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NUCLEOCYTOPLASMIC TRANSPORT: The Soluble Phase

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ABSTRACT

Active transport between the nucleus and cytoplasm involves primarily three classes of macromolecules: substrates, adaptors, and receptors. Some transport substrates bind directly to an import or an export receptor while others require one or more adaptors to mediate formation of a receptor-substrate complex. Once assembled, these transport complexes are transferred in one direction across the nuclear envelope through aqueous channels that are part of the nuclear pore complexes (NPCs). Dissociation of the transport complex must then take place, and both adaptors and receptors must be recycled through the NPC to allow another round of transport to occur. Directionality of either import or export therefore depends on association between a substrate and its receptor on one side of the nuclear envelope and dissociation on the other. The Ran GTPase is critical in generating this asymmetry. Regulation of nucleocytoplasmic transport generally involves specific inhibition of the formation of a transport complex; however, more global forms of regulation also occur.

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INTRODUCTION: WHAT'S THE PROBLEM?

The compartmentation of eukaryotic cells gives rise to a need for intercompartmental transport of macromolecules. Specialized systems have evolved that allow proteins to be imported into membrane-bound organelles such as mitochondria, chloroplasts, lysosomes, the endoplasmic reticulum, and nuclei. Nuclear transport is unusual in that both import into and export out of the organelle are major processes, whereas in other organelles transport is largely unidirectional. All nuclear proteins are made in the cytoplasm and must be imported to the nucleus. In cells with an open mitosis these proteins must be reimported after each nuclear division. RNAs transcribed in the nucleus are almost all exported to the cytoplasm, in the form of ribonucleoproteins (RNPs). Many proteins shuttle continuously between the nucleus and cytoplasm. In total, this gives rise to an enormous level of nucleocytoplasmic traffic. Even a conservative estimate suggests that more than 1 million macromolecules are transferred between the two compartments each minute in a growing mammalian cell (1).

The nuclear envelope (NE) consists of a double lipid bilayer with an intervening lumen. The lumen and the outer bilayer are continuous with the endoplasmic reticulum and, thus, with the cellular secretory system (2). The NE is penetrated by nuclear pore complexes (NPCs). These huge structures, 125 million Daltons in vertebrates (3), form aqueous channels through which all nucleocytoplasmic transport is thought to occur. The NPC is composed of between 50 and 100 distinct polypeptides (4) that are often called nucleoporins. Molecules of up to approximately 9 nm in diameter, corresponding to a globular protein of approximately 60 kDa, can in principle enter or leave the nucleus by diffusion through the NPC, although in practice very few proteins and no known RNAs do so. Rather, nucleocytoplasmic transport is an active, signal-mediated process.

Very large complexes, like ribosomal subunits or even larger RNPs (5), are actively transported through the NPC. The functional pore size for active transport is somewhat greater than 25 nm (6). The difference between the size of the diffusion and active transport channels means that active transport must be accompanied by large conformational changes in the NPC. How this happens

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and, more generally, the mechanism of active translocation through the NPC are fascinating topics. Many recent reviews have covered NPC composition, assembly, structure, and function (7–13). The size of the diffusion pores means that ions and small metabolites must, in the free state, be able to cross the NPC unhindered. This does not mean that they all equilibrate between the nucleus and cytoplasm, since their distribution will be determined by the location of the macromolecules to which they bind on either side of the nuclear envelope.

Until recently, it was not clear whether nucleocytoplasmic transport represented active movement against a chemical concentration gradient or facilitated movement through the NPC followed by binding of the transported substrate and consequent retention in the target compartment. This was obviously a critical point in interpreting mechanistic studies of transport (see 14). Experiments in both mammalian and yeast cells have recently confirmed that at least one form of nucleocytoplasmic transport conforms to the active transport paradigm. This involved showing that molecules that were free to diffuse, and thus not retained in the nucleus by binding interactions, could be pumped into the nucleus against a concentration gradient when energy was provided. Substrates that were small enough to diffuse between the nucleus and cytoplasm and that carried a signal for nuclear import were constructed. When cells were depleted of energy, either by using a combination of energy poisons and low temperature or by low-temperature treatment alone, these substrates diffused throughout the nuclear and cytoplasmic compartments. Restoring ATP production or increasing the temperature caused the proteins to reaccumulate in the nucleus (15, 16). These studies demonstrate that the class of nuclear import signal tested does not bind tightly to an immobile nuclear phase, but rather is recognized by an active transport system capable of carrying the substrate against a concentration gradient. A further logical consequence is that the signal acts in one direction only, from the cytoplasm to the nucleus.

Given the similarities among the transport systems described in this review, it is likely that this conclusion will hold for most transport substrates. This review examines our current understanding of nuclear import and export signals, the transport mediators that recognize them, and the regulation of signal-mediator interactions. During import or export, transport mediators are found at the NPC, but they also spend some time free in the cytoplasm or nucleoplasm, and that soluble phase is the focus of this review.

NUCLEAR IMPORT

Import Signals and Receptors: How Much Diversity?

Signal sequences involved in targeting proteins into either the endoplasmic reticulum or mitochondria are generally removed during transit. As noted above,

in many cell types nuclear proteins have to reaccumulate in the nucleus after each mitotic division. This means that nuclear targeting signals must be part of the mature nuclear protein rather than being removed on use (17, 18). The definition of what came to be called nuclear localization signals (NLSs) began with the study of proteolytic fragments of nucleoplasmin (19), but DNA-based technology soon took over.

The two best defined NLSs are those of SV40 large T antigen (SV40 TAg) and nucleoplasmin (20, 21; see Table 1). In this review we use the term NLS to refer only to the class of nuclear import signal represented by these two examples. They are both short and contain several critical basic amino acids. The discovery that a sequence as short as seven amino acids could direct nuclear import (20) allowed the synthesis of artificial import substrates by chemical cross-linking of the SV40 TAg NLS to human serum albumin (22). These conjugates, at high concentration, competitively inhibited nuclear import of NLS-bearing proteins, demonstrating that NLS-protein import is saturable. Together with earlier data on the saturability of tRNA export (23) and on the energy dependence of both NLS-protein import and tRNA export (23, 24) these results confirmed that nucleocytoplasmic transport processes are not only active but require saturable mediators.

NLS conjugates have also been used to determine whether all nuclear import substrates require the same saturable mediators. Initially, it seemed that most nuclear proteins might do so since the import of many members of a radiolabeled mixture of nuclear proteins was affected by saturating levels of SV40 TAg NLS (25). In contrast, the NLS conjugate had no effect on import of U snRNPs (26) that assemble in the cytoplasm and are imported to the nucleus (27). Various techniques were used to demonstrate that saturation of either NLS-protein import or U snRNP import did not affect the transport of the other karyophile and thus to suggest that these two substrates did not require the same saturable import mediator (26, 28).

More recent work has revealed the existence of a variety of protein import signals whose activity is not affected by saturation of the NLS import mediator (Table 1). The stage to which these signals have been characterized varies and is discussed later. In general, Table 1 shows that earlier ideas on the limited diversity of import pathways for proteins may have to be revised. A significant and growing number of well-defined import signals interact with import mediators that do not recognize the classical type of NLS. In addition, the signals listed at the foot of Table 1 do not appear to correspond to any of the better-characterized examples described in the body of the table. This should alert us to the possibility that the diversity of signals and transport mediators remaining to be discovered may still be extensive. Limitations to these arguments are discussed in a later section. There is at present a single example of nuclear uptake

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Table 1 Import signals: an overview[a]

Protein/signal	Nature of signal	Import receptor (plus adaptor)
SV40 large T antigen (simple basic NLS)	PKKKRKV Short sequences containing a single cluster of basic amino acids, often preceded by an acidic amino acid or a proline residue	Importin β together with members of the importin α family
Nucleoplasmin (bipartite basic NLS)	KRPAATKKAGQAKKKK Two interdependent clusters of basic amino acids separated by a flexible spacer (21); neutral and acidic residues flanking the motif contribute (282)	Importin β together with members of the importin α family
hnRNP A1 (M9-domain)	Amino acids 265–303 of human hnRNP A1, a region rich in glycines and aromatic residues. Also an export signal in mammalian cells	Transportin
STAT 1	Not known; formed after tyrosine phosphorylation and dimerization (263, 283)	Importin β together with one of the importin α family, NPI-1
hnRNP K (KNS)	Amino acids 323–361 of human hnRNP K. HnRNP K also carries a bipartite NLS. The KNS is not conserved in evolution. Also an export signal in mammalian and avian cells	Unknown, but experimental evidence excluding importin α and β and transportin
U snRNPs	Complex: RNA-bound Sm core proteins and the trimethyl cap structure of the RNA	Importin β is necessary; Snurportin binds to the m_3GpppN cap
Some ribosomal proteins	Accumulation of basic amino acids. Currently presumed to be distinct from the simple basic or bipartite NLS	In yeast Kap123p/Yrb4 or Pse1p
U1A	Amino acids 94–204 of human U1A protein	Importin α involvement unlikely
TFIIIA	Not yet well-defined; located within the zinc fingers (284)	Importin α involvement unlikely
Lamin B receptor	Within the N-terminal 204 amino acids. This signal targets membrane proteins to the inner nuclear membrane (285)	Importin α involvement unlikely (286)
Suppressor of white apricot	SR domain, i.e. a domain containing serine-arginine repeats (143, 287)	Not characterized
Glycoconjugates	Glucose, fucose, or mannose residues (288)	Importin α involvement unlikely; physiological significance unclear

[a]Examples of NLSs that do not conform to the classes above have been found in the HTLV-1 Tax transactivator (289), the yeast Mat α2 protein (290, 291), the yeast Gal 4 transactivator (292), Adenovirus E1A protein (293), and the c-myc protein (282). It is unclear whether these signals use as yet unknown import pathways, are divergent members of one of the classes above, or lead to nuclear accumulation via a piggyback mechanism, as has been shown for E2F/D1 transcription factors (294), the CBP20 protein (248), yeast Cdc2 protein kinase (295), and others. References not present in the table are given in the text.

of a protein that requires a mediator but does not seem to be energy dependent. This involves calmodulin, whose nuclear accumulation is saturable but appears to require no energy in the form of nucleotide triphosphate hydrolysis (29) and can thus be considered facilitated, rather than active, transport.

Recognition of the NLS by Importin

The NLS-conjugate saturation experiments provided early evidence for the existence of saturable mediators of import, the import receptors. Several such receptors have been characterized molecularly (Table 1). The initial experimental approach used to identify them was to search for proteins that would bind to the well-characterized SV40 TAg NLS (reviewed in 30). Concurrently, in vitro assays for protein import were established (24, 31, 32). These assays were based either on nuclei added to or assembled in *Xenopus* egg extracts or on mammalian cultured cells that had been gently extracted with detergent to permeabilize the plasma membrane and remove soluble cytoplasmic components but leave the nucleus intact. Import in the latter system was dependent on the readdition of cytosol to the permeabilized cells (32). Fractionation of the added cytosol was the major technique used in the identification of the soluble factors required for NLS-protein import.

Researchers identified a protein from reticulocyte lysate (the NLS receptor) that could be cross-linked to the SV40 TAg NLS and functioned in NLS import together with a second protein (p97) (33–35). The NLS receptor from *Xenopus* was cloned and called importin α (36). Importin α was related in sequence to the previously identified yeast Srp1p protein (37), and Srp1p was subsequently shown to be the functional homologue of importin α (38). Although yeast has a single importin α protein, cloning of homologues from other species (e.g. 39–43) demonstrated that multicellular eukaryotes encode a family of closely related importin α–like proteins (reviewed in 1, 44). In contrast, the second subunit of the heterodimer that functions in NLS-protein import, importin β, is unique (38, 45–48). There are several alternative nomenclatures for the importin subunits, such as nuclear pore targeting complex (PTAC)58 and PTAC97, Karyopherin α and β, and NLS receptor and p97. In addition, some individual members of the importin α family have been given several names (1, 44). Where possible, we use the importin α and β nomenclature to avoid confusion.

NLS-protein import has been grossly divided into two stages: energy-independent docking at the cytoplasmic face of the NPC and energy-dependent NPC translocation (49, 50). In the permeabilized cell assay, the importin α/β heterodimer is both required and sufficient for the docking step (45, 51–53). The two importin subunits have specialized functions in docking. Importin α binds the substrate protein through recognition of the NLS, whereas importin β interacts with the NPC (33–35, 41, 51–53). Although this has been shown

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formally only for the importin-NLS protein complex, all import receptors are likely capable of docking at the NPC with their substrate.

The primary docking sites for importin were identified by electron microscopic examination of the docking of NLS substrates that had been conjugated to colloidal gold (6). The docking sites were found on fibers that extend from the NPC into the cytoplasm. Although there may be only one high-affinity docking site per fiber (89), considerable arrays of colloidal gold particles, apparently attached to the NPC fibers, were seen when the substrate was present at high concentrations (6, 49). This observation suggests that the importin complex, via importin β, can bind to multiple sites on the fibers. The existence of multiple docking sites could concentrate the import complex close to the site of NPC translocation.

NPC translocation of the importin-NLS protein complex requires two additional soluble proteins, the Ran GTPase and p10/NTF2 (see below). Dissection of importin α led to the important conclusion that the only functions of α in NLS-protein import are its binding to the NLS and importin β (54, 55). The N-terminal basic region of importin α that is responsible for binding to β (the importin β–binding or IBB domain) includes sequences that look remarkably like a bipartite basic NLS (Table 1). In spite of this, the IBB domain binds to importin β, not to α, and when fused to a reporter protein, is sufficient to target that protein to the nucleus in a β-dependent and α-independent manner (54, 55). Thus it is possible to consider importin β as the genuine import mediator or receptor, and α as an adaptor that joins β and the NLS substrate.

Other Import Signals and Receptors

An interesting mechanistic variation concerns the STAT1 protein, one member of the signal transducer and activator of transcription protein family. STATs are transcription regulators that are cytoplasmic in the resting state but are activated by various extracellular stimuli to move to the nucleus (56, 57). STAT1 activation involves tyrosine phosphorylation–dependent dimerization, which is required to generate a nuclear targeting signal. Unlike proteins that contain an NLS, which seem capable of utilizing any of the importin α family members for import (1, 58), STAT1 import is more selective. STAT1 can be imported by one human importin α, namely hSRP1/NPI-1, but not by another, hSRP1α/Rch1 (59). NPI-1 can also import NLS proteins, but the region of NPI-1 required for NLS binding is distinct from that needed for STAT1 binding (59).

This observation has two major implications. The first implication is practical. We should not expect to be able to recognize all substrates of a particular import receptor by looking at the sequence of their nuclear import signals since STAT1 has no obvious NLS-like sequence. Thus examples of divergent import signals do not necessarily imply the existence of novel import receptors.

Indeed, it is unclear if saturation by NLS-conjugates blocks STAT1 import, and it may be that this test does not unambiguously assign a substrate to a specific transport receptor or adaptor.

The second implication is evolutionary. Given the existence of multiple import (and export) receptors that are capable of shuttling between the nucleus and cytoplasm (see below), it might be relatively easy to generate a domain on a potential substrate protein that can bind somewhere on the surface of one of the receptors, and thus to target a new protein for import or export. Import or export requires both association with a receptor on one side of the NPC and dissociation from the receptor on the other side, and evolution of new substrates therefore is made more difficult.

U snRNP import was mentioned previously. The nuclear import signal of these RNPs is bipartite (Figure 1; Table 1). The first feature consists of an ill-defined component that entails binding of the so-called Sm core proteins, a collection of eight proteins, to an RNA element found only in these imported U snRNAs (27, 60). Assembly of the Sm core structure in vivo may not occur spontaneously, but rather appears to require the mediation of at least two assembly factors, one of which is encoded by the gene whose mutation causes the common inherited genetic disorder spinal muscular atrophy (61, 62). In addition to the Sm core structure, the trimethylguanosine cap that is formed when the Sm core proteins bind the snRNA is also part of the U snRNP import signal (63–67).

As mentioned above, U snRNP uptake was the first example of import proposed to require a different saturable mediator than did NLS proteins. The saturable adaptor that binds to the NLS is importin α, and direct study of in vitro import of U snRNPs has confirmed that importin α is not required (68). However, a variety of approaches led to the conclusion that importin β is needed for U snRNP import. Most directly, depletion of importin β blocked U snRNP import, and readdition of recombinant β reversed this defect (68). The protein that recognizes the trimethyl cap structure during import, Snurportin 1, is a distant relative of importin α and, like α, has an N-terminal IBB domain (J Huber, C Marshallsay, U Crontzhagen, M Sekine, R Lührmann, manuscript submitted). The role of importin β in U snRNP import may therefore involve only its interaction with Snurportin 1. However, Snurportin 1 does not appear to interact with the Sm core structure (J Huber, C Marshallsay, U Crontzhagen, M Sekine, R Lührmann, manuscript submitted). This means that the U snRNP–containing import complex contains minimally one additional essential component, and this may also interact with importin β (68). The advantage of having two or more separate adaptors that interact with the same import receptor, as exemplified by the mutually exclusive interactions of importin α and Snurportin 1

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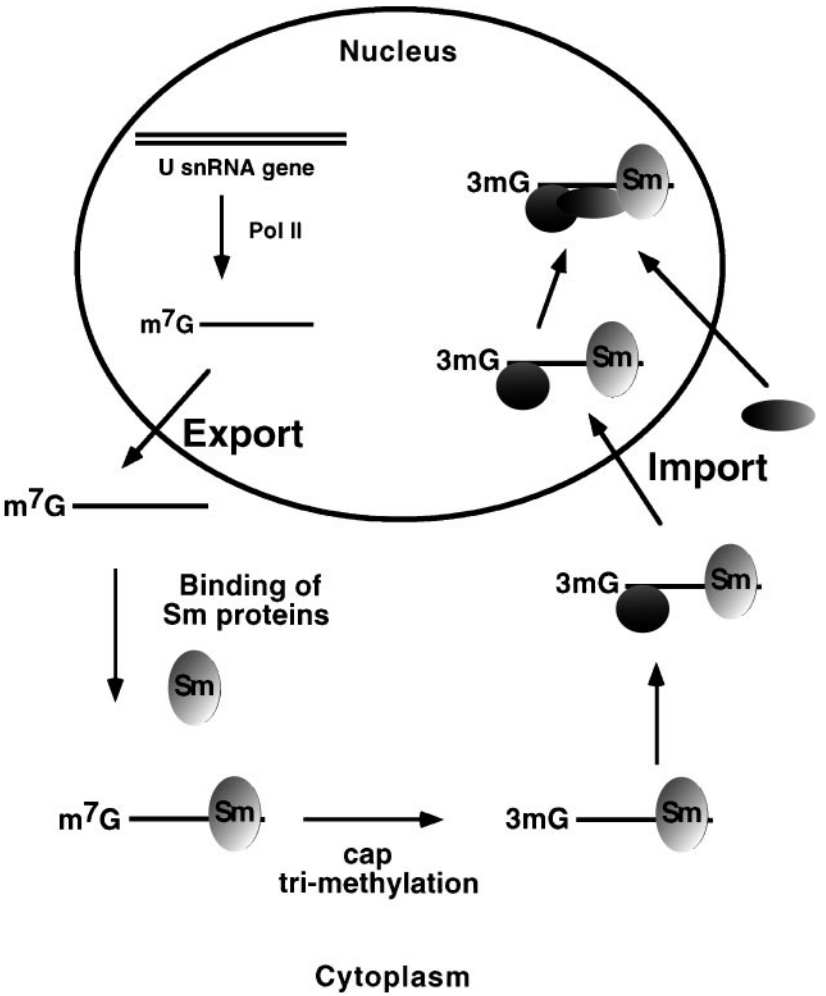

Figure 1 A simplified scheme of U snRNP assembly. This scheme applies to RNA polymerase II–transcribed spliceosomal U snRNAs. They are transcribed in the nucleus and obtain a monomethylguanosine cap cotranscriptionally. After nuclear export, they bind the Sm core proteins. Maturation steps occur, including formation of the trimethylguanosine cap. The U snRNP can then be imported to the nucleus. Other U snRNP–specific proteins can join the RNP either in the cytoplasm or, more likely (79, 300–302), in the nucleus.

Table 2 Importin β-like proteins[a]

Mammalian transport factor	Yeast homologue	Function
Importin β	Importin β/Kap95p	Import of NLS-proteins; import of U snRNPs in vertebrates
Transportin	Transportin/Kap104p	Import of mRNA-binding proteins
Ran BP5 (296)/ Karyopherin β3 (297)	Probably Pse1p	Pse1p thought to mediate import of ribosomal proteins in yeast
?	Yrb4p/Kap123p	Import of ribosomal proteins in yeast
RanBP7 (71)	Possibly Sxm1p or Nmd5p	Unknown
CRM1	Crm1p/Xpo1p	Export of NES-containing proteins
CAS	Cse1p	Export of importin α

[a]Sxm1p, Mtr10p, and Los1p are additional importin β–like proteins for which experimental evidence of an involvement in nucleocytoplasmic transport exists (see text). Other members of the importin β family (70, 71) have not been further characterized. References not present in the table are given in the text.

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with importin β, is presumably to allow separate regulation of import of the two distinct classes of import substrates.

Either by coincidence or for unknown functional reasons, the remaining well-defined examples of import signal-receptor pairs in Table 1 all involve RNA-binding proteins. The receptors involved in the transport of hnRNP and ribosomal proteins characterized to date are distantly related to importin β (Table 2; 13, 70, 71) but do not follow the importin paradigm of division of function between two subunits. Rather, they are either known or thought to bind their substrates directly rather than via an α-like adaptor.

Transportin is the import receptor for hnRNP A1 (72, 73). The hnRNP A1 import signal, the M9 domain, is much longer than the SV40 TAg NLS at 38 amino acids (74, 75) and is rich in glycine and aromatic amino acids rather than being basic in character. Transportin binds directly to the M9 domain (72, 73). Mutation of yeast transportin, Kap104p (76, 77), leads to defective import of one hnRNP protein, Nab2p, and yeast transportin has also been implicated in the import of a second one, Hrp1p/Nab4p (76). The import signals of the yeast transportin substrate proteins have not been delineated, but they are not closely related in sequence to the M9 domain. Similarly, human transportin interacts with a variety of hnRNP proteins, not all of which contain regions that resemble M9 (77a).

The human hnRNP K protein has two import signals. One is a bipartite basic NLS and the second an unrelated element called the KNS (78). KNS-mediated in vitro import is not inhibited by saturating amounts of either the IBB domain (which blocks both NLS-protein and U snRNP import) or the

M9 domain and thus is not likely to be dependent on either importin β or transportin (78). Similarly, the U1A protein of U1 snRNP has a complex nuclear import signal of roughly 100 amino acids in length (79). Import driven by the U1A import signal is not affected by saturation with the IBB, M9, or KNS domains (M Hetzer, unpublished data). These two examples, plus others in Table 1, offer strong evidence that not all the import mediators have been defined.

Almost all ribosomal proteins have to be imported into the nucleoplasm, and then to the nucleolus, for ribosomal subunit assembly. It is unclear whether all ribosomal proteins are imported individually or if some may enter the nucleus as preassembled complexes. Studies of a few yeast and human ribosomal proteins have revealed that some proteins carry more than one functional nuclear import signal (e.g. 80–85). This is probably easier to rationalize by proposing that complexes containing more than one ribosomal protein assemble prior to import. If this were the case, some of these signals would in fact represent interaction domains with other ribosomal proteins required to form a complex. Each complex of ribosomal proteins would require only one "real" import signal, that is, one sequence capable of interaction with an import receptor. All ribosomal protein import signals will presumably have to be covered up on assembly of a ribosomal subunit to prevent their recognition by the import machinery in the cytoplasm.

The best-characterized ribosomal protein import signals either include, or consist of, short, basic peptides (80–85) and therefore were initially presumed to be imported via the NLS-importin pathway. The first indication that this might not be the case was the observation that import directed by the signal derived from the yeast L25 protein (81) was not affected in cells carrying a mutant of the yeast NPC protein, or nucleoporin, Nsp1p (86). In the same cells, import mediated by signals from other yeast nuclear proteins was inhibited (86).

More recently, proteins carrying the L25 import signal have been shown to associate with two members of the importin β family in yeast cells, namely Pse1p and Yrb4p/Kap123p (Tables 1 and 2; 87, 88). Genetic evidence for roles of these proteins in nuclear uptake mediated by the L25 import signal has been presented (87, 88). Pse1p is essential in yeast, while Yrb4p/Kap123p is not. These two import receptors have therefore been proposed to act in a partially redundant way to bring about ribosomal protein import. Many interesting questions remain regarding ribosomal protein import. Apart from the uncertainty about the mono- or oligomeric state of the proteins during import, it also remains to be seen whether all ribosomal proteins will use the same import receptors. In vitro studies with pure proteins and receptors would help to resolve this issue.

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Ran

The energy-dependent step of NPC translocation occurs through the center of the membrane-embedded part of the NPC, the so-called transporter or central plug (6, 89, 90). Translocation of substrate-receptor complexes requires the Ran GTPase. Ran is a critical component of almost all known nucleocytoplasmic transport pathways (91–97) and is discussed at several points in this review. But first, we provide some essential background information (see also 98, 99).

Ran is extremely abundant and at steady state is mainly nuclear, although it is believed to move between the nucleus and cytoplasm. Like many other regulatory GTPases, Ran has low intrinsic activity. It has to interact with both a GTPase-activating protein (RanGAP1 in humans, Rna1p in yeast) and a small Ran-binding protein (RanBP1/Yrb1) to achieve maximal GTPase activity. Once hydrolysis has occurred, Ran also needs a cofactor to dissociate from the GDP formed, the guanine nucleotide exchange factor, or GEF, RCC1/Prp20p. Although other Ran-binding proteins exist, these are the four major components involved in the parts of Ran function that are elucidated thus far. A critical aspect of Ran's function relies on the fact that RCC1 is nuclear and is stably bound to chromatin in the nucleus (100), while both RanBP1 and RanGAP1 are found either on the cytoplasmic side of the NPC or in the cytosol (98, 99). This distribution predicts that RanGTP concentration will be high in the nucleus and low in the cytoplasm. Treatments that collapse this RanGTP concentration inequality, such as increasing the cytoplasmic RanGTP:RanGDP ratio or decreasing the nuclear RanGTP concentration, block nucleocytoplasmic transport (91–97, 101, 102).

The mechanism of the block of import when the cytoplasmic RanGTP: RanGDP ratio is increased seems straightforward. RanGTP binds directly to import receptors like importin β or transportin. In these cases, and apparently also for the Yrb4p-L25 import complex, the result of RanGTP binding is to cause substrate-receptor complex disassembly (77a, 88, 97, 102–105). This interaction may cause dissociation of import complexes in the nucleus after NPC translocation (1, 99, 102, 107), although other possibilities have also been raised (103). It therefore seems logical that cytoplasmic RanGTP would inhibit nuclear import, as it will cause import complexes to disassemble before they reach the NPC. In regulating the formation of import complexes by allowing assembly to occur in the cytoplasm and causing disassembly in the nucleus, Ran provides asymmetry, a property that is needed for transport across the NPC against a concentration gradient. Ran thus helps impart directionality to the process of import. This is the first critical function that Ran is proposed to play in nuclear import.

Crucial though this is, it does not seem to be the only function of Ran in import. Energy-dependent import is inhibited by nonhydrolyzable GTP

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analogues, but not by nonhydrolyzable ATP analogues, and is also inhibited in a dominant way by Ran mutants that can bind GTP but are unable to perform hydrolysis (91–93, 95, 108, 109). Although all these treatments will generate cytoplasmic RanGTP, and thus cause inhibition via disruption of import receptor-substrate complexes, the results have often been interpreted as suggesting that GTP hydrolysis by Ran would play a direct role in nuclear import.

The best evidence for this came from the use of a mutant form of Ran that binds to and hydrolyzes xanthine triphosphate (XTP) rather than GTP. This Ran XTPase supported importin-mediated nuclear transport when tested in permeabilized cells. Instead of cytosol, the cells were supplemented only with the soluble factors required for NLS-protein import in recombinant form (101). Import in this case required both XDP and small amounts of XTP and was not inhibited by nonhydrolyzable GTP (or ATP) analogues in quantities that inhibited cytosol-mediated import (101). The inescapable conclusion of this experiment seems to be that if nucleotide triphosphate hydrolysis is at all required for the nuclear import observed, then XTP (or GTP) hydrolysis by Ran can fulfill the energy requirement.

It should be borne in mind that other studies with the XTPase Ran mutant, also carried out with permeabilized cells but using Ran-depleted cytosol as a source of import factors, led to the proposal that at least one additional GTPase plays a role in NLS-protein import (110). Resolution of this issue will require either functional characterization of the second putative GTPase activity or the development of a simpler in vitro system for NLS-protein import. A candidate for the putative second GTPase is the yeast GTP-binding protein, and possible GTPase, Gtr1p. A mutant form of Gtr1p suppressed mutations in both yeast RanGAP1 (Rna1p) and RanGEF (Prp20p) suggesting that this protein may be involved in nuclear transport (111).

Several other factors have been proposed to play a role in nuclear import. We next discuss additional proteins whose function in relation to import is either poorly understood or has not been firmly established.

p10/NTF2

This small protein is required for efficient NLS-protein nuclear import in permeabilized cells (112, 113). The yeast homologue Ntf2p is essential (114–116), and genetic evidence supports its role in nuclear import (115–117). p10/NTF2 interacts with Ran in the GDP-bound state (114, 118, 119), and it also binds both to a number of nucleoporins and to the Ran-binding protein Yrb2p/RanBP3, which is located in the nucleus (see below) (113, 114). Overexpression of Ntf2p suppresses certain gsp1p (Ran) mutants in yeast (117). The interesting result of the converse experiment, namely that the lethality caused by deletion of the

NTF2 gene can be suppressed by Gsp1p (Ran) overexpression (119) further suggests that the critical role of the protein is to increase the efficiency of one or more of the functions of Ran. For example, this role could involve localizing Ran to a particular place on the NPC, although more information is required before drawing firm conclusions about p10/NTF2 function.

RanBP1/Yrb1p

RanBP1 binds to RanGTP and increases the rate of RanGAP1-induced GTP hydrolysis (120). Both RanBP1 and RanGAP1 are cytoplasmic and therefore maintain cytoplasmic RanGTP at low levels, allowing interaction between import receptors and their adaptors and substrates (101, 102, 104, 107, 121, 122).

Some properties of RanBP1 suggest an additional function, although this function is not yet well characterized. RanBP1 is capable of forming a stable complex with RanGDP and importin β. RanGDP sits between the two larger proteins and is needed to mediate complex formation since either RanBP1 or importin β alone binds well to RanGTP but very poorly to RanGDP (104, 105). Unlike the RanGTP–importin β complex, the RanBP1–RanGDP-β heterotrimer is capable of binding importin α and α-associated NLS proteins (104, 105, 122). Indirect evidence suggests a role for the ternary complex in NLS-protein import (105).

There are some problems with this theory (see also 99). First, RanBP1 contains a signal that retains it in the cytoplasm, presumably through binding to a nontransportable partner. Only when this retention signal is mutated can RanBP1 be detected in the nucleus (123). Further, RanBP1 also contains a signal for active nuclear export (123, 124). If one RanBP1 molecule were carried into the nucleus (as the ternary complex) with each importin β, then an equal and opposite amount of export, and export receptor, would be necessary for each round of import. This is possible, but it is not economical. Third, nuclear RanBP1 is toxic, at least in part because it inhibits many types of nuclear export (97, 124). Given the nuclear toxicity of RanBP1, and the dominance of the cytoplasmic retention signal over RanBP1 nuclear import (123), the nuclear export signal in RanBP1 may be required to prevent trapping of even small amounts of RanBP1 in the reforming nucleus after mitosis.

Yrb2p/RanBP3

There is no direct evidence that this protein is involved in nucleocytoplasmic transport. On the basis of its sequence it was originally designated Nup36p, for nucleoporin of 36 kDa, and shown to interact with p10/Ntf2p (114). However, both the yeast (Yrb2p) and human (RanBP3) homologues are located in the nucleoplasm rather than at the NPC (125; L Mueller, VC Cordes,

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FR Bischoff, H Ponstingl, manuscript submitted). Yrb2p makes a Ran-dependent interaction with Prp20p, and their human counterparts, the RanBP3 and RCC1 proteins, behave in a similar way (125; L Mueller, VC Cordes, FR Bischoff, H Ponstingl, manuscript submitted). Yrb2p/RanBP3 also binds to RanGTP on its own, although this interaction is weak (125; L Mueller, VC Cordes, FR Bischoff, H Ponstingl, manuscript submitted). Analysis of Yrb2p mutants has provided no evidence to date of a role in transport, although yeast cells lacking Yrb2p are cold sensitive for growth (125).

RanBP2/Nup358

RanBP2/Nup358 is an extremely large and interesting nucleoporin. It is located on the cytoplasmic fibers of the NPC (126, 127). It includes four RanBP1-like Ran-binding domains that function in a similar way to RanBP1 in in vitro assays that measure interaction with and modification of Ran activity (121, 126). A fraction of RanGAP1 is found at the NPC rather than in the cytoplasm because it binds to RanBP2 (128, 129). This fraction is modified by the addition of a small, ubiquitin-like peptide, and this modification is required for tight RanBP2-RanGAP1 interaction (128–130). RanBP2 therefore potentially provides a site on the NPC to which both RanGTP and RanGAP1 are bound. Furthermore, the RanGTP, through binding to the RanBP1-like domains, would be in its most sensitive configuration for GTPase activation.

Two possibilities exist for the function of RanBP2 and the bound proteins. First, RanBP2 might bind RanGTP as it leaves the nucleus (perhaps together with export receptors; see below) and hydrolyze it, thus increasing the efficiency by which the cytoplasmic RanGTP concentration is maintained at a low level (99, 107). Alternatively, RanBP2 could function in coupling RanGTP hydrolysis to NPC translocation (128, 129). Since the latter function should be an essential one, the fact that the soluble, cytoplasmic Yrb1p is the only essential RanBP1-like RanGTP-binding protein in yeast speaks against this idea. The only other two RanBP1-like proteins in yeast are Nup2p, a nucleoporin (131, 132), and Yrb2p. They both bind RanGTP poorly (99, 125; L Mueller, VC Cordes, FR Bischoff, H Ponstingl, manuscript submitted), and they are not essential for yeast growth. Even a strain lacking both proteins is viable (133). Unfortunately, it seems as if the function of RanBP2 will have to be elucidated without the benefit of comparative yeast genetics.

Hsp70/hsc70

A final factor with a rather ill-defined role in nuclear import is the heat shock protein, hsp70, and its constitutively expressed cognate, hsc70. Inhibition experiments involving either microinjection of anti-hsc70 antibodies into cells or their addition to in vitro assays provided first evidence for a role of hsc70 in

NLS-protein import (134, 135). More persuasively, depletion of the cytosolic extract used in an import assay with ATP agarose, which would remove hsc70 among other things, inactivated the extract, and this effect could be reversed by addition of recombinant hsp70 or hsc70 (135). More recent experiments in yeast showed that overexpression of hsp70 increased import rate (16).

Although it is hard to rule out nonspecific effects of the hsps in all of these experiments—because they are able to help keep all the proteins required for import in an active conformation—these results may indicate a direct role for hsps in import. This would not have been an a priori expectation since, given both the size of the active NPC transport channel and the fact that RNP substrates such as ribosomal subunits cross the NPC intact, proteins clearly do not need to unfold during NPC translocation. One additional finding that might help to illuminate the role of hsps in import is the observation that not all NLS substrates are affected by hsc70 depletion (136), leading to the suggestion that hsc70 might be required in some cases to allow presentation of the NLS to importin α. A final problem in proposing a role for hsp/hsc70 in nuclear import is that although the known functions of these proteins require ATP hydrolysis, import, at least in vitro, does not (101, 108).

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NUCLEAR EXPORT

Nuclear Export and Nuclear Retention

Many substrates for protein import are individual proteins that carry a nuclear import signal and, thus, are relatively small and simple. This is probably not the case for nuclear export substrates, many of which are ribonucleoprotein particles (RNPs) that can be both large and complex in composition. Ribosomal subunits are one obvious example and mRNPs another (137). A favorite experimental system for microscopic study illustrates this point. The Balbiani Ring (BR) mRNAs of *Chironomus tentans* encode secreted proteins of roughly 10^6 Daltons that are made in larval salivary gland cells. The BR transcripts are 35–40 kb in length, and each is estimated to associate with roughly 500 protein molecules (reviewed by 5, 138). The BR RNPs are large enough to allow easy identification in electron microscope sections, and their passage through the NPC has been studied in detail. The particles, which form during transcription and fold into regularly shaped structures of 50-nm diameter, have to partially unfold at the NPC to permit translocation (139, 140). The unfolded RNP that is translocated has a diameter of 25 nm, roughly equivalent to the maximal size of substrates for nuclear import.

Although different in size from other mRNPs, studies of the protein composition of the BR RNPs have revealed that in this respect they seem to be representative of mRNPs as a class. For example, BR mRNAs are associated

with hnRNP proteins, SR proteins, and the nuclear cap-binding complex, CBC (5). The SR proteins are a family of splicing factors containing repeated serine-arginine dipeptides. They share many properties with hnRNP proteins, being highly abundant, binding to RNA with relatively weak sequence discrimination, and including both members that are constitutively nuclear as well as ones that shuttle between nucleus and cytoplasm (141–143). Further, the BR mRNAs contain introns that must be removed before they are exported from the nucleus; thus, the mRNAs must also associate with the pre-mRNA splicing machinery. Conclusions from the study of BR RNPs are likely to be generally applicable to mRNPs.

One of the most significant observations made thus far is that individual proteins leave the BR RNP at different stages during its formation and passage through the nucleoplasm and NPC into the cytoplasm. Some splicing factors, such as the U snRNPs, are associated with only the nascent or newly mature RNPs (144) and thus probably dissociate soon after splicing has occurred. Other proteins whose mammalian homologues have roles in splicing behave differently. The homologue of hnRNP A1 remains with the RNA at all steps in its maturation and transport and is still detected on the mRNA even when translation is occurring (145). The nuclear CBC stays on the RNA through NPC translocation, but is then removed (146). This is particularly interesting since BR RNPs always exit the nucleus in a 5′ to 3′ orientation, that is, with the capped end, to which CBC is bound, in the lead (139, 140). Finally some proteins, such as the homologue of the SR splicing factor ASF/SF2, dissociate prior to NPC translocation and thus remain in the nucleoplasm (147).

Removal of some RNP proteins is likely to be a general prerequisite for RNP export, due to the phenomenon of nuclear retention. Certain RNAs do not leave the nucleus (except during mitosis), and these RNAs are actively retained in the nucleus by binding to specific, saturable factors (148–151). Similarly, splicing factors that bind to the splice sites of introns in pre-mRNAs prevent these RNAs from leaving the nucleus until the introns have been removed and the mature mRNA released (152–154). How spliced mRNAs are released from the retaining splicing complexes is not understood, although Prp22, a putative RNA helicase of the DEAD/H box class, is required for this process in mammalian cells (155).

With one exception, protein sequences directly involved in nuclear retention are poorly characterized. This exception involves the hnRNP C protein, which is constitutively nuclear. Since hnRNP C associates with many polyA-containing mRNAs and pre-mRNAs in the nucleus (156, 157), it must be removed from them before mRNA export. The nuclear retention sequence (NRS; 158) of the protein plays a role in this process. The NRS has been defined as a 78-amino-acid segment of hnRNP C (158). The NRS is dominant when fused

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to a protein carrying a nuclear export signal (see below), but it is unclear how it functions. Two classes of explanation for NRS activity seem possible. An NRS might interact with some untransportable structure in the nucleus, such as the nuclear lamina, or an NRS might block the activity of, or physically mask, export signals and thus need to be removed to allow transport to occur. It is possible that NRSs may be used for the regulation of export of specific mRNAs since the adenovirus E4 34K protein has an hnRNP C-like NRS that is somehow involved in the selective export of viral RNA, as opposed to cellular mRNAs, from the nuclei of infected cells (159).

It will be interesting to learn how hnRNP C, the SF2/ASF homologue on the BR RNP, and other nuclearly retained mRNA-binding proteins are removed from mRNPs before export, and if there is indeed a single mechanism that achieves this for the different retained proteins. Retention and export factors can be easily distinguished experimentally since saturation of a factor required for retention allows export to happen while saturation of a factor required for export competitively inhibits export (see e.g. 151). However, an important corollary of the seeming generality of nuclear retention is that factors that can be shown experimentally to be required for active export may be needed to release retention rather than in a more direct positive sense.

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Export Signals on RNA Are Generally Adaptor-Binding Sites

As noted previously, the export of tRNA was the first nucleocytoplasmic transport process shown to be saturable and thus receptor mediated (23). Later work revealed that other classes of RNA that are exported from the nucleus, like ribosomal RNA (in the form of ribosomal subunits), U snRNA, 5S rRNA, and mRNAs, also require saturable mediators (160–162). These mediators appear to be class specific. For example, saturable factor(s) required for tRNA export are different from those needed for mRNAs, U snRNAs, 5S rRNA, or ribosomal subunits (161, 162). It has been shown (see below), or is believed, that the class-specific saturable proteins detected in these experiments are not export receptors per se, but rather are RNA-binding proteins that recognize the specific RNAs and mediate their interaction with the actual export receptors. In other words, they are adaptors that are analogous to importin α, which recognizes NLS proteins and mediates their interaction with the actual import receptor, importin β.

Before discussing these adaptors we describe a few features of RNAs that are recognized during export (Table 3). Some of these features are simple: For example, the export of U snRNAs depends upon recognition of their monomethylguanosine cap structures (154, 161, 163, 164). Others are complex and redundant: For example, nonoverlapping regions of yeast heat-shock mRNAs are

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Table 3 Export signals[a]

Export signals on proteins protein/signal	Nature of signal	Export pathway
HIV-1 Rev, PKI (leucine-rich NES)	L-$X_{2\text{-}3}$-(F,I,L,V,M)-$X_{2\text{-}3}$-L-X-(L,I); consensus is not exclusive[b]	Receptor: CRM1
Importin α	Unknown	Receptor: CAS
HnRNP A1 (M9-domain)	See Table 1	Receptor unknown; tested in mammalian cells
HnRNP K (KNS)	See Table 1	Receptor unknown; tested in mammalian and avian cells
CBC	Unknown, formed when CBC binds capped RNA	Receptor: CRM1; CBC conserved in evolution, but evidence for transport function so far in vertebrates and insects
Export signals on RNA		
Rev responsive element of HIV-1 (RRE)	A 234 nt region within the HIV *env* gene with a complicated secondary structure	RRE is bound by Rev, which carries the NES
Constitutive transport element (CTE) of simple retroviruses	Mapped in the Mason-Pfizer monkey virus (MPMV); a 154 nt sequence that forms a long, imperfectly paired stem	Interaction with a component of the cellular mRNA export pathway
U snRNAs	Monomethyl guanosine cap structure	m_7G cap is bound by CBC
5SRNA	Binding sites for TFIIIA or ribosomal protein L5	Ability to bind to either TFIIIA or L5 is necessary for 5SRNA export
Histone mRNA	Acquired during 3′ end formation; a second signal in the mature histone mRNA	

[a]An increasing number of proteins are known to shuttle between the nucleus and cytoplasm, including transcription factors (258, 259), nucleolar proteins (299), other RNA-binding proteins (see text), and protein phosphatases and kinases (185, 258). Whether these proteins all carry signals for active export and what these signal are remains to be elucidated.

[b]Like bipartite basic NLSs, leucine-rich NESs can also be divergent from the consensus, e.g. the equine infectious anemia virus (EIAV) Rev protein NES, GPLESGQWCRVLRQSLPE (298). References to other data in the table are in the text.

recognized during their export (165), and multiple features of polyadenylated mRNAs including the cap structure, the polyA tail, and the "body" of the mRNA (161) seem to contribute to their efficient export and to combine to produce maximal export rates. Similar conclusions with respect to recognition of several RNA features by export factors were obtained in studies of non-polyadenylated histone mRNA export (166; Table 3).

As mentioned above, intron-containing cellular mRNA precursors (pre-mRNAs) are generally retained in the nucleus. For nuclear viruses, for example, the retroviruses, this presents a problem since some viral mRNAs, as well as genomic RNAs, contain sequences that either are or are not spliced out of different individual transcripts to produce mRNAs with different coding capacities (167, 168). Thus these viruses need to export unspliced as well as spliced RNAs. Simple retroviruses like Mason-Pfizer monkey virus (MPMV) or Rous sarcoma virus (RSV) have evolved RNA structures called constitutive transport elements (CTEs; Table 3). These elements allow export of the unspliced mRNAs to which they are attached (169–173). Experiments carried out in *Xenopus* oocytes have shown that saturating quantities of CTE-containing RNAs prevent the export of mRNAs but do not inhibit export of the other classes of RNA tested (U snRNAs, tRNA), suggesting that CTEs are bound by a cellular factor required for mRNA export (172, 173). The identity of this factor is of considerable interest, not least because the CTE acts in a dominant manner to direct the export of an excised intron that would normally be retained in the nucleus (172) whereas mRNA sequences do not have this property. The CTE binds to a number of cellular proteins in vitro (173, 174), but there is no direct evidence that these proteins are transport factors.

Complex retroviruses have developed a more complete solution to the problem of exporting nonspliced RNAs. The best studied example is HIV-1. The RNA element in this case (see Table 3) is predicted to be a complicated collection of hairpin loops called the Rev Response Element, or RRE (175). The RRE, as its name suggests, binds directly to the Rev protein (176).

Export Adaptors

Rev is the best-understood example of an RNA export adaptor. A direct demonstration that Rev promoted RNA export from the nucleus came from experiments in *Xenopus* oocytes (177). As previously discussed, introns are retained in the nucleus, even after being spliced out of pre-mRNAs. When the RRE was inserted into an intron, Rev-dependent export of the intron took place, that is, like the CTE, the RRE-Rev complex is dominant over nuclear retention mechanisms. Rev binds to the RRE through an arginine-rich motif (178) and requires an additional peptide region located C-terminal to this RNA-binding domain for function (179). Initially this region was called the activation domain, and it was

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defined by extensive mutagenic analyses as an eight-amino-acid leucine-rich peptide (179, 180). Later work showed that peptides corresponding to the activation domain of the Rev protein (Table 3), when cross-linked to BSA, directed nuclear export of the conjugate, identifying the leucine-rich peptide as an NES, or nuclear export signal (181). Simultaneously, a cellular protein called PKI (protein kinase A inhibitor) was shown to contain a similar leucine-rich NES (182).

PKI is an example of the use of protein nuclear export for regulation. The cyclic AMP–dependent protein kinase (PKA) exists in unstimulated cells as an inactive heterotetramer of four subunits, two catalytic (C) and two regulatory (R). On stimulation, the complex dissociates and the active C subunits enter the nucleus by diffusion and can phosphorylate substrate proteins there (183, 184). PKI is a small protein that binds to the C subunit, and its NES directs the C subunit–PKI complex back to the cytoplasm, thereby limiting the time in which the C subunit can remain active in the nucleus (182). Mutations in several components of the nucleocytoplasmic transport machinery give rise to complex phenotypes, including the common observation of cell-cycle-related defects. We therefore suspect that transport-determined activation or inactivation of regulatory molecules like kinases or protein-degrading enzymes, including those involved in regulating cell cycle progression (see e.g. 185), may be rather common.

Leucine-rich NESs have been implicated in transport of RNA substrates other than those that carry an RRE. The first evidence for this involvement was that saturation of NES-dependent export with BSA-NES peptide conjugates blocked the export of both 5S rRNA and U snRNAs in *Xenopus* oocytes (181); however, the conjugates had no effect on mRNA export.

To be transported out of the nucleus of *Xenopus* oocytes, 5S has to be able to bind to either TFIIIA or ribosomal protein L5 (186). TFIIIA contains a leucine-rich sequence that, as an isolated peptide, is a functional NES (181, 187). The block to 5S rRNA export caused by saturating levels of BSA-NES conjugates, and the presence of a NES in TFIIIA, further suggest that TFIIIA might mediate 5S rRNA export. However, since 5S mutants that do not bind TFIIIA but do bind to the L5 protein are also exported (186), this cannot be a complete explanation for the export of this RNA.

Although the BSA-NES conjugate experiments indicate that leucine-rich NESs do not play an essential role in mRNA export from oocyte nuclei, some data suggest that the situation may be different in yeast cells. Two *S. cerevisiae* proteins, Gle1p (or Rss1p) and Mex67p, were identified by their genetic interactions with the nucleoporins Nup100p and Nup85p, respectively, and are located at the NPC (188–190). Mutation of either of these proteins can cause the accumulation of polyA in yeast nuclei, an assay that was developed specifically

to allow identification of yeast genes involved in some aspect of mRNA export (e.g. see 191–193). This assay is extremely useful, although its major weakness is that polyA does not necessarily equate with mRNA. Conditional (temperature-sensitive) mutants of both Gle1p and Mex67p were used to show that polyA accumulation, that is, the presumed mRNA export defect, occurred rapidly after inactivation of the proteins (188–190).

Further evidence that Mex67p could be involved directly in RNA export came from the observation that the protein can be cross-linked to RNA in vivo and that it interacts with RNA-binding proteins (190). Both Mex67p and Gle1p contain sequences that resemble a leucine-rich NES (188, 190), although these putative NESs are not conserved in their vertebrate homologues (190; L Englmeier, unpublished data). Nevertheless, tested as short peptides, the NES-like sequences from both Gle1p and Mex67p do signal export on microinjection into vertebrate cell nuclei (188, 190). Moreover, deletion of these peptides or point mutations in the NES-like sequences gives rise to either nonfunctional or only conditionally functional proteins, respectively, with consequent accumulation of polyA in the nucleus. This observation suggests that NESs may be involved in yeast mRNA export. To confirm this conclusion, it must be shown that the sequences function as NESs in the context of either Gle1p or Mex67p in yeast cells, and that this function is required for mRNA export.

The hnRNP family of proteins is implicated in mRNA export (157, 194). HnRNP proteins associate with polyA-containing RNA in the nucleus. There are roughly 20 proteins in the human hnRNP family, although neither the size of the family, nor the sequences of individual members, are highly conserved in evolution. Since hnRNP proteins are located in mammalian cell nuclei at steady state, they were not initially considered as potential export mediators. The demonstration that some hnRNP proteins shuttle continuously between the nucleus and cytoplasm (195, 196) changed this perception. The best-studied member of this protein family to date is hnRNP A1. Both the number of hnRNP A1 molecules per HeLa cell nucleus (10^8) and the amount of A1 shuttling (10^5 molecules per min) is enormous (197). As noted above, the *C. tentans* hnRNP A1 homologue leaves the nucleus bound to the BR mRNA (145). These results suggested a role for hnRNP A1 in mRNA export.

This proposal was further strengthened when a region of hnRNP A1, the M9 domain, was found not only to direct nuclear import of the protein (Tables 1 and 3) but also to be sufficient for nuclear export (15). Note that a different shuttling signal has since been identified in hnRNP K (Tables 1 and 3), which must also be considered a candidate mRNA export mediator (78). Microinjection of saturating amounts of hnRNP A1 into *Xenopus* oocyte nuclei blocked export of some mRNAs (198; see also 97, 172). The export block presumably

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reflects titration of an export mediator by hnRNP A1, and the variation between RNAs may be explained by the fact that different mRNAs preferentially bind different hnRNP proteins (199, 200). Some mRNAs might, for example, have sufficient hnRNP K bound not to need the factor that recognizes hnRNP A1 for their export.

Evidence suggests that the export mediator titrated by hnRNP A1 might be distinct from transportin, the M9 import receptor (Table 1). A mutation of the M9 domain that prevents its recognition by transportin and blocks hnRNP A1 nuclear import (15, 72, 198) does not abrogate the effect of saturating levels of hnRNP A1 on mRNA export, although deletion of the whole M9 domain does so (198). Further, transportin is dissociated from M9-containing proteins by RanGTP (77a, 97). RanGTP is found at high concentration in the nucleus; therefore, it might prevent transportin-M9 interaction from occurring there. Definitive proof that transportin is not involved in mRNA export will require identification of the export receptor that recognizes hnRNP A1.

Yeast also contains hnRNP-like proteins: Np13p/Nop3p/Nab1p (201–205), Nab2p (205), Nab3p (201), and Hrp1p (206). Npl3p is the most extensively studied. It bears a distant resemblance to hnRNP A1, as does Hrp1p, and its mutation results in polyA accumulation in the nucleus (202, 204). Like hnRNP A1, Npl3p shuttles between the nucleus and the cytoplasm (203, 207). These characteristics indicate a level of functional homology between Npl3p and hnRNP A1; however, critical differences exist. First, although hnRNP A1 export is transcription independent (195), movement of Npl3p to the cytoplasm requires ongoing transcription and is blocked by mutation in an RNA-binding domain of Npl3p (207). This observation might suggest that Npl3p is carried to the cytoplasm by RNA rather than the reverse, although Npl3p might carry an export signal that is exposed only on RNA binding. Further, mutants of Npl3p prevent NLS-mediated protein import (208), whereas hnRNP A1 has no obvious connection to this import pathway. The implication of this finding for Npl3p function is not obvious. Again, as for hnRNP A1, identification of the hnRNP export receptors in yeast will help to resolve Npl3p's functional role and further clarify whether the apparent differences with hnRNP A1 are significant.

Although the earliest RNA export studies were with tRNA, the export of this RNA is still not well-understood. Evidence suggests that tRNA export in vertebrates exhibits differences from that of other RNA species, not only in terms of the saturable export mediators with which it interacts but also in relation to NPC proteins involved in its export (161, 209, 210; but see below). However, no proteins specifically involved in tRNA export have been identified. Yeast genetic studies have demonstrated interaction between the nucleoporin Nsp1p and two proteins that are involved in tRNA production (211). The proteins are

a pseudouridine synthase (Pus1p) that modifies certain uridine nucleotides in tRNAs to pseudouridine and Los1p. Los1p localizes to the NPC (211) and had previously been implicated in pre-tRNA splicing (212). Los1p is distantly related to importin β (Table 2; 70, 71). These facts suggest that Los1p could be involved in tRNA export and, more generally, that tRNA maturation and export could be coupled events.

The last part of this section is devoted to CBC, the nuclear cap-binding complex that functions in U snRNA export. This heterodimeric complex, composed of the CBP80 and CBP20 proteins (163, 213–217), binds to capped nuclear RNAs. CBC translocates through the NPC attached to the 5′ end of BR mRNPs (146). Since these mRNPs always traverse the pore 5′ end first, CBC might have a role in orienting the RNPs for efficient translocation. However, it does not appear that the role of CBC in mRNA export is more than a minor one (161, 215).

In contrast, preventing the interaction of CBC with newly synthesized U snRNAs disrupts their nuclear export (154, 161, 163, 215) and thus prevents their assembly with U snRNP proteins in the cytoplasm (Figure 1). A likely explanation for the difference in the relative importance of the role of CBC for U snRNA and mRNA export is that additional proteins that bind to the latter, such as the hnRNP proteins, are sufficient to allow their export even in the absence of CBC. CBC is multifunctional, with roles in pre-mRNA processing as well as in RNA export (214, 215, 218, 219). Although CBC is also found in yeast (220–222) and functions in pre-mRNA processing there, no evidence exists to indicate that yeast CBC plays a direct role in U snRNA or mRNA export.

Export Receptors, the Exportins

The previous section discussed adaptors that we proposed would mediate interaction between RNAs and "real" export receptors. Two such receptors have just been identified, although only one, CRM1, has thus far been shown to mediate RNP export. The second, CAS1, was initially identified as the human homologue of an *S. cerevisiae* gene, *CSE1*, whose mutation caused abnormal chromosome segregation in mitosis (223, 224). Mutants of *S. pombe* CRM1, in contrast, caused abnormal chromosome morphology (225). These phenotypes underline the previously discussed problem of identifying transport factors on the basis of the visible defects they cause when mutated. Clearly, blocking nucleocytoplasmic transport of a class of macromolecules can affect virtually any aspect of cell growth and morphology.

Other information on the CRM1 and CAS proteins provided more direct evidence of their function. Both are distantly related to importin β, particularly in its N-terminal Ran-binding region (70, 71). Mammalian CRM1 had furthermore been identified as an NPC-associated protein that binds directly to at least

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one nucleoporin, CAN/Nup214 (70). Therefore, there were good reasons to expect a functional relationship between the proteins and importin β.

This turned out to be the case. CAS binds importin α in a RanGTP-dependent way (226), that is, under conditions thought to exist in the nucleus. An in vitro assay showed that importin α re-export from the nucleus depends on the presence of CAS (226). Although the region of importin α that interacts with CAS is not defined, previous work had shown that the N-terminal importin β–binding (IBB) domain was sufficient to direct nuclear import but not export (54, 55), indicating that CAS binding will require other regions of importin α.

CRM1 also binds to the substrates it transports cooperatively with RanGTP (227). These substrates are proteins like Rev and PKI that carry a leucine-rich NES (Table 3) (227–229). Leptomycin B was identified as a potential fungicide and later shown to be an inhibitor of CRM1 function (227, 230, 231). As expected from the role of CRM1 as an export receptor for NES proteins, leptomycin B inhibits Rev export from the nucleus (227, 232). Consistent with the fact that saturation of the NES pathway also blocks U snRNA export, leptomycin β also inhibits export of these RNAs in *Xenopus* oocytes (227), although it is unclear whether CRM1 interacts directly with CBC, the export adaptor of these RNAs.

One mutant form of *S. cerevisiae* Crm1p blocks mRNA export (229), which recalls our prior discussion of the role of proteins containing NES-like sequences in mRNA export in yeast and somewhat strengthens the argument that leucine-rich NESs may be involved in mRNA transport in this organism. Some caution is required, however. Mutation of importin β, an import receptor, can give rise to dominant negative effects that block transport of many substrates whose import or export does not require importin β (233). At least one mutation in human CRM1 (one that changes only two amino acids of the protein) blocks mRNA export when expressed in *Xenopus* oocytes even though CRM1 is not required for this form of export (M Ohno, unpublished data). Furthermore, several additional yeast Crm1p mutants that block Rev NES-mediated transport in *S. cerevisiae* do not affect mRNA export (234).

Proteins other than CRM1 had previously been found to interact with the Rev NES. Might they also have a role in export? One of these proteins was eukaryotic initiation factor (eIF)5A (235, 236). This protein was thought to be involved in translation initiation, but its role was poorly defined (see 235). We must admit to skepticism about the involvement of eIF5A in Rev function. First, it was identified by a five-step procedure that would require co-migration on anion exchange chromatography, (isoelectric) chromatofocusing, and two-dimensional gel electrophoresis of the native 16.7-kDa eIF5A protein and of the protein after its cross-linking to a Rev NES peptide of 2 kDa in mass and net charge of -3. Furthermore, the most direct experimental evidence for a role of

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eIF5A in Rev function was based on the authors' contention that overexpression of eIF5A was required for Rev to show export activity in *Xenopus* oocytes (235). This was later shown not to be the case (172, 177).

The second pair of candidate Rev NES-binding proteins were yeast Rip1p and the human Rip/Rab protein, which are distantly related in sequence (237–239). The proposed interaction between these proteins and the Rev NES was based almost entirely on the results of two-hybrid screens. As previously discussed (227), the Rip proteins are related to nucleoporins and in particular bear a striking resemblance to CAN/Nup214, the protein with which mammalian CRM1 interacts in vivo (70). The resemblance with Rip1p/Rip/Rab is in the domain of CAN/Nup214 required for CRM1 binding (240). It was therefore proposed that the two-hybrid data might be explicable if endogenous yeast Crm1p mediated the interaction between the Rev NES and Rip proteins. Indeed, no two-hybrid interaction was seen between Rip1p and the Rev NES in yeast strains carrying mutant CRM1 genes (234). This observation strongly supports the view that Crm1p mediates NES-Rip1p interaction. Thus, as suggested by the original studies (237–239), Rip1p may well have a function in Rev NES export, but this function will involve interaction with Crm1p.

We end this section with a short description of three additional importin β–related proteins for which there is genetic evidence of a role in nuclear transport (Table 2). Los1p (211) was already discussed, and the indirect evidence for its possible role in tRNA export described. Los1p is unlikely to be the only import or export receptor for specific substrates, because it is not an essential gene (211, 212).

The proposal (87, 88) that Pse1p and Yrb4p/Kap123p may be redundant import receptors for ribosomal proteins (Table 2) is also a topic treated previously. Surprisingly, combination of a YRB4/KAP123 gene deletion that is viable with a conditional allele of PSE1 leads to polyA accumulation in the nucleus in nonpermissive conditions, that is, presumably to a defect in mRNA export (241). This result may indicate additional functions for either of these importin β relatives. The alternative explanation raised earlier in relation to CRM1, that is, the possibility of dominant effects of the mutant Pse1p protein on transport events not directly mediated by Pse1p, also applies here. Nevertheless, further study of the basis of these effects is clearly of interest. There was no defect in NLS protein or hnRNP protein import in these strains (241), consistent with the conclusion that the yeast importin and transportin homologues are their required import receptors (Table 1).

Possession of the doubly mutant yeast strain allowed the identification of Sxm1p as a high-copy suppressor of the phenotype caused by combination of the conditional Pse1p mutant and the deletion of *YRB4/KAP123* (241). Sxm1p could also rescue the lethality caused by *PSE1* deletion (241). This is evidence

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for a functional relationship between Sxm1p and Pse1p. Like *YRB4/KAP123*, *SXM1* is not an essential gene (241).

Finally, Mtr10p was identified by a mutation that caused polyA accumulation in the nucleus (193). Further study revealed abnormal 18S rRNA production in the mutant strain, indicative of pleiotropic defects (193). Nevertheless, the correlation between relatedness to importin β and function in nucleocytoplasmic transport (Table 2) means that Mtr10p and the other importin β family members (70, 71) are valuable objects of further study.

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ASYMMETRY AND RECYCLING

Ran and the Real Difference Between the Nucleus and the Cytoplasm

We now discuss the interaction between Ran and exportins, emphasizing the likely importance of this interaction for our understanding of nucleocytoplasmic transport. Two previously discussed points bear repeating here: First, RanGTP interacts with at least three import receptor-substrate complexes and causes their dissociation (88, 97, 102, 103, 105, 107; Figure 2); and second, high nuclear and low cytoplasmic RanGTP concentrations are predicted (98, 99). These points make obvious the significance of the observation that the two identified export receptors, CAS and CRM1, bind tightly to their substrates only through cooperative binding involving both the substrates and RanGTP (Figure 2). Export complexes form in the nucleus in the presence of RanGTP. Their dissociation in the cytoplasm is likely to involve the hydrolysis of GTP by the receptor-bound Ran under the influence of the RanBP1, RanBP2, and RanGAP1 proteins that are either cytosolic or associated with the cytoplasmic face of the NPC (99, 107, 226, 242). In contrast, import complexes are stable only in the absence of receptor-bound RanGTP, that is, in the cytoplasm, and they dissociate in its presence (Figure 2). Therefore, the differential RanGTP concentrations in the nucleus and cytoplasm are likely to be a fundamental cause of directionality in receptor-mediated nucleocytoplasmic transport events and, thus, of the difference between the macromolecular composition of the nucleus and the cytoplasm. Ran is unlikely to be the only determinant of transport asymmetry; other contributions to directionality are likely to include those of the structurally asymmetric NPC.

Although the nuclear and cytoplasmic concentrations of RanGTP are unknown, strong indirect evidence indicates the importance of the RanGTP concentration gradient across the NPC. The studies reviewed here focus on vertebrate systems, but studies in yeast (see 98, 243) have also contributed greatly to this conclusion.

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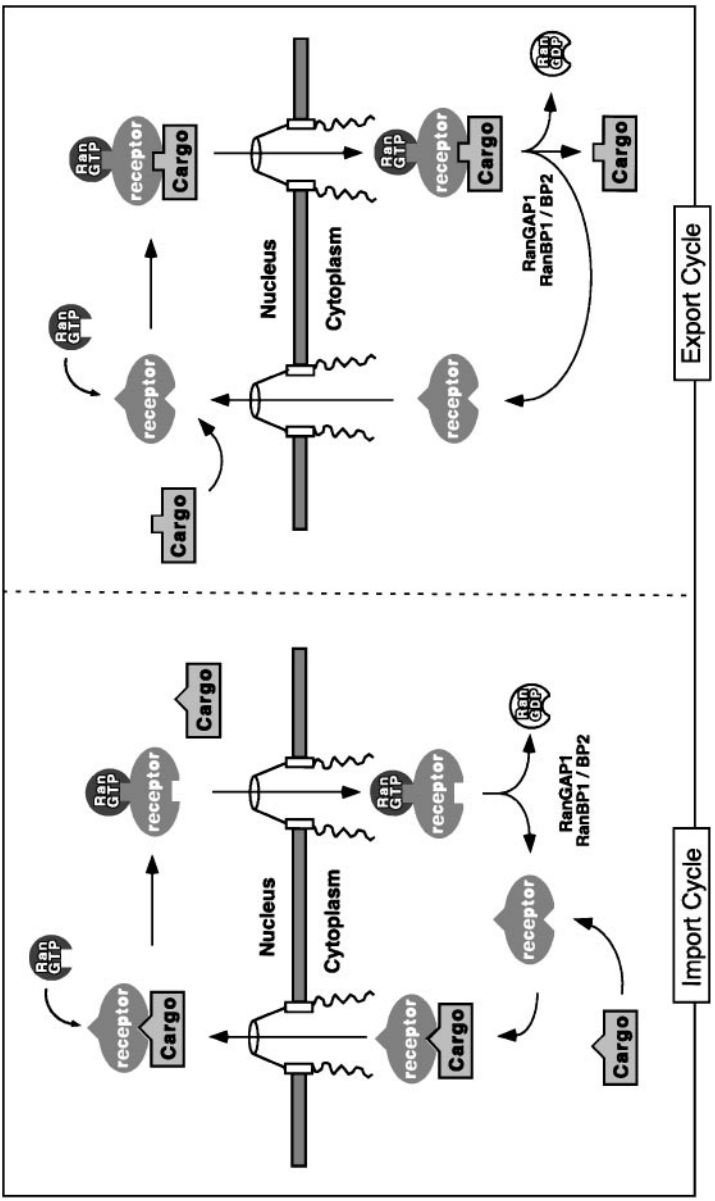

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A first line of evidence has come from the use of the tsBN2 Chinese hamster ovary cell line. In these cells RCC1, the nuclear Ran guanine nucleotide exchange factor, is temperature sensitive and unstable at the nonpermissive temperature (244). The loss of RCC1 protein at high temperature will result in reduced regeneration of RanGTP from RanGDP in the nucleus and also results in relocation of Ran to the cytoplasm. As a consequence, tsBN2 cells at the nonpermissive temperature are defective in NLS-protein import (245), in the export of polyA-containing (m)RNA and U snRNAs (210, 246), and in ribosomal RNA production, perhaps as a result of defective ribosomal protein import (210). Some forms of transport, probably those that are either most rapid or have the lowest requirement for RanGTP, and are thus most difficult to inhibit by RanGTP depletion, still go on in these cells. Examples are tRNA export and Rev export (96, 210). However, Rev export is inhibited by further reduction of nuclear RanGTP in the nuclei of these cells induced by direct injection of RanGAP1 (96).

Similarly, a series of experiments designed to decrease nuclear RanGTP concentration in the nuclei of *Xenopus* oocytes caused a direct block of all tested forms of nuclear export of proteins and RNPs, including those containing tRNA (97). That the effect was direct, and not an indirect consequence of a block of import of adaptor proteins, was shown by the fact that export could be inhibited in conditions that did not block protein import (97). The conclusion of these studies is that high nuclear RanGTP concentration is critical for all forms of nucleocytoplasmic transport examined. The one apparent exception, involving heat shock, is discussed later.

The vesicular stomatitis virus M protein, when injected into or expressed in vertebrate cells (247), results in a complex phenotype similar to that seen in tsBN2 cells at the nonpermissive temperature or in *Xenopus* oocytes into whose nuclei high concentrations of RanGTP in a nonhydrolyzable form have been injected (97, 247). If the hypothesis (247) can be confirmed that M protein directly affects some aspect of the RanGTP cycle, it will be an extremely useful tool in the study of Ran function.

←

Figure 2 Simplified scheme of the interactions between import and export receptors, their cargo, and RanGTP. RanGTP causes assembly of export complexes and disassembly of import complexes in the nucleus. The cooperative action of either cytosolic RanBP1 and RanGAP1 or NPC-associated RanBP2 and RanGAP1 combine to cause hydrolysis of Ran-bound GTP and thus release export cargo or regenerate import receptor in the active conformation. In many cases, adaptors (such as importin α) are required to mediate receptor-substrate interaction. For simplicity's sake we have considered these adaptors as part of the cargo. Note, however, that like the receptors these adaptors must also be recycled to their original starting compartment. This involves receptor-mediated transport. See text for references.

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If the RanGTP distribution across the NPC is so important, how is it set up and maintained through mitotic or meiotic division? Here, little concrete evidence has been presented, but it seems probable that the tight association of RCC1 with chromosomes (100, 244) may be critical. Since chromosomes are first separately surrounded by nuclear envelopes at telophase to form individual mini-nuclei before fusing to form the nucleus (2), chromosome-associated RCC1 might be incorporated directly into nuclei at the first assembly stage, leaving RanBP1 and RanGAP1 in the cytoplasm and thus automatically leading to the re-establishment of the RanGTP gradient.

An interesting speculation arises from the differential effects of RanGTP on the binding between import and export receptors and their substrates (Figure 2). In theory, a single receptor could bind to an import substrate in the absence of RanGTP, that is, in the cytoplasm, and release it into the nucleoplasm on binding RanGTP. At the same time, RanGTP could induce a conformation in the receptor that would allow association with an export substrate, which would be carried to the cytoplasm and released on hydrolysis of the Ran-bound GTP. Thus a single receptor could function both as an import and an export receptor. There seems no obvious logical reason why this should not occur, even if there are currently no examples of such behavior.

Recycling of Adaptors and Receptors

We have discussed both import and export as unidirectional events. In order to function, however, both adaptors and receptors have to be recycled in the empty state to the compartment (nucleus or cytoplasm) where they will pick up a new cargo molecule. Current evidence suggests that adaptors function as adaptors in one direction and as substrates in the other. For example, importin α mediates interaction between NLS proteins and importin β during import. For export, importin α binds to CAS in a RanGTP-dependent way (226). CAS prefers to bind importin α in the absence of a bound NLS (226). Whether this, and the lower affinity of importin α for the NLS after separation from β (102–105), is enough to explain α-NLS dissociation in the nucleus remains to be seen. An active dissociation mechanism might be required. In any event, CAS is the receptor that recycles the "empty" importin α adaptor back to the cytoplasm.

HnRNP A1, proposed to mediate mRNA export, can be imagined in a similar way. It leaves the nucleus bound to mRNA (145, 195, 196) and after dissociation is recycled to the nucleus via transportin-mediated import (72, 73). The CBC cycle is both more complex than this and economical in a rather elegant way. CBC leaves the nucleus bound to capped RNA (146; M Ohno, unpublished data). This export is mediated by CRM1 (227), and CBC is presumably bound either directly or indirectly to CRM1. The association between CBC and CRM1 is thought to be broken in the cytoplasm via hydrolysis of the GTP on

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the CRM1-bound Ran (226, 227). However, CBC also makes an unusual interaction with importin. CBC, through its NLS-containing CBP80 component, remains stably bound to importin α in the nucleus rather than dissociating like other NLS proteins (220). Thus the CBC-U snRNA-CRM1 export complex also contains importin α. When this complex leaves the nucleus and, thus, the region of high RanGTP concentration, importin β can interact with the CBC-bound α molecule. This interaction simultaneously causes CBC to release the bound RNA (220) and targets CBC for import back into the nucleus by the normal importin α/β-mediated mechanism (248). In this way, two receptors, CRM1 and importin β, combine to ensure the directionality of CBC-mediated U snRNA export.

On purely logical grounds, the adaptor-recycling mechanism seems extremely unlikely to apply to the receptors, the importin β family, themselves. The reason for this is simple: If each molecule of importin β that moves into the nucleus needed a receptor, say CRM1, to carry it back out, then CRM1 would need an import receptor (X) to carry it back in, and so on in a never-ending cycle of receptors in one direction turning into cargo in the other. This scenario is clearly absurd, as an infinite number of transport events and an infinite amount of energy would be needed to import a single NLS protein. Although an NES-like sequence in yeast importin β is required for its function (249), we consider it very unlikely that this sequence will direct CRM1-dependent export. Rather, the export of importin β and the import of CRM1, as examples, should follow a different principle. It seems most likely that the receptors, devoid of adaptors and transport substrates, will be recycled directly via interactions with the NPC.

In a formal sense, receptor recycling is the reverse of the functional import or export step but in the absence of bound cargo (adaptors plus substrate). Thus it represents a form of the next critical problem that needs to be solved in the field of nucleocytoplasmic transport, the mechanism by which NPC translocation occurs. Although many interactions between importin β family members and individual nucleoporins have been reported (47, 70, 76, 87, 103, 250, 251), little evidence exists for the functional significance of most of these (discussed in 13, 251).

Ran is also involved in the control of receptor-nucleoporin interactions. RanGTP binding to importin β dissociates nucleoporin–importin β complexes in vitro (103). In addition, a mutant form of importin β from which the RanGTP-binding site is deleted is capable of NPC translocation but appears to bind very tightly to specific sites on the nuclear side of the NPC (102). This mutant is a dominant inhibitor of all tested forms of active nucleocytoplasmic transport (233). This shows that importin β can be translocated through the NPC without bound Ran and suggests that RanGTP is required for clearance of importin β from a critical site or sites that it occupies once nucleus-directed translocation

is complete. The dominant effect on other transport events could indicate that these sites are commonly used by different transport receptors, but other explanations are also possible (discussed in 233). Perhaps the most interesting possibility is the idea that the site to which the mutant form of β binds is some kind of "checkpoint," whose occupancy would in some way close down the pore for the transport of other receptor complexes. If true, this might reflect a mechanism to prevent traffic jams in the pore (233).

It is very likely that RanGTP binding to importin β at the sites occupied by the mutant is not only the last (or termination) step of NLS-protein import but also the first step in importin β re-export (102). Thus importin β would be expected to exit the nucleus bound to RanGTP. Conversely, export receptors would be expected to exit the nucleus bound to both cargo and RanGTP, but would return to the nucleus without either. Further details on the mechanism of these recycling processes should be forthcoming.

It is possible that the NPC clearance or termination step of importin-mediated transport is the only step of the process that requires RanGTP, in other words, that RanGTP is needed to dissociate importin β from the inner side of the NPC and from importin α. Similarly, there is no definitive argument against the possibility that GTP hydrolysis by Ran is required only to prevent the build-up of cytoplasmic RanGTP, which would prevent interaction between importin β and the α-NLS protein complex. Thus more data are required to prove that NPC translocation per se is an energy-requiring process.

A mutant form of Ran that cannot hydrolyze GTP is able to support several forms of nuclear export (97). This observation again raises the possibility that NPC translocation is not obligatorily coupled to hydrolysis of GTP by Ran. This does not mean that we hold the view that translocation will be found to be uncoupled from the provision of energy. It seems unlikely that the aqueous NPC channel could be opened from 9 to 25 nm without energy input. Similarly, transport of large substrates such as the BR RNPs seems on the one hand inconceivable without energy and on the other not fundamentally different from transport of smaller mRNP substrates. Where and how energy is needed for translocation is a critical area for further study. It may be that receptor recycling, since it involves simpler transport substrates, is the experimental system of choice to answer these questions.

REGULATION OF NUCLEOCYTOPLASMIC TRANSPORT

This section describes examples of regulated nuclear transport, involving both protein and RNA substrates. The examples, while not comprehensive, illustrate themes in transport regulation, such as changes in transport that are responses to

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growth state, extracellular signals, developmental stage, or stress. Furthermore, they illustrate the theme that the many possible targets of regulation in the transport machinery are all likely to be used in one case or the other.

Feldherr and colleagues (reviewed in 252) have observed that cells have different nuclear "import capacities" when they are in different states. This observation reveals itself in two ways: First, rates of import change; and second, when import substrates are coupled to colloidal gold particles of different sizes, the ability of cells to import large- versus small-sized conjugates varies. For example, proliferating cells import faster and have a larger apparent active NPC diameter than do quiescent cells (253). Similar differences are seen between transformed and nontransformed cells or between cells immediately after mitosis and at later times in the cell cycle (252). Although the molecular basis of these effects is still unknown, it is hard to see how they could be explained without proposing changes in the NPC itself. Further progress in understanding this global regulation is eagerly awaited.

At a more specific level, the nuclear accumulation of many proteins, including many transcription regulators, is controlled in response to signals that result in a change in their phosphorylation. One of the first examples discovered was the yeast Swi5p protein, which helps control expression of the endonuclease responsible for mating-type switching, a prerequisite for sexual reproduction in *S. cerevisiae*. Swi5p is nuclear in G1 but cytoplasmic at other times in the cell cycle. A 50-amino-acid fragment from Swi5p confers this regulated import behavior on a reporter protein (254). The 50 amino acids contain a bipartite basic NLS and two serine residues whose phosphorylation regulates the activity of the NLS. Their mutation to alanine results in a constitutively nuclear protein (254). Thus the activity of the Swi5p NLS is negatively regulated by the phosphorylation of critical serine residues.

A similar vertebrate example is the NF-AT family of proteins, involved in regulating genes involved early in mounting an immune response. These proteins are competent for both DNA binding and transcriptional activation when isolated from cytosol and, thus, appear to depend on control of nuclear import to regulate their activity (255). NF-AT activation occurs as a result of signal-mediated changes in calcium concentration. Elevated intracellular calcium ion concentration activates calcineurin, a phosphatase that dephosphorylates critical serine residues in the NF-AT proteins. Dephosphorylation exposes two basic NLSs, allowing nuclear accumulation and target gene activation (256). An interesting facet of this regulation is that calcineurin enters the nucleus with NF-ATs and must continuously dephosphorylate the proteins, antagonizing a nuclear kinase, to maintain their nuclear localization (257, 258). Thus, phosphorylated NF-ATs must be substrates for (phosphate-regulated) nuclear export.

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The best-characterized example of regulated protein export concerns yeast Pho4p, a transcription activator involved in turning on genes involved in phosphate metabolism. These genes are switched off rapidly when yeast cells are exposed to extracellular phosphate. The nuclear localization of Pho4p is regulated by the cyclin/cyclin-dependent kinase pair Pho80p-Pho85p (259) as part of this phosphate response. Phosphorylated Pho4p is cytoplasmic and therefore inactive as a transcription activator, whereas the dephosphorylated, functional form of the protein is nuclear. It was recently shown that the Pho80p-Pho85p kinase is constitutively nuclear, and that Pho4p is nuclear until Pho80p-Pho85p is activated in response to extracellular phosphate, at which time Pho4p is phosphorylated and rapidly exported (E O'Shea, personal communication).

Another common strategy for controlling the nuclear import of transcription factors is to regulate interactions with inhibitory factors that cause their cytoplasmic retention. Examples are the *Drosophila* heat shock transcription factor (260), the mammalian glucocorticoid receptor (261) and STAT factors (262, 263). The best-characterized case is NFκB, which is ubiquitously expressed but seems to have a particularly significant role in the immune system. In unstimulated cells NFκB is present in trimeric cytoplasmic complexes like the one that consists of the p50 and p65 subunits of NFκB bound to IκBα. I stands for inhibitor, and IκB prevents nuclear entry of the active heterodimeric NFκB by occluding its NLS (264). One pathway of NFκB activation involves tyrosine phosphorylation of IκB. This is sufficient to cause IκB to dissociate from NFκB and to allow NFκB nuclear translocation (265). The more commonly used pathway is more complex. It involves phosphorylation of two serine residues on IκB (266, 267). This targets IκB degradation by proteasome-mediated hydrolysis (268–271), releasing NFκB for nuclear entry.

Transport regulation is not confined to proteins, and examples of RNA export regulation have been described. Several viruses have evolved mechanisms that allow selective export of viral mRNAs (reviewed in 272), but for a long time the only example of regulated cellular mRNA export was that of maternal histone mRNAs in certain sea urchin species (273, 274). In this case, maternal histone messages are stored in the nuclei of the eggs and released shortly before maturation to allow the bulk histone protein synthesis required during the first, very short, cleavage-stage cell cycles of embryo development. The basis of this retention and release mechanism has not been studied.

Recently, a second example of regulated RNA export has been discovered that points to the existence of a transport mechanism quite different from any of those discussed here so far. The regulated mRNAs are those encoding the *S. cerevisiae* heat shock proteins. Despite their name, these mRNAs are not only synthesized in response to excessive heat but also in response to signals induced by multiple forms of cellular stress. The discussion that follows is relevant to

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the stress response in general, not just to heat shock. We focus on the effect of stringent heat shock, for *S. cerevisiae* at 42°C, as the situation appears different after a milder heat shock at 37°C (165, 275).

The basic observation on which this work was based was the finding that heat shock (hs) prevents bulk polyA (i.e. mRNA) export from the nucleus (276). Cole and colleagues realized, however, that since induction of some hs genes involved turning on their transcription, the newly made hs mRNAs must be exported to allow hs protein synthesis (165). By comparing the export of bulk polyA and specific hs mRNAs they proved that hs mRNA was indeed exported in stress conditions (165). The specific mRNA they examined, encoded by the *SSA4* gene, carries at least two different nonoverlapping *cis*-acting signals for export under stress conditions. The signals were defined by fusing them to reporter mRNAs and demonstrating export of the fusion transcripts after heat shock (165). The conclusion of this experiment was that hs mRNAs might bind different proteins than bulk polyA-containing RNA.

Further evidence for this conclusion came from studying cellular factors required for hs mRNA export. As discussed above, mutants of the hnRNP-like Npl3p protein are dominant inhibitors of polyA RNA export in yeast (reviewed in 277); however, hs mRNA export was normal in *npl3* mutant strains (275). This observation might indicate that Npl3p does not interact with hs mRNAs. An even greater surprise came from analysis of hs mRNA export in strains mutant in the Gsp1p (Ran), Prp20p (RanGEF), or Rna1p (RanGAP) components of the yeast Ran system. As described above, these three components of the Ran system are essential for all other studied forms of nuclear import and export. In contrast, hs mRNA export was not affected in these mutant strains (165, 275), suggesting a fundamental difference between hs mRNA export and the better understood nucleocytoplasmic transport pathways described in this review.

A first glimpse of what is required for hs mRNA export came from the observation that Rip1p, the nonessential nucleoporin previously implicated in Rev-NES-mediated nuclear export (237), is required for hs mRNA export (275, 278). The relationship between hs mRNA export and NES-dependent export is still unclear (275, 278), but further studies of this fascinating form of transport are to be expected. In our opinion, the major question is, What substitutes for Ran in this export pathway?

PERSPECTIVES

The field of nucleocytoplasmic transport is booming. We expect further rapid progress in identification of the functions of the new import and export receptors that are members of the importin β family. A further expansion in the collection of well-defined import and export signals is also expected. Further examination

of energy consumption during different import and export processes, using in vitro transport systems and yeast genetics, is another area with open questions and experimental possibilities. Comparison of conventional transport pathways with unusual examples such as the heat shock mRNAs should be productive. The specific roles of the different Ran-binding proteins that are not related to importin β also need to be studied further. We expect such studies to lead to the discovery of Ran functions that are not related directly to nucleocytoplasmic transport. Some transport substrates, specifically mRNPs, may not leave the nucleus isometrically, but rather might be exported out of the nucleus in a directional way (reviewed in 279). If this is true, transport could be studied in terms of nuclear architecture. The atomic structures of both p10/NTF2 (280) and Ran in its GDP-bound state (281) have been elucidated, but several fascinating problems of protein structure that relate to nucleocytoplasmic transport remain unsolved. These problems include how proteins such as importin α, transportin, or CRM1 can accurately bind to such diverse, but seemingly simple, transport signals, and the nature of the conformational changes induced by RanGTP binding to transport receptors.

The critical current question in the field, in our opinion, concerns the NPC translocation step of import and export. We have not discussed this topic in depth in this review because less is known about translocation than about other aspects of nucleocytoplasmic transport. Nevertheless, we are confident that the translocation mechanism will be elucidated through the enormous effort of the various scientists investigating this area.

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