are virtually all selected by base pairing with the snoRNAs. Less abundant are the base modifications. These are also essentially modified by methylation (mN) and rely, as far as we know, on protein-specific enzymes rather than the snoRNPs.

3.2.5.1 Ribose Methylation, Pseudouridines formation and the snoRNAs

There are essentially two families of snoRNAs, the box C+D (involved in sugar 2′–O methylation) and the box H+ACA (required for pseudouridines formation) (Fig. 3.2-5). A third class is defined by the related RNAse P/RNAse MRP RNAs. Yeast snoRNAs range in size from about 60 to about 600 nucleotides.

Box C+D snoRNAs consist of a stem-loop structure with boxes C (UGAUGA) and D (CUGA) flanking a terminal helix; duplicated boxes C′ and D′ are also observed

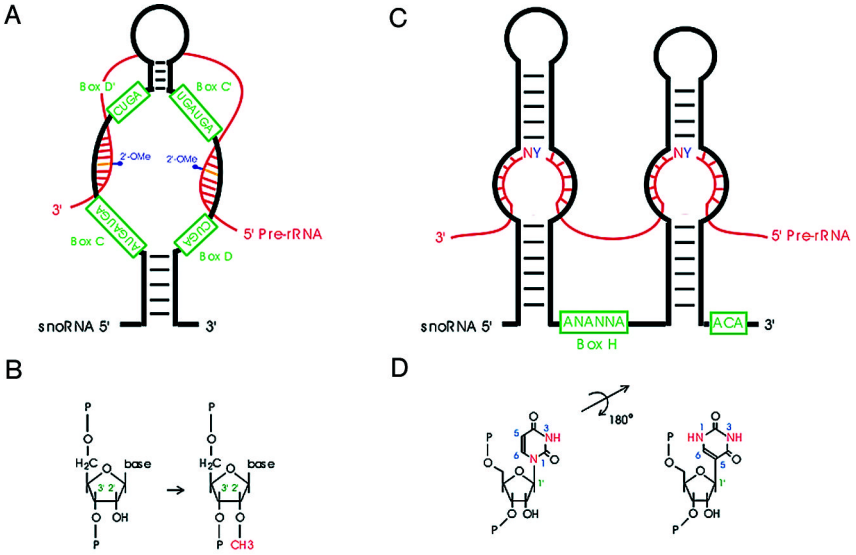


Figure 3.2-5 snoRNA in pre-rRNA modification. SnoRNA/pre-rRNA hybrids at sites of 2′–O methylation (a) and pseudouridine formation (c). Sugar methylation (b) and pseudouridines (d). See main text. Adapted from Ref. [140].

(reviewed in Refs. [72, 140, 320]) (Fig. 3.2-5a). Box H+ACA RNAs show two consecutives hairpin structures, bridged by a conserved H-box (ANANNA, where N is any residue) or hinge motif (hence its name); the triplet ACA is always located 3 nucleotides upstream to the 3′-end of the RNA (Fig. 3.2-5c).

At each site of modification, a duplex is formed by Watson–Crick base-pair interactions between a specific snoRNA and the RNA substrate. This results in the formation of a snoRNA/pre-rRNA hybrid that precisely position the residue to be modified on the substrate with respect to conserved boxes on the snoRNA. For the box C+D snoRNAs, the guide or 'anti-sense' elements are located upstream of boxes D or D′ and provide the potential to form between 10 and 21 consecutive base-pairs with the pre-rRNAs; including the site of 2′–O methylation invariably located five nucleotides upstream of boxes D or D′ (reviewed in Refs. [11, 124]). For the box H+ACA snoRNAs, the 'anti-sense' motifs are within internal bulges (also known as 'pseudouridylation pockets') in the hairpin stems, and target the formation of two short helices of 3–10 base-pairs with the substrate; these are interrupted by the uridine to be altered by rotation into ψ (reviewed in Refs. [12, 125]). This uridine is usually located at about 14 residues from boxes H or ACA. SnoRNAs show a high degree of divergence outside the conserved boxes; including, obviously, the 'anti-sense' elements.

SnoRNAs are associated with a limited set of specific core proteins. Snu13p (15.5K in humans), Nop1p (yeast *Fibrillarin*) and the related Nop56p and Nop58p-KKD/E containing proteins are associated with all box C+D snoRNAs [83, 144, 146, 237, 316]. The human 15.5K is expected to nucleate box C+D snoRNP assembly through direct binding to a conserved RNA-fold (K-turn, see below) generated by interactions between boxes C and D [316]. Remarkably, Snu13p is also a component of the spliceosomal U4/U6•U5 tri snRNP [316]. Cbf5p (NAP57 in rodents, *Dyskerin* in humans), Gar1p, Nhp2p and Nop10p are all associated with the H+ACA snoRNAs [90, 97, 141, 315]. Concurring evidences support that Nop1p and Cbf5p are the methyltransferase and the pseudouridine synthase, respectively [102, 141, 201, 271, 307, 333]. The localization of the snoRNAs and their associated core proteins in the DFC of the nucleolus suggest that this is the site of rRNA modification [97, 156, 197].

Telomerase is an RNP reverse transcriptase that maintains telomere length by adding telomeric DNA repeats onto the ends of eukaryotic chromosomes [23, 36, 165]. In humans, the telomerase RNA (hTERT) has a canonical H+ACA motif at its 3′-end that is bound by the core H+ACA proteins [53, 57, 174, 217]. In yeast, the TEL RNA is bound by the spliceosomal Sm proteins [241]; another interesting evolutionary crosstalk. Human telomerase also interacts with La and SMN (see below and Refs. [10, 73]).

Strikingly, several self-immune and genetic human diseases map to key nucleolar RNA-processing factors and snoRNP proteins, such as the exosome, *fibrillarin*, *dyskerin* and the RNAse MRP [6, 30, 96, 159, 175, 227, 231, 274, 334].

3.2.5.2 The Emergence of the snoRNAs

Eukaryotes have about 10 times (in the range of the hundred) more modifications in their ribosomal RNAs than prokaryotes do. An observation that, a *posteriori,* seems to fully justify the emergence of the snoRNAs. Why would a cell evolve and produce several dozens of protein enzymes with distinct substrate specificities when it can rely on a single snoRNA-associated protein? In addition, the snoRNA-based system of RNA modification is very flexible as the guide sequences are not conserved (they have little, if any, functions in snoRNA synthesis, stability, and nucleolar targeting) and are therefore prone to rapid evolution. The accumulation of point mutations in snoRNAs generates new 'anti-sense' elements that, eventually, will find new RNA targets.

In fact, there is a steady increase in the number of modification across evolution with bacteria and eukaryotes on both ends of the range and the archaea showing intermediate distributions. This raised the possibility that these too may rely on a 'snoRNA-like mechanism' to select their sites of RNA modification (discussed in Ref. [140]). An assumption that turned out to be correct as a large family of archaeal box C+D and H+ACA sRNAs (archaea lacking a clear nucleolar structure) and a full set of core sRNPs proteins has now been described ([82, 137, 200, 201]; reviewed in Refs. [51, 266]). Remarkably, in archaea, the snRNAs not only target the modification of rRNAs but also of tRNAs [45]. A model, based on the assumption that 2′–O methylation confers extra thermostability, has been proposed that correlates the distribution of archaeal rRNA modification with the temperature of their ecological niches [196].

Archaeal box C+D sRNAs, active in methylation, have been reconstituted *in vitro* from individually produced components [201]. In these experiments, assembly appeared to follow a strict order with the aL7a (archaeal Snu13p) binding first to the RNAs, followed by aNOP56 binding (archaeal Nop56/58p) and then finally association with aFib (archaeal Nop1p). These analysis led further support to the predictions that Snu13p may nucleate the step-wise assembly of box C+D snoRNPs and that Nop1p carries the methyltransfersae activity (mutations in aFib catalytic motifs were inactive in methylation). In yeast, Nop56p was dependent on Nop1p for binding to the snoRNAs whereas Nop58p was found to bind independently [146].

Recent studies have revealed that archaea assemble symmetric sRNPs with a complete set of core proteins (L7a, the single Nop56/58p homolog and fibrillarin) at both box C+D and C′+D′ motifs [275]. In contrast, eukaryotes snoRNPs appeared asymmetric with a distinct set of core proteins bound to each motifs; 15.5K, Nop58p, and fibrillarin were all detected at the terminal C+D motif, whereas Nop56p, fibrillarin, but no apparent 15.5K, were present at the internal C′+D′ position [31, 321]. A rationale to this key difference in protein composition is provided by the observation that during evolution, the 15.5K seemed to have lost its ability to recognize internal C′+D′ motif [275]. The box C′+D′ motif is degenerated and suboptimal for tight association with the core proteins. Significantly, the recent resolution of the 3D structure of an archaeal Nop58p–fibrillarin complex bound to

S-adenosyl methionine (SAdoMet), the universal methyl donor, strongly suggests that the C-terminal coiled-coil domain of Nop58p may promote its homodimerization and allow the assembly of a core complex at the suboptimal C′+D′ motif ([2]; reviewed in Ref. [70]). In eukaryotic snoRNPs, this interaction would take place between the C-terminal tails of Nop58p and Nop56p at the C+D and C′+D′ motifs, respectively, and possibly compensate for the absence, at this site, of the nucleation activity carried out by 15.5K.

Snu13p belongs to a family of related RNA-binding proteins including several RPs of both subunits: yeast L30 (which binds to its own mRNA for autoregulation, see Refs. [303, 304]) and human L7a and S12, the box H+ACA snoRNP protein Nhp2p [97], SBP2 (which binds to the stem-loop SECIS element in the 3′-UTR of selenocysteine protein-encoding mRNAs, see Refs. [3, 132]) and eRF1 (a subunit of the translation termination release factor). These proteins have been shown, or predicted to, bind to a ubiquitous RNA structural motif, known as 'kink-turn' (K-turn, [126]) or 'GA motif' suggesting that they share a similar strategy for binding to their substrates.

Interestingly, archaeal Snu13p not only binds to the box C+D sRNAs but also to the LSU (23S) rRNA contacting a K-turn and suggesting that ancestors to small stable RNAs may have evolved from rRNA segments; an assumption further supported by the identification of an archaeal box C+D sRNA within a non-coding rRNA spacer region [261].

In the widely accepted concept of the 'prebiotic RNA world', RNAs preexisted proteins and most essential functions were carried out by 'RNA-based machines'. In contrast, the model proposed for the emergence of the snoRNAs is a case where a function initially performed by individual proteins has slowly been taken over by RNPs to achieve greater efficiency (see Ref. [140] for further discussions).

3.2.5.3 **Non-ribosomal RNA Substrates for the snoRNAs**

Although originally described in pre-rRNA modification, snoRNAs and alike (archaeal sRNAs, human scaRNAs, see below) have now been demonstrated to work on other RNA substrates, including spliceosomal U RNAs U1, U2, U4 and U5 (Pol II transcripts) and U6 (Pol III), tRNAs and possibly mRNAs [32, 45, 46, 81]. An interesting case of putative mRNA guide is a tissue-specific (brain) snoRNA, expressed from an imprinted region of the genome that is linked to the neurodegenerative genetic disease Prader-Willi syndrome [32–34]. Remarkably, this snoRNA is expected to target a site of RNA 2′–O methylation on a serotonin receptor mRNA at a position that is also subjected to A to I editing.

Orphan snoRNAs are waiting for their RNA target to be identified and many more classes of RNAs are expected to use a similar strategy for their modification. Viral RNAs are particularly interesting to consider in this respect, as these would require additional co-evolution with their hosts.

3.2.5.4 Possible function(s) of RNA modifications

Several structural and thermodynamic effects have been proposed for RNA modifications, including altered steric properties, different hydrogen-bonding potential and increased local base stacking (ψ), increased structural rigidity (ψ and Nm) and protection from hydrolysis of inter-nucleotides bonds (Nm) [147, 198]. However, the precise function of these modifications is not known and we have failed to identify a single modification that is essential for ribosome synthesis or function, although the selective loss of the ψ's surrounding the peptidyl transferase center significantly reduce translation efficiency [123]. It is therefore probable that each modification contributes a little benefit and that it is the overall modification pattern that significantly improves ribosome synthesis and/or function. It is quite remarkable that three sites of ψ and three sites of 2′–O methylation are common to bacteria and eukaryotes; these have been selected independently twice during evolution and are made by distinct mechanisms (snoRNPs versus protein-specific enzymes; see Ref. [140]). In addition, most known modification enzymes carry additional, presumably indirect, essential functions in ribosome synthesis, notably in pre-rRNA cleavage (e.g., Nop1p, Cbf5p, Dim1p; reviewed in Ref. [145]).

An attractive hypothesis certainly remains that RNA modifications are simply 'by-products' reflecting the involvements of the snoRNAs in pre-rRNA processing and pre-rRNP assembly. Through extensive base pairing with the rRNA precursors, snoRNAs dictate specific pre-rRNA structures and fold them into conformations that are competent for processing and assembly. Modifications could then be seen as mere triggers to unleash the snoRNAs from the pre-ribosomes following the precise kinetics of ribosome assembly. In yeast, methylation of the rRNA occurs immediately after the completion of transcription [226, 283], implying that the snoRNAs are associated with the growing chain as it is being transcribed and potentially circumvent early unwanted folding.

3.2.5.5 Base methylation

Several putative base methyl-transferases have been described and, as far as we know, do not involve the snoRNAs for their function [103, 131, 212, 249, 327].

A well-characterized example of base methylation is the 18S rRNA dimethylation carried out by Dim1p (KsgAp in *E. coli*). Both the site of modification (the 3′-terminal SSU hairpin located at the subunit interface where interactions important for ribosome function occur) and the modification itself (a twin methylation at position 6 on two adjacent adenosine residues) are highly conserved in evolution [145, 291]. Methylation of the pre-rRNAs by Dim1p is a fairly late event in the SSU assembly pathway, possibly linked to 40S subunit export and occurring in the cytoplasm. However, Dim1p binds to the pre-rRNAs in the nucleolus and is required for early cleavages at sites A_1 and A_2 [138, 139, 142]. This is further evidence for the existence of 'quality control' mechanisms in ribosome synthesis. Processing does not occur on pre-rRNAs that have failed to bind Dim1p and will consequently not be methylated. Consistently, the Dim1p methylation is essential for ribosome function *in vitro* and is

favorable to translation *in vivo* (reduced rates of frame-shifting and misreading; D. Demonté and D.L.J. Lafontaine, unpublished results).

A thermosensitive conditional mutation in Dim1p is suppressed on overexpression of RPL23, a primary binding protein of the large ribosomal subunit (D. Demonté and D.L.J. Lafontaine, unpublished results). This indicates that alteration in the kinetics of LSU assembly (the process is presumably prematurely triggered on RPL23 overexpression) overcomes the need for the 'quality control' exerted by Dim1p in early pre-rRNA processing and small subunit synthesis.

In bacteria, the methylation is conserved but KsgAp is not essential, indicating that eukaryotic Dim1p evolved an additional function in ribosome synthesis.

3.2.5.6 **U3 snoRNP, the 'SSU Processome', and the Central Pseudoknot**

Several snoRNAs are involved in pre-rRNA cleavage rather than pre-rRNA modification. In yeast, these include the box C+D snoRNAs U3 and U14 and the box H+ACA snoRNAs snR10 and snR30. U3, U8, U14 and U22 are also involved in pre-rRNA cleavage in metazoans (reviewed in Ref. [270]). In yeast, U3, U14, snR10, and snR30 are required for the first three pre-rRNA cleavages at sites A_0–A_2; these are either delayed (snR10) or inhibited (U3, U14, and snR30) [105, 153, 184, 269]. U14 and snR10 are also required for pre-rRNA modification. For snR10, a point mutation in the guide sequence could efficiently uncouple its requirement for pre-rRNA processing and modification [123]. Metazoans have an additional member (U8) involved in ITS2 processing [204, 205, 272, 273]; no equivalent has thus been found in yeast.

U3 is undoubtedly the best-characterized member of this class of snoRNAs both in structure, function, and synthesis (see below). U3 is larger than most box C+D snoRNAs (333 nucleotides in yeast) and carry, in addition to the conserved core motifs, sequences (including a protruding 5′-extension, largely unfolded, and ending with a stem-loop) that are known, or presumed, binding sites for about a dozen of U3-specific proteins: Mpp10p, Imp3p, Imp4p, Sof1p, Dhr1p, Lcp5p, Rrp9p/h55K, Rcl1p, and Bms1p [22, 44, 60, 112, 151, 216, 300, 317, 324].

Recently, U3 has been isolated in association with 28 proteins [56, 319]; 10 of which were known U3-specific RRPs, another was a known RRP involved in early and late pre-rRNA processing (Rrp5p), the remaining 17 (Utp1-17p) were all nucleolar and required for 18S rRNA synthesis. This complex is now referred to as the 'SSU processome' and on the basis of its calculated mass (>2 200 000 kDa) and large size (~80S; roughly the size of a mature ribosome or the spliceosome) has been proposed to correspond to the terminal balls visualized at the 5′-ends of nascent transcripts in chromatin spreads [172, 187]; depletion of several 'SSU processome' components led to the disappearance of these structures [56].

The function of U3 in pre-rRNA processing is mediated through at least two Watson–Crick base-pair interactions between U3-specific motifs and the pre-rRNAs. An interaction between an essentially unstructured region of the 5′-extension of U3 and the 5′-ETS (at site +470) is required for cleavages at sites A_0–A_2 [18–20]. A second interaction between a conserved motif (box A) in the 5′-stem-loop of U3 and the

pre-rRNA at the 5′-end of the mature 18S rRNA is necessary for cleavage at sites A_1 and A_2 [242]. The interaction between box A and the 18S rRNA 5′-end is mutually exclusive with the formation of the central pseudoknot, a conserved long-range interaction, which brings together, in the mature particles, sequences that are located more than a kb apart. The formation of the central pseudoknot is a major structural rearrangement in the SSU rRNA and as such is most probably an irreversible step in ribosomal assembly. Dhr1p, a U3-specific DEAH putative RNA helicase required for pre-rRNA processing at sites A_1 and A_2, has been proposed to be involved in this RNA isomerization [44]. One possibility is that the action of Dhr1p is regulated such as to leave sufficient time for early pre-rRNP assembly to occur prior to the formation of the central pseudoknot. Growing yeast cells have about enough copies of the U3 snoRNP to support ribosome synthesis for only ~1 min in the absence of recycling (considering a production rate of ~2000 ribosomes/min). A function of Dhr1p in recycling the U3 snoRNP and in SSU-processome assembly is therefore also probable. This is currently under investigation.

3.2.6
SnoRNA Synthesis and Intranuclear Trafficking

3.2.6.1 SnoRNAs Synthesis

SnoRNAs have adopted a large range of strategies for their expression. Their synthesis, in the nucleoplasm, can either proceed from individual Pol II (most snoRNAs) or Pol III (U3 in plants) promoters and produce mono- (most yeast snoRNAs) or poly-cistronic units (many plants snoRNAs; several yeast snoRNAs) or be expressed from introns of house-keeping genes (most vertebrates snoRNAs; several yeast snoRNAs) (reviewed in Refs. [72, 164, 320]). Host genes are often somehow related to ribosomal synthesis or function and, in extreme cases, do not seem to have any additional function than to carry the snoRNAs, i.e., no proteins are expressed from the spliced mRNAs [26, 32, 207, 251, 281].

SnoRNA maturation is complex. Processing of independently encoded or polycistronic units is initiated by endonucleolytic, possibly co-trancriptional, cleavage in the 3′-portion of the primary transcript and requires Nrd1p, the Sen1p helicase and the cleavage factor IA activity of the mRNA polyadenylation machinery [69, 182, 257]. SnoRNAs encoded in polycistronic units are separated by the endonucleolytic activity of Rnt1p/yeast RNase III [38, 39, 221]; precursors transcripts containing a single snoRNA may also be cleaved at their 5′-ends by Rnt1p [38].

Intron-encoded snoRNAs are usually synthesized from the excised intron lariat following splicing and debranching by Dbr1p, and exonucleolytic trimming on both ends [202, 210]. In a minor, splicing-independent pathway, the pre-mRNA is directly cleaved endonucleotically to provide entry sites for exoribonucleases.

In all cases, final pre-snoRNA maturation steps require exonucleolytic digestions to the mature ends. This involves 3′ to 5′ exonucleolytic digestion (exosome) [4, 288] and, at least in the case of intronic or polycistronic snoRNAs, 5′ to 3′ exonuclease digestion (Rat1p, Xrn1p) [210, 221].

The best-characterized pre-snoRNA processing pathway is for the box C+D snoRNA U3 (Fig. 3.2-6). As for many other snoRNAs, U3 is synthesized with 3′-extensions; these require endonucleolytic cleavage (Rnt1p) to provide an entry site for a processing 'hand over' by the exosome subunits [134]. This processing is literally 'timed' by the binding of yeast Lhp1p (human La) to poly(U)-rich tracks located close to the RNA 3′-ends [134]. Displacement of La is concomitant with snoRNP assembly (the core snoRNP proteins bind to the RNA, presumably conferring 3′-ends protection) and allows final trimming by the exosome to produce the mature 3′-ends. The binding of La to the pre-snoRNAs presumably provides sufficient time for snoRNP assembly to occur prior to the final action of the exosome complex. U3 additionally requires the concomitant splicing of an intron.

Individually expressed Pol II snoRNA precursors are produced with a 5′-terminal 7-monomethylguanosine (m^7G) cap that is retained in many snoRNAs and hypermethylated to 2,2,7-trimethylguanosine ($m^{2,2,7}G$ or TMG) by Tgs1p [186]; the timing of this modification is not known. Tgs1p is also active on snRNAs. For U3, cap trimethylation is dependent on boxes C and D and is concomitant with 3′-end formation and snoRNP assembly as 3′-extended forms of U3 are not bound by the core proteins and are not precipitated by anti-TMG antibodies [134, 252, 253, 264]. In plants, U3 is transcribed by Pol III and carries a γ-monomethyl phosphate cap [245].

3.2.6.2 Non-core snoRNP Proteins required for snoRNA Accumulation

Besides the core components, several proteins have been linked physically or functionally to the snoRNPs but are not found in mature snoRNPs. Such proteins are the Rvb2p(p50)/p55 putative NTPases [122], the putative DEAD-box helicase Sen1p [285], the Naf1p/Shq1p complex [54, 68, 330] and Nopp140 [331]. These are required for snoRNA accumulation, through presumed transient interactions, and are potentially involved in snoRNA synthesis, snoRNP assembly, and/or nucle(ol)ar trafficking.

The nucleoplasmic p50/p55 complex is required for the stability of both box C+D and box H+ACA snoRNAs as well as for proper nucleolar localization of the core proteins Nop1p and Gar1p. Mammalian orthologs have DNA unwinding activity *in vitro* and have been linked to chromatin remodeling and transcription (see Ref. [122] and references therein).

Sen1p is required for snoRNA accumulation of both families as well as several other classes of RNAs (including rRNAs, tRNAs, and snRNAs) [223, 285]. Nop1p is mislocalized on Sen1p inactivation [284].

The Naf1p/Shq1p complex is specific to box C+D snoRNAs accumulation. Naf1p is mostly localized to the nucleoplasm and can be co-precipitated at low levels with several snoRNP components [54, 68, 330]. Naf1p interacts directly with the RNA *in vitro*, and most interestingly, is found in association with the phosphorylated form of the C-terminal domain (CTD) of Pol II. This provides a further link to RNA synthesis [68].

Nopp140 (yeast Srp40p) [166, 168], a highly phosphorylated nucleolar- and CB-specific protein, is found in association with both box C+D and box H+ACA

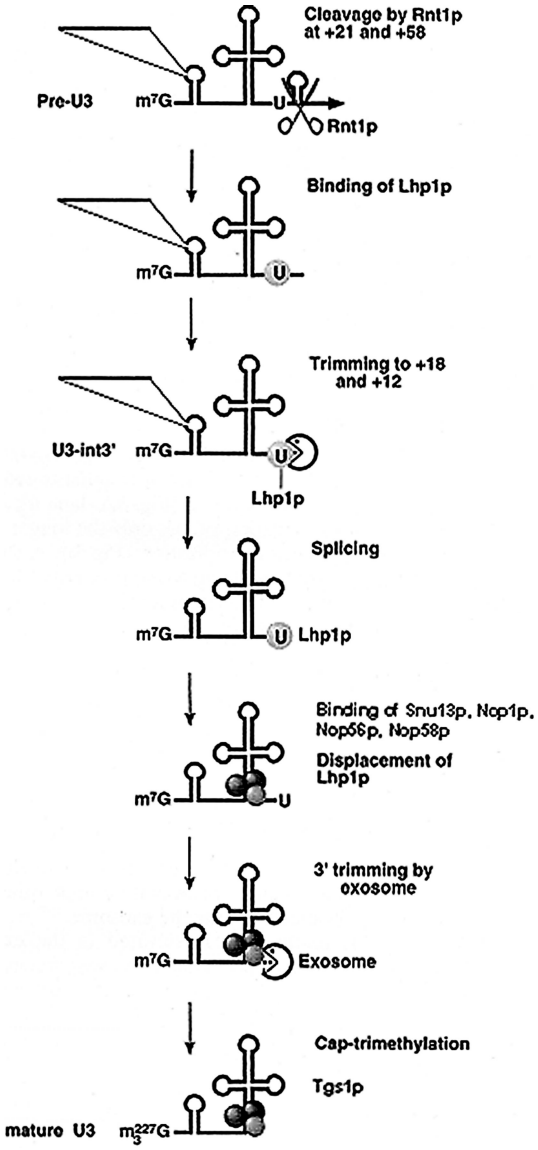


Figure 3.2-6 U3 synthesis pathway. The box C+D snoRNA U3 is synthesized with 3′-extensions; these are cleaved co-transcriptionally by Rnt1p/yeast RNase III. Yeast La (Lhp1p) binds to 3′-terminal poly(U) tracks. Lhp1p-bound precursors are monomethylated and are not assembled with the core proteins. SnoRNP assembly is concomitant with the displacement of La and the production of mature 3′-ends by the exosome; the cap is trimethylated by Tgs1p. The yeast U3 genes are unusual in that they contain an intron; this is spliced out from the 3′-extended precursors. Adapted from Ref. [134].

snoRNPs [108, 331]; association with the box H+ACA is more avid. The interaction with the snoRNPs is dependent on Nopp140 phosphorylation [306]. The expression of a dominant-negative allele of Nopp140 depletes core snoRNP proteins (NAP57, Gar1p, and fibrillarin) from nucleoli and inactivates Pol I transcription [331]. Nopp140 was also coimmunoprecipitated with the largest subunit of Pol I; strengthening a link between snoRNP metabolism and transcription [41]. NAP57/Cbf5p was originally isolated as a Nop140-associated protein [167]; Nopp140 is however not required for *in vitro* pseudouridine formation [306]. Box H+ACA snoRNAs are lost on *srp40* deletion in a yeast synthetic lethal background [331].

3.2.6.3 Interactions between Cleavage Factors and Core snoRNP Proteins

Interaction between Rnt1p and Gar1p is required for optimal Rnt1p activity in pre-rRNA processing, nucleolar localization of the core H+ACA proteins and pseudouridylation. This provides a link between snoRNP synthesis and transport and between RRPs involved in 3′-ETS co-transcriptional cleavage (Rnt1p) and 5′-ETS pre-rRNA processing (Gar1p) [278]. This possibly ensures proper pre-rRNA kinetics and coordinated cleavages on both ends of the primary transcript and prevents processing of incomplete molecules.

In addition, Rnt1p accurately cleaves most of the snoRNA substrates *in vitro* in the absence of other cofactors, with the exception of the U18 intron-encoded snoRNA, which requires the additional presence of the box C+D snoRNP protein Nop1p; Rnt1p and Nop1p interact with each other in pull-down experiments [89].

3.2.6.4 SnoRNAs Trafficking

The synthesis of the snoRNAs in the nucleoplasm but their function, in pre-rRNA processing and/or modification, in the DFC of the nucleolus raise interesting questions as to their localization pathway. Nucleolar targeting and localization of the snoRNAs is probably achieved by diffusion through the nucleoplasm followed by retention through multiple interactions with nucleolar components.

The cis-acting elements involved in this nucleolar targeting have been identified and, unsurprisingly, precisely map to the conserved boxes C and D and H and ACA [148, 149, 192, 193, 232]. These are the only sequences conserved in the snoRNAs and are, known or presumed, protein-binding sites. The ACA element in the telomerase RNA is also required for its nucleolar trafficking [158, 192].

Trans-acting factors involved in this process have only started to be addressed in yeast, with most attention being paid to the box C+D snoRNAs. All core proteins are required as well as several nucleolar proteins of previously ill-defined or unknown functions such as Srp40p (Nopp140 in rodents) and Nsr1p (human nucleolin). The Ran cycle is not involved [191].

Nucleolar routing involves transit through the CB in plants and vertebrates and their recently identified homolog in yeast, the NB [193, 243, 301, 302] (Fig. 3.2-7).

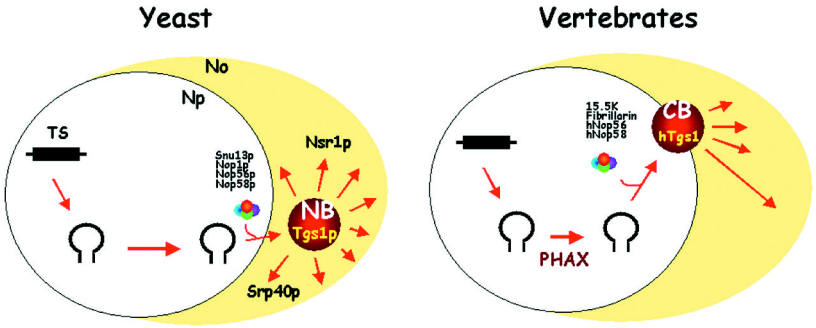


Figure 3.2-7 Intra-nuclear trafficking of box C+D snoRNAs. A comparison between the yeast and vertebrate systems is provided. Box C+D snoRNA nucleolar targeting involves transit through a conserved nuclear locale, the NB and CB, in yeast and vertebrates, respectively. The cap trimethyl-transferase (Tgs1p/hTgs1) is a specific antigen of this cellular compartment. SnoRNA nucleolar routing is a multi-step process. In mammals, PHAX, the phosphorylated adaptor for snRNA export, drives the snoRNAs from their transcription sites (TS) to the CB (E. Bertrand, pers. Comm.). SnoRNP assembly and cap-trimethylation presumably occur in the NB/CB. Np, nucleoplasm; No, nucleolus.

Overexpression of artificial box C+D snoRNAs in yeast led to their accumulation in a single, roughly spherical structure of ~200 nm in diameter always contiguous to the fibrillar component of the nucleolus [302]. This structure was highly reminiscent to the CBs, also often found in close association with the nucleolus and functionally linked to this nuclear locale ([24, 215, 250]; reviewed in Ref. [80]). Expression of a human GFP-SMN reporter construct (a CB-specific antigen) specifically co-localized with the NB strongly supporting this assumption. In addition, the cap trimethyl-transferase Tgs1p, specifically localized to the CB and NB in yeast and humans, respectively, providing further supporting evidences [301]. Most importantly, NBs were later detected with endogenous snoRNAs, in the absence of snoRNA overexpression, supporting the physiological importance of this novel nuclear compartment [301].

Survival of motor neurons (SMN) is the causative agent for spinal muscular atrophy, a neurodegenerative disease and most frequent genetic cause of infant mortality ([152]; reviewed in Refs. [76, 194]). SMN is present in multiple RNP complexes and notably interacts with core snoRNP proteins of both families (Nop1p and Gar1p), the human telomerase RNP and the human cap trimethyl-transferase, hTgs1 [10, 116, 185, 208]. In the best-described complex, SMN is associated with Gemins 2–6 and is involved in snRNP metabolism and pre-mRNA splicing ([209]; reviewed in Refs. [169, 203, 265]). In a Gemin 3 (a putative DEAD-box helicase), gemin 4 and eiF2C-specific complex, SMN has also recently been linked to the metabolism of the micro RNPs (miRNPs) [188].

The accumulation of snoRNAs in NBs on RNA overexpression suggested that nucleolar targeting is a saturable, multi-step process (Fig. 3.2-7); snoRNAs would first transit from transcription sites (TS) to NB/CB before being redistributed to the entire nucleolus. Both Nsr1p and Srp40p were involved in the emergence of the NB [302].

The first step in the nucleolar routing pathway has recently been successfully uncoupled from the subsequent nucleolar distribution and imaged in time-lapsed microscopy [27]. Transcription sites and CBs were relatively static as to their locations (at least within the time frame used, ~1 h); snoRNPs appeared to transit from TS to the vicinity of CBs within minutes but strikingly lagged for up to 60 min before being incorporated into this compartment [27].

PHAX (phosphorylated adaptor for snRNA export; [199]) is localized in the nucleoplasm and the CBs, binds specifically to box C+D 3′-extended precursors, and is able to target artificial RNA substrates from their transcription sites to CB, supporting a direct role for this protein in the first step of nucleolar routing (Fig. 3.2-7). PHAX interacts with the 15.5 K (human Snu13p) *in vitro* and contact the snoRNPs, at least in part in an hSnu13p-dependent fashion (E Bertand, pers. comm.). 15.5K is also present in the spliceosomel U4 snRNP (see Sect. 3.2.5.4), raising interesting questions as to the discrimination of snRNAs and snoRNAs for their trafficking. Studies on the U3 box B+C motif, which is also bound by 15.5 K, indicate that specific flanking sequences and/or structure, surrounding a conserved 15.5 K-binding site, probably provide the specificity for the recruitment of additional complex-specific proteins [92].

The recent identification of box C+D and/or box H+ACA containing small RNAs localized at steady-state in the CB [46], hence their name scaRNAs (small cajal bodies specific RNAs) and active in snRNAs modifications raise additional questions as to the presence of specific cis- or trans-acting determinants in these RNAs for CB retention.

3.2.6.5 **CB/NB are Conserved Sites of Small RNP Synthesis**

Our current view is that NBs/CBs are conserved sites of small RNPs biogenesis; maturation steps occurring in NBs/CBs include snoRNA cap trimethylation (presence of Tgs1p), snRNA internal modification (identification of the scaRNAs) and snoRNA 3′-end formation and snoRNP assembly (occurrence of unassembled 3′-end-extended snoRNA precursors and core snoRNP proteins).

3.2.7
Ribosome Intranuclear Movements and Ribosome Export

Once released from the nucleolus, pre-ribosomes transit through the nucleoplasm to reach the NPC. The precise mechanisms of ribosomes intranucle(ol)ar movements are unknown. This presumably occurs by diffusion and may involve unleashing the pre-ribosomes from successive nucle(ol)ar retention sites.

Interestingly, three related couples of proteins, originally identified in a large Pol I transcription-related nucleolar complex [66], have recently been involved in this process. In these, Noc1p (Mak21p), Noc2p (Rix3p), and Noc4p share a 45-amino-acid-long domain (Noc domain) [170, 171]. Noc2p organizes two distinct nucleolar complexes, Noc1p/Noc2p and Noc2p/Noc3p (a related nucleolar protein which does not show a Noc motif). The Noc complexes differ both in their intranuclear

localization and association with the pre-ribosomes. Noc2p/Noc3p is mainly nucleoplasmic and interacts with 66S particles; Noc1p/Noc2p is nucleolar-enriched and associates with the 90S and 66S pre-ribosomes [170]. The Noc1p/Noc2p and Noc2p/Noc3p complexes are required for pre-60S export. The Noc1p homolog, Noc4p, is associated with Nop14p (another unrelated nucleolar protein) [157]; the Noc4p/Nop14p complex is nucleolar, associated with 90S and presumably 43S pre-ribosomes and is involved in pre-40S export [171]. The dynamic intranuclear distribution of the Noc proteins (potential to shuttle between the nucleolus and the nucleoplasm) and their association with distinct species of pre-ribosomes supports a role in intranuclear movements.

A problem faced with many RRP mutants defective in ribosome export (also referred to as Rix, for ribosome export) is that they are, in addition, impaired in pre-rRNA processing. Typically, strains defective for pre-60S export show inhibitions in early pre-rRNA processing reactions (sites A_0–A_2). This suggests that efficient pre-rRNA processing is dependent on ongoing ribosome export. Most importantly, in this respect, overexpression of the Noc domain results in a dominant-negative phenotype for growth and nuclear accumulation of the pre-ribosomes in the absence of pre-rRNA processing defects [170]. In this case, pre-rRNA processing and transport defects were efficiently uncoupled, strongly supporting a direct involvement of the Noc proteins in intranuclear movement and nuclear exit of the ribosomes. Another RRPs, the ribosomal-like protein Rlp7p, has also been recently involved in pre-60S subunits release from the nucleolus [79].

Export assays based on microinjections in *Xenopus* oocytes and the use of isolated *Tetrahymena* nuclei concluded that ribosome nuclear exit is a unidirectional, saturable (involvement of trans-acting factors, including components of the NPC), energy- and temperature-dependent process [17, 88, 120, 218, 328]; subunits are believed to transit to the nucleoplasm independently.

In yeast, the intranucle(ol)ar accumulation of pre-ribosomes is either monitored *in vivo* by the use of fluorescent reporter RPs (e.g. Rps2p-eGFP, Rpl11p-GFP, and Rpl25p-eGFP) [78, 106, 171, 56] or on fixed samples by FISH (e.g., a probe specific to the 5′-portion of ITS1 has been used to follow pre-40S export) [189, 190]. Although none of these strategies is entirely satisfactory (the RPs assay relies on proper incorporation of the reporter constructs in strains that are also potentially defective for assembly; the FISH assay largely used a *xrn1*Δ strain that accumulates high levels of cytoplasmic 20S and/or ITS1 D-A_2 fragment but with a *plethora* of associated phenotypes in unrelated processes as diverse as mRNA turnover, microtubule function, DNA replication, telomere length, karyogamy, etc.; see discussion in Ref. [189]), they nevertheless succeeded in identifying a role in ribosome export for a subset of RPs, several nucleoporins, the Ran-system, as well as a, very large number of known or novel RRPs.

A well-characterized set of Rix proteins is the Rpl10p/Nmd3p/Xpo1p complex. Rpl10p binds late to the pre-60S ribosomes and interacts with Nmd3p, a nucleo-cytoplasmic shuttling protein which serves as a transport adaptor providing a leucine-rich nuclear export signal (NES) to the exportin Xpo1p/Crm1p ([78, 100, 255];

reviewed in Refs. [1, 115]); the RRP Rsa1p was involved in facilitating the loading of Rpl10p onto pre-ribosomes [129]. The Nmd3p-mediated pathway of LSU export is conserved in metazoans [267, 279]. It is most probable that additional such NES are provided, either directly or not, by the RPs. Consistently, Yrb2p, a Ran-GTP-binding protein required for the efficient export of NES-containing protein has recently been involved in 40S subunit export [190, 263]. In addition, a specific conditional inactivation of Mtr2p, which is required for mRNA export [233], led to the nuclear accumulation of pre-60S ribosomes and was synthetic lethal with Nmd3p [14]; the mechanism underlying these observation is not known at present.

Proteomic analyses of late nuclear pre-60S complexes revealed the presence of the Rpl10p complex as well as several RRPs that were also isolated in NPC purifications [14, 229].

Since the size of the NPC is just about enough (~20–25 nm in diameter) to accommodate that of individual ribosomal subunits (25–30 nm), it is anticipated that extensive remodeling is needed prior to, during passage through the pore, and following nuclear exit of such large RNPs. The recently identified AAA-ATPase Rix7p is a good candidate to be involved in such structural rearrangements [77].

How late pre-rRNP cleavage, modification and assembly are coupled to intranuclear movements and translocation of pre-ribosomes through the NPC is the subject of ongoing research.

3.2.8 The Cytoplasmic Phase of Ribosome Maturation

Following nuclear exit, both the small and large ribosomal subunits undergo final cytoplasmic maturation steps; these include structural rearrangements, the addition of late RPs, and possibly, late pre-rRNA processing and modification reactions. These steps underlie the long-standing observation that ribosomal subunits undergo a significant cytoplasmic lag before their incorporation into polysomes [133, 276, 282, 314]. The recent identification of the first trans-acting factors involved in these reactions led to an important novel concept in the field, several RRPs follow the pre-ribosomes to the cytoplasm and, at least for some of them, are recycled to the nucle(ol)ar pre-rRNA processing machinery [78, 100, 195, 240, 332]. Such nucleo-cytoplasmic shuttling was observed more than a decade ago for nucleolin/C23 and No38/B2, two important vertebrate nucleolar antigens [25]. However, the interpretation of these data was not clear at that time.

The 20S pre-rRNA is exported to the cytoplasm where cleavage at site D, by an unknown RRP, generates the 18S rRNA. A conclusion largely based on cell fractionation experiments [276, 282] and indirectly supported by the following concurring evidences: (i) strains deleted for the major cytoplasmic 5′–3′ exoribonucleolytic activity (Xrn1p) accumulates high levels of the D-A_2 fragment in the cytoplasm [189, 258]; (ii) strains genetically depleted for Rio1p or Rio2p, two putative protein kinases, accumulate increased amounts of cytoplasmic 20S pre-rRNAs [295, 296]; and (iii) deletion of the translation initiation factor eIF3j (Hcr1p) slightly impairs 20S pre-rRNA processing [286].

Although cleavage at site D is certainly very closely linked to small subunit export, it should be kept in mind that (i) Xrn1p works cooperatively with Rat1p in multiple nuclear reactions (see above) and, consistently, Xrn1p has recently been copurified with nucle(ol)ar pre-60S subunits [195], and that (ii) the D-A_2 fragment, or the 20S pre-rRNA (none of which has ever been directly detected in the cytoplasm in a wild-type strain, see Refs. [189, 190]) could be leaking through the NPC in *xrn1*Δ backgrounds or *rio* mutants. Furthermore, although mostly located in the cytoplasm, eIF3j is also detected within the nucleus. The formal possibility that cleavage at site D occurs shortly prior to nuclear exit or during passage through the NPC prevails. The Dim1p dimethylation was also reported to be a late, cytoplasmic event based on crude cell fractionation and fingerprint analysis [28, 127, 160, 161]. Nucle(ol)ar pre-rRNA precursors are dimethylated when pre-rRNA processing kinetics is altered [98, 139] and dimethylation too could still formally be a late nucleoplasmic event closely linked to export in wild-type strains.

Elongation factor-like 1 (Efl1p), a cytoplasmic GTPase homologous to the ribosomal translocases EF-G and EF-2, has recently been involved in nucleolar pre-rRNA processing at sites A_0–A_2 [240]. It turned out that in strains deficient for Efl1p, Tif6p (a nuclear protein involved in early pre-rRNA processing [16]) is mislocalized to the cytoplasm. We proposed that the pre-ribosomes exit the nucleus in association with Tif6p and that the latter is unleashed from the particles and allowed to recycle to the nucle(ol)us following a structural rearrangement mediated by the GTPase activity of Efl1p [240] (Fig. 3.2-8). The homology between Efl1p and ribosomal translocases further suggests that Efl1p may check on the pre-ribosomes that the binding sites for the

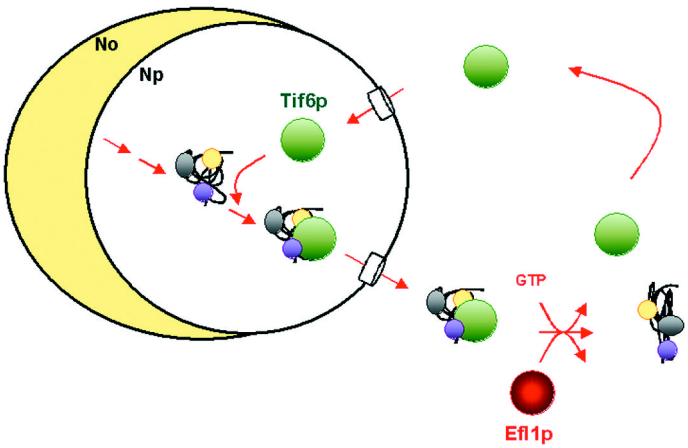


Figure 3.2-8 The cytoplasmic phase of ribosome maturation. Several RRPs follow the pre-ribosomes to the cytoplasm during the assembly process. A case is provided here for Tif6p. A structural rearrangement in cytoplasmic pre-ribosomes, mediated by the GTPase activity of Efl1p, is proposed to facilitate the release of Tif6p and its recycling to the nucle(ol)ar pre-rRNA processing machinery.

elongation factors have the proper configuration for interaction before ribosomes engage in translation. Furthermore, the nuclear exit of Tif6p has recently been shown to be dependent on phosphorylation [15]. Finally, Lsg1p/Kre35p, another cytoplasmic GTPase, may also be involved in recycling RRPs to the nucle(ol)us [118].

3.2.9
Regulatory Mechanisms, all along

Many examples of what we currently interpret as 'quality control' mechanisms have been provided here (coupling between early and late cleavages, the Dim1p dimethylation, involvement of Dhr1p in pseudoknot formation, Efl1p and late LSU structural rearrangement, Rlp's versus RPs binding, Noc's in intranuclear movements, Rix's in nuclear exit, etc.).

In most cases, 'quality control' steps potentially circumvent premature, irreversible events to occur such as to drive properly the pre-rRNPs from one assembly step to the next. To put it simply, cells have evolved complex strategies to keep the 'assembly line' in good order. In other instances, checkpoints possibly signal upstream processing events to abort the production of what would be unfaithful and non-productive synthesis. In wild-type cells, synthesis is presumably only delayed until the proper event occurs (i.e., RRP or RP binding, a specific structural rearrangement, a *clivage*, modification, or transport reaction).

3.2.10
And Now ... What's Next?

The next few years will undoubtedly refine the ribosomal assembly pathway. Much attention needs to be paid to the RPs; and as mass-spectrometry techniques develop, to quantitation of the various components in distinct pre-rRNP particles.

It is probable that several dozens of novel RRPs will be identified adding to the over increasing list of such factors and that, eventually, the endoribonucleases will uncover. Their identification may, however, await the availability of *in vitro* reconstitution assays.

It is quite surprising, considering the amount of work put into the functional characterization of the snoRNAs, that we still barely have a clue to what they do in pre-rRNA processing and ribosome assembly.

A major challenge will be to try to understand what the known RRPs are doing and, as further connections between ribosome synthesis and other biosynthetic pathways unfold, it will become essential to distinguish properly the primary versus secondary effects of these trans-acting factors.

It will also become necessary to better define the relationships between the various morphological subnucle(ol)ar compartments and the biochemical reactions that occur during ribosome synthesis.

Ribosome turnover has not been properly addressed yet. (Pre)-ribosomal assembly studies indicate that RRPs probably recycled but it is presently unclear whether this also applies to some mature ribosomal components.

Recent observations are suggesting the existence, in higher eukaryotes, of a nuclear translation-like mechanism [107]. Are the particles involved fully matured, considering the essential cytoplasmic synthesis steps described in yeast – these steps are not known to occur in humans? What is the relationship, if any, between this currently ill-defined process and ribosome synthesis? Does this add a further level of complexity in the assembly process through a connection with pre-mRNA metabolism?

3.2.11
Epilogue

It is becoming more and more evident that ribosome synthesis is fully integrated with respect to most other essential cellular pathways. The importance of these connections is only starting to emerge and so far evidences have been provided for a link to transcription, pre-mRNA splicing, mRNA turnover, translation and telomere function (see above), as well as to the secretory pathway [179, 180, 280] and the cell cycle (see, e.g., Refs. [58, 246, 247, 259, 305]). This is a promising and exciting area of research for the future.

Remarkably, two proteins encoded within rDNA or rDNA-like sequences (Tar1p and *Ribin*, respectively) have recently been identified; these are transcribed in the antisense direction with respect to 25S or 25S-like sequences [43, 119]. Yeast Tar1p is a mitochondrial protein that is capable of rescuing respiration-deficient strains. Mouse *Ribin* is linked to rDNA transcription; its expression is regulated by physiological changes. These are fascinating observations suggesting stringent coevolution between these short proteins (14 and 32 kDa for Tar1p and *Ribin*, respectively) and rDNA sequences and providing compelling evidences for a high level of integration between ribosome synthesis and other biosynthetic pathways.

3.3.12
Useful WWW links

>http://www.expasy.org/linder/proteins.html

- A comprehensive list of the yeast RRPs with a short description of their known or putative functions.

>http://www.pre-ribosome.de/; http://yeast.cellzome.com/; http://genome-www.stanford.edu; http://biodata.mshri.on.ca/yeast_grid/Servlet/Search Page

- A list of physical and functional interactions between RRPs and between RRPs and proteins involved in unrelated biosynthetic pathways. These mostly rely on data sets from extensive co-immunoprecipitation and two-hybrid schemes.

>http://www.umass.edu/molvis/pipe/ribosome/opinion/index.htm

- 3D maps of rRNA modifications.

>http://www.bio.umass.edu/biochem/rna-sequence/Yeast_snoRNA_Database/snoRNA_DataBase.html

- A most useful database of the yeast snoRNAs.

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