Comment

The Road to Ribosomes: Filling Potholes in the Export Pathway

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The genetic material of eukaryotes is surrounded by a nuclear envelope, which acts as a barrier to the free exchange of proteins and nucleic acids between the nucleoplasm and cytoplasm. Although the resulting physical separation of transcription and translation provides cells with a level of control of gene expression not available to prokaryotes, it also demands the presence of a transport system to selectively move macromolecules between the two compartments. Such a system must be capable of discriminating between a wide variety of structurally and functionally distinct molecules and may also respond to differing growth and environmental conditions.

The biosynthesis and transport of ribosomes in a rapidly growing cell (such as the yeast *Saccharomyces cerevisiae*) presents a dramatic paradigm for the magnitude of the problems faced by the nucleocytoplasmic transport system. Each mRNA encoding a ribosomal protein must first be transported to the cytoplasm, where it is translated. The resulting ribosomal proteins are then imported into the nucleus and then to the nucleolus, where they associate with newly synthesized ribosomal RNA (rRNA)[1]. The preribosomes, so formed, then undergo a series of complicated modifications before they are exported back to the cytoplasm, to perform their function in translation. In a rapidly growing culture of yeast that double their ribosomal content every 1.5 h, each cell must import at ~150,000 ribosomal proteins per minute across the nuclear envelope (while simultaneously exporting ~4,000 ribosomal subunits per minute; Warner, 1999).

Tremendous recent advances in methodology have allowed us to trace a sketchy sequential path through the components and mechanisms of ribosome biogenesis (Kressler et al., 1999). This path begins with the production of ribosomal proteins, continues via their import into the nucleus and association with nascent rRNA in the nucleolus, and heads out towards the nuclear envelope again during the still ill-defined steps of preribosomal subunit maturation. However, until now, the path has had a glaring pothole: the proteins specifically mediating ribosomal subunit export have remained elusive. In this issue, insight into this process has been gained from studying the process of ribosome biogenesis in yeast (Ho et al., 2000a). This finding has identified players in this export process, and has potentially linked the final stages of ribosomal subunit maturation with their nuclear export, underscoring the continuum of the ribosome life cycle from its birth and maturation in the nucleus to its functional role in translation in the cytoplasm.

The Nuclear Transport System

Transport of proteins and RNA between the nucleus and cytoplasm is accomplished by soluble transport factors that bind their cargoes and carry them through numerous pores embedded in the nuclear envelope. Within the pores are huge macromolecular assemblies, termed nuclear pore complexes (NPCs), that act as the gatekeepers of the nucleus (Wente, 2000). NPCs freely allow the diffusion of small molecules (such as water and ions), but exclude all macromolecules above the diffusion limit size (~9 nm) except those carrying specific nucleocytoplasmic targeting sequences. Thus, proteins carrying a nuclear localization sequence (NLS) can be specifically imported through the NPC, while macromolecules to be exported from the nucleus harbor nuclear export sequences (NESs). The signals are recognized by karyopherins (abbreviated as kaps, and also known as importins, exportins, and transportins; Mattaj and Englmeier, 1998). Kaps bind to the import or export signals in their cargoes, mediate the docking of these cargoes to the NPC, and chaperone them through the pore. The GTPase Ran is also required for nucleocytoplasmic exchange. Ran is maintained by cofactors in its GTP-bound state in the nucleus, and in its GDP-bound form in the cytoplasm. This distribution sets up an energy gradient across the NPC, powering nucleocytoplasmic transport, and can also be used by transport factors to determine which side of the NE they are on. Thus, the formation of an import complex between a kap and its cargo is stable in the presence of cytoplasmic Ran–GDP, but in the nucleoplasm, Ran–GTP triggers its disassembly. In contrast, the formation of an export complex is stabilized in the nucleus
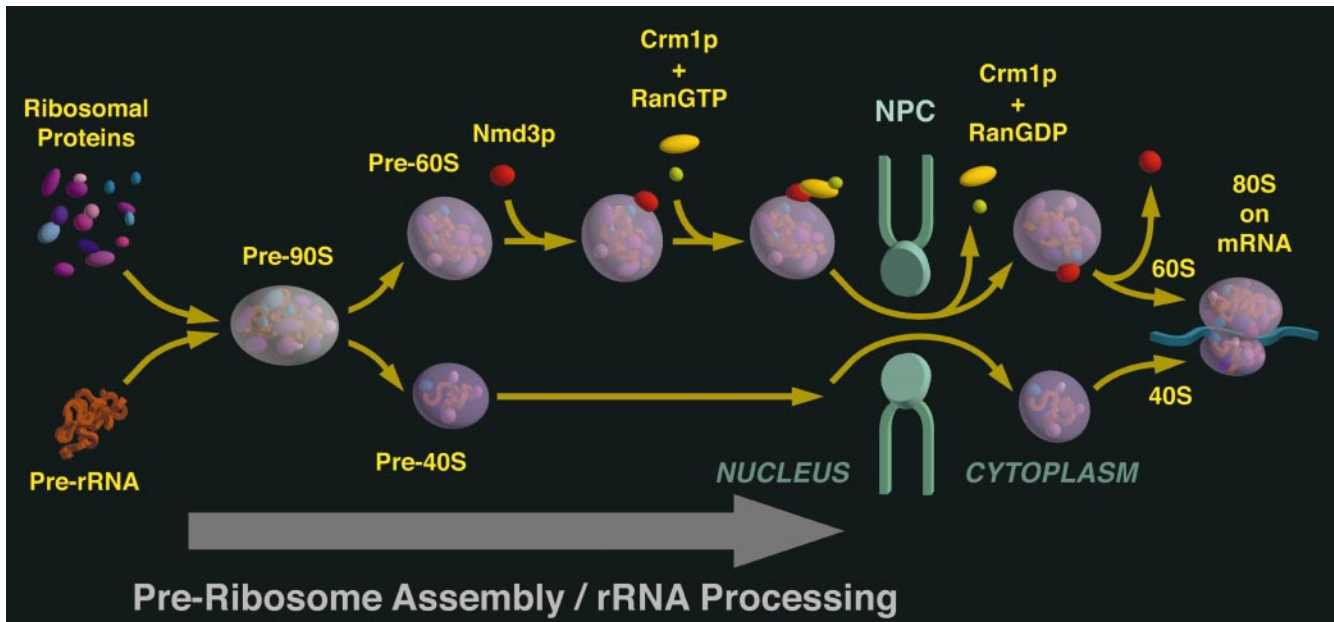
Figure 1. Diagram of ribosome biogenesis in *Saccharomyces*, and a model for 60S subunit export from the nucleus. See text for details.

by Ran–GTP, but as this complex reaches the cytoplasm, the GTP on Ran is hydrolyzed and the complex disassembles. It is now clear that there are distinct transport pathways, using distinct cognate kaps. In yeast there are at least 14 structurally related karyopherins that mediate the import and export of different classes of molecules, and this number is likely to be substantially higher in metazoans (Wozniak et al., 1998). Thus, many different but partially redundant and overlapping transport pathways all converge at the NPC. Among the most heavily trafficked pathway is the export of ribosomal subunits.

The Ribosome

Ribosomes are one of the most fundamental complexes common to all organisms. Descendants of an ancient RNA world, they are primarily composed of a catalytically active rRNA skeleton upon which ∼80 proteins are assembled. It seems that the primary role of most proteins in the ribosome (at least for the large subunit of the bacterium *Halarula marisomortui*) is stabilization of the three-dimensional structure (Ban et al., 2000). However, it is also likely that many proteins associated with the ribosome are involved in directing it both temporally and spatially through the many steps in its biogenesis. Ribosomal proteins, like other proteins, are synthesized in the cytoplasm. Although many of them are small and thus below the theoretical exclusion limit of the NPC, they are nonetheless actively imported by kap-mediated processes. In yeast, this is accomplished primarily by Kap123p, but it is evident that the process is redundant and other karyopherins can pick up much of the slack in the absence of Kap123p (Rout et al., 1997). Upon import into the nucleus, ribosomal proteins associate with newly transcribed rRNA within the nucleus to generate a preribosomal particle.

Because the mechanism of ribosome export is difficult to separate from its biogenesis, it is important to appreciate the complexity of this process. In yeast, there are >60 trans-acting factors necessary for efficient ribosome biogenesis (Kressler et al., 1999). The majority of these protein and small RNA factors are located within the nucleolus, but they are also present in the nucleoplasm, the cytoplasm, and even at the NPC. The precise roles of many of these are unknown, and some carry out other nuclear functions (for example, pre-mRNA splicing). Furthermore, it is not clear when and where all the ribosomal proteins assemble onto the rRNA. However, it is clear that the assembly of preribosomes is intimately coordinated with rRNA maturation and transport, and can be considered a continuum of successive steps leading from the sites of nucleolar rRNA transcription through the nucleoplasm to the NPC and beyond (reviewed in Kressler et al., 1999) (see Fig. 1).

In all eukaryotic cells, three of the four rRNAs are transcribed as a single RNA polymerase I transcript, which is extensively spliced, trimmed and otherwise covalently modified, to yield the 18, 5.8, 25/28S rRNA species. The fourth (5S RNA) is also heavily modified, but is transcribed by RNA polymerase III, and recruited separately to the assembling ribosome. Initially, the primary RNA polymerase I transcript assembles with many different large and small subunit proteins to form a pre-90S particle. The transcript is subsequently cleaved to yield precursors of the 60S and 40S subunits. These are then apparently separately exported to the cytoplasm. The pre-40S particle undergoes further cytoplasmic maturation of its 20S rRNA to yield the mature 18S rRNA, whereas it is believed that the rRNA within the 60S particle matures just before export. In the cytoplasm, the 40S subunit threads onto the 5′ end of the mRNA and scans to the initiation codon, where this translation initiation complex waits for the 60S subunit to attach and begin translation. The 60S subunit must undergo a number of cytoplasmic maturation events before it can carry out translation. This fine tuning likely involves the addition of several proteins, including Rpl10p. Addition of the 60S subunit to the translation complex is also coordinated with the release of factors, including

eIF2 from the initiation complex. In the absence of efficient 80S ribosome formation, for example due to insufficient numbers of functional 60S subunits, the 40S subunit stalls leading to the accumulation of halfmers. These kinetic intermediates of polysomes with a stalled 40S-containing initiation complex are characterisitic of 60S biogenesis defects.

Fundamentals of Export

Studying the process of ribosome export in vivo has been complicated by the fact that the biogenesis is a series of consecutive steps. Thus, alteration of any particular step leads to a backlog in the assembly process. Perhaps because of this, some of the most fundamental principles underlying ribosome export were established by microinjection of radioactively labeled ribosomal subunits into the nucleus of *Xenopus* oocytes (Bataille et al., 1990). These and other studies established that export is energy dependent, receptor-mediated, and not competed by other major export pathways (tRNA, snRNA, and tRNA) (Mattaj and Englmeier, 1998). However, in this system it is difficult to identify the cellular factors that specifically mediate the transport. One recent key advance was the development of a GFP-tagged ribosomal protein that faithfully integrates into ribosomes, and thus can be used to monitor the movement of preribosomes out of the nucleus in yeast (Hurt et al., 1999). These studies demonstrated a dependence of ribosome subunit export on Ran and proteins regulating its GTP-bound state. Furthermore, temperature sensitive mutants of the Ran cycle lead to aberrant rRNA processing mutants (Cheng et al., 1995). In addition, such studies identified specific nucleoporins (Nup49p, Nic96p, and Nsp1p) that seem to be required for ribosome export (Hurt et al., 1999). Together, these data suggested that members of the karyopherin family are involved in the export process, but fell short of identifying the factor.

Now, studies by Ho et al. (2000b) provide compelling evidence that Nmd3p acts as an adapter between 60S ribosome subunits and the nuclear export pathway mediated by the karyopherin Crm1p (Fig. 1). Nmd3p is a nonribosomal protein that has an essential role in late stages of large ribosomal subunit biogenesis, and is highly conserved from archea to metazoans, but absent from eubacteria. This is particularly interesting, given that archaeal ribosome biogenesis and translation share many features with eukaryotic processes and are distinct from eubacterial mechanisms. In yeast, Nmd3p is cytoplasmic and when immunoprecipitated, yields apparently mature 60S subunits from the cytoplasmic pool (Ho et al., 2000b), suggesting that it functions in a highly conserved cytoplasmic phase of either the 60S biogenesis process or in the formation of the translation complex, perhaps (for example) in coordinating the joining of the 40S and 60S subunits. This possibility is supported by the observation that mutants in Rpl10p, which are defective in subunit joining, are suppressed by overexpression of wild-type NMD3. To identify eukaryotic-specific functions for the Nmd3p, Ho et al. (2000a,b) concentrated on a COOH-terminal extension found within the yeast protein but absent from the archeal orthologues. Interestingly, expression of a truncated version of Nmd3p lacking this extension (Nmd3Δ100) gave a dominant negative phenotype (see also Belk et al., 1999); the mutant protein accumulated in the nucleus, where it bound to 60S subunits and impaired

their export. The export defect was observed both by the nuclear accumulation of GFP-tagged version of the large ribosomal subunit protein Rpl25p, and by the accumulation of cytoplasmic halfmers as detected by sucrose gradient centrifugation analysis. Further studies showed that the truncated protein still contains an NLS directing it to the nucleus, but lacks a putative leucine-rich NES, the likes of which are exported from the nucleus by the karyopherin family member Crm1p (Stade et al., 1997). Furthermore, the addition of an exogenous NES (but not a mutant NES) to the truncated protein restored its function. The authors then show that Nmd3p shuttles between the nucleus and cytoplasm, that its export requires the canonical NES-substrate export factor Crm1p, and that it can form a complex with Ran–GTP and Crm1p. Together, these data suggest that Nmd3p binds to 60S subunits in the late nuclear stages of its biogenesis, and through an NES tethers the preribosomal subunit to the Crm1p nuclear export pathway.

Of course, results from the dominant negative studies do not directly demonstrate that Nmd3p is required for 60S subunit export, nor that Crm1p actually mediates the export of 60S subunits through Nmd3p. If these were so, then it might be expected that recessive mutations such as temperature sensitive yeast mutants of either *crm1* or *nmd3* would lead to a 60S subunit export defect. Curiously this is not the case. In fact, Hurt's group directly tested this possibility with a strain carrying a temperature-sensitive allele of *crm1*, and found no such defect (Hurt et al., 1999). Furthermore, Johnson's group found no nuclear accumulation of 60S subunits in *nmd3* mutant cells. However, absence of the effect is also not conclusive. The problem with yeast mutants is that they can have pleiotropic effects, and the effects can be masked by other phenotypes. This is particularly relevant in complex interacting pathways like those of nuclear transport and ribosome biogenesis. Thus, it has been demonstrated that temperature shifts such as those used to induce the restrictive phenotype of the *crm1* temperature sensitive strain also result in a transient reduction in ribosome biogenesis (Warner, 1999), potentially explaining the lack of an export phenotype in these cells. Similarly, the *nmd3* mutant strains also cause a drop in ribosomal subunit production, presumably upstream of the export step in the ribosome biogenesis pathway, again perhaps accounting for the inability to detect any nuclear subunit accumulation (Ho and Johnson, 1999). On the other hand, experiments using a drug-sensitive version of Crm1p have perhaps provided more direct and less pleiotropy-prone evidence for its involvement in ribosomal subunit export. Leptomycin B (LMB) is an antibiotic that binds to Crm1p and inhibits its ability to bind to NES-containing proteins (Fornerod et al., 1997). Normally, yeast Crm1p is LMB-insensitive, but the introduction of a single cysteine in the protein renders it susceptible to the drug (Neville and Rosbash, 1999). Addition of LMB to the LMB-sensitive Crm1p strains, but not the insensitive controls, led to the accumulation of both Nmd3p and pre-60S particles in the nucleus (Ho et al., 2000a); the result expected if Crm1p mediates the export of the Nmd3p-60S preparticle complex from the nucleus. Thus the model proposes that Nmd3p associates with the pre-60S subunit at the late stages of its nuclear assembly, licensing it for nuclear export. The Nmd3p NES is then bound by Crm1p, which also binds Ran–GTP. Crm1p then chaper-

ones the subunit across the NPC to the cytoplasm, where the Ran–GTP is hydrolyzed to Ran–GDP, causing both Crm1p and Ran to dissociate. The subsequent loss of Nmd3p from the 60S particle would be expected to be one the penultimate stages before the formation of a fully translation-competent ribosome (Fig. 1).

What Now?

Ribosome subunit export has been the focus of much study, and the results from Johnson's group point the finger directly at Crm1p and Nmd3p as primary mediators of 60S subunit export. However, many gaps remain to be filled to fully understand the role of Nmd3p in the ribosome life cycle. For example, it is curious that mutations in *nmd3* do not lead to an accumulation of ribosomal subunits. As discussed above, this can be explained by the instability of the subunit, but it may also point to other redundant transport pathways or factors that can compensate for the loss of Nmd3p or even Crm1p. In the case of protein import into the nucleus, this is almost certainly the case (Rout et al., 1997), and so an analogous situation of redundant pathways for ribosomal subunit export should be given serious consideration. It also remains to be shown how Nmd3p interacts with the ribosome, and how it interfaces with both ribosome biogenesis and nonsense-mediated mRNA decay. A clue may come from physical evidence that Nmd3p interacts with Upf1p, a component of a multiprotein complex that mediates this decay (Belk et al., 1999). The genetic interaction between Mex67p, a mediator of mRNA export (Segref et al., 1997), and Nmd3p also provides a link between mRNA and ribosomal biogenesis. There are emerging data hinting that 40S subunit export also uses some factors involved in other karyopherin-mediated transport pathways (Moy and Silver, 1999). It will be interesting to determine if the principles established for the 60S subunit apply to the 40S subunit, and what factors distinguish the two pathways.

Another major remaining question is: how do the ribosomal subunits physically traverse their biogenesis pathway? As proposed for mRNPs, ribosomes may simply diffuse along this pathway, from their site of synthesis in the nucleolus through the nucleoplasm to the NPC (Politz et al., 1999). However, electron microscopy evidence has suggested that the subunits may actually be exported along tracks to the cytoplasm (Leger-Silvestre et al., 1997). In addition, RNA helicases have been detected in association with the NPC. Perhaps, as in the case of large mRNA molecules, they are partially unwound and threaded through the NPC channel. More mysterious still is the question of how this translocative pathway was retrofitted onto the ribosome during the evolution of eukaryotes from their protoplasmically unsegregated prokaryotic ancestors. Ribosomes may be old, but they've certainly not gone simple.

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