Nuclear RNA export

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Summary

Eukaryotic cells export several different classes of RNA molecule from the nucleus, where they are transcribed, to the cytoplasm, where the majority participate in different aspects of protein synthesis. It is now clear that these different classes of RNA, including rRNAs, tRNAs, mRNAs and snRNAs, are specifically directed into distinct but in some cases partially overlapping nuclear export pathways. All non-coding RNAs are now known to depend on members of the karyopherin family of Ran-dependent nucleocytoplasmic transport factors for their nuclear export. In contrast, mRNA export is generally mediated by a distinct, Ran-independent nuclear export pathway that is both complex and, as yet, incompletely understood. However, for all classes of RNA molecules, nuclear export is dependent on the assembly of the RNA into the appropriate ribonucleoprotein complex, and nuclear export therefore also appears to function as an important proofreading mechanism.

Key words: Gene expression, mRNA, Nuclear pore complex, Nuclear RNA export

Introduction

Unlike prokaryotes, eukaryotic cells segregate the vast majority of their RNA and protein synthesis into two distinct cellular compartments, i.e. the nucleus and the cytoplasm. This division necessitates nucleocytoplasmic transport pathways that can rapidly and specifically transport newly made macromolecules from their site of synthesis to their site of use. In addition, this subdivision also implies the existence of communication systems that can allow the nucleus and cytoplasm to respond in concert to changes affecting the cell. It has, in fact, now become clear that regulated changes in the subcellular localization of specific proteins form a key component of the cellular response to the activation of many signaling pathways (Hood and Silver, 1999).

Although the nucleocytoplasmic transport of proteins and RNAs share many features in common, and indeed all nuclear RNA export is protein mediated, here I nevertheless focus exclusively on the subset of nuclear export pathways used by different classes of RNA molecule. Although I deal primarily with nuclear RNA export in metazoan cells, it is clear that these export pathways are highly conserved among eukaryotes and I will therefore also rely extensively on data obtained in the yeast *Saccharomyces cerevisiae*, which has proven to be a powerful genetic system to identify critical components of several nuclear RNA export pathways. Readers interested in the mechanisms underlying protein nuclear export and, particularly, import are directed to reviews in this area (Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999).

Fundamental aspects of nucleocytoplasmic transport

With the exception of the short period of nuclear envelope breakdown that accompanies mitosis in higher eukaryotes, all macromolecular transport between the nucleus and cytoplasm must occur via nuclear pore complexes (NPCs) (Allen et al., 2000; Ryan and Wente, 2000). Although the average nucleus present in cultured human cells is decorated with ~4000 NPCs, this number is strongly influenced by cell size and by the level of biosynthetic and proliferative activity. Vertebrate NPCs, which display an eightfold rotational symmetry in electron micrographs, are large, ~125 MDa structures embedded in the nuclear membrane. NPCs feature eight fibrils that extend ~50 nm into the cytoplasm and also feature a nuclear basket-like structure that extends up to 100 nm into the nucleoplasm. These NPC extensions may function as initial cargo-docking sites during nucleocytoplasmic transport.

Careful proteomic analysis has shown that the yeast NPC consists of ~30 distinct proteins termed nucleoporins (Rout et al., 2000); the somewhat larger metazoan NPCs may consist of ~50 or more components. Although the majority of these nucleoporins are stationary, some NPC components are mobile and might, therefore, interact with nucleocytoplasmic transport factors prior to docking. A subset of NPC components contain characteristic domains featuring multiple phenylalanine-glycine (FG) repeats. These function as transient hydrophobic docking sites for nucleocytoplasmic transport factors. The NPCs contain an aqueous channel that is ~9 nm in diameter when at rest but can expand up to ~25 nm during active transport. The fact that the NPC remains partially open when not engaged in active transport means that small (≤40 kDa) proteins and RNAs can move between the nucleus and cytoplasm by passive diffusion. However, this process is inefficient, and even small proteins and RNAs that need to cross the nuclear membrane are generally actively transported (Zasloff, 1983; Breeuwer and Goldfarb, 1990).

The bulk of cellular nucleocytoplasmic transport is mediated by factors that belong to a single family of nuclear transport receptors termed karyopherins or importins/exportins. Different members of this protein family bind to distinct cargo

molecules, or to adapter proteins that in turn bind cargo, and also share the ability to interact with specific nucleoporins. However, the most important characteristic of karyopherins is their shared dependence on the biological activity of a key cofactor, the small GTPase Ran (reviewed by Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999; Allen et al., 2000).

The Ran-specific GTPase-activating protein (RanGAP) is localized to the cytoplasm whereas the Ran-specific guanine-nucleotide-exchange factor (RanGEF) is localized in the nucleus. As a result, cytoplasmic Ran exists predominantly in an inactive, GDP-bound form, whereas nuclear Ran is largely bound to GTP. Nuclear import factors belonging to the karyopherin family bind their protein cargo in the cytoplasm, in the absence of Ran-GTP, translocate through the NPC and then release their cargo upon binding to nuclear Ran-GTP (Fig. 1). Conversely, nuclear export factors of the karyopherin class, such as Crm1 and Exportin t (Exp-t), require Ran-GTP for cargo binding. Therefore, these factors bind their cargo in the nucleus and then release it upon translocation to the cytoplasm, where hydrolysis of the Ran bound GTP is induced by Ran-GAP (Fig. 1). Because the large majority of nuclear import and export is mediated by karyopherin family members, interference with the Ran-GTP gradient across the nuclear membrane inhibits many transport processes, including the nuclear export of most RNAs. The major exception to this generalization is bulk mRNA export, which is not dependent on Ran and is, in fact, mediated by nuclear export factors that are not members of the karyopherin family of proteins (Clouse et al., 2001; Herold et al., 2001).

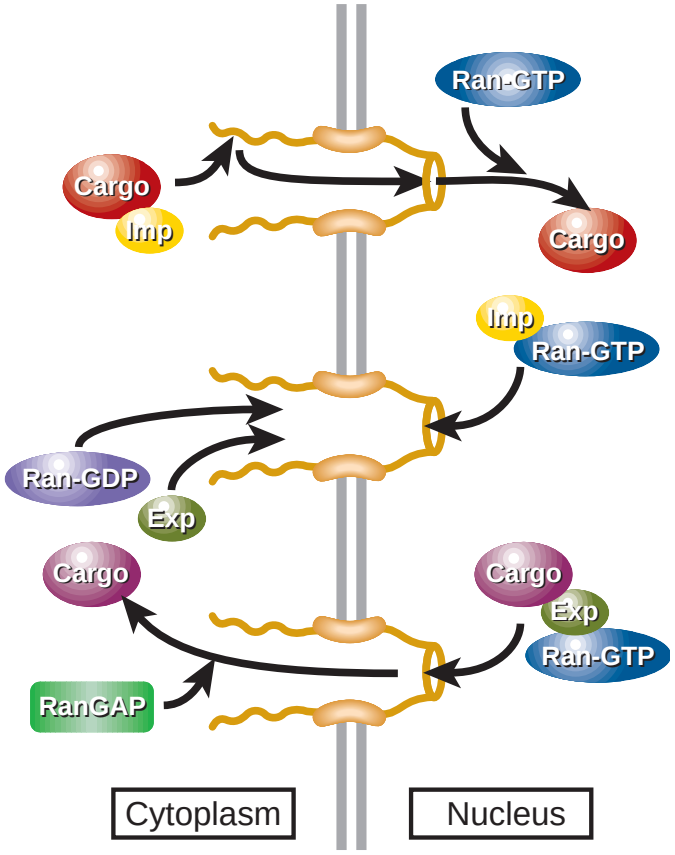

Fig. 1. The Ran-GTP gradient governs the directionality of nucleocytoplasmic transport mediated by members of the karyopherin family of nuclear transport factors. The key role played by the GTP-bound form of Ran in mediating cargo binding and release by karyopherins functioning in nuclear import (importins) or nuclear export (exportins) is illustrated. See text for more detailed discussion. Imp, importin; Exp, exportin.

Several different types of RNA are exported from the nucleus, including transcripts synthesized by RNA polymerase I (Pol I; large rRNAs), RNA polymerase II [Pol II; mRNAs and some uridine-rich small nuclear RNAs (U snRNAs)] and RNA polymerase III (Pol III; tRNAs and 5S rRNA). Competition experiments in microinjected *Xenopus* oocytes have shown that these RNAs largely use distinct pathways to exit the nucleus [e.g. an excess of microinjected tRNA inhibits tRNA export but not U snRNA export (Jarmolowski et al., 1994)]. Efforts to identify the critical protein components that define each of these distinct nuclear RNA export pathways have used a number of distinct yet complementary approaches, including microinjection assays in *Xenopus* oocytes, genetic analysis in yeast cells, biochemical analyses of vertebrate cell extracts and, more recently, RNA interference to inactivate specific genes in metazoan cells. One system that has proven unexpectedly fruitful in identifying human nuclear RNA export factors has been the analysis of the nuclear mRNA export pathways accessed by different primate retroviruses (Cullen, 1998).

Crm1-dependent nuclear RNA export

Initial evidence identifying the karyopherin Crm1 as a nuclear RNA export factor derived from efforts to identify the cellular cofactor for the human immunodeficiency virus type 1 (HIV-1) Rev protein, which is essential for the nuclear export of the incompletely spliced HIV-1 mRNA species expressed late in the viral life cycle (Malim et al., 1989). Rev specifically interacts with a cis-acting target site, the Rev response element (RRE), found on these viral mRNAs, and also contains a short leucine-rich nuclear export signal (NES) (Fischer et al., 1995). The Rev NES, which forms the prototype of the most common form of protein NES, directly interacts with the nuclear, Ran-GTP bound form of Crm1 (Fornerod et al., 1997; Neville et al., 1997; Stade et al., 1997) (Fig. 2). It is now clear that Crm1 is the workhorse karyopherin for nuclear export of cellular proteins and RNAs. That is, Crm1 not only mediates the export of the majority of proteins that undergo nucleocytoplasmic shuttling but also is responsible for the export of several U snRNAs, of all rRNAs and of a small but significant subset of mRNAs.

Crm1-dependent nuclear export of mRNAs

It is perhaps ironic that Crm1 was first identified as a factor required for the nuclear export of late HIV-1 mRNAs because Crm1, although required for the export of a wide variety of cellular protein and RNA substrates, actually plays at most a very limited role in cellular mRNA export (Fischer et al., 1995; Fornerod et al., 1997; Bogerd et al., 1998; Neville and Rosbash, 1999). In addition to HIV-1, several other retroviruses encode adapter proteins that recruit Crm1 to late viral mRNAs. These include the Rev proteins encoded by all members of the lentivirus family, the distinct human T-cell leukemia virus Rex

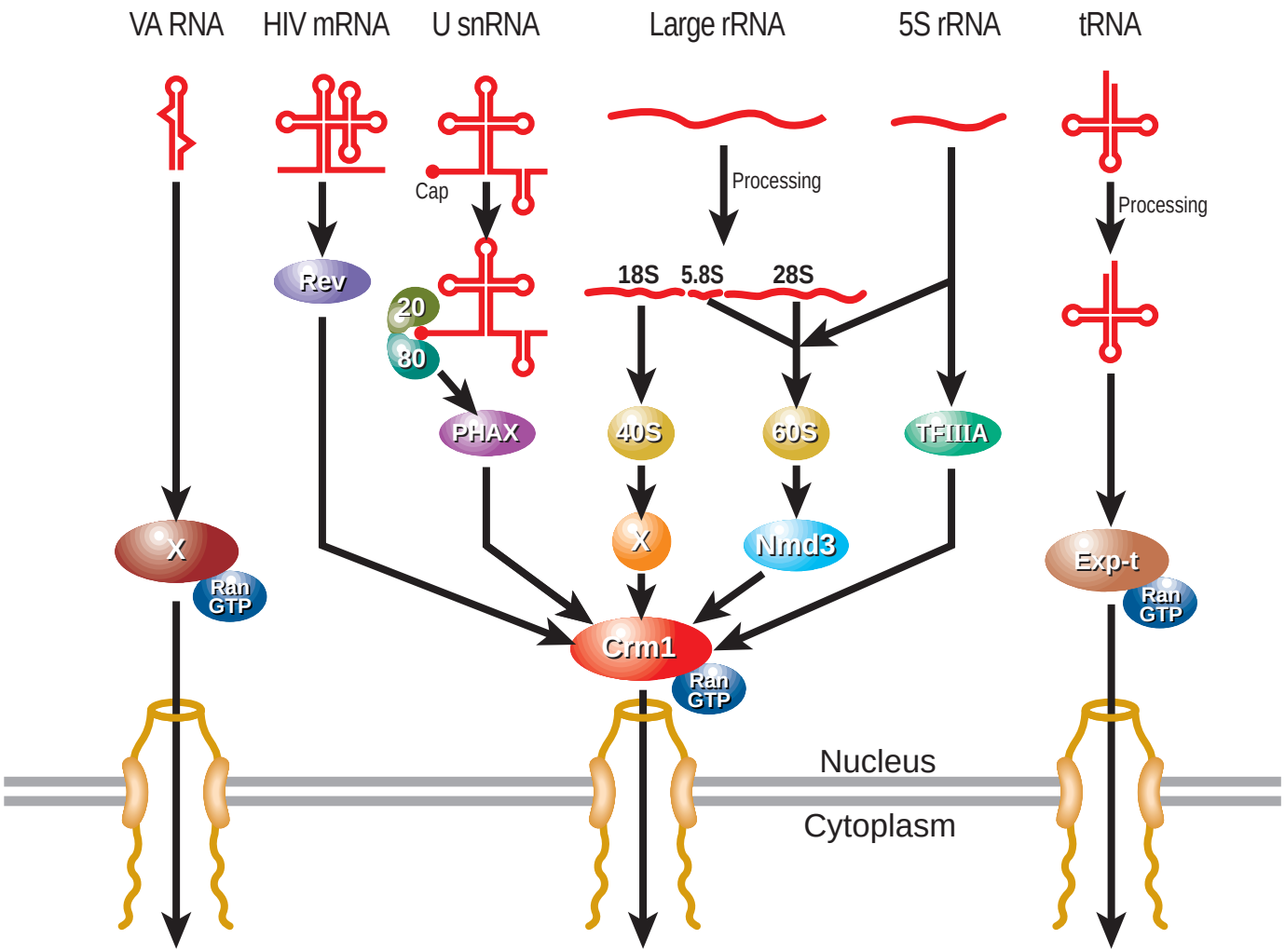

Fig. 2. Karyopherin-mediated nuclear RNA export pathways. This schematic provides an overview of the key factors involved in mediating the nuclear export of different classes of non-coding RNA, as well as the minor class of mRNAs that use the Crm1 nuclear export factor. Hypothetical factors that remain to be identified are indicated by 'X'. In addition to late HIV mRNAs, a small number of cellular mRNAs may also be exported from the nucleus in a Crm1-dependent manner. Candidate cellular Rev-like adapter proteins include APRIL, pp32 and NXF3. The nuclear export pathway used by VA RNAs is also used by other small, non-coding RNAs including Y RNAs and, possibly, pre-miRNAs.

protein (Bogerd et al., 1998) and the K-Rev protein encoded by a family of unrelated human endogenous retroviruses termed the HERV-Ks (Yang et al., 1999). The reason these retroviruses encode adapter proteins that recruit Crm1 to incompletely spliced retroviral mRNAs is that eukaryotic cells have a stringent proofreading mechanism to ensure that mRNAs that retain complete introns (i.e. pre-mRNAs) do not leave the nucleus. This primarily reflects the recognition of splice sites by a subset of splicing factors, termed commitment factors, that can retain pre-mRNAs in the nucleus (Chang and Sharp, 1989; Legrain and Rosbash, 1989). Although advantageous to the cell, this proofreading mechanism presents a problem for retroviruses that, as an integral part of their life cycle, must express both fully spliced and incompletely spliced variants of the same initial transcript in the infected cell cytoplasm (Cullen, 1998). Retroviruses therefore have developed mechanisms that overcome nuclear retention by promoting the sequence-specific recruitment of cellular nuclear export factors to these mRNAs.

Because Crm1 function can be specifically inhibited by the drug leptomycin B (LMB), it is simple to demonstrate that nuclear export of the late mRNAs encoded by HIV-1 depends on Crm1 function whereas nuclear export of bulk cellular poly(A)$^+$ RNA does not (Fornerod et al., 1997). Nevertheless, export of a small subset of cellular mRNAs might also rely on Crm1. One line of evidence supporting this hypothesis comes from studies of the AU-rich elements (AREs) found in the 3′ untranslated regions of many human genes involved in cell signaling (Brennan et al., 2000). AREs bind to the protein HuR, which in turn interacts with two nucleocytoplasmic shuttle proteins, pp32 and APRIL. Both pp32 and APRIL contain leucine-rich NESs and interact with Crm1. Importantly, Brennan et al. reported that inhibition of Crm1 function using LMB results in the selective nuclear accumulation of mRNAs that contain AREs (e.g. *fos* mRNA) whereas the subcellular distribution of bulk poly(A)$^+$ RNA is unaltered (Brennan et al., 2000). These data imply that a specific subset of cellular mRNAs are substrates for Crm1-

mediated nuclear mRNA export, at least under certain conditions.

A second line of evidence suggesting that Crm1 functionally interacts with some cellular mRNAs comes from an analysis of members of the NXF family of human nuclear export factors (Yang et al., 2001). Bulk nuclear mRNA export in metazoan cells is mediated by a member of the NXF protein family termed NXF1 or, more commonly, Tap (Table 1, see below). Tap is not a karyopherin, and Tap-mediated nuclear export does not require its interaction with any karyopherin. Instead, Tap contains two domains that can directly interact with components of the NPC, including one located at the C-terminus. Both of these NPC-binding domains play a critical role in mediating Tap-dependent nuclear mRNA export (Kang and Cullen, 1999; Braun et al., 2002).

While Tap is expressed ubiquitously in all cells and tissues, two closely related proteins, NXF2 and NXF3, display highly tissue-specific expression patterns (Yang et al., 2001). Analysis of its biological activity showed that NXF3, like Tap, is a nucleocytoplasmic shuttle protein that can export nuclear mRNA efficiently when tethered via a heterologous RNA-binding motif. However, NXF3 entirely lacks the C-terminal NPC-binding domain that is critical for Tap function. This conundrum was resolved by the demonstration that NXF3, unlike Tap, contains a leucine-rich NES that directly interacts with Crm1 (Yang et al., 2001). NXF3-dependent, but not Tap-dependent, nuclear mRNA export is therefore effectively blocked by LMB. NXF3 associates with poly(A)$^+$ mRNA in vivo and therefore probably functions as a tissue specific, Crm1-dependent nuclear mRNA export factor. However, no mRNAs dependent on NXF3 for their nuclear export have been identified thus far.

Crm1-dependent nuclear export of U snRNAs

The small nuclear ribonucleoprotein particles (snRNPs) are RNA-protein complexes that play a critical role in the splicing of nuclear pre-mRNAs. In higher eukaryotes, the U snRNA components of the snRNPs are synthesized in the nucleus but, with the exception of U6, then assembled into mature snRNPs in the cytoplasm and reimported into the nucleus. The snRNAs that undergo this cytoplasmic assembly step (snRNAs U1, U2, U4 and U5) are synthesized by Pol II and, like mRNA, acquire a 7-methylguanosine (m^7G) cap cotranscriptionally. This cap serves as the critical export signal for these U snRNAs (Izaurralde et al., 1995).

The m^7G cap is bound by the nuclear cap-binding complex (CBC), consisting of the proteins CBP20 and CBP80, and CBC binding is required for U snRNA export (Izaurralde et al., 1995). Because U snRNA export depends on Crm1 function, one or both components of the CBC heterodimer might in theory serve as targets for Crm1 binding. However, neither CBP20 or CBP80 contains a leucine-rich NES and neither protein directly interacts with Crm1 in vitro. It was therefore clear that the CBC:Crm1 interaction must involve at least one additional component. The identity of this factor, termed PHAX for phosphorylated adapter for RNA export, was recently resolved in a series of elegant experiments performed by the Mattaj laboratory (Fig. 2).

Ohno et al. showed that the Ran-GTP-bound form of Crm1 and PHAX bind, in a highly cooperative manner, to the CBC, which is in turn bound to the U snRNA m^7G cap (Ohno et al., 2000). Importantly, PHAX binding requires phosphorylation at as-yet-undefined residues. Once this U snRNA ribonucleoprotein complex migrates through the NPC to the cytoplasm, it is disassembled following hydrolysis of the Ran-GTP component and de-phosphorylation of PHAX. PHAX is then reimported into the nucleus, where it is re-phosphorylated and can again participate in U snRNA binding and export (Ohno et al., 2000). The directionality of U snRNA export is therefore assured not only by the Ran cycle (Fig. 1) but also by the compartmentalization of the as-yet-unidentified nuclear kinase and cytoplasmic phosphatase that act on PHAX.

Crm1-dependent nuclear export of rRNAs

Three rRNAs (28S, 18S and 5.8S) are transcribed in the nucleus by RNA polymerase I as a single large pre-rRNA that is then extensively processed to yield mature rRNAs. The fourth rRNA, 5S rRNA, is transcribed by RNA Pol III and requires only modest post-transcriptional processing. In a complex and highly ordered process, the 28S, 5.8S and 5S rRNA species are then assembled, together with 50 ribosomal proteins, to form the 60S preribosomal subunit; the 18S rRNA is assembled with 33 ribosomal proteins to give the 40S preribosomal subunit (reviewed by Venema and Tollervey, 1999). These subunits are then separately exported to the cytoplasm.

Initial clues to the mechanism underlying nuclear export of the preribosomal subunits came from genetic screens in *S. cerevisiae*, which showed that export of both subunits depends on Ran and is therefore likely to be mediated by one or more karyopherins (Hurt et al., 1999; Moy and Silver, 1999). Genetic screens also identified a protein called Nmd3p as critical for a late step in the biosynthesis of 60S, but not 40S, preribosomal subunits (Ho and Johnson, 1999). Although Nmd3p is not a ribosomal protein per se, it associates with 60S preribosomal subunits in the nucleus through a direct interaction with the large subunit ribosomal protein Rpl10p (Gadal et al., 2001). Nmd3p shuttles between nucleus and cytoplasm in a Crm1-dependent manner and contains two C-terminal NESs similar to that in HIV-1 Rev (Ho et al., 2000; Gadal et al., 2001). Importantly, nuclear export of 60S preribosomal subunits can be inhibited not only by the Crm1-specific inhibitor LMB but also by overexpression of Nmd3p mutants lacking a functional NES. Nuclear export of the 60S preribosomal subunit is therefore mediated by the Nmd3p-dependent recruitment of Crm1 (Fig. 2). Because assembly of Nmd3p onto the 60S preribosome is a very late step in 60S preribosome assembly, Crm1 is only recruited to 60S preribosomes that are ready for export and assembled appropriately.

Nuclear export of the 40S preribosomal subunit is also Crm1 dependent (Moy and Silver, 2002). However, Nmd3p does not bind to 40S subunits and does not play a direct role in mediating their export. Efforts to demonstrate a direct interaction between 40S preribosomal subunits and Crm1 have so far been unsuccessful. Recruitment of Crm1 to nuclear 40S preribosomal subunits therefore probably depends on an as-yet-unidentified adapter that has an analogous role to Nmd3p (Fig. 2).

Although 5S rRNA is normally assembled directly into large ribosomal subunits in the cell nucleus and therefore does not reach the cytoplasm on its own, an interesting exception exists

in amphibian oocytes (Nakielny et al., 1997). These highly specialized cells express an oocyte-specific 5S rRNA that migrates to the cytoplasm, where it is stored pending the onset of vitellogenesis, when it is reimported into the nucleus and incorporated into 60S subunits. A major cytoplasmic 5S rRNA storage particle, the 7S particle, consists of 5S rRNA and the transcription factor TFIIIA, which also plays a critical role in the transcription of 5S rRNA. Interestingly, amphibian TFIIIA contains a functional leucine-rich NES that is apparently absent in mammalian TFIIIA (Fridell et al., 1996). Nuclear export of 5S rRNA is specifically inhibited in *Xenopus* oocytes upon saturation of the Crm1 export pathway by nuclear injection of high levels of a leucine-rich NES peptide (Fischer et al., 1995). In this differentiated cell type, Crm1 thus probably also mediates nuclear export of 7S storage particles containing TFIIIA and 5S rRNA (Fig. 2).

Other karyopherin-dependent nuclear RNA export pathways

Nuclear export of tRNAs

The formation of mature tRNAs from the pre-tRNAs generated by Pol III transcription requires many post-transcriptional modifications, all of which occur in the nucleus (De Robertis et al., 1981). Specifically, all tRNAs are trimmed at the 5′ end by RNase P, and uracil residues at the 3′ end are replaced by the CCA sequence found on all mature tRNAs. In addition, many bases present in the tRNA are post-transcriptionally modified, and some pre-tRNAs contain an intron in the anticodon loop that must be removed by splicing. Because detectable levels of pre-tRNAs do not normally reach the cytoplasm, the tRNA export mechanism probably selects for mature tRNAs.

The first clue to the identity of the nuclear export factor for tRNAs was the identification, in a yeast genetic screen, of Los1p, a protein that is important for production of suppressor tRNAs (Hopper et al., 1980). Los1p is a member of the karyopherin family, and its human homolog, Exp-t, binds directly and specifically to mature tRNA molecules, but only in the presence of Ran-GTP (Arts et al., 1998a; Kutay et al., 1998) (Fig. 2). Although the ability of Exp-t to bind to an RNA, rather than to a protein target, so far appears to be unique among karyopherins, in common with other karyopherins, Exp-t can bind to specific nucleoporins and shuttle between nucleus and cytoplasm.

A key prediction for the tRNA export factor is that it should be specific only for mature tRNAs. In fact, Exp-t binds very poorly to tRNAs that bear incorrect 5′ or 3′ ends or are inappropriately modified (Arts et al., 1998b; Lipowsky et al., 1999). Unexpectedly, Exp-t does bind to tRNAs that retain an intron and can export these from injected oocyte nuclei. However, because intron removal normally occurs prior to 5′- and 3′-end processing at physiological levels of tRNA expression (Lund and Dahlberg, 1998), this may not normally present a problem. Two groups have also suggested that Exp-t selectively binds to and exports tRNAs that have been aminoacylated in the nucleus (Lund and Dahlberg, 1998; Sarkar et al., 1999), although aminoacylation is clearly not an absolute requirement for export (Arts et al., 1998b). Because aminoacylation would occur efficiently only with mature tRNAs, this would, however, represent an effective proofreading step to ensure that only functional tRNAs are exported from the nucleus.

Nuclear export of micro RNAs and other small non-coding RNAs

Eukaryotic cells, and particularly metazoan cells, encode a wide range of other small non-coding RNAs, whose functions are frequently unknown. Several of these are predominantly localized to the cytoplasm: the ~100 nt long human Y RNAs, which form part of the Ro ribonucleoprotein particle; the ~160 nt long VA RNAs encoded by adenoviruses, which act as inhibitors of host antiviral responses; and micro RNAs (miRNAs), a diverse class of small non-coding RNAs that appear to play important roles in post-transcriptional gene regulation (Peek et al., 1993; Liao et al., 1998; Ambros, 2001). Y and VA RNAs are transcribed by Pol III, but the polymerase(s) responsible for miRNA transcription is not yet known.

Although both Y and VA RNAs are small, highly structured molecules, they do not share any significant sequence similarity. However, both feature a terminal RNA helix that is necessary and sufficient for nuclear export (Gwizdek et al., 2001; Rutjes et al., 2001). The sequence of this ‘terminal minihelix’ appears to be irrelevant, although a double-stranded region of >10 bp, and its location at the end of the RNAs, are essential. Export of the Y and VA RNAs depends on the integrity of the Ran system, which implicates a karyopherin, but is not dependent on Crm1 or Exp-t (Gwizdek et al., 2001; Rutjes et al., 2001). The mechanism underlying the nuclear export of these small, non-coding RNAs and other small RNAs bearing a terminal helical region therefore remains largely unclear.

All known miRNAs are single stranded and ~22 nt in length when fully mature but are initially transcribed as longer precursor RNAs (pri-miRNAs), that can be several hundred nucleotides in length. Embedded within the pri-miRNA is the structured miRNA precursor (pre-miRNA), which is an ~70 nt RNA stem-loop structure containing the mature miRNA sequence as part of the stem (Lee et al., 2002). miRNA synthesis involves the initial excision of the ~70 nt pre-miRNA from the longer pri-miRNA, which gives rise to a short RNA stem-loop bearing a terminal RNA helix (Lee et al., 2002). This pre-miRNA is then exported to the cytoplasm, where it is further processed, by the unusual dicer ribonuclease, to yield the mature ~22 nt miRNA. At present, nothing is known about the mechanism underlying the nuclear export of the pre-miRNA precursor. However, the close structural similarity of this small, structured RNA to other small RNAs that are actively exported through terminal minihelix recognition strongly suggests that the pre-miRNA utilizes the same nuclear export pathway as the Y and VA RNAs (Fig. 2). Indeed, the integrity of the pre-miRNA basal stem, but not the sequence, is essential for miRNA expression in transfected cells (Zeng and Cullen, 2003).

Nuclear export of mRNAs

The Tap-Nxt heterodimer mediates bulk mRNA nuclear export

Export of most mRNAs is independent of the Ran system and karyopherins (Clouse et al., 2001). The key mediator of bulk

mRNA export is instead a heterodimer of Tap and a small cofactor termed Nxt or p15 (Fig. 3). In yeast cells, the Tap ortholog is termed Mex67p while the small cofactor is termed Mtr2p (Table 1).

Evidence implicating Mex67p as a potential nuclear mRNA export factor initially came from the observation that yeast cells lacking functional Mex67p rapidly accumulate poly(A)$^+$ RNA in the nucleus (Segref et al., 1997). Mex67p was subsequently found to bind to a second essential export factor, Mtr2p. This in turn induces binding of Mex67p-Mtr2p to nucleoporins and is required for nucleocytoplasmic shuttling of the Mex67p-Mtr2p heterodimer and export of poly(A)$^+$ RNA (Santos-Rosa et al., 1998; Strässer et al., 2000).

Meanwhile, investigation of the nuclear export of the incompletely spliced mRNA encoded by the retrovirus Mason Pfizer Monkey Virus (MPMV) had identified Tap as a key nuclear mRNA export factor in metazoan cells. Like other retroviruses, MPMV requires nuclear export of incompletely spliced mRNAs (see above). Rather than encoding adapter proteins such as Rev that recruit Crm1, however, several simple retroviruses, such as MPMV, instead encode a cis-acting RNA sequence, the constitutive transport element (CTE), that is sufficient to induce nuclear export in the absence of any viral gene product (Bray et al., 1994). Evidence suggesting that the cellular cofactor that binds to the CTE also functions in cellular mRNA export came from microinjection experiments in *Xenopus* oocytes, which showed that an excess of CTE RNA blocks cellular mRNA export but does not affect Crm1-dependent RNA export or tRNA export (Pasquinelli et al., 1997; Saavedra et al., 1997). Grüter et al. subsequently identified the cellular cofactor for the MPMV CTE as Tap, the human ortholog of Mex67p, using a biochemical approach (Grüter et al., 1998).

The binding partner for Tap is Nxt (Katahira et al., 1999). Although Nxt does not show any obvious sequence similarity to Mtr2p, it is nevertheless functionally closely analogous. Thus, nucleoporin binding by Tap is greatly enhanced by formation of a Tap-Nxt heterodimer, as is nucleocytoplasmic shuttling (Fribourg et al., 2001; Lévesque et al., 2001; Wiegand et al., 2002). Indeed, Tap mutants that cannot form Tap-Nxt heterodimers do not support nuclear mRNA export. Finally, although human Tap and Nxt can at least partially rescue nuclear mRNA export in Mex67p- and/or Mtr2p-deficient yeast cells, both human proteins are required (Katahira et al., 1999). This strongly suggests that Tap and Nxt are the true orthologs of Mex67p and Mtr2p, respectively (Table 1).

Table 1. Nomenclature of candidate nuclear mRNA export factors

Metazoan cells		
Common name	Alternative name	Yeast cells
Tap	Nxf1	Mex67p
Nxt	p15	Mtr2p
Aly	Ref	Yralp
UAP56	Hel	Sub2p

Tap and Mex67p proteins share a similar domain organization: an N-terminal leucine-rich region (LRR), a central domain that shows sequence similarity to the nuclear import receptor NTF2 and finally a C-terminal domain that displays a UBA (ubiquitin associated) fold. The central domain mediates the interaction with Nxt and Mtr2p. The UBA domain is critical for interaction with nucleoporins and hence Tap-mediated nucleocytoplasmic transport (Kang and Cullen, 1999; Katahira et al., 1999; Bachi et al., 2000). Interestingly, recent data suggest that the nucleoporin-binding functions of the central Nxt-binding domain and the UBA domain are functionally equivalent, that is, Tap variants containing two central domains but no UBA domain are partially active in mediating nuclear mRNA export, and vice versa (Braun et al., 2002). In contrast, mutants of Tap containing only one copy of either domain are inactive. The N-terminal LRR domain is required for CTE RNA binding by Tap and is likely also to be critical for the sequence-non-specific recruitment of Tap to cellular mRNAs (Kang and Cullen, 1999; Liker et al., 2000). Indeed, the LRR domain becomes dispensable for Tap-

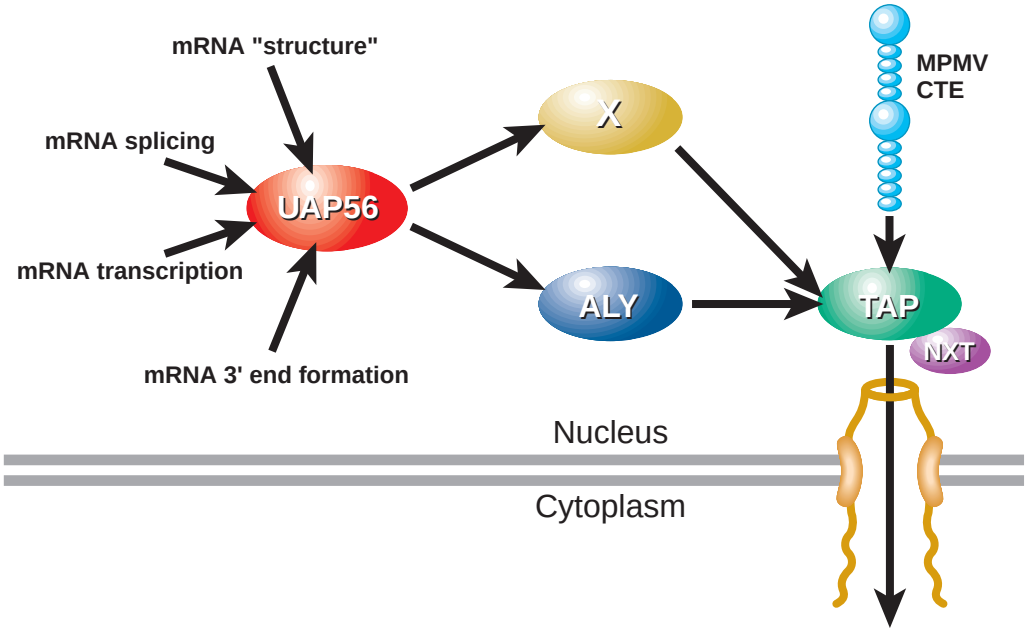

Fig. 3. Key factors involved in bulk mRNA export in metazoan cells. Recruitment of UAP56 to mRNA molecules, either by splicing or possibly via one of the other indicated mechanisms, likely represents the key initial step in inducing nuclear mRNA export. UAP56 may then recruit Aly, which in turn binds the Tap-Nxt nuclear RNA export factor. The MPMV CTE RNA binds the Tap-Nxt heterodimer directly, thus obviating the need for upstream factors. UAP56, Tap and Nxt are all essential for bulk mRNA nuclear export, but Aly is not, thus implying that a second, unknown factor may also mediate recruitment of the Tap-Nxt heterodimer by mRNA-bound UAP56 molecules.

mediated nuclear RNA export when Tap is tethered to target RNAs via a heterologous RNA-binding domain. In contrast, both the central NTF-2-like domain and the C-terminal UBA-like domain remain essential in this fusion protein context (Wiegand et al., 2002).

RNA interference has now revealed that, like Mex67p and Mtr2p, Tap and Nxt are essential for poly(A)$^+$ RNA nuclear export (Tan et al., 2000; Herold et al., 2001; Wiegand et al., 2002). The Tap-Nxt heterodimer thus plays a critical, evolutionarily conserved role in mediating the nuclear export of cellular mRNAs in a wide range of eukaryotic organisms (Fig. 3).

How Tap-Nxt is recruited to mature nuclear mRNAs remains uncertain. Unlike non-coding RNAs, mRNAs do not share any obvious RNA sequence or structure except for the 5′ m^{7}G cap and the 3′ poly(A) tail. However, neither of these two post-transcriptional modifications is critical for mRNA export in injected oocytes (although the process of 3′ end formation is important, see below) (Jarmolowski et al., 1994). Recruitment of the Tap-Nxt mRNA exporter is therefore likely to occur during the synthesis and/or the post-transcriptional processing of mRNAs (Fig. 3).

Coupling of mRNA splicing with nuclear export

The large majority of genes encoded by humans and other metazoans contain introns. In general, mRNAs containing introns give rise to a higher level of the encoded protein than do the equivalent intronless, cDNA-derived mRNAs. The mechanisms underlying this difference are complex because splicing can strongly influence not only the efficiency of mRNA 3′ end formation but also mRNA stability and even translation (Ryu and Mertz, 1989; Niwa et al., 1990; Matsumoto et al., 1998). Nevertheless, for most human genes, splicing is not essential for detectable mRNA and protein synthesis. In fact, a recent survey of 15 genes showed that the presence of an excisable intron enhanced gene expression in human cells by an average of 6.3± 4.7 fold with a range of from 1.5- to over 20-fold (S. Lu and B. R. Cullen, unpublished).

The first evidence suggesting that splicing also enhances the efficiency of mRNA export came from the demonstration that several intron-containing mRNAs are exported more efficiently from microinjected *Xenopus* oocyte nuclei than the same RNAs injected in an intronless, cDNA form (Luo and Reed, 1999). Luo and Reed also compared the export of an RNA that had been subjected to splicing in a nuclear extract with those of a cDNA version of the same RNA that had simply been incubated in nuclear extract. Remarkably, ribonucleoprotein (RNP) complexes formed on these in principle identical RNA molecules displayed distinct mobilities on non-denaturing gels, the spliced RNP complex migrating more slowly. In addition, the RNA contained within the spliced RNP complex was exported more efficiently from injected oocyte nuclei than the RNA contained within the RNP that lacked splicing factors. Intron removal thus appeared to lead to the deposition of specific proteins that targeted the spliced mRNA for efficient nuclear export.

That splicing somehow tags an mRNA was confirmed by the discovery of the exon junction complex (EJC), a protein complex deposited 20-24 nucleotides 5′ of the site of intron removal (Kataoka et al., 2000; Le Hir et al., 2000). Although the composition of the EJC remains to be fully determined, it minimally consists of the proteins SRm160, RNPS1, Y14, Magoh and a protein termed Aly or Ref. SRm160 appears to facilitate appropriate mRNA 3′ end formation (McCracken et al., 2002) whereas RNPS1 and Y14 appear to be involved in the regulation of nonsense-mediated decay (reviewed by Wagner and Lykke-Anderson, 2002). Aly has been proposed to facilitate nuclear mRNA export by directly interacting with Tap (Fig. 3).

The first evidence that Tap and Aly interact came from studies in yeast. Strässer and Hurt, and Stutz et al. demonstrated a direct interaction between Yralp, the yeast ortholog of metazoan Aly (Table 1), and Mex67p in vitro and also showed that yeast Yralp mutants are impaired in nuclear poly(A)$^+$ RNA export (Strässer and Hurt, 2000; Stutz et al., 2000). Yralp was also shown to bind to mRNA molecules both in vitro and in vivo (Strässer and Hurt, 2000; Lei and Silver, 2002). Zhou et al. subsequently demonstrated that human Aly is a nucleocytoplasmic shuttle protein that is selectively recruited during in vitro mRNA splicing (Zhou et al., 2000). Most importantly, recombinant Aly was found to selectively enhance nuclear mRNA export in microinjected oocytes and to specifically interact with recombinant human Tap in vitro (Zhou et al., 2000; Rodrigues et al., 2001). Finally, Le Hir et al. showed that the EJC provides a binding site for the Tap-Nxt heterodimer, thus providing a potential explanation for how splicing could enhance nuclear mRNA export (Le Hir et al., 2001).

An interesting question that had remained unresolved was how splicing leads to the deposition of the EJC and, more specifically, how Aly is recruited to mRNAs during splicing. Again, yeast genetics provided the initial breakthrough. Strässer and Hurt demonstrated a genetic interaction between Yralp, the yeast Aly homolog, and Sub2p (Strässer and Hurt, 2001), a member of the DEAD-box family of RNA helicases that had previously been shown to play an important role in spliceosome assembly (Fleckner et al., 1997; Libri et al., 2001). Sub2p and Yralp directly interact both in vitro and in vivo, and loss of Sub2p function blocks poly(A)$^+$ RNA nuclear export in yeast cells. In metazoan cells, Aly specifically interacts with UAP56, the human homolog of Sub2p, and recruitment of Aly to spliced mRNAs depends on this interaction (Luo et al., 2001) (Fig. 3). Gatfield et al. subsequently showed, using RNA interference, that UAP56 is also essential for nuclear mRNA export in *Drosophila* (Gatfield et al., 2001). UAP56 associates with mRNAs in the nucleus and accompanies the resultant mRNP complexes to the NPC (Kiesler et al., 2002).

On the basis of these data, one can envision a simple hypothesis for how Tap-Nxt is recruited to mRNAs in a splicing-dependent manner in metazoan cells (Fig. 3). During splicing, UAP56 facilitates spliceosome assembly and then recruits Aly to the resultant EJC. Aly in turn recruits the Tap-Nxt heterodimer, which then targets the spliced mRNP complex to the NPC and, hence, to the cytoplasm. Unfortunately, considerable recent evidence suggests that this attractive hypothesis is at best an oversimplification.

One problem with the above proposal is that it is not clear that splicing is essential for nuclear mRNA export. Although Luo and Reed reported that splicing can markedly enhance mRNA export in microinjected oocytes (Luo and Reed, 1999),

a difficulty with this experimental approach is that only the nuclear export of preformed RNAs was analyzed. Therefore, if the effect of splicing on mRNA export factor recruitment is largely or entirely redundant with the recruitment of the same export factors during transcription and/or mRNA 3′ end formation (Fig. 3), this assay will give the misleading result that splicing is uniquely critical for nuclear mRNA export. More importantly, there is no agreement that splicing is indeed essential for nuclear export of mRNA molecules, even in *Xenopus* oocytes. Rodrigues et al. reported that splicing does not enhance the nuclear export of mRNAs derived from the *Ftz* or *β-globin* genes, although splicing does enhance the nuclear export of a very short RNA derived from the adenovirus major late region (Rodrigues et al., 2001). Ohno et al. have also presented evidence against a critical role for splicing in mRNA nuclear export in *Xenopus* oocytes (Ohno et al., 2002).

In transfected human cells, splicing can result in a significant enhancement in the expression of the majority of genes (see above). However, the absence of introns reduces mRNA levels equivalently in both the nucleus and cytoplasm; a selective defect in nuclear mRNA export should affect cytoplasmic levels more significantly. Also, the poor expression of intronless mRNAs is not significantly enhanced by insertion in cis of the MPMV CTE, a high affinity-binding site for Tap. Splicing thus does not appear to have a major effect on the nuclear export efficiency of mRNAs transcribed in metazoan cells (S. Lu and B. R. Cullen, unpublished; A. Nott, S. H. Meislin and M. J. Moore, personal communication). A final argument against a critical role for the EJC in mediating nuclear mRNA export comes from recent RNA interference experiments (Gatfield and Izaurralde, 2002) showing that known components of the EJC, including SRm160, Y14, RNPS1 and even Aly, are not required for bulk export of nuclear poly(A)$^+$ RNA in *Drosophila*. In contrast, Tap, Nxt and UAP56 are all clearly essential. These data therefore again suggest that splicing, and the EJC, are not critical for nuclear mRNA export and further imply that the role of metazoan Aly in mediating the recruitment of Tap-Nxt to mature mRNAs must, at minimum, be redundant (Fig. 3).

A second problem with the hypothesis that mRNA nuclear export depends on splicing is that recruitment of UAP56, and probably also of Aly and Tap, to mRNA transcripts appears to occur independently of splicing in vivo. Sub2p and Yralp are essential for nuclear export of the intronless mRNAs encoded by most yeast genes (Strässer and Hurt, 2000; Strässer and Hurt, 2001; Jensen et al., 2001a), and Yralp binding to mRNAs can clearly occur in the absence of splicing (Lei and Silver, 2002). In metazoan cells, recruitment of UAP56 to mRNAs has also been shown to occur independently of splicing (Kiesler et al., 2002), and elimination of either UAP56 or Tap expression by RNA interference again blocks the nuclear export of not only spliced but also intronless mRNAs (Gatfield et al., 2001; Herold et al., 2001). Finally, in *Xenopus* oocytes, microinjection of antibodies against Aly inhibits the nuclear export of both spliced and intronless mRNAs (Rodrigues et al., 2001). In total, the above experiments therefore strongly argue that splicing is not a prerequisite for the recruitment of nuclear mRNA export factors.

If splicing does not direct mRNAs into the export pathway, then what does? Surprisingly, a recent paper suggests that an unstructured region of sufficient length can act as a dominant determinant of mRNA identity. Ohno et al. inserted an intron into U1 snRNA and found that the U1 snRNA was spliced and then exported exclusively through the Tap-dependent mRNA export pathway, rather than the Crm1 pathway (Ohno et al., 2002). This result is obviously consistent with the proposal that splicing facilitates Tap recruitment. Surprisingly, however, insertion of random exonic sequences also targeted U1 snRNA into the mRNA export pathway, regardless of whether these sequences were inserted in a sense or antisense orientation. Unstructured RNA, inserted into the otherwise highly structured U1 snRNA, therefore appears to be sufficient to induce the recruitment of Tap-Nxt (Fig. 3). These data suggest that the mRNA nuclear export pathway could represent the default nuclear export pathway followed by unstructured RNA molecules that are either not recognized by other RNA export factors and/or not actively retained in the nucleus by proofreading proteins, such as splicing commitment factors.

Coupling of transcription with mRNA nuclear export

Pre-mRNA processing is coupled to transcription, the C-terminal domain (CTD) of Pol II providing a platform for recruitment of RNA-processing factors during transcription (reviewed by Neugebauer, 2002). Transcription of mRNAs facilitates their capping, splicing and polyadenylation and might also directly promote the recruitment of nuclear export factors. The first evidence for this hypothesis came from chromatin immunoprecipitation assays, which showed that yeast proteins involved in mRNA export are recruited to mRNAs co-transcriptionally (Lei et al., 2001). More recently, Strässer et al. have shown that Yra1p and Sub2p are stoichiometrically associated with a yeast protein complex, THO, that plays a key role in transcription elongation (Strässer et al., 2002). Similarly, human Aly and UAP56 associate with the human THO complex. Strässer et al. proposed that this complex of mRNA export and transcription factors, which they termed the transcription/export (TREX) complex, plays an important role in mediating the co-transcriptional recruitment of nuclear export factors such as UAP56 and Aly, and hence presumably Tap-Nxt, to mRNAs (Strässer et al., 2002) (Fig. 3).

Although the hypothesis that export factor recruitment occurs co-transcriptionally is attractive, it leaves unresolved the issue of how pre-mRNAs avoid being exported to the cytoplasm. For example, the incompletely spliced mRNAs encoded by the retrovirus MPMV depend on the cis-acting CTE RNA for nuclear export, and the CTE acts as a high-affinity binding site for Tap-Nxt (Grüter et al., 1998) (Fig. 3). If the Tap-Nxt heterodimer is indeed recruited to mRNAs, including MPMV RNAs, co-transcriptionally, then it is unclear why the nuclear export of this mRNA would be dependent on the subsequent recruitment of the same Tap-Nxt heterodimer by the CTE.

Coupling of 3′ end formation with mRNA nuclear export

Microinjection assays in *Xenopus* oocytes indicate that insertion of a stretch of adenine residues at the 3′ end of an mRNA does not significantly enhance its export (Jarmolowski et al., 1994). Conversely, experiments in transfected human cells have demonstrated that mRNA molecules bearing 3′ ends formed by ribozyme cleavage are not efficiently exported to

the cytoplasm (Eckner et al., 1991; Huang and Carmichael, 1996). Together, these data suggest that it is the process of mRNA 3′ end-formation, rather than the poly(A) stretch itself, that facilitates nuclear mRNA export (Fig. 3). Moreover, in yeast, mutational inactivation of nuclear export factors such as Mex67p or Mtr2p results in the accumulation of transcripts at the site of transcription and in their hyperadenylation (Hilleren and Parker, 2001; Jensen et al., 2001b). Conversely, several yeast mutants that express defective forms of mRNA 3′ processing factors, including for example the poly(A) polymerase, exhibit defective mRNA export (Hammell et al., 2002). Recently, Lei and Silver have demonstrated that recruitment of Yra1p to yeast mRNAs is dependent on appropriate 3′ end formation, even if the mRNA contains an intron (Lei and Silver, 2002). Although these data clearly indicate some form of tight mechanistic coupling between mRNA 3′ end formation and nuclear export, its molecular basis remains unresolved.

Conclusions

Although the karyopherin-dependent nuclear export factors used by non-coding RNAs are now quite well defined (Fig. 2), much remains to be learned about how nuclear mRNA export is regulated. Specifically, it remains unclear how the critical nuclear mRNA export factors that have been identified thus far (Fig. 3) are recruited to mature mRNA molecules and, conversely, how pre-mRNAs are effectively prevented from interacting with these factors and reaching the cytoplasm (i.e. what are the mechanisms involved in proofreading mRNAs prior to their nuclear export)? It seems probable that nuclear mRNA export will prove to be tightly coupled to mRNA transcription and processing, just as the various steps in mRNA processing are in turn tightly coupled to transcription and to each other.

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