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Review

Mapping the cleavage sites on mammalian pre-rRNAs: Where do we stand?

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ABSTRACT

Ribosomal RNAs are produced as lengthy polycistronic precursors containing coding and non-coding sequences, implying that extensive pre-rRNA processing is necessary for the removal of non-coding spacers. Remarkably, this feature is conserved in all three kingdoms of life and pre-rRNA processing has even become more complex during the course of evolution. While the need for such extensive processing remains unclear, it likely offers increased opportunities to finely regulate ribosome synthesis and to temporally and spatially integrate the various components of ribosome synthesis. In this review we discuss our current understanding of pre-rRNA processing pathways in mammals (human and mouse), with a particular focus on the known and putative cleavage sites, and we compare it to budding yeast, the best eukaryotic model, thus far, regarding ribosome synthesis. Based on the emerging research, we suggest that there are likely more pre-rRNA processing sites and alternative processing pathways still to be identified in humans and that a certain level of functional redundancy can be found in the *trans*-acting factors involved. These features might have been selected because they increase the robustness of pre-rRNA processing by acting as "back-up" mechanisms to ensure the proper maturation of rRNA.

1. Introduction

The eukaryotic ribosome is a large, evolutionarily conserved ribonucleoprotein complex composed of four rRNA molecules (18S rRNA in the small subunit and the 25/28S, 5.8S, and 5S rRNAs in the large subunit) and approximately 80 ribosomal proteins. Three of the four rRNAs are transcribed by RNA polymerase (Pol) I in the nucleolus as a single polycistronic transcript comprising the 5′-external transcribed spacer (ETS)–18S rRNA–internal transcribed spacer (ITS) 1–5.8S rRNA–ITS2–25/28S rRNA–3′-ETS (Fig. 1). The 5S rRNA gene is transcribed separately by RNA Pol III [1,2]. Maturation of the pre-rRNA transcripts includes cleavage (processing) steps, chemical modification (pseudouridylation and methylation) of specific residues, assembly (folding of the RNA and association with proteins into pre-ribosomes), and transport (within the nucleus and through the nuclear pore complexes). All of these processes involve scores of protein *trans*-acting factors and small nucleolar (sno) RNAs [1,3] (see Box 1).

For two decades, research in the field of ribogenesis has been dominated by work on budding yeast. Thirty years of genetics, followed by the recent purification of pre-ribosomes, have led to the identification and characterization of about 200 ribosome synthesis factors; much fewer human ribosome synthesis factors have been characterized to date [4–6]. There are several reasons why ribogenesis is bound to be more complex in humans than in budding yeast. Firstly, human ribosomes are larger and contain additional ribosomal proteins and non-conserved rRNA extensions [7]. The mature human rRNAs are comparable in size to yeast, except for human 28S rRNA which has increased in size by ~1.5 fold. A greater difference in size is seen in the non-coding spacers, which underwent remarkable expansion, being extended 5-fold or more (see Fig. 1). The transcribed spacers contain a plethora of mono- and di-nucleotide repeats that act as hotspots for expansions potentially caused by replication slippage [8]. Secondly, human nucleoli, the sites of rRNA transcription and early pre-rRNA processing, have three, rather than two, subcompartments [9,10]. Thirdly, the human nucleolar proteome comprises at least ten times more proteins than its yeast counterpart (~200–300 estimated nucleolar proteins in budding yeast and up to ~4500 in human) [11,12]. Despite these differences, it was generally assumed, until recently, that extrapolation of the work carried out in yeast could be sufficient to understand how ribosome are synthesized and assembled in human cells, but this prediction is probably an

Abbreviations: Pol, polymerase; ETS, external transcribed spacer; ITS, internal transcribed spacer; RACE, random amplification of cDNA ends; RPS, proteins of the small subunit; PCR, polymerase chain reaction; siRNA, small interfering RNA.

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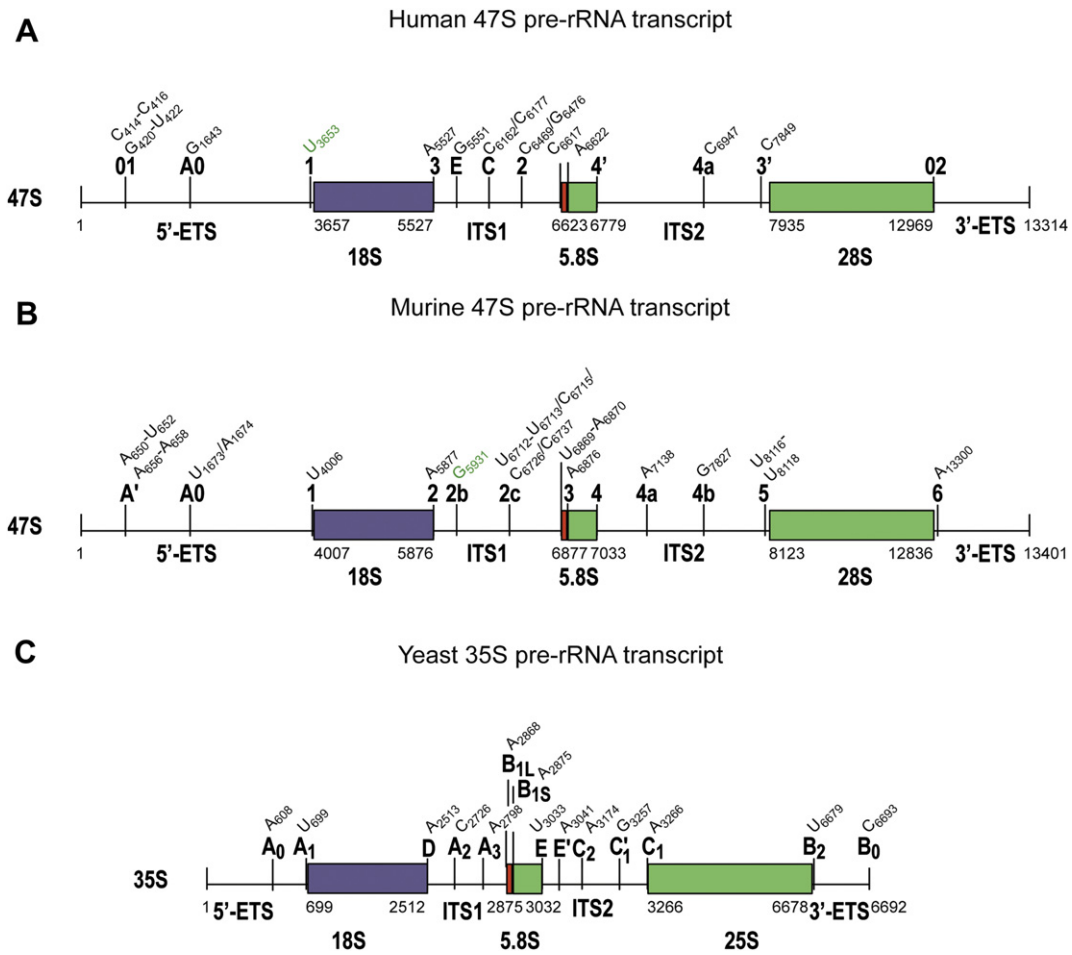

Fig. 1. Schematic of the human, mouse and yeast RNA polymerase I transcript. A, Human 47S pre-RNA; B, Mouse 47S pre-rRNA; C, Yeast 35S pre-rRNA. Three out of the four ribosomal RNAs, the 18S, 5.8S, and 25S (in yeast)/28S (in human and mouse), are encoded in a single long RNA Pol I transcript (47S in human and mouse; 35S in yeast). The fourth rRNA (5S) is synthesized independently by RNA Pol III (not represented). The coding sequences for the mature rRNAs are embedded in non-coding spacers, namely the 5′- and 3′-external transcribed spacers (5′- and 3′-ETS) and the internal transcribed spacers 1 and 2 (ITS1 and ITS2). Relative positions of known and predicted processing sites are indicated. The size of the mature rRNAs is largely conserved, except for the 28S which is about ~1.5 fold longer than the 25S. The size of the non-coding spacers has witnessed a dramatic expansion during evolution being up to ~5 fold larger in human than yeast. All processing sites were determined experimentally *in vivo*, except those color-coded in green, which were established in *in vitro* reconstituted systems.

oversimplification because of the frequent functional redundancy and increased complexity in higher eukaryotes.

Even though much is still unknown regarding the protein factors involved in pre-rRNA processing in humans, a number of the cleavage sites have been mapped over the last thirty years and a consensus processing pathway is available (Fig. 2A [13,14], reviewed in [4,15]). Using a combination of biochemical approaches, including Northern blotting, primer extension, and 3′ random amplification of cDNA ends (RACE) in combination with DNA sequencing techniques, processing sites have been mapped in the 5′-ETS, ITS1, ITS2, and 3′-ETS regions, yet recent analyses indicate that additional cleavage sites likely exist (e.g. [16–19]). In this review we discuss the known cleavage sites in mammalian (human and murine) rRNA transcripts, compare these sites to those known in budding yeast, and highlight the sites that require further study to resolve the location at the nucleotide level.

2. General overview of pre-rRNA processing in human cells

Pre-rRNA processing begins on the 47S primary transcript by snipping both ends of the molecule at sites 01 (sometimes called A′) and 02 in the 5′- and 3′-ETS segments, respectively, generating the 45S pre-rRNA (Fig. 2A). Previous studies indicated that the 45S precursor is matured following two major alternative pre-rRNA processing pathways [13,14]. In pathway 1, the initial cleavage occurs in the 5′-ETS at site A0 and is soon followed by cleavage at site 1. In pathway 2, the first cleavage event takes place at site 2 within ITS1. In cultured HeLa cells, the major contributor to ribosome synthesis is pathway 2. Each pathway offers an additional, optional "loop" that results from uncoupling at sites A0 and 1 (Fig. 2A). These "loops" generate the 43S and 26S pre-rRNAs in pathways 1 and 2, respectively. As far as we know, the two major pathways appear to differ not in the nature of the actual processing sites used but rather in the kinetics and order of cleavage. Similarly, two major pathways have been described in mouse (Fig. 2B). In addition to this post-transcriptional processing, it is quite possible that a fraction of the pre-rRNA molecules are cleaved co-transcriptionally, akin to the situation recently described in budding yeast. In fast-growing yeast cells up to 70% of pre-rRNA molecules are cleaved at site A_2, midway through ITS1 (see Fig. 3), while RNA Pol I is still actively transcribing the rDNA and approaching the 5′-end of the 25S gene [20–22]. Yeast site A_2 separates the precursors destined to be incorporated into the small and large subunit and is equivalent to site 2 in human and site 2c in mouse (Figs. 2 and 3). While co-transcriptional cleavage remains to be documented in mammals, we suggest that it might increase the overall efficiency of ribosome synthesis and, as such, be

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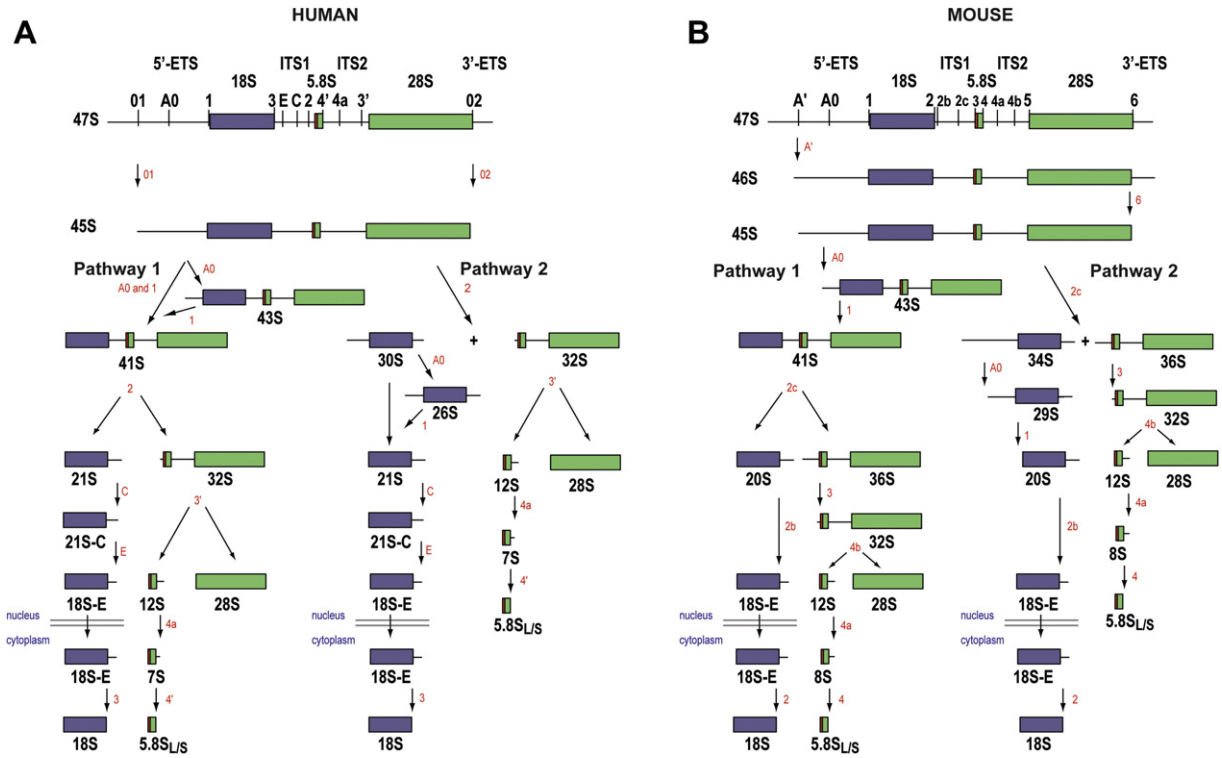

Fig. 2. Pre-rRNA processing in mammals. Simplified pre-rRNA processing schemes in human (A) and murine (B) cells. A, Pre-rRNA processing in human cells. The primary transcript, the 47S pre-rRNA, is initially cleaved at both ends of the molecule, at sites 01 and 02, generating the 45S precursor which is primarily processed by two alternative pathways. In pathway 1 processing is initiated in the 5′-ETS by concomitant cleavage at sites A0 and 1; whereas in pathway 2 processing starts in ITS1 at site 2. In pathway 1 the 41S pre-rRNA is cleaved at site 2 into 21S and 32S pre-rRNAs. Note that uncoupling at sites A0 and 1 (shown as a "loop") generate the 43S pre-rRNA. The 21S is successively trimmed to sites C and E; the latter processing site is situated only 24 nt downstream of the mature 18S rRNA 3′-end. The 18S-E pre-rRNA is exported to the cytoplasm where it is cleaved at site 3 to generate the 18S rRNA. In parallel with 21S processing, the 32S pre-rRNA is matured at site 3′ in ITS2, generating the 12S pre-rRNA and the 28S rRNA. The 12S is cut at site 4a into 7S and then at site 4′ to generate the 5.8S rRNA. Recent work indicate that there are additional processing sites both within ITS1 and ITS2 which are not represented here for simplicity (see text for details). There are two forms of 5.8S rRNA (short and long) differing by a ∼7 or 8 nt extension at the 5′ terminus (represented in red). In pathway 2, the 30S is either directly matured into 21S by simultaneous processing at sites A0 and 1, or through the formation of a 26S pre-rRNA intermediate, when sites A0 and site 1 are uncoupled (shown as a "loop"). B, Pre-rRNA processing in mouse cells. The primary 47S transcript is first cleaved in the 5′-ETS at site A′, generating the 46S pre-rRNA, which is then processed in the 3′-ETS at site 6, leading to the formation of the 45S (note that in HeLa cells, cleavages at sites 01 and 02 are strongly coupled temporally). In mouse cells, sites A′ and 6 are sufficiently uncoupled so as to allow the detection of the 46S pre-rRNA. Mouse 45S processing also proceeds mainly through two alternative pathways which are very similar to those described in human.

particularly pertinent to cells with enhanced growth properties, such as aggressive cancer cells. It is important to note that in *Xenopus*, both major alternative processing pathways have been shown to co-exist in a single cell [23]. It is also worth noting that there are conditions that favor alternative pre-rRNA processing pathways. For example, the order of cleavages in pre-rRNA is changed by a temperature sensitive mutation in BHK cells [24]. Moreover, the cleavage order is altered after mutations in U3 or U8 snoRNAs in *Xenopus* [23,25] (see below and Box 1). In addition, the order of cleavage has also been shown to vary according to species, cell type, physiological and developmental stages, and even, more recently, disease state [14,15,26,27]. In the following sections the processing steps are described in detail in the context of the nucleotide position of the cleavage sites. Throughout this manuscript, pre-rRNA processing sites are numbered with respect to the transcription start site (+1), unless stated otherwise. In Box 2, we discuss why we think that pre-rRNAs are universally processed.

3. Initial processing events: cleavage sites within the 5′- and 3′-external transcribed spacer segments

3.1. Generation of the 45S precursor: sites 01 and 02

Initial cleavage of the 47S pre-rRNA transcript occurs several hundred nucleotides downstream of the transcriptional start at site 01, equivalent to site A′ in the mouse rRNA transcript (Figs. 1 and 2). Site 01 was mapped by Kass et al. in the human rRNA transcript with total RNA isolated from human cervical cancer (HeLa) cells using S1 nuclease mapping to position ∼ C414-C416[1] [28]. A second cleavage site was identified 6 (±3) nucleotides (nt) downstream at residue G420-U422 (Fig. 1 and Table 1).

Primer extension and S1 nuclease analyses were used to map the murine primary cleavage site A′ *in vivo* with total RNA isolated from murine L1210 and Erlich ascites cells [28]. Primary processing of mouse rRNA was also examined *in vitro* using pulse-chase analysis of rDNA transcribed by T7 RNA Pol, followed by incubation with an S-100 extract of murine L1210 cells. Similar to the observations with HeLa cells, site A′ was shown to consist of two processing sites, separated by approximately 6 (±3) nt, around positions A650-U652 and A656-A658[2] (Fig. 1). This site was recently remapped *in vivo* by Kent et al. in murine NIH 3T3 fibroblast cells by primer extension analysis [29]. Several bands were detected; the predominant one corresponded to a transcriptional stop at A650 and minor bands were observed at C651, U655, A656,

[1] Numbering according to GenBank sequence U13369.1.

[2] Numbering according to the Pestov laboratory and Genbank sequence X82564. The boundaries of the 18S, 5.8S, and 28S mouse genes were identified by aligning the rRNA transcript against the human one.

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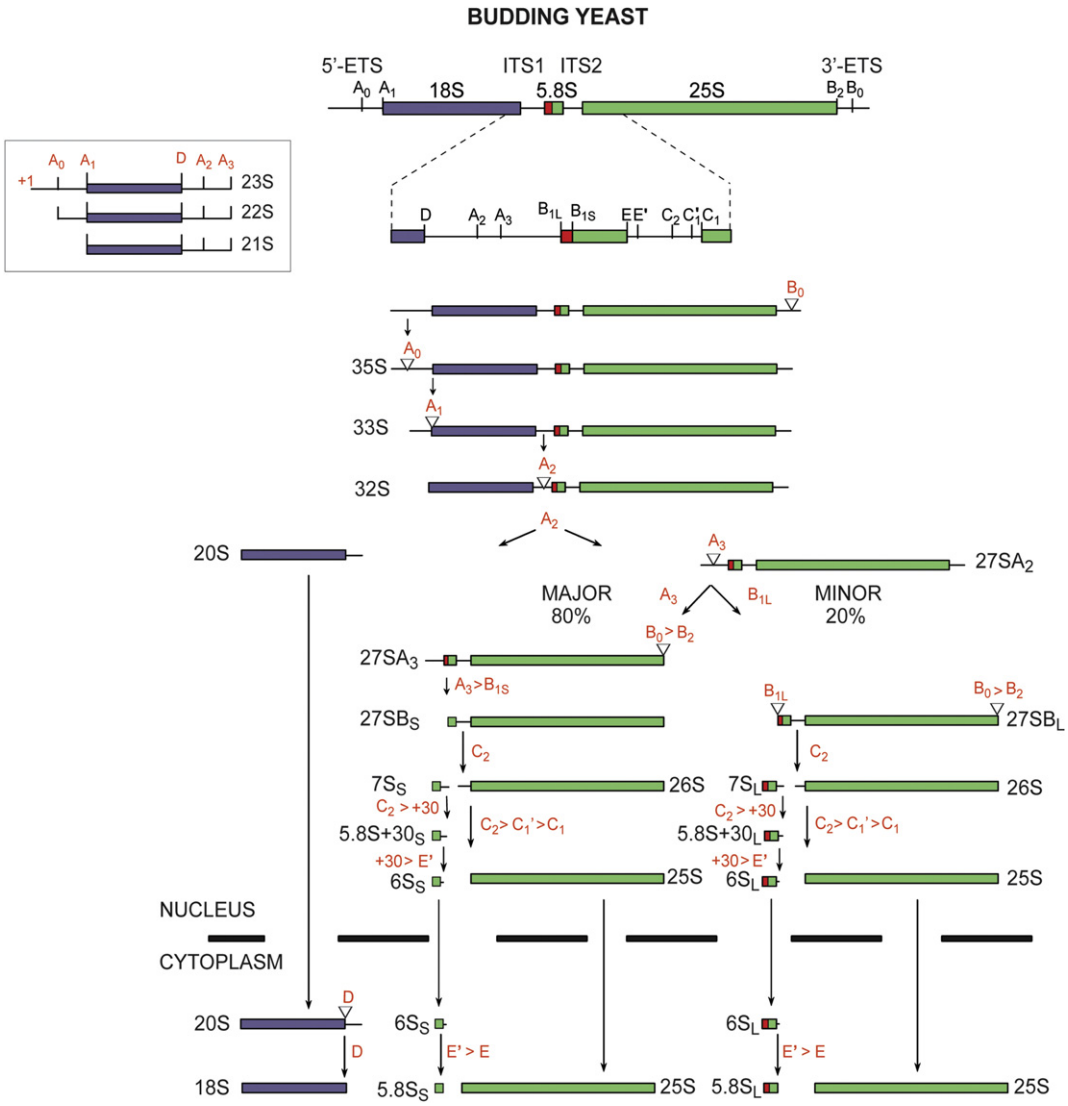

Fig. 3. Pre-rRNA processing in budding yeast. Full-length primary transcripts, 35S pre-rRNAs, are released by cotranscriptional cleavage by Rnt1 (homologous to *E. coli* RNase III) in the 3′-ETS at site B_0. Alternatively, nascent transcripts are cleaved co-transcriptionally in ITS1 at site A_2 (not represented). In fast-growing yeast cells, cotranscriptional cleavage in ITS1 occurs in up to 50–70% of cases. The 35S RNA is initially cleaved at sites A_0, A_1, and A_2 by the SSU-processome, a large snoRNP, primarily organized around the box C + D snoRNA U3, and visualized on chromatin spreads as "terminal balls". Recently, it was suggested that the PIN-domain protein Utp24 might be the elusive endoribonuclease responsible for cleavages at site A_1 and/or A_2 [51]. In an independent work, Rcl1 was also suggested as the endoribonuclease that carries out cleavage at site A_2 [110]. The resulting 20S and 27SA_2 pre-rRNAs are destined to become the small and large subunits, respectively. The 20S pre-rRNA is exported to the cytoplasm, where it is converted to 18S rRNA, following 3′-end endonucleolytic cleavage at site D, likely by Nob1. The 27SA_2 pre-rRNA is matured following two alternative pathways. This results in the production of two forms (short and long) of 5.8S rRNA, differing in size by about ~7/8 nucleotides at their 5′-ends. In the major pathway, representing ~80% of the molecules, 27SA_2 is endonucleolytically cleaved at site A_3 by RNase MRP and digested to site B_{1S} by the 5′–3′ exoRNases Rat1-Rai1 or Rrp17. The 5′–3′ exoRNase Xrn1 can also contribute to this step. In the minor pathway, corresponding to ~20% of cases, the 27SA_2 is cleaved endonucleolytically at site B_{1L} by an unknown RNase. Note that processing at sites B_1 and B_2 is concurrent. Both forms of 27SB pre-rRNA are cleaved at site C_2 within ITS2, generating the 7S pre-rRNA, precursors of the 5.8S rRNA, and 26S pre-rRNA, precursor of the 25S rRNA. The 7S pre-rRNA is digested to site E, corresponding to the 3′-end of 5.8S, by an extremely complex succession of reactions involving the core exosome, the nucleus-specific exosome subunit Rrp6, Ngl2, and the Rex exoRNases. Discrete intermediates corresponding to 5.8S rRNA precursors extended at their 3′-ends by ~7/8 nucleotides (site E′, 6S) are detected in wild-type cells. The final step in formation of the 3′-end of 5.8S rRNA occurs in the cytoplasm and is performed by Ngl2. The 26S pre-rRNA is digested to site C_1, the 5′-end of the 25S rRNA, by the Rat1-Rai1 complex or Rrp17. Xrn1 can also contribute to this step. Discrete intermediates corresponding to 25S rRNA precursors extended by ~7/8 (site C'_1) are detected in wild-type cells. The inset shows an example of aberrant RNAs resulting from altered pre-rRNA kinetics and uncoupling of cleavage at A_0 to A_2.

and G657. The latter three bands are in vicinity of the secondary cleavage site described by Kass et al. [28].

The results of the pulse-chase study of murine rRNA using an *in vitro* reconstituted system indicated that the two fragments generated by cleavage at site A′ were present simultaneously and that their relative abundance did not change over a chase period of 10–90 min [28]. This prompted the authors to propose that processing at site A′ consists of two concomitant events, rather than an initial cleavage reaction followed by a second one.

Interestingly, sites 01 in humans and A′ in mouse are located immediately upstream of a ~200-nt conserved region that shares 80% sequence homology between the two organisms [28]. This region is important for the initial cleavage and is involved in the binding of the box C + D snoRNA U3. In humans, U3 was shown to interact with the 5′-ETS region using *in vivo* psoralen cross-linking studies and quantitative hybridization selection/depletion experiments with rDNA clones and cross-linked nuclear RNA [30]. U3 was found to interact with a segment downstream of the primary cleavage site (at

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Table 1
Summary of data for the mapping of cleavage sites in human and mouse.

Spacer segment	Site		Nucleotide position		Cell line[a]		Technique		Reference	
	Human	Mouse	Human[b]	Mouse[c]	Human	Mouse	Human	Mouse	Human	Mouse
5′-ETS	01	A′	~C414–C416, G420–U422	~A650–U652, A656–A658	HeLa	L1210, Ehrlich ascites, NIH 3T3	S1 nuclease mapping (sequencing by Maxam and Gilbert)[d]	S1 nuclease mapping (sequencing by Maxam and Gilbert)[d] Primer extension (sequencing ladder) [d,e]	[28]	[28,29]
3′-ETS	02	6	N/A[f]	~A13300	N/A	Balb 3T3	N/A	High-resolution agarose gel electrophoresis	N/A	[37]
5′-ETS	A0	A0	~G1643	U1673/A1674	HeLa	NIH 3T3	Primer extension (sequencing ladder)	Primer extension, cDNA sequencing[g]	[39]	[29]
5′-ETS (5′ end of 18S)	1	1	U3653	U4006	HeLa, HEK293	L	2-dimensional RNase T_1 fingerprinting[h], secondary nuclease, primer extension (MW marker)	S1 nuclease mapping, sequencing by Maxam and Gilbert	[59,17]	[60]
3′ region of ITS1	2	2c	C6469–C6476	U6712–U6713/C6715 /C6726/G6737	HeLa	NIH 3T3-derived LAP3	Northern hybridization	Primer extension (sequencing ladder)	[61]	[62]
ITS1	E	2b	~G5551	~G5931	HeLa	*in vitro*	3′ RACE	S1 nuclease mapping (MW marker)	[39]	[69]
ITS1 (3′ end of 18S)	3	2	A5527	A5877	N/A	L	Transfection of human mini-genes in murine L929 cells. Northern hybridization, S1 nuclease mapping	S1 nuclease mapping, sequencing by Maxam and Gilbert	[72,74]	[61]
ITS1 (5′ end of 5.8S)	N/A	3	U6618, C6623	U6869/A6870, A6876	HeLa, HEK293	L	Primer extension (MW marker)	S1 nuclease mapping, sequencing by Maxam and Gilbert	[79,74]	[60]
3′ region of ITS2	3′	4b	~C7849	G7827	HeLa	L, NIH 3T3-derived LAP3 cells	Northern hybridization	Northern hybridization	[14]	[60]
5′ region of ITS2	4a	4a	~C6947	A7138	UT7-Epo	L	Northern hybridization	S1 nuclease mapping, fingerprinting, sequencing, Northern hybridization	[19]	[60,83–85]
ITS2 (5′ end of 28S)	N/A	5	N/A	U8116–C8118	N/A	L	N/A	Northern hybridization, S1 nuclease mapping, sequencing by Maxam and Gilbert	N/A	[60]

[a] HeLa, human cervical cancer; L1210, lymphocytic leukemia; Erlich ascites, tumor; NIH 3T3, embryonic fibroblast; Balb 3T3, embryonic fibroblast; HEK293, human embryonic kidney cells; mouse L cells, fibroblast; UT7-Epo, megakaryoblastic leukemia cell line.

[b] Numbering according to GenBank sequence U13369.1.

[c] Numbering according to the Pestov laboratory and Genbank sequence X82564. The 5′- and 3′ termini of the 18S, 5.8S, and 28S were identified by aligning the murine 47S transcript against the human sequence.

[d] Processing was examined *in vitro* by Kass et al. (1987) using an S-100 extract from murine L1210 cells and *in vivo* using L1210 cells and Ehrlich ascites. Processing in HeLa cells was examined *in vivo*. The reaction products were migrated alongside a sequencing ladder generated using the Maxam and Gilbert method and the authors corrected for a migration difference of 1.5 nt between the bands in the sequencing ladder and those generated by the S1 nuclease and primer extension mapping, owing to the different ends generated during the mapping (3′OH) and chemical sequencing (removal of the terminal residue and addition of a 3′P) procedures.

[e] *In vivo* processing at mouse site A′ was also mapped in NIH 3T3 cells by Kent et al. (2009) using primer extension analysis and the products were migrated alongside a sequence ladder generated using SequiTherm EXCEL™ II (Epicentre).

[f] N/A, not applicable or data not available.

[g] An anchor was ligated to the 3′ end of RNA obtained from murine NIH 3T3 cells, fragments were amplified by reverse transcriptase (RT) PCR with primers complementary to the region between position 1441 in the 47S transcript and the 3′ anchor, and the cDNA was cloned and sequenced.

[h] Precursor RNA was synthesized *in vitro* using plasmid DNA as a template for transcription by SP6 polymerase. RNase T_1 cleaves 3′ of G residues. The following enzymes were used the secondary nuclease analysis: pancreatic RNase A (cleaves 3′ of Y); RNase U2 (cleaves 3′ of A residues); nuclease P1 (cleaves 5′ of N); and RNase T_2 (cleaves 3′ of N).

~414/415 nt downstream of the transcriptional start) between nt +438 and +695 [30]. In budding yeast, the catalytic activity that carries out the initial cleavages (sites A_0, A_1, and A_2, see Fig. 3) is still unknown but is strictly dependent upon U3 [31]. In yeast, U3 contacts pre-rRNA at multiple positions in the 5′-ETS (upstream of site A_0) and within the 18S rRNA sequence (at the 5′-ETS/18S rRNA boundary and at the site of central pseudoknot formation more than >1 kb downstream within the 18S rRNA) (discussed in [32,33]). Importantly, the base-pairing interaction between U3 snoRNA and the 5′-ETS was also nicely worked out in *Xenopus* [34,35], and that work in *Xenopus* oocytes further indicated that cleavage at site A′ is not mandatory in all organisms [36].

In mouse there is evidence from several studies suggesting that the 47S precursor is cleaved first at site A′, generating a 46S species, and then at site 6 (corresponding to 02 according to the human rRNA nomenclature), producing the 45S species (Fig. 2) [37,38]. The "45S" fraction of RNA labeled with either [^{3}H] or [^{14}C] uridine obtained from L5178Y mouse lymphoma cells was separated by high resolution gel electrophoresis; the fraction was separated into three distinct peaks, designated "47S", "46S", and "45S" [38]. In the same study examining the labeled "45S" fraction from HeLa cells only two major peaks were revealed [38]. A subsequent study of rRNA from mouse 3T3 cells indicated that the mouse "45S" species consisted of three species of about 13.9 kb (47S primary transcript), 13.3 kb (46S, following cleavage at site A′), and 12.8 kb (45S, following cleavage at site 6) in size [37], suggesting that murine site 6 is in the vicinity of position A13300. While it is likely that the cleavage order is first A′ and then site 6 in the mouse transcript, it is unclear at this stage whether in humans the cleavages at sites 01 and 02 occur simultaneously or sequentially. In yeast, pre-rRNA processing in the 3′-ETS is initiated co-transcriptionally by cleavage at site B_0 by Rnt1 (homologous to *E coli* RNase III) and cleavages at sites A_0, A_1 and A_2 are closely coupled (Fig. 3, and see below section 5.4).

3.2. Generation of the 43S species: site A0

Investigation of 18S rRNA maturation by Rouquette et al. [39] revealed a second cleavage site, A0, within the 5′-ETS segment of the human rRNA transcript, located between sites 01 and 1 (the 5′-end of the 18S rRNA); this site was mapped to position ~G1643 by primer extension analysis. Cleavage of the 45S precursor at site A0 generates a 43S species. Under normal conditions, cleavage at sites A0 and 1 are tightly coupled, directly producing the 41S pre-rRNA. A low level of 43S is detected, however, by pulse-chase analysis in control HeLa cells [40], indicating that it is a normal intermediate. It is striking that the 43S accumulates in an aggressive cancer model cell line (the breast adenocarcinoma cell line MCF-7) that had been transfected with small interfering RNA against ADP-ribosylation factor-like 2 [27], as well as in HeLa cells depleted for a number of ribosomal proteins of the small subunit (RPS), including those encoded by *RPS2*, *RPS3*, *RPS10*, *RPS17*, *RPS20*, *RPS21*, *RPS26*, *RPS27a*, and *RPS29* [41]. This indicates that under those conditions, cleavages at sites A0 and 1 are sufficiently uncoupled so that the 43S RNA becomes detectable (see also "loops" in Fig. 2A).

Cleavage site A0 was also mapped in murine NIH 3T3 embryonic fibroblast cells [29]. Primer extension analysis revealed two major transcriptional stops at positions U1673 and A1674. The position of the site was confirmed in cells depleted for ExoSC10 (Rrp6 in yeast), a subunit of the exosome that degrades some of the cleaved spacer fragments. An anchor was ligated to the 3′ end of RNAs obtained from these cells, fragments were amplified by reverse transcriptase PCR, and the cDNA was cloned and sequenced. The longest amplified product corresponded to cleavage at position U1673. It is worth mentioning that historically within the vertebrate group processing at site A_0 was first identified in *Xenopus*, a model organism that often offered breakthrough observations when it comes to pre-rRNA processing studies [42].

In budding yeast, a single cleavage site, designated A_0, was identified in the 5′-ETS (Figs. 1 and 3) [43]. Cleavage at sites A_0, A_1, and A_2 are tightly coupled in yeast. However, similar to some extent to the situation in HeLa cells (accumulation of 43S and 26S), some uncoupling is possible; e.g. upon depletion of the box H + ACA snoRNA snR10, or in cells expressing a truncation of Mpp10 (a U3-associated protein), or upon depletion of the 18S rRNA dimethyl transferase Dim1 [44–46]. Uncoupling, or delaying, early nucleolar cleavages in yeast generates aberrant RNA species extending from the +1 transcriptional start site to site A_3 (23S), from site A_0 to A_3 (22S), and from site A_1 to A_3 (21S) (Fig. 3, see inset). In principle, these aberrant RNAs are not faithfully processed to 18S rRNA – at least not quantitatively, as they are hardly detected by pulse-chase labeling in unperturbed wild-type cells. For the 23S RNA, it was indeed shown that it is rapidly degraded by a nucleolar surveillance pathway involving the addition of short poly-A tails at its 3′-ends by TRAMP complexes, followed by digestion by the exosome (e.g. [21,47–49]). There are conditions where the 23S RNA can be processed into 18S rRNA: for example, when a subgroup of U3-associated protein, the so-called UTP-A subcomplex, is absent (which slows down the initial steps of ribosome assembly) and when nucleolar surveillance is inactivated ([21]; discussed in [50]). However, in such conditions and despite a restoration of pre-rRNA processing, the cells are still defective for growth, which suggests that encroaching the assembly of defective ribosomal precursors otherwise destined for degradation does not allow the generation of functional ribosomes [21]. It has also been suggested that the 23S RNA is a normal pre-rRNA intermediate, which is faithfully processed into mature 18S rRNA possibly through the successive formation of the 22S, 21S and 20S (see e.g. [51,52]). Finally, note that RNAs equivalent to the yeast A_0 to A_3 species and to the A_1 to A_3 species have also been detected in *Xenopus* (here called 19S and 18.5S, respectively) where they are found in low amounts in unperturbed cells and increased amounts after U3 snoRNA alterations [42].

What exactly the ribosomal proteins do during the assembly process is not known. They are thought to stabilize secondary structures in the rRNA, promote the formation of tertiary structures, contribute along with the assembly factors to folding the pre-rRNA in higher order structure competent for cleavage and prevent particle misfolding [53,54]. Recently, proteins of the small ribosomal subunit were systematically investigated for their requirement in pre-rRNA processing both in cultured human cells and in budding yeast [41,55–57]. It was shown in HeLa cells that 32 ribosomal proteins fall in two distinct categories with half of them being required for the initial cleavages, and the other half for cleavages further downstream in the processing pathway [41,55]. The involvement of ribosomal proteins in early or late cleavages correlates well with the expected timing of their incorporation into pre-ribosomes; a conclusion which is evolutionarily supported by work on budding yeast [56,57]. That the general principle of subunit assembly in successive intermediary modules is an evolutionarily conserved feature is also supported by the observation that the bacterial homologs of the proteins involved in early cleavage steps in eukaryotes were characterized as primary binders in *in vitro* reconstitution assays in prokaryotes ([58] and references therein), while homologs to those involved in later cleavage steps are known as secondary or tertiary binders (discussed in [41]).

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4. Maturation of the 18S species: cleavage sites in the 5′-ETS and ITS1 regions

4.1. Initial processing at sites 1 and 2

4.1.1. Site 1

Historically, several pre-rRNA processing sites, particularly those used early, were mapped *in vitro* in reconstituted systems. Maturation of the 5′-end of the 18S rRNA, site 1, was examined *in vitro* in HeLa cell nucleolar extracts using 5′- or 3′-end labeled RNA transcribed by SP6 Pol [59]. Processing *in vitro* was found to consist of an initial endonucleolytic reaction followed by exonucleolytic trimming. Three cleavages corresponding to site 1 processing were identified using two-dimensional RNase T_1 fingerprinting and secondary nuclease analysis. Two major sites were mapped in the 5′-ETS at 8 and 3 nt upstream of the start of the 18S sequence, and a minor one was identified 1 nt into the mature 18S sequence, corresponding to positions U3648, U3653, and U3657, respectively. Strikingly, all three sites are located 5′ to the adenosine residue (in bold) in the sequence motif U**A**CCU, repeated three times in tandem. The authors incubated purified 3′ end-labeled products corresponding to RNA cleaved at positions −8, −3, and +1 (with respect to the start of the 18S gene sequence) in HeLa nucleolar, nuclear, and cytoplasmic extracts. Only the product cleaved at position U3653 (3 nt upstream from the start of the 18S sequence) was accurately processed *in vitro* by components of the HeLa cytoplasmic extract to form the mature 5′ terminus of the 18S rRNA at position U3657 [59].

Morello et al. recently re-examined the 5′ terminus of the 18S sequence *in vivo* in human embryonic kidney (HEK) 293 cells by primer extension analysis [17]. Extension of a primer complementary to positions 3685-3714 (nt 29-58 of the mature 18S sequence) yielded two products differing in size by ~1 nt and migrating at the expected size for U3657.

The position of site 1 was also identified in mouse L cells by mapping the 5′ terminus of the 41S species and the 3′ end of the 24S fragment (the 5′-ETS-containing spacer fragment resulting from cleavage of 45S at site 1, not represented in Fig. 2), using an S1-nuclease protection assay [60]. To determine whether the cleavage at site 1 was the result of an endonucleolytic cut or of trimming from an upstream site, the authors mapped the 3′ terminus of the 24S fragment and recovered 12 major bands, one of which abutted the 5′ terminus of the 18S sequence at position U4006, suggesting that processing at site 1 is the result of an endonucleolytic activity and the remaining 5′ ETS fragment is digested 3′ to 5′ [60].

4.1.2. Site 2, separating the RNAs destined to the small and large ribosomal subunits

In HeLa cells, site 2 was narrowed down on 21S pre-rRNA to a position lying between C6469 and G6476 by differential Northern blot hybridization [61]. This is consistent with a former mapping also carried out in HeLa cells on 26S and 21S pre-rRNA, which placed site 2 more broadly between A5687 and C6613 [39].

Cleavage at site 2c in mouse transcripts ultimately produces the 20S species, equivalent to the human 21S. The location of 2c was recently mapped in murine NIH 3T3-derived LAP3 cells using primer extension analysis of total RNA isolated from cells depleted in Xrn2, a 5′–3′ exonuclease. Reverse transcription of a primer complementary to positions 6876-6895 generated several stops at U6712-U6713/C6715/C6726/G6737 [62].

4.2. Site C, from 21S-C to 18S-E

The recent characterization of *Bystin*, a novel human pre-40S ribosome synthesis factor homologous to yeast Enp1, in HeLa cells led to the identification of shorter versions of the 21S pre-rRNA for which the name 21S-C was coined [16] (Fig. 2A). The 3′-end of 21S-C was mapped by differential Northern blot hybridization and 3′ RACE and shown to largely correspond to positions +635 and +650 (ITS1 numbering, corresponding to positions G6162 and C6177 in the full length transcript). Notably, 21S-C was also detected in the nuclear fraction of unperturbed control HeLa cells, indicating that it is, indeed, a *bona fide* processing intermediate [16].

siRNA-mediated depletion of *RPS19* also led to the accumulation of shorter forms of 21S, similar to the situation described for *Bystin* [61]. Here, the novel intermediate was tentatively named “20S” pre-rRNA [which might lead to some confusion as it is equivalent neither to the mouse nor to the yeast 20S pre-rRNAs, see Figs. 2B and 3]. The 3′-end of this so-called “20S” pre-rRNA was mapped to positions +755/+766 (ITS2 numbering, C6282/C6293 in 47S, not represented in Figs. 1,2) making it a moderately longer species than 21S-C. Unlike 21S-C, the human “20S” species was not detected in untreated cells but rather only in cells depleted for *RPS19* [16,61]. On the other hand, this “20S” was readily detected in pulse-chase analysis, attesting of its “precursor-product” relationship to mature 18S rRNA [61].

Finally, Morello et al. [17] performed a primer extension analysis on total RNA extracted from HEK293 cells and demonstrated that a primer specific to positions 6299–6324 was extended by 5–9 nt, suggesting the presence of a processing site in the vicinity of C6290 and C6294, which the authors named site 2b (according to murine nomenclature, not represented in Figs. 1,2). The position of site “2b” identified by Morello et al. [17] precisely corresponds to the 3′-end of the “20S” RNA described by Idol et al. [61] (see above). From this, one can infer that site “2b” in humans, as described by Morello et al. is not, in fact, equivalent to murine site 2b. In HEK293 cells, site “2b” was detected in untreated cells and shown to require the *trans*-acting factor Nip7 [17]. The suggestion is thus that the 21S-C results from the progressive 3′–5′ digestion of the 21S pre-rRNA, which is consistent with the detection of intermediate species with somewhat heterogeneous 3′-ends, such as the “20S” detected by Idol et al. [61] and RNAs ending at site “2b” identified by Morello et al. [17].

4.3. Final maturation takes place in the cytoplasm: sites E and 3

Processing site E was identified by Rouquette et al. while characterizing the kinase Rio2 [39]. 3′-RACE analysis on total RNA extracted from HeLa cells transfected with siRNAs against Rio2 mRNA, which led to the accumulation of an 18S-E species, positioned site E at approximately nucleotide +24 nt within the ITS1 segment (corresponding to G5551 on the primary transcript) [39]. As discussed above for 21S-C, the 18S-E pre-rRNA was readily detected in untreated control cells, making it a genuine intermediate. The 18S-E is more abundant than the 21S-C because it has a longer half-life. 80% of 18S-E was found to accumulate in the cytoplasmic fraction in both HeLa and murine L929 cells indicating that the final maturation of the 18S rRNA in humans and mouse occurs in the cytoplasm, as had previously been shown in *Saccharomyces cerevisiae* [63–65]. Interestingly, 18S-E was not detected in polysomes. This is unlike the situation in yeast where a fraction of the 20S pre-rRNA (the immediate precursor to the 18S rRNA, see Fig. 3) has been detected in polysomes [66]. The functional role of the yeast 20S species in polysomes remains unclear, since late ribosomal assembly factors clearly hinder functional ribosomal sites [67].

HeLa cells depleted of either of four individual ribosomal proteins from the small subunit (*RPS18*, *RPS19*, *RPS21* or *RPSA*) are defective for 21S to 18S-E conversion and, quite interestingly, the resulting abortive pre-40S ribosomes are no longer released from the nucleolus into the nucleoplasm [41]. In HeLa cells, the 18S-E

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RNA was shown to accumulate in the cytoplasm upon depletion of most members of the class of late-acting ribosomal proteins of the small subunit [39,41] (see above paragraph 3.2). For *RPS2*, *RPS15* and *RPS17* the 18S-E was enriched in the nucleus indicating a further requirement for pre-40S export [39,41]; depletion of yeast *RPS15* also led to the nuclear enrichment of the 18S rRNA precursor (here called 20S pre-rRNA, see Fig. 3) [56,68].

It was previously shown *in vitro* in mouse that the ITS1 segment is cleaved 55 nt downstream of the 3′-terminus of the mature 18S sequence at position G5931 using S1 nuclease-mapping of *in vitro*-synthesized transcripts [69]. It was suggested that this cleavage is carried out by an endoribonuclease that is also active in the nucleolus [69]. This cleavage site might be equivalent to human site E.

Just as the 21S pre-rRNA is likely progressively trimmed down to 21S-C by 3′−5′ exoribonucleolytic digestion, site E might also be produced by exoribonucleolytic digestion, as the 18S-E species sometimes appears as a "fuzzy" smear on gels ([16,39,41,55] and our unpublished data). In yeast, the 20S pre-rRNA is directly converted to the 18S rRNA by endoribonucleolytic cleavage at site D, presumably by the PIN-domain-containing endoribonuclease Nob1 [70]. The released D-A_2 fragment is readily detectable as a discrete band in cells inactivated for the 5′−3′ exoribonuclease Xrn1, which normally degrades it [71]. The human genome encodes a putative ortholog of Nob1, so the detection of heterogeneous 3′-extended 18S precursors suggests that human cells potentially follow a strategy involving direct cleavage, like in yeast, and have adopted additional, redundant strategies that rely on exoribonucleolytic digestion to generate the 3′-end of 18S rRNA.

Processing of the 3′-end of the 18S rRNA in humans was examined using truncated 3′-18S-ITS1 sequences inserted into constructs containing mouse promoter and termination elements [72,73]. These "mini-genes" were transfected into murine L929 fibroblast-like cells and expressed. Northern hybridization analysis and S1 nuclease protection assays demonstrated that the mini-genes were accurately processed at the 18S-ITS1 junction at position A5527 [72,73].

In mouse, the 3′-end of 18S rRNA, called site 2 (see Fig. 1B), was mapped by S1 nuclease protection assays in L cells using nuclear and cytoplasmic pre-18S species in [60]. The site was found to be located at position A5877.

5. Processing of the large subunit components: cleavages in the ITS segments

5.1. Maturation of the 5′ end of the 5.8S rRNA

Heindl et al. and Morello et al. recently remapped the 5′-end of 5.8S rRNA by primer extension in HeLa and HEK293 cells, respectively [17,74]. In both analyses, two products were identified, differing in size by ~7–8 nt, indicating that in humans, like in yeast, there are two versions of the mature 5.8S rRNA that differ at their 5′-terminus; a long (designated $5.8S_L$) and a shorter form ($5.8S_S$). At present, it is not clear whether these two forms of 5.8S rRNA harbor distinct functions. Heindl et al. reported that the two forms of 5.8S end respectively at position U6618 and C6623 ([74]). In yeast, the ratio of short to long forms is generally ~80:20, but shows some variation [18]; in HeLa cells, this ratio is between 60:40 and 70:30 [18,74].

The 5′ terminus of the murine 5.8S rRNA is also heterogeneous. The termini were mapped in mouse L cells using S1 nuclease mapping and sequencing by the Maxam and Gilbert method and were found to be at positions U6869/A6870 and A6876 for the $5.8S_L$ and $5.8S_S$ forms, respectively [60].

5.2. Processing of the 3′-terminus of the 5.8S gene

5.2.1. Generation of the 12S intermediate: cleavage at 3′

Cleavage in the 3′-region of ITS2 generates the 12S species, the longest known precursor of the 5.8S rRNA. The 3′-end of the 12S was characterized in HeLa cells by Hadjiolova et al. by differential Northern blot hybridization [14]. The probing indicated that the 3′ terminus of the 12S species lies at approximately nucleotide +1070 of the ITS2 region (corresponding to ~ C7849 on the 47S pre-rRNA). In mouse L cells, S1 nuclease mapping of the 3′ terminus of the 12S fragment was used to determine the location of site 4b which was mapped to approximately G7827. No fragment was detected whose 5′ end abutted the 12S species [60], which is compatible with the 5′-end of the 28S rRNA being formed by 5′−3′ exoribonucleolytic digestion.

5.2.2. Generation of the 7S intermediate: processing at 4a

Farrar et al. [19] examined defects in 60S subunit biogenesis in the megakaryoblastic leukemia cell line UT7-Epo upon depletion of large subunit ribosomal protein RPL35a. In addition to the 12S, another 3′-extended precursor of the 5.8S rRNA, the 7S, was detected in an untreated control by Northern blotting with a probe targeting the 5′-end of ITS2 (complementary to positions 6794–6824). The detection of the 7S pre-rRNA indicated the existence of an additional processing site within ITS2, which the authors designated site 4a. The 7S was lost upon Rpl35a depletion. Farrar et al. estimated the 7S pre-rRNA to be of ~325 nt, which would position its 3′ end at around nucleotide C6947.

In addition to site 4a there are likely additional processing sites within human ITS2, as recently illustrated by Schillewaert et al. ([18]; see also [75–78]). A detailed analysis by high resolution Northern blotting of total RNA extracted from HeLa cells depleted of either the exosome subunit ExoSC10 (yeast Rrp6), or the exosome cofactor Skiv2L2 (yeast Dob1/Mtr4) indicated that formation of the 3′-end of the 5.8S in humans proceeds mainly via progressive 3′−5′ exoribonucleolytic trimming, similar to the situation in yeast. Interestingly, many 3′-extended forms of 5.8S rRNA detected in cells deprived of either ExoSC10 or Skiv2L2 were readily detected in untreated control cells. In particular, 5.8S precursors 3′-extended in the ITS2 by ~40/50 nt and by ~170/200 nt were particularly abundant in cells depleted for ExoSC10 and Skiv2L2, respectively [79]. The size of the 5.8S rRNA precursor extended by ~170/200 nt is compatible with that of the 7S species described by Farrar et al. The presence in untreated control cells of the shorter species, being extended by only ~40/50 nt, indicates that there is at least another processing site involved in the formation of the mature 3′-end of 5.8S rRNA [18]. Additional exoribonucleases, such as Eri-1 and Isg20L2 have also been shown to contribute to the formation of the 3′-end of the 5.8S rRNA [80–82], and the challenge is now to delineate a complete pathway of maturation and establish how these proteins relate functionally to each other.

In mouse, a 12S precursor ending at position G7827 and an 8S precursor ending at position A7138 were identified by S1 nuclease mapping, fingerprinting, sequencing and hybridizations [60,83–85]. The expected cleavage sites for the rodent 8S and 12S RNAs sites lie in highly conserved structural elements of ITS2 [84], which make it tempting to extrapolate these cleavage sites to their equivalent positions in human; these rodent positions would correspond to nucleotide A6879 (for 8S) and G7578 (for 12S) in the human sequence. The predictive value of this analysis, however, appears to be limited, since the proposed positions do not exactly correspond to the experimentally determined 3′-ends of human 12S and 7S (see above). Interestingly, Joseph et al. [84] showed in their phylogenetic analysis that yeast cleavage site C_2 (which generates yeast 7S, see Fig. 3) is located in the same conserved stem

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in ITS2 as rodent site 4b (which generates rodent 12S), making these two processing sites likely equivalent.

5.3. Maturation of the 5′ terminus of the 28S

It is interesting to note that a nuclear precursor of the 28S rRNA with an extension of 4–6 nt at its 5′-end was identified early on in mouse using S1 nuclease mapping, suggesting the presence of a cleavage site between positions U8116-U8118 [60]. This precursor might be related to the recently discovered yeast 25S′ pre-rRNA corresponding to the 25S rRNA extended in 5′ by ~7 or 8 nt (C_1′ -like) (discussed in [18] and see Fig. 3).

5.4. Maturation of the 3′ terminus of the 28S: processing at site 02

Currently it is thought that in mammals the generation of the 3′-end of the 28S rRNA occurs following direct cleavage at site 02, which happens at the same time or soon after cleavage at site 01 (see section 3.1). It has been shown in *Xenopus* and in mouse that this cleavage requires the displacement of U8 from the 5′-end of the 28S rRNA [86,87]. In mouse, the putative DEAD box helicase Ddx51 and its interacting partner the GTPase Nog1 have recently been shown to contribute to displacing U8 from pre-ribosomes [87].

In budding yeast, cotranscriptional cleavage at site B_0, carried out by Rnt1 (yeast RNase III), releases the primary transcript [88,89]. Processing at site B_2, generating the mature 3′-end of the 25S rRNA, is then delayed and occurs concomitantly with the formation of the 5′-end of 5.8S rRNA [89].

6. Concluding remarks: where do we go from here?

There are currently several pathways available in the literature describing the processing of human and murine pre-rRNA transcripts. These pathways are schematics representing a simplified, and likely caricature, overview of the actual processing steps. Recent research indicates that novel intermediates and processing sites are currently being identified in mammals. It is also clear that several processing sites in human require re-mapping to achieve identification with a nucleotide-level resolution. Moreover, some cleavage sites in the human transcripts are occasionally referred to using the mouse nomenclature (e.g. site 01 in the 5′-ETS is also referred to as A′ or 0 by some authors, and site 3′ is sometimes referred to as 4b), some cleavage sites were named similarly across eukaryotes but are not equivalent, and likewise, some pre-rRNA species were named identically across species but represent different intermediates. One major challenge ahead is to construct a comprehensive processing pathway that includes all physiologically-relevant intermediate species. In addition, it would be beneficial to consider a standard nomenclature for existing cleavage sites in human rRNA transcripts and to propose guidelines for the designation of newly identified sites and intermediate species. This requires a joint effort between the research teams involved.

A conserved feature of the eukaryotic pre-rRNA processing pathways is the use of alternative, “back-up”, routes. It is tempting to speculate that the relative use of such alternative pathways, or “loops”, might be modulated according to cell type, physiological state and/or developmental stages. A tantalizing hypothesis is that the relative use of these alternatives and the respective kinetics of some cleavage reactions might be altered under specific disease situations. In support of this idea, it was recently reported that in an aggressive cancer model cell line, a cryptic processing pathway is activated leading to the generation of the 43S pre-rRNA [27]. This study also showed that under these circumstances, pre-rRNAs are differentially methylated, and ribosomes with altered translational capacities (decreased fidelity and reduced IRES-dependent translation) produced [27]. Of similar interest, global defect in rRNA pseudouridylation was also shown recently to affect translation fidelity in yeast and mammals, and to impair IRES-dependent translation [90]. This raises the exciting possibility that what we have coined “renegade” ribosomes might accumulate under pathological conditions, implying that these aberrant ribosomes have somehow escaped the surveillance mechanisms that normally degrade defective ribosomes.

Box 1. A quick recap on snoRNAs

Ribosomal RNA is extensively modified, mostly by scores of snoRNAs. SnoRNAs are short (60–300 nucleotides long) stable RNAs that localize within the nucleolus at steady-state [91]. The vast majority of snoRNAs act as anti-sense guides in RNA modification carrying either *Fibrilarin*, a 2′-O ribose methyltransferase (box C + D snoRNAs) or *Dyskerin*, a pseudouridine synthase (box H + ACA) to the site of RNA modification. It was suggested that during evolution, a strategy involving snoRNPs rather than proteinaceous enzymes, might have allowed for testing many more nucleotide positions for possible selective advantage upon modification as, intuitively, it seems easier to evolve new complementarities within an RNA guide rather than to develop a new protein-only enzyme with topological specificity for each new site of modification [92]. Although rRNA modifications are restricted to coding sequences and strikingly cluster around functional ribosomal sites [93,94], their precise function in ribosome synthesis and ribosome function has only started to be unveiled. It is now evident that rRNA modification influences translation performance, and therefore, growth. For example, wild-type yeast cells outcompete cells defective for several rRNA pseudouridylation around the peptidyl transferase centre on the large subunit [95] (see also: [96–98]). Global rRNA pseudouridylation was recently shown to be important for the binding of ligands to the ribosome (e.g. the binding of tRNAs), as well as for translation fidelity and IRES-dependent translation [90] and global methylation is now also known to be important for IRES-dependent translation [27,99]. In addition, few snoRNAs are involved in pre-rRNA processing (e.g. U3, U8, U17, U22 in mammals; U3, U14 and snR10 and snR30 in yeast) and it is understood that this also requires base-pairing with the pre-rRNAs. In vertebrates, U8 is involved in ITS2 processing and 3′-ETS removal and U22 in 18S rRNA formation [25,86,100,101]. For U8, it was shown that its involvement in pre-rRNA processing involves its transient base-pairing with the 5′-end of the 28S rRNA, followed by its displacement to allow formation of a highly conserved stem between the 3′-end of 5.8S rRNA and the 5′-end of 28S rRNA, as found in mature 60S subunits [86]. The suggestion is that U8 contributes to regulating the timing of ribosome assembly. Several years ago, it was proposed that the few snoRNAs involved in pre-rRNA processing might provide a function in *trans*- that was originally provided in *cis*- by the long complementary spacer sequences elements that flank the coding regions in Bacteria and Archaea and which brings together the mature ends of the rRNA molecules (discussed in [102] and [103]). It is worth bearing in mind that snoRNAs involved in rRNA modification also offer strong potential for bringing together sequences distant on pre-rRNA molecules through Watson-Crick base-pairing, and thereby for folding precursor RNA into defined conformations, possibly contributing to making them “competent” for RNA cleavage. It is even possible that on occasions the rRNA modification itself might be a mere side-product of such

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a requirement of snoRNAs in pre-rRNA folding. As a testimony to their ancient origin and deep-rooted nature in the RNA world, snoRNA-like molecules have been shown to be processed from Archaeal tRNA introns [104,105], and snoRNAs are bound by core proteins using strategies similar to those extensively used within the ribosome (K-turn). The future holds promises when it comes to snoRNAs as some of them have recently been shown to be processed into microRNAs [106] and there are emerging evidences that point to their role in cell fate and oncogenesis [107].

Box 2. Why rRNAs are matured?

It is not clear at this stage why rRNA, and in fact most cellular RNAs, are produced as precursors rather than mature molecules. We, and others, have discussed that the requirement for a succession of multiple steps and the involvement of numerous *trans*-acting factors, with sometime the absence of just one stalling the whole assembly process, likely allows the fine regulation of the synthesis of mature rRNA termini. It was also suggested that pre-rRNA processing might integrate the various ribosome assembly reactions, such as RNA folding, RNA modification, protein binding, ribonucleoprotein particle structure remodeling and pre-ribosome transport by providing precise window frames for these reactions to occur, i.e. acting as "quality control". Put simply, pre-rRNA processing might act as a timekeeper, a "metronome": cleavages in the spacer occurring according to a strict timetable might provide an efficient and robust means of keeping the ribosome "assembly line" in check and slowly assembling particles, which are not cleaved in a timely fashion, targeted to degradation pathways [108]. The complementary view that RNA cleavage affords the energy stored in phosphodiester bonds to the RNP assembly process, and that it imparts directionality (rRNA cleavages are irreversible) to the assembly pathway has also been discussed ([5,109]).

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