Binding of the Mex67p/Mtr2p Heterodimer to FXFG, GLFG, and FG Repeat Nucleoporins Is Essential for Nuclear mRNA Export

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Abstract. It is not known how Mex67p and Mtr2p, which form a heterodimer essential for mRNA export, transport mRNPs through the nuclear pore. Here, we show that the Mex67p/Mtr2p complex binds to all of the repeat types (GLFG, FXFG, and FG) found in nucleoporins. For this interaction, complex formation between Mex67p and Mtr2p has to occur. *MEX67* and *MTR2* also genetically interact with different types of repeat nucleoporins, such as Nup116p, Nup159p, Nsp1p, and Rip1p/Nup40p. These data suggest a model in which nuclear mRNA export requires the Mex67p/Mtr2p heterodimeric complex to directly contact several repeat nucleoporins, organized in different nuclear pore complex subcomplexes, as it carries the mRNP cargo through the nuclear pore.

Key words: nucleocytoplasmic transport • Nup116 • Nup159 • Rip1 • nuclear pore complex

Introduction

Eukaryotic cells are subdivided into different compartments. Since transcription occurs in the nucleus and translation in the cytoplasm, mRNAs, among other export cargoes, need to be transported from the site of transcription to the site of translation. All these transport processes occur solely through the nuclear pore complexes (NPCs)[1], which reside within the nuclear envelope. Whereas a mechanistic view has emerged of how proteins are transported back and forth through the nuclear pores (for review see Mattaj and Englmeier, 1998; Ohno et al., 1998), only little is known about the mechanism of nuclear RNA export, in particular mRNA export.

However, quite a number of proteins that are involved in the mRNA export process were identified in the past (for review see Sträßer and Hurt, 1999). Not unexpectedly, some of the nucleoporins (Nups), the proteins that constitute the nuclear pores, were shown to be involved in mRNA export. These proteins could provide the stationary phase for nuclear transport. An example for a Nup with a role in mRNA export is *NUP116* (Wente and Blobel, 1993). Nup116p, which is homologous to Nup100p and Nup145p-N (Wente et al., 1992; Fabre et al., 1994), interacts genetically with Nsp1p (Wimmer et al., 1992). Common to Nup116p, Nup100p, and Nup145p-N is the GLFG repeat domain at the NH_2 terminus (N domain), which can bind to karyopherin/importin β-family transport receptors (Iovine et al., 1995; Iovine and Wente, 1997). Thus, repeat sequences of Nups were suggested to provide the binding sites for karyopherin/importin β transport receptors for their passage through the pore channel (Rexach and Blobel, 1995). In addition, these three GLFG Nups contain a so-called NRM (Nup RNA binding motif) in their COOH-terminal domains (C domains), which can bind in vitro to homopolymeric RNA (Fabre et al., 1994). Only Nup116p harbors an evolutionarily conserved sequence, called the GLEBS (Gle2p binding sequence), which binds another mRNA export factor, Gle2p (Wente and Blobel, 1993; Murphy et al., 1996; Bailer et al., 1998), which is evolutionarily conserved and was also studied in *Schizosaccharomyces pombe* (called Rae1p; Brown et al., 1995; Bharathi et al., 1997) and human (called mrnp41; Kraemer and Blobel, 1997). It is worth mentioning that Nup98, the higher eukaryotic homologue of Nup116p, plays a broad role in RNA export mechanisms, including the export of mRNA (Powers et al., 1995; Radu et al., 1995; Pritchard et al., 1999; Zolotukhin and Felber, 1999). Another Nup in yeast involved in mRNA export, Rip1p (Nup42p), contains FG repeats and is thought to participate in the export of heat shock mRNAs (Saavedra et al., 1997).

There are two large subcomplexes at the NPC, the Nup82p and the Nup84p complex, that are essential for

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[1]*Abbreviations used in this paper:* C domain, COOH-terminal domain; CTE, constitutive transport element; hc, high-copy; LMB, leptomycin B; LRR domain, leucine-rich repeat domain; M domain, middle domain; N domain, NH_2-terminal domain; NES, nuclear export activity; NPC, nuclear pore complex; Nups, nucleoporins; ORF, open reading frame; ts, temperature-sensitive.

nuclear mRNA export. The Nup82p complex consists of three Nups: Nup159p, Nsp1p, and Nup82p (Grandi et al., 1995; Hurwitz and Blobel, 1995; Belgareh et al., 1998; Hurwitz et al., 1998). Mutants of *NUP159* (*RAT7*) and *NUP82* are strongly impaired in mRNA export (Gorsch et al., 1995; Grandi et al., 1995; Hurwitz and Blobel, 1995; Del Priore et al., 1997; Hurwitz et al., 1998). Interestingly, the NH_2 terminus of Nup159p binds to Dbp5p (Hodge et al., 1999; Schmitt et al., 1999), an ATPase with RNA helicase activity, shown to be essential for nuclear export of mRNA (Snay-Hodge et al., 1998; Tseng et al., 1998). The Nup82p/Nsp1p/Nup159p complex is held together by coiled-coil protein–protein interactions, since all subunits exhibit heptad repeats in their COOH termini (Grandi et al., 1995; Belgareh et al., 1998; Hurwitz et al., 1998). Indeed, it has been shown experimentally that the C domains of Nup159p and Nup82p participate in complex formation and assembly into the NPCs (Kraemer et al., 1995; Belgareh et al., 1998; Hurwitz et al., 1998). Interestingly, the Nup82p complex was localized exclusively to the cytoplasmic side of the NPCs (Kraemer et al., 1995; Hurwitz et al., 1998). In terms of evolutionary conservation, the Nup82p complex seems to be the yeast counterpart of the higher eukaryotic Nup214/CAN complex, in which CAN/Nup214 is homologous to yeast Nup159p, and vertebrate Nup88/84 to yeast Nup82p (Bastos et al., 1997; Fornerod et al., 1997). Similar to the Nup82p complex, the Nup84p complex, which consists of six subunits, contains several proteins with a crucial role in nuclear mRNA export, such as Nup85p (Rat9p), Nup120p (Rat2p), and Nup145p-C (Rat10p) (Fabre et al., 1994; Wente and Blobel, 1994; Aitchison et al., 1995; Heath et al., 1995; Siniossoglou et al., 1996; Goldstein et al., 1996; Dockendorff et al., 1997). Interestingly, none of the proteins of this subcomplex contains repeat sequences.

Since proteins bind to the RNA during, or shortly after, transcription, it is widely accepted that the RNP particle is the substrate for export, not the naked RNA (for review, see Nakielny and Dreyfuss, 1999). Since some of these proteins shuttle between the nucleus and the cytoplasm, it was suggested that these proteins provide the signals for export and are exported in an exportin-dependent manner. One protein, though, that is directly involved in the export of mRNA is Mex67p. Mex67p, which forms a tight complex with Mtr2p and interacts with the Nup84p complex, localizes to nuclear pores in vivo and can bind directly to RNA (Segref et al., 1997; Santos-Rosa et al., 1998). Thus, this complex meets the two requirements, namely binding to the nuclear pore and the transport cargo RNA, to be an mRNA exporter on its own.

This notion is underlined by the human homologue of Mex67p, TAP. TAP is the cellular cofactor for export of CTE (constitutive transport element), containing RNAs of simple type D retroviruses (Grüter et al., 1998; Braun et al., 1999). These RNAs access the mRNA export pathway, since injection of CTE-containing RNA into *Xenopus laevis* oocytes blocks mRNA export, but not the export of other RNAs (Pasquinelli et al., 1997). Thus, TAP is believed to play an essential role in cellular mRNA export. TAP can bind both directly to RNA and to the FG repeats of the Nups CAN/Nup214 and hCG1 (Katahira et al., 1999). In addition, TAP associates with a small protein called p15, which has homology to the RanGDP-binding protein, NTF2 (Katahira et al., 1999). Interestingly, TAP and p15, when expressed in yeast, can complement the otherwise lethal gene disruptions of *mex67*, *mtr2*, or both (Katahira et al., 1999). Thus, the mRNA export pathway is conserved from yeast to human.

Although the players specifically involved in mRNA export are beginning to be unraveled, the way they interact and thus contribute to the overall mRNA export mechanism still remains unclear. Here, we show that the Mex67p/Mtr2p complex can bind directly to the repeat sequences of different Nups. Unlike TAP, which appears to interact with repeat Nups in the absence of p15, Mex67p requires complex formation with Mtr2p to become competent for binding to repeat sequences of Nups. Thus, the direct binding of Mex67p/Mtr2p to all of the repeat types (GLFG, FXFG, and FG) found in Nups appears to be crucial for the mechanism of transport of mRNP cargoes through the pores.

Materials and Methods

Yeast Strains, DNA Recombinant Work, and Microbiological Techniques

The strains used in this study are listed in Table I. Microbiological techniques, plasmid transformation, plasmid recovery, gene disruption, mating, sporulation of diploids, and tetrad analysis were done essentially as described (Santos-Rosa et al., 1998). DNA recombinant work, such as restriction analysis, end-filling, ligations, and PCR amplifications was performed according to Maniatis et al. (1982). Leptomycin B (LMB) treatment of CRM1T539C cultures was done as described (Neville and Rosbash, 1999).

Plasmids

For this study, the following previously described plasmids were used: pRS314-MEX67, pRS316-Mex67, pUN100-MEX67, pRS314-mex67-5, pRS314-mex67 (Δ544-559), pRS314-mex67(L[552]>P), pRS314-mex67 (LL>EE), pET8c-HIS$_6$-MEX67 (Segref et al., 1997), pNOPGFP-MTR2, pET9d-MEX67, pPROEX-MTR2 (Santos-Rosa et al., 1998), pRS314-mex67-6 (Sträßer and Hurt, 1999), pVDP16 (pLEU2-Nup159 Δrepeats), pVDP17 (pLEU2-Nup159 C domain; Del Priore et al., 1997), pGEX4T-3 (Amersham Pharmacia Biotech), pSW304 (pGEX-3TX-NUP116 GLFG-repeat; amino acids 161–730 (Iovine and Wente, 1997), pFS410 (pGEX-4T-1 + RIP1-FG; Strahm et al., 1999), pSB32-ala6-nsp1-C (Wimmer et al., 1993), pUN100-Nup116 (Wimmer et al., 1992), pDC-CRM1, and pDC-CRM1T539C (Neville and Rosbash, 1999).

An NcoI-PstI fragment coding for *NSP1* amino acids 274–564 plus the ADH1 terminator was amplified by PCR and cloned into the pET9d vector containing a TEV-cleavage site to obtain pET9d-GST-TEV-NSP1. For construction of pGEX-4T-3-FG$_{Nup159}$, an EcoRI-XmaI PCR fragment coding for amino acids 457–900 of *NUP159* was cloned into pGEX-4T-3.

An NheI restriction site was generated by site-directed PCR mutagenesis just 3′ of the stop codon of *MEX67* (pRS314-MEX67-NheI). This site was convenient for generation of temperature-sensitive (ts) mutants within the C domain (see below). pRS315-mex67ΔC2 was derived from pRS314-mex67ΔC2 (see below) by subcloning the mex67ΔC2 open reading frame (ORF)-containing SacI fragment into SacI-cut pRS315. For construction of an NH_2-terminally GFP-tagged version of Mex67p, the ORF of *MEX67* was amplified by PCR, creating an NdeI and a BamHI site, cloned into the same sites of pNOPPATA1L, and subcloned into pNOPGFPA1L (Hellmuth et al., 1998). pRS315-GFP-mex67ΔC1 and pRS315-GFP-mex67ΔC2 were cloned the same way, but with mex67ΔC1 or mex67ΔC2 as templates for the PCR reaction. For cloning pRS424-mex67(Δ546–558) and pRS424-mex67(L[552]>P), the SacI fragment of pRS314-mex67(Δ546–558) or pRS314-mex67(L[552]>P) containing the corresponding mutant *MEX67* ORF was subcloned into pRS424 linearized with SacI.

For expression of GFP-Mex67p-C-NLS and GFP-Mex67p-LRR-NLS

Table I. Yeast Strains

Strain	Genotype	Literature
NSP1 shuffle strain	*MATa, ade2, his3, leu2, trp1, ura3, nsp1::HIS3* (pRS316-NSP1)	Hurt et al., 1999
mex67-5 strain	*MATα, ade2, his3, leu2, trp1, ura3, mex67::HIS3* (pUN100-mex67-5)	Segref et al., 1997
FSY17	*MATa, ade2, his3, leu2, trp1, ura3, rip1::KanMX*	Stutz et al., 1997
LGY108 (NUP159 shuffle strain)	*MATa, his3, leu2, ura3, Nup159::HIS3* (pLG4)	Gorsch et al., 1995
nup116 null strain	*MATα, ade2, his3, leu2, trp1, ura3, nup116::HIS3*	Bailer et al., 2000
MEX67 shuffle strain	*MATa, ade2, his3, leu2, trp1, ura3, mex67::HIS3* (pRS316-MEX67)	Segref et al., 1997
nup116Δ/MEX67 shuffle strain	*MATα, ade2, his3, leu2, trp1, ura3, nup116::HIS3, mex67::HIS3* (pRS316-MEX67)	This study
rip1Δ/MEX67 shuffle strain	*MATa, ade2, his3, leu2, trp1, ura3, rip1::KanMX, mex67::HIS3* (pRS316-MEX67)	This study
NUP159/MEX67 double shuffle	*MATa, his3, leu2, trp1, ura3, nup159::HIS3* (pRS316-NSP1), *mex67::HIS3* (pRS316-MEX67)	This study
NSP1/MEX67 double shuffle	*MATa, ade2, his3, leu2, trp1, ura3, nsp1::HIS3* (pRS316-NSP1), *mex67::HIS3* (pRS316-MEX67)	This study
Mtr2-GFP/Mex67 shuffle strain	*MATa, ade2, his3, leu2, trp1, ura3, mtr2::HIS3* (pRS315-Mtr2-GFP), *mex67::HIS3* (pRS316-MEX67)	Santos-Rosa et al., 1998
LLY1044	*MATa, his3, leu2, trp1, ura3, crm1::KAN^R* (pLDB396 [CRM1^{HA} URA3 CEN])	Yan et al., 1998

in yeast, the sequence coding for the corresponding amino acids (483–599 for the C domain, and 155–264 for the leucine-rich repeat [LRR] domain) was amplified by PCR creating PstI sites and cloned into the PstI site pRS315-NOP1::GFP-NLS$_{Npl3p}$ (Senger et al., 1998).

Recombinant Mex67p or individual domains without the His$_6$ tag were overexpressed in *Escherichia coli* by inserting the entire *MEX67* ORF, the ORF coding for the ΔC mutants, the N+LRR domain (residue 3–308), the middle (M) domain (residue 252–494), the ΔM+C domain (residue 303–599) of *MEX67*, or the M domain of thermosensitive *mex67-5*, carrying a single point mutation (H[400]>Y), into pET9d (Novagen).

Isolation of the mex67ΔC Alleles

Mutations in the C domain of *MEX67* were generated by mutagenic PCR, generation of a library in *E. coli*, and transformation into the MEX67 shuffle strain as described (Siniossoglou et al., 2000). In detail, pRS314-MEX67-NheI was amplified by PCR under suboptimal conditions (5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 1 μg template DNA, 5 U Taq DNA polymerase). The PCR product was gel-purified and digested with NheI and EcoNI to release the C domain of MEX67, and inserted into pRS314-MEX67-NheI cut with the same enzymes. The mutagenized library was first transformed into *E. coli*. 5,600 transformants were obtained, from which the plasmid DNA was isolated. Approximately 55% of the plasmids contained an insert. The plasmids were transformed into the MEX67 shuffle strain. About 2,500 transformants were restreaked onto plates containing 5-FOA, to shuffle out the plasmid pRS316-MEX67. Ura$^-$ colonies were restreaked onto YPD and incubated at 23 and 37°C. From ts colonies, the plasmids were recovered and retransformed into the shuffle strain, to confirm the plasmid dependency of the ts phenotype. Two different ts *mex67* alleles were isolated and sequenced. pRS314-mex67ΔC1 and pRS314-mex67ΔC2 encode COOH-terminally truncated versions of Mex67p (amino acids 1–524 and 1–493, respectively).

Purification of the Mex67p/Mtr2p Complex from E. coli

The pellets of BL21 expressing HIS$_6$-tagged Mtr2p and untagged Mex67p were resuspended in universal buffer (Künzler and Hurt, 1998), cells were lysed by sonification, and the supernatant incubated with Ni-NTA agarose (Qiagen). The beads were washed with universal buffer and with universal buffer containing 50 mM imidazole. Proteins were eluted with universal buffer containing 150 mM imidazole.

Purification of GST-tagged Proteins

GST-tagged proteins were expressed in BL21. The *E. coli* pellet was dissolved in universal buffer (Künzler and Hurt, 1998) and cells were lysed by sonification. The supernatant was incubated with 200 μl glutathione Sepharose 4B (Amersham Pharmacia Biotech), the beads were washed, and the bound proteins were eluted with univeral buffer containing 20 mM GSH.

In Vitro Binding Assay

The GST-fusion proteins (1:10 diluted) were rebound to 25 μl GSH-bead slurry per binding assay. After incubation for 1 h at 4°C, the beads were washed with universal buffer and the partially purified Mex67p/Mtr2p complex, Mex67p alone, Mtr2p alone, or supernatant from BL21 expressing HIS$_6$-Mtr2p and Mex67p (centrifuged in an ultracentrifuge for 1 h at 100,000 *g*) was added to the GSH-beads and incubated for 1 h at 4°C. The beads were finally washed with universal buffer and the proteins were bound to the beads eluted with sample buffer.

Preparation of Double Disruption Strains

The NUP159 and NSP1 shuffle strains were mated to the *mex67::HIS3* strain complemented by pLEU2-mex67-5 and diploids selected on SDC (−ura, −leu) plates, respectively. After sporulation and tetrad analysis, haploid progeny *mex67::HIS3/nup159::HIS3* containing pURA3-NUP159 and pLEU2-mex67-5, and *mex67::HIS3/nsp1::HIS3* containing pURA3-NSP1 and pLEU2-mex67-5, respectively, were transformed with pURA3-MEX67 plasmids and transformants selected at 37°C. Finally, colonies were selected, which lost pUN100-mex67-5, thereby yielding the corresponding double shuffle strains (see Table I).

Double disruption strains, *mex67::HIS3/nup116::HIS3* and *mex67::HIS3/rip1::KanMX*, were constructed by mating the MEX67 shuffle strain with the corresponding *nup116::HIS3* and *rip1::KanMX* null strains, respectively, followed by tetrad analysis.

To analyze the genetic interaction between *MEX67* and *NUP-X* mutant alleles, the double shuffle strains were transformed with various plasmid combinations of *NUP-X* and *MEX67* mutants, and their growth phenotype was assessed on 5-FOA plates at 23°C. No growth on 5-FOA indicates synthetic lethality.

Analytical Ultracentrifugation of the Mex67p/Mtr2p Complex

For further purification of the Mex67p/Mtr2p complex, the eluate from the Ni-NTA column was purified by FPLC on a MonoS column (Amersham Pharmacia Biotech). The peak fractions, which eluted from the MonoS column at ~700 mM NaCl, were pooled, concentrated, and further purified by gel filtration. Gel filtration was performed with buffer containing 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM DTT, and a Superdex-200 26/100 column (Amersham Pharmacia Biotech). The peak fractions were again pooled and concentrated. For analytical ultracentrifugation, 150 μl of the complex, which was diluted to ~0.1 mg/ml with the flow-through of the concentration step, was used. As reference sample, it also served as the flow-through of the concentration step. Analytical ultracentrifugation was performed according to Laue et al. (1984) using a Beckman XL-A centrifuge and a 60 Ti Beckman rotor. The sample was analyzed at 4°C in 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM DTT. The equilibrium spin run was performed at a equilibrium spin speed of 12,000 rpm as determined using Ultrascan II, Version 4.1. The data was collected using the Beckman XL-A package. Using linear regression analysis, an average molecular weight of 85 ± 3 kD for the recombinant Mex67p/Mtr2p complex was determined.

Miscellaneous

SDS-PAGE and Western blot analysis were performed according to Siniossoglou et al. (1996). In situ hybridization of poly(A)$^+$ RNA and fluorescence microscopy of GFP-fusion protein expressing yeast cells were performed as described (Doye et al., 1994; Santos-Rosa et al., 1998). Multiple sequence alignment of NTF2 proteins from human, *Saccharomyces cerevi-*

siae, *Neurospora crassa*, and a Ras-GAP binding protein: p15 (human), Mex67p (*S. cerevisiae*), Mex67p (*S. pombe*), and TAP proteins from human and *Drosophila melanogaster*, was done using ClustalW1.8′ (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Results

The Middle Domain of Mex67p Binds to Mtr2p

Mex67p is a multidomain protein, which exhibits an N, LRR, M, and C domain (Fig. 1; see also Segref et al., 1997). Previous work indicated that a single point mutation within the M domain (H[400]>Y, *mex67-5*) impairs both the association of Mex67p with the nuclear pores and interaction with Mtr2p in vivo (Segref et al., 1997; Santos-Rosa et al., 1998).

Since coexpression of His_6-Mtr2p and untagged Mex67p in *E. coli* allows heterodimer formation and subsequent affinity purification of a stable complex, we could test which domain of Mex67p can associate with His-tagged Mtr2p. This revealed that the M domain (Mex67p-M [252–494]) can associate with Mtr2p, allowing affinity purification of a His_6-Mtr2p/Mex67-M (252–494) complex from *E. coli* (Fig. 1). The other domains seem to not interact with His-Mtr2p in this in vivo assembly assay. However, we cannot exclude this with certainty, since for example, most of the N+LRR domain precipitates in *E. coli* and is thus present in lower amounts in the soluble supernatant as compared with the other domains (Fig. 1, compare lanes H and S). However, since we coexpress His-Mtr2p and the N+LRR domain in *E. coli*, in principle they are able to interact, which could render the N+LRR domain soluble as it does for the full-length protein. Therefore, although we cannot exclude that a more soluble N+LRR domain would interact with Mtr2p, our data show that the M domain of Mex67p directly binds to Mtr2p.

Since the mex67-5 mutation causes dissociation from Mtr2p in yeast, we tested whether this mutant M domain does not interact with Mtr2p in *E. coli* any longer. As anticipated, Mex67-M (H[400]>Y) or full-length Mex67p (H[400]>Y) can no longer bind to His_6-Mtr2p in the *E. coli* system (data not shown). This shows that the M domain of Mex67p directly interacts with Mtr2p, and that this interaction is required for the in vivo interaction of Mex67p with the nuclear pores, since a mutant in the M domain in the context of the full-length protein mislocalized to the cytoplasm (Segref et al., 1997).

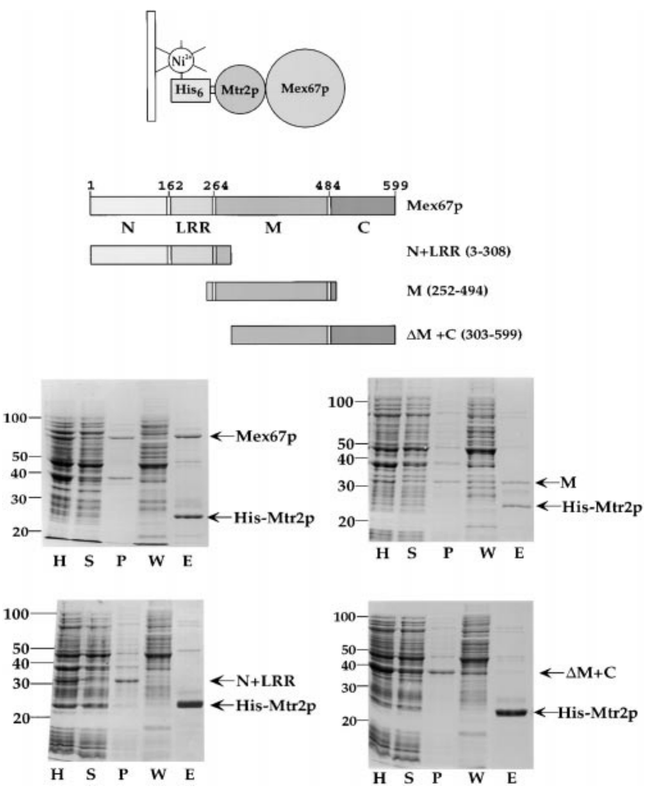


Figure 1. Binding of Mtr2p to the M domain of Mex67p. His_6-Mtr2p and untagged full-length Mex67p or the various truncation constructs (top) were expressed and purified from *E. coli* as described in Materials and Methods. The homogenate, soluble supernatant, insoluble pellet, wash, and His_6-Mtr2p eluate derived from the Ni-NTA agarose beads were analyzed by SDS-PAGE and Coomassie staining (bottom). Copurification of the full-length Mex67p and the M domain, respectively, with His_6-Mtr2p is seen. H, Homogenate; S, soluble supernatant; P, insoluble pellet; W, wash; E, eluate. The expression levels of the various Mex67p truncation constructs in *E. coli* were similar.

The C Domain of Mex67p, which Is Not Essential for Cell Growth, Is Important for mRNA Export and Exhibits NES Activity

To study the role of the C domain of Mex67p, we searched for ts mutants mapping to this part of Mex67p. We were able to isolate two new ts alleles, which encode Mex67p proteins lacking most of the C domain (Fig. 2, A and B). *mex67ΔC1* and *mex67ΔC2* cells grow more slowly at permissive temperatures and are thermosensitive for growth at 37°C. However, both *mex67ΔC* mutants are complemented by overexpression of *MTR2* (Fig. 2 B). Thus, *MTR2* overproduction can compensate for the lack of the Mex67p C domain (see Discussion). This shows that the C domain of Mex67p is important, but not essential, for Mex67p function. This result was unexpected, since we previously showed that a short deletion within the C domain (Δ544–559; see also Fig. 2 A) or a single point mutation (L[552]>P) renders yeast cells nonviable at all temperatures (Segref et al., 1997). However, this is not due to a dominant-negative phenotype, because the *mex67ΔC2* mutant expressing the *mex67* (Δ544–559) allele is viable (Fig. 2 C). When the expression of Mex67p (Δ544–559) or (L[552]>P) was analyzed by Western blotting, these mutant proteins are barely detectable (Fig. 2 D, lanes 2 and 3), but are seen when overexpressed from high-copy (hc) plasmids (Fig. 2 D, lanes 4 and 5). Overproduced Mex67p (Δ544–559) or (L[552]>P) are able to complement the *mex67*$^-$ strain (data not shown). We could not analyze the expression of Mex67pΔC1 or Mex67pΔC2 by Western blotting (see Fig. 2 D, lanes 2 and 3), since the Mex67p antibodies are mainly directed against the C domain (data not shown; Sträßer, K., unpublished results). However, other data suggest that Mex67pΔC1 or Mex67pΔC2 are more stably expressed than Mex67p (Δ544–559) or Mex67p L[552]>Π.

To find out whether the mutations in the C domain affect nuclear mRNA export, poly(A)$^+$ RNA localization was analyzed by in situ hybridization. Already at permissive temperature, ~50% of the mex67ΔC2 cells accumulate poly(A)$^+$ RNA inside the nucleus (Fig. 3 A). This percentage further increases when cells are shifted to the restrictive temperature (Fig. 3 A). However, the inhibition of mRNA export is only partial in this mutant, since a significant cytoplasmic poly(A)$^+$ signal is still evident. This is in contrast to other mex67 mutants (e.g., mex67-5), which exhibit a rather fast onset and a complete block of mRNA export (Hurt et al., 2000). When tagged with GFP and expressed in mex67$^-$ cells (for expression see Fig. 3 B, right, Western blot), GFP-Mex67pΔC fusion proteins no longer exhibit a nuclear envelope staining, but distribute to the nucleus and the cytoplasm with a tendency for a higher accumulation in the nucleus in most of the cells (Fig. 3 B, left). Interestingly, the steady state location of GFP-tagged Mtr2p changes in mex67ΔC cells; Mtr2p is also no longer concentrated at the nuclear envelope, but tends to accumulate in the nucleus (Fig. 3 C). When the intracellular localization of GFP-Mex67pΔC2 was analyzed in cells that express hc MTR2, Mex67pΔC2 no longer accumulates in the nucleus. Although a ring-like staining of GFP-Mex67pΔC2 is not reestablished by hc MTR2, it appears that GFP-Mex67pΔC2 is more efficiently reexported into the cytoplasm (Fig. 3 D). This would be consistent with our in vitro findings that Mtr2p faciliates Mex67p interaction with repeat Nups, which could speed up nuclear export (see below). Taken together, these data show that the C domain of Mex67p is required for efficient nuclear mRNA export and nuclear envelope localization at steady state.

Since the C domain of TAP contains a nuclear export activity (NES; Bear et al., 1999; Braun et al., 1999; Kang and Cullen, 1999; Truant et al., 1999), we wanted to test whether Mex67p exhibits such an activity as well, which can contribute to nuclear export of Mex67p. Previously, we have shown that Mex67p contains a short sequence in its C domain, which resembles the leucine-rich Rev NES, and indeed acts as NES in the *Xenopus* oocyte system (Segref et al., 1997). The entire C domain of Mex67p, including this NES-like motif, was attached to an NLS-containing GFP reporter. The NLS corresponds to the RGG domain of Npl3p (Senger et al., 1998). When expressed in yeast, GFP-Mex67p-C-NLS was predominantly detected in the cytoplasm by fluorescence microscopy (Fig. 4 A, expression is at the top and localization is at the bottom). In contrast, the same GFP-NLS construct lacking the C domain of Mex67p (GFP-NLS) strongly accumulated in the nucleus (Fig. 4 A). When the Mex67p C domain carrying the short deletion (Δ544–559) or the single point mutation L(552)>Π in the NES-like sequence was fused to GFP-

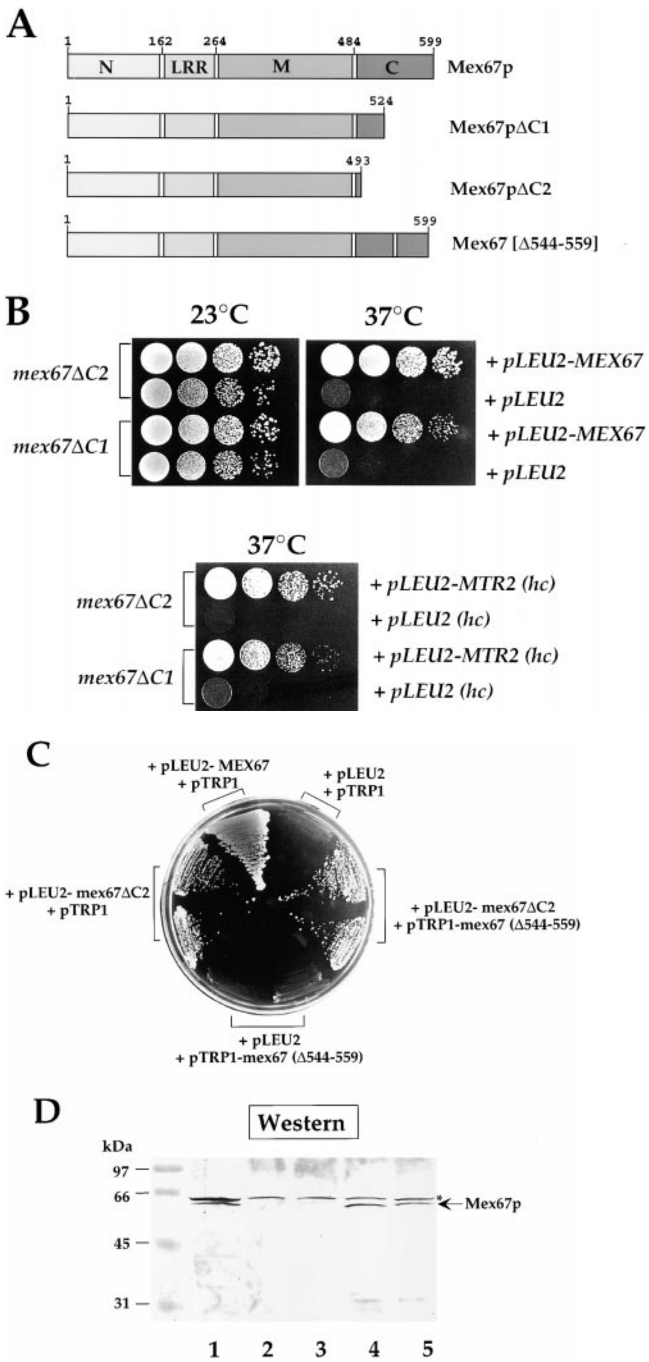


Figure 2. The C domain of Mex67p is not essential for cell growth. A, Domain organization of Mex67p and amino acid truncations/point mutations in the C domain. B, Growth of the *mex67*ΔC1 and *mex67*ΔC2 thermosensitive mutants at 23 and 37°C. The same amount of cells was diluted in 10^{-1} steps, spotted onto YPD plates, and grown for 3 d at the indicated temperatures. The mutants were transformed with empty, *MEX67*, or *MTR2* containing plasmids (pLEU2, either single or hc plasmid). C, The *mex67*(Δ544–559) allele is not dominant-lethal. The MEX67 shuffle strain (*mex67::HIS3*, pURA3-MEX67) was transformed with the pLEU2 and pTRP1 plasmids coding for the indicated gene constructs. Transformants were then streaked onto 5-FOA plates (+uracil, −leucine, −tryptophane) and grown for 5 d at 30°C. The nonviable mex67(Δ544–559) allele has been described recently (Segref et al., 1997). D, Western blot analysis of whole cell yeast lysates expressing *mex67*ΔC2 deletion constructs using anti-Mex67p antibodies. 1, full-length Mex67p; 2, *mex67*ΔC2 and mex67(Δ544–559) (low-copy); 3, *mex67*ΔC2 and mex67(L[552]>P) (low copy); 4, *mex67*ΔC2 and mex67(Δ544–559) (hc); 5, *mex67*ΔC2 and mex67(L[552]>P) (hc). The band marked by an asterisk is a protein cross-reacting with the anti-Mex67p immune serum. A molecular weight marker standard is also shown.

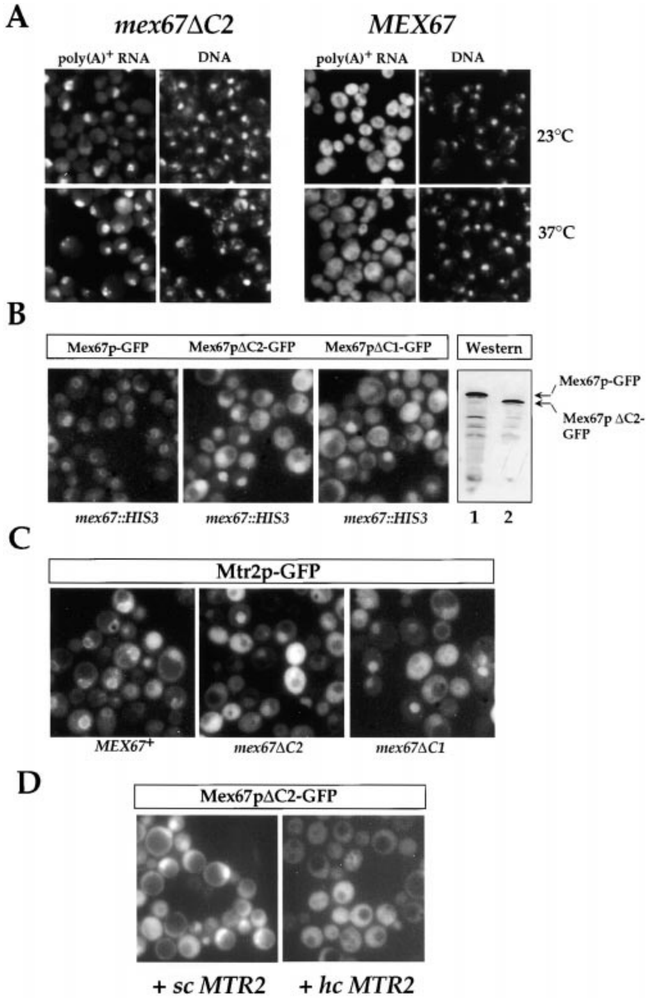


Figure 3. Inhibition of mRNA export in *mex67*ΔC cells. A, Subcellular localization of poly(A)$^+$ RNA was analyzed by in situ hybridization, in *MEX67* and *mex67*ΔC2 cells, either grown at 23°C or shifted for 1 h to 37°C. Nuclear DNA was stained with DAPI. B, Localization of GFP-tagged wild-type Mex67p and Mex67pΔC1 and Mex67pΔC2 mutants as revealed by fluorescence microscopy (left). Expression of Mex67p-GFP and Mex67pΔC2-GFP was analyzed by Western blotting using anti-GFP antibodies (right). C, Localization of GFP-tagged Mtr2p in *MEX67*, *mex67*ΔC1, and *mex67*ΔC2 strains as revealed by fluorescence microscopy. D, Localization of GFP-tagged Mex67pΔC2-GFP in a single copy (+ sc *MTR2*) and hc (+ hc *MTR2*) strain background at 23°C, as revealed by fluorescence microscopy.

NLS and expressed in yeast, the corresponding fusion accumulated in the nucleus. This suggests that the C domain of Mex67p has nuclear export or cytoplasmic retention activity. To further distinguish between these possibilities, the GFP-Mex67p-C-NLS construct was expressed in the *xpo1-1* mutant, which is defective in nuclear export of cargoes containing a Rev-type NES (Stade et al., 1997). Interestingly, GFP-Mex67p-C-NLS accumulates in the nucleus of *xpo1-1* cells already after a five-minute shift to the restrictive temperature (Fig. 4 B). This could mean that the NES-receptor, Xpo1p–Crm1, is involved in the nuclear export of the C domain of Mex67p. To show this in an independent way, GFP-Mex67p-C-NLS was expressed in a *Saccharomyces cerevisiae* strain, in which Xpo1p has been made LMB-sensitive by site-specific mutagenesis of a critical residue within Xpo1p (Neville and Rosbash, 1999). Likewise, nuclear export of GFP-Mex67p-C-NLS is inhibited in this engineered *S. cerevisiae* LMB-sensitive strain when LMB is added to the growth medium (Fig. 4 C). Whether this NES in the C domain of Mex67p is also active in the context of the intact protein is not clear, since full-length Mex67p-GFP does not significantly accumulate inside the nucleus in *xpo1-1* cells, but remains predominantly located at the nuclear envelope (Santos-Rosa et al., 1998). However, Mex67p also can associate with nuclear pores by direct interaction of the Mex67p/Mtr2p complex with repeat Nups (see below).

The Mex67p/Mtr2p Complex Binds to Repeat Sequences of Nup116p, Nup159p, Rip1p, and Nsp1p

The data above indicate that the C domain of Mex67p plays a role for the in vivo association of the Mex67p/Mtr2p complex with nuclear pores, and that Xpo1p may contribute to this process. However, Mex67p may associate directly with NPCs, since TAP, the human homologue of Mex67p, interacts with FG-repeat sequences of human CAN/Nup214 and hCG–Rip1p (Katahira et al., 1999). We tested the interaction with different yeast repeat Nups in an in vitro assay. First, FG repeats derived from Nup159p were tested for binding to the Mex67p/Mtr2p complex. Nup159p is essentially involved in nuclear mRNA export and homologues to human CAN/Nup214 (Gorsch et al., 1995). The Mex67p/Mtr2p complex binds strongly to immobilized GST-FG$_{Nup159}$ beads, but not at all to GST, which served as a negative control (Fig. 5 A). The binding of Mex67p/Mtr2p to GST-FG$_{Nup159}$ beads is stable up to a salt concentration of at least 500 mM KAc.

To test whether repeat sequences from other Nups can associate with the Mex67p/Mtr2p complex, GLFG repeats from Nup116p, FXFG repeats from Nsp1p, and FG repeats from Rip1p/Nup42p, were all fused to GST and immobilized on GSH-beads, and were tested in the in vitro binding assay. In this case, a whole cell lysate from *E. coli* expressing moderate levels of both recombinant Mex67p and His$_6$-Mtr2p served as a source for the Mex67p/Mtr2p complex (Fig. 5 B, L). This lysate was first cleared from unsoluble proteins by ultracentrifugation, before it was incubated with GST-beads carrying the different Nup repeat sequences. Strikingly, among the many different *E. coli* proteins, only the recombinant Mex67p/His$_6$-Mtr2p complex bound to the various Nup repeats (Fig. 5 B). To determine whether Mex67p and Mtr2p bind as a heterodimeric complex to Nup repeats, we compared it side by side to the Mex67p/Mtr2p complex purified by gel filtration chromatography (see Materials and Methods) after SDS-PAGE and Coomassie staining (Fig. 5 C). The exact molecular weight of the Mex67p/Mtr2p complex was determined to be 85 ± 3 kD by analytical ultracentrifugation, indicating a 1:1 stoichiometry of the complex (see Materials and Methods). Furthermore, the Mex67p/Mtr2p complex eluted slightly faster from an FPLC Superdex 200 column than a defined 92- and 95-kD protein complex, respectively (Sinning, I., and M. Groves, unpublished data), again con-

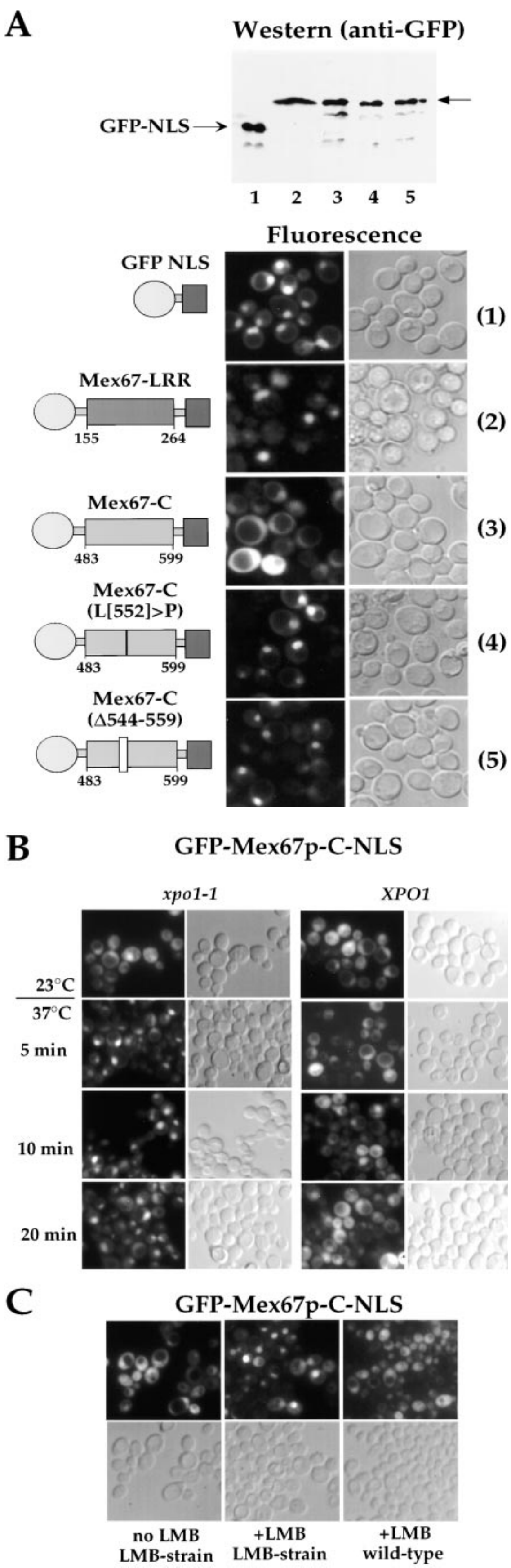


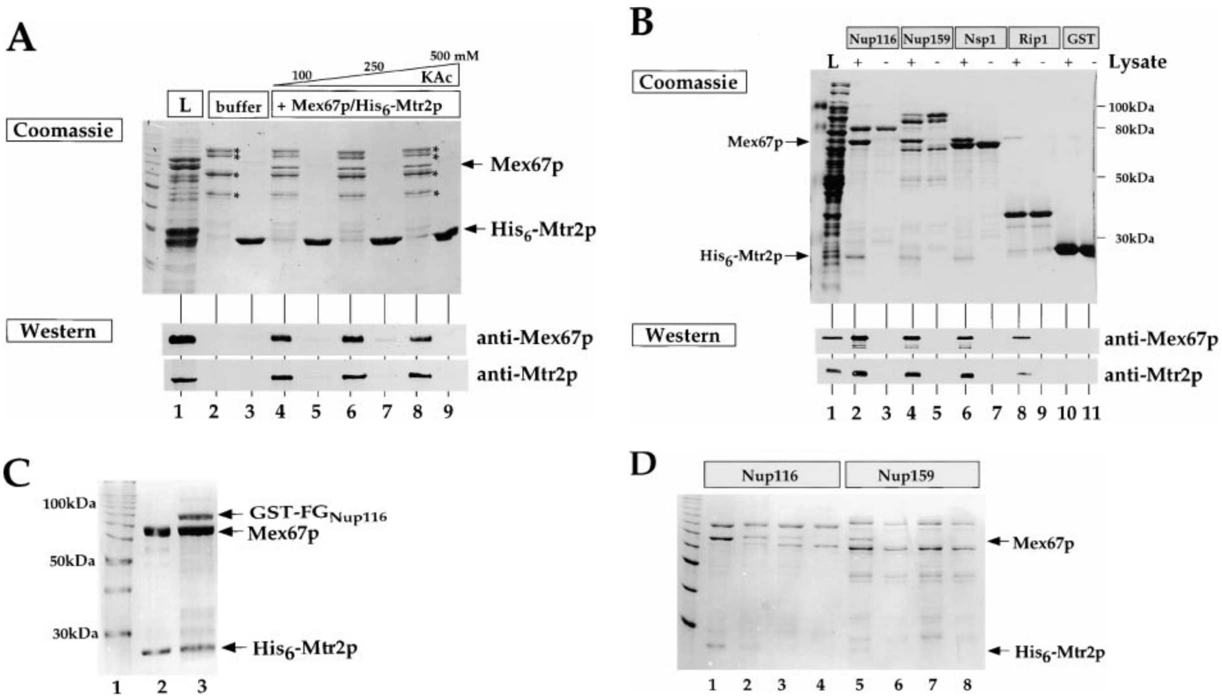
Figure 4. A nuclear export activity in the C domain of Mex67p. A, The C domain of Mex67p (3) and its derived mutant forms mapping in the NES-like sequence (4 and 5) or the LRR domain (2) of Mex67p were fused to a GFP-NLS reporter construct (1) and expressed in wild-type yeast cells. Expression of the GFP constructs was tested by Western blotting using anti-GFP antibodies (top, lanes 1–5; note that in lane 2, five times as much as in the other lanes was loaded, which corresponds to the GFP signal for the fusion protein containing the LRR domain as compared with the other fusion proteins) and the intracellular localization determined by fluorescence microscopy (bottom, lanes 1–5). B, Nuclear export of GFP-Mex67p-C-NLS is impaired in *xpo1-1* cells. Strain *xpo1*Δ (Stade et al., 1997) complemented either by plasmid-borne *xpo1-1* or *XPO1* were transformed with a plasmid harboring the *GFP-Mex67-C-NLS* reporter construct. Cells were grown in selective medium at 23°C before shift for 5, 10, and 20 min to the nonpermissive temperature (37°C). The intracellular localization of GFP-Mex67p-C-NLS was determined by fluorescence microscopy. C, Nuclear export of GFP-Mex67p-C-NLS is impaired in a *S. cerevisiae* strain, which is sensitive to LMB. LMB treatment of the LMB-sensitive (LMB-strain) and of a control strain (wild-type) was done as described (Neville and Rosbash, 1999). Cells were incubated for 1 h in the presence of LMB.

sistent with a heterodimeric complex. The intensities of the Coomassie-stained Mex67p and Mtr2p bands are similar for the highly purified complex and the complex, respectively, which is pulled down by Nup116p repeats (Fig. 5 C). We also quantified the intensity of the Mex67p and Mtr2p bands derived from the Coomassie-stained gel of Fig. 5 B, lane 2, and correlated these values with the relative mass of both proteins. This determination revealed a 1:1 stoichiometry of the Mex67p and Mtr2p bands when bound to Nup116 repeats (data not shown). Thus, Mex67p and Mtr2p bind as a heterodimer to repeat sequences.

To get a first hint about the relative affinities of the Mex67p/Mtr2p complex to the different repeat sequences (GLFG, FXFG, and FG), we varied the concentration of the Mex67p/Mtr2p complex within the *E. coli* lysate by dilution with cold lysate (a lysate that does not contain the Mex67p/Mtr2p). These lysates, with decreasing concentrations of Mex67p/Mtr2p, but the same amount of total protein, were then incubated with GST repeats derived from Nup116p, Nup159p, Nsp1p, or Rip1p. Clearly, Nup116p repeats exhibit the highest binding affinity for the Mex67p/Mtr2p complex (binding is observed up to a 1:10 dilution), whereas Nup159p and Nsp1p repeats already lost binding to the complex when the lysate was diluted 1:5 (Fig. 5 D).

In contrast to the complex, His_6-Mtr2p or His_6-Mex67p alone did not bind to (for example) GST-FG_{Nup159} (Fig. 5 E) or GST-$GLFG_{Nup116}$ (data not shown). Although we cannot exclude that the single subunits are correctly folded in *E. coli*, notably Mtr2p alone is soluble in high concentrations and Mex67p can also be recovered in the soluble supernatant of *E. coli* lysates in reasonable amounts (Fig. 5 E). All this shows that the Mex67p/Mtr2p heterodimer, but not the single subunits, exhibits a significant affinity for different Nup repeats, since the complex is specifically and efficiently selected on Nup repeats from a whole cell lysate. This binding of Mex67p/Mtr2p to repeat Nups could be a crucial step during nuclear mRNA export through the nuclear pores (see Discussion).

To find out whether the COOH-terminal part of



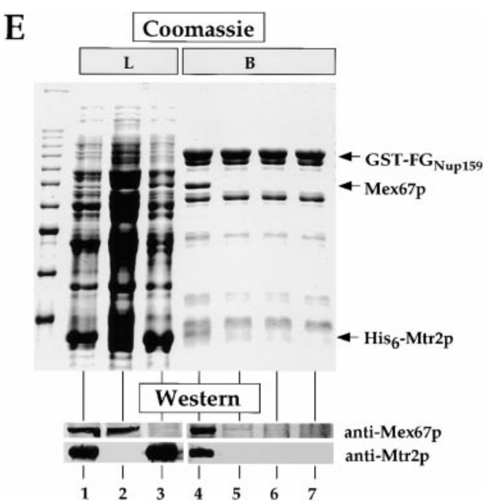


Figure 5. Recombinant Mex67p/Mtr2p complex binds to different Nup repeat sequences. A, Nup159p-derived FG repeat sequences were expressed as a GST-fusion protein in *E. coli* and immobilized on GSH-beads, before incubation with partially purified Mex67p/His$_6$-Mtr2p complex, also derived from *E. coli*. Bound proteins were eluted with SDS-sample buffer and analyzed by SDS-PAGE and Coomassie staining (top) or Western blotting (bottom) using anti-Mex67p and anti-Mtr2p antibodies, respectively. The pull-down experiment was carried out under different salt concentrations. Partially purified Mex67p/His$_6$-Mtr2p complex (lane 1; L, load); GST-FG$_{Nup159}$ immobilized on GSH-beads (lane 2; buffer); GST immobilized on GSH-beads (lane 3; buffer); immobilized GST-FG$_{Nup159}$ incubated with Mex67p/His$_6$-Mtr2p complex (lanes 4, 6, and 8) at the indicated salt concentrations; immobilized GST incubated with Mex67p/His$_6$-Mtr2p complex (lanes 5, 7, and 9) at the indicated salt concentrations. The asterisks indicate GST-FG$_{Nup159}$ and its breakdown products. B, Repeat sequences derived from Nup116p, Nup159p, Nsp1p, and Rip1p were expressed as GST-fusion proteins in *E. coli* and immobilized on GSH-beads, before incubation with *E. coli* whole cell lysates containing the recombinant Mex67p/His$_6$-Mtr2p complex. After the binding reaction, GST-beads were eluted with SDS-sample buffer and analyzed by SDS-PAGE and Coomassie staining or Western blotting using anti-Mex67p and anti-Mtr2p antibodies. L, *E. coli* lysate containing the Mex67p/His$_6$-Mtr2p complex; + and −, incubation of beads with lysate or buffer alone, respectively. Soluble *E. coli* lysate (L) containing the Mex67p/His$_6$-Mtr2p complex (lane 1); GST-GLFG$_{Nup116}$ immobilized on GSH-beads incubated with lysate (lane 2) or buffer (lane3); GST-FG$_{Nup159}$ immobilized on GSH-beads incubated with lysate (lane 4) or buffer (lane 5); GST-FXFG$_{Nsp1}$ immobilized on GSH-beads incubated with lysate (lane 6) or buffer (lane 7); GST-FG$_{Rip1}$ immobilized on GSH-beads incubated with lysate (lane 8) or buffer (lane 9); GST immobilized on GSH-beads incubated with lysate (lane 10) or buffer (lane 11). The positions of Mex67p and His-Mtr2p are indicated. C, Comparison of the highly purified Mex67p/Mtr2p complex and Mex67p/Mtr2p bound to GST-Nup116p repeat sequences. 1, Protein standard; 2, recombinant Mex67p/His-Mtr2p complex, expressed in *E. coli*, and purified by Ni-NTA affinity, FPLC-MonoS, and gel filtration chromatography; 3, Mex67p and Mtr2p bound to GST-Nup116p repeats. In lane 2 and 3, similar amounts of Mex67p were loaded and analyzed by SDS-PAGE and Coomassie staining. D, Concentration-dependent binding of Mex67p/Mtr2p to Nup repeat sequences. An *E. coli* lysate with Mex67p/His-Mtr2p complex was used undiluted or diluted 1:5, 1:10, or 1:50 with cold lysate lacking Mex67p/Mtr2p, and the derived lysates were incubated with GST-Nup116p or GST-Nup159p repeat sequences. The amount of bound Mex67p/Mtr2p was analyzed by SDS-PAGE and Coomassie staining. 1–4, GST-Nup116p repeats; 5–8, GST-Nup159p repeats; 1 and 5, undiluted lysate; 2 and 6, 1:5 diluted lysate; 3 and 7, 1:10 diluted lysate; 4 and 8, 1:50 diluted lysate. A protein standard is also shown. Note that a protein from the *E. coli* lysate binds to GST-Nup16p repeats when little Mex67p/Mtr2p complex is present. E, GST-FG$_{Nup159}$ containing GSH-beads (4–7; B, bound) were incubated with soluble *E. coli* cell lysates (1–3; L, load) that contain the recombinant Mex67p/His$_6$-Mtr2p complex (lanes 1 and 4), recombinant His$_6$-Mex67p (lanes 2 and 5), and recombinant His$_6$-Mtr2p (lanes 3 and 6) or buffer (lane 7). After a 1-h incubation, proteins were eluted with SDS-sample buffer and analyzed by SDS-PAGE and Coomassie staining (top) or Western blotting (bottom) using anti-Mex67p and anti-Mtr2p antibodies.

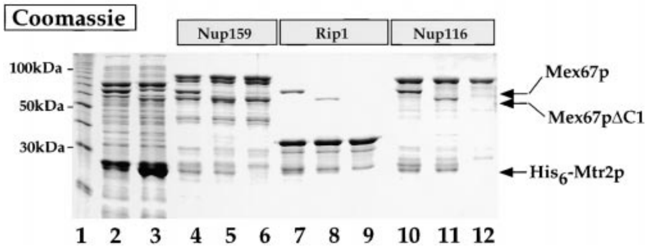


Figure 6. Recombinant Mex67p/Mtr2p complex lacking the C domain of Mex67p binds to Nup repeat sequences. Partially purified Mex67p/His$_6$-Mtr2p complex, containing full-length Mex67p (lane 2) or Mex67pΔC1 (lane 3) was incubated with GST-Nup159p, GST-Rip1p, or GST-Nup116p repeats. After binding, GST-beads were eluted with SDS-sample buffer and analyzed by SDS-PAGE and Coomassie staining. GST-FG$_{\mathrm{Nup159}}$ incubated with Mex67p/His$_6$-Mtr2p (lane 4), Mex67pΔC1/His$_6$-Mtr2p (lane 5), or buffer (lane 6); GST-FG$_{\mathrm{Rip1}}$ incubated with Mex67p/His$_6$-Mtr2p (lane 7), Mex67pΔC1/His$_6$-Mtr2p (lane 8), or buffer (lane 9); GST-GLFG$_{\mathrm{Nup116}}$ incubated with Mex67p/His$_6$-Mtr2p (lane 10), Mex67pΔC1/His$_6$-Mtr2p (lane 11), or buffer (lane 12). The positions of Mex67p, Mex67pΔC1, and His$_6$-Mtr2p are indicated. Molecular weight protein standard (lane 1).

Mex67p is necessary for the interaction of the Mex67p/Mtr2p complex with Nup repeat sequences, we deleted the C domain from Mex67p according to the ts COOH-terminal deletion mutants. When these Mex67pΔC constructs are coexpressed with His$_6$-Mtr2p in *E. coli*, a corresponding Mex67pΔC/Mtr2p complex is assembled and still binds to all the different Nup repeat sequences (Fig. 6 and data not shown).

Repeat Nups NUP116, NUP159, RIP1, and NSP1 Interact Genetically with MEX67 and MTR2

Since the Mex67p/Mtr2p heterodimer binds directly to Nup repeats, we wanted to test for genetic interactions between the corresponding repeat Nups and *MEX67* or *MTR2*. Interestingly, mutations in the C domain of Mex67p, which lead to a nuclear mislocalization of Mex67p and Mtr2p, and partial inhibition of nuclear mRNA export (e.g., *mex67*ΔC1; see also Fig. 3), cause synergistic growth defects or synthetic lethality when combined with mutant alleles mapping in the different repeat Nups (Fig. 7). The strongest genetic interaction was found with the *nup116::HIS3* null allele, which gives synthetic lethality not only with Mex67p COOH-terminal mutations, but also with the *mex67-6* allele (Fig. 7 B), which has mutations in the N+LRR domain and interacts genetically and physically with the RNA–RNA annealing protein, Yra1p (Sträßer and Hurt, 2000). Synthetic lethal relationships were also detected between Mex67p COOH-terminal mutations and truncation alleles of *NUP159* (e.g., lacking the N and FG domains), the *rip1* null allele, or the *nsp1-ala6* mutation (Fig. 7, B and C). In contrast, other mutant alleles of *MEX67* mapping in the M (*mex67-5*) and N+LRR (*mex67-6*) domains did not cause synthetic lethality when combined with these various Nup mutant alleles, with exception of the *nup116::HIS3* allele (Fig. 7 B). In a similar way, *mtr2* mutant alleles were tested for synthetic lethal relationships with these Nup mutants. This showed that (for example) the *mtr2-26* allele (Santos-Rosa et al., 1998)

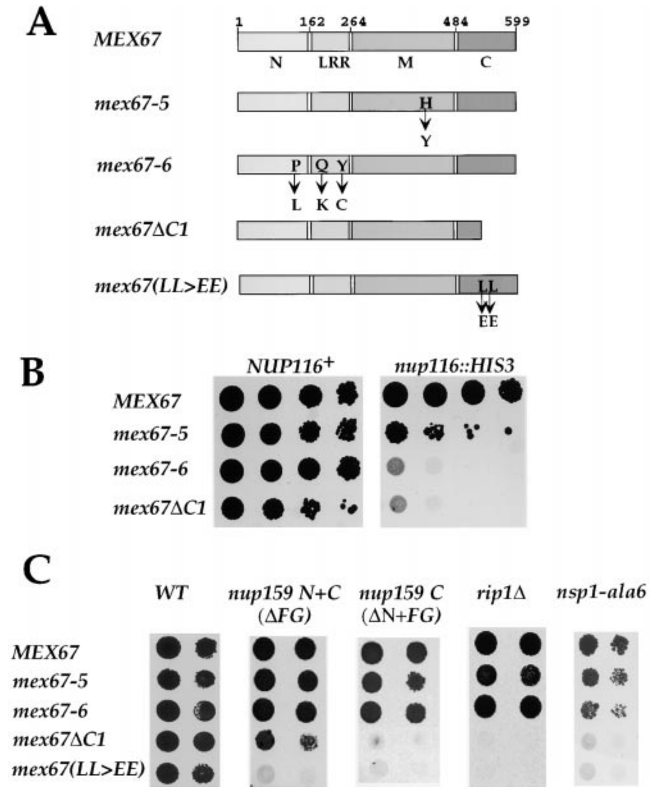


Figure 7. Genetic interactions between *MEX67* and Nups carrying repeat sequences. A, Schematic drawing of wild-type and mutant *MEX67* alleles. *mex67-5* and *mex67-6* are impaired in the interaction with *MTR2* (Segref et al., 1997) and *YRA1* (Sträßer and Hurt, 2000), respectively. The *mex67(LL>EE)* maps in the C domain (Segref et al., 1997). B, Genetic interaction between *nup116* null *(nup116::HIS3)* and different *mex67* ts alleles. The *nup116::HIS3/mex67::HIS3* shuffle strain containing pURA3-MEX67 (Table I) was transformed as indicated with plasmids containing *NUP116*, *mex67-5*, *mex67-6*, and *mex67*ΔC1 gene constructs. The synthetic lethal relationship was tested by spotting transformants on 5-FOA–containing plates in 10^{-1} dilution steps. Plates were incubated at 23°C for 6 d. Growth inhibition on 5-FOA plates indicates synthetic lethality. C, Genetic interaction between *nup159*, *rip1*, and *nsp1* mutant alleles with different *mex67* ts alleles. The corresponding mutant alleles are described in Materials and Methods. *nupX::HIS3/mex67::HIS3* strains containing the complementing pURA3 plasmids (Table I) were transformed as indicated with plasmids containing *nup159* or *nsp1-ala6* mutants and the *mex67-5*, *mex67-6*, *mex67ΔC1*, or *mex67(LL>EE)* constructs. The synthetic lethal relationship was tested by spotting transformants on 5-FOA containing plates in 10^{-1} dilution steps. Plates were incubated at 23°C for 6 d.

is synthetically lethal with the *nup116::HIS3* null allele (data not shown). Thus, the in vivo analysis revealed a genetic network of interactions, in which *MEX67* and *MTR2* are functionally linked to repeat Nups and also interact physically.

Discussion

The Mex67p/Mtr2p complex and its mammalian TAP/p15 counterpart act as a shuttling receptor for nuclear mRNA export, but the mechanism of movement through the nuclear pores is still unknown. In the past, we have shown that the intact Mex67p/Mtr2p complex exhibits in vivo a distinct NPC distribution, but impairment of the interaction between Mex67p and Mtr2p (e.g., by mutating either *MEX67* or *MTR2*) causes dissociation of Mex67p from the pores and nuclear accumulation of mRNA (Segref et al., 1997; Santos-Rosa et al., 1998). Both biochemical and genetic data suggested that the Nup84p complex is one of the targets at the NPC with which the Mex67p/Mtr2p complex functionally interacts (see below).

We now show that additional docking sites at the nuclear envelope contribute to the in vivo localization of the Mex67p/Mtr2p complex at the NPCs. Strikingly, the Mex67p/Mtr2p heterodimer, but not the single subunits, binds directly to all of the repeat types (GLFG, FXFG, and FG) found in Nups, as revealed in our in vitro reconstitution assay. For this in vitro binding, the C domain of Mex67p is not necessary in the context of the Mex67p/Mtr2p heterodimeric complex, since a Mex67pΔC/Mtr2p complex efficiently assembles in *E. coli* and still binds to the different Nup repeat sequences. This suggests that a core Mex67p/Mtr2p complex lacking the C domain of Mex67p can still interact with repeat Nups. This could explain why in vivo this core complex still supports cell growth at physiological temperatures (e.g., 30°C). Consistent with this finding is the observation that the human Mex67p homologue, TAP, when lacking the C domain is able to export CTE-containing RNA from the nucleus to the cytoplasm (Braun et al., 1999). Thus, the C domain of Mex67p may modulate the in vivo interaction of a core Mex67p/Mtr2p complex to Nup repeat sequences. Since the C domain exhibits a nuclear export activity, the entire domain, or part of it, may also contribute to efficient nuclear export of the shuttling Mex67p/Mtr2p complex by interacting with additional nuclear export factors, such as the NES-export receptor, Xpo1p/Crm1. Accordingly, the role of Xpo1p in nuclear mRNA export could be auxiliary, but not essential. Such a model would be consistent with earlier findings that the *xpo1-1* mutant shows a significant mRNA export defect at the restrictive temperature (Stade et al., 1997), yet Xpo1p is not the major mRNA export receptor in *S. cerevisiae* (Neville and Rosbash, 1999).

Our data have clearly shown a distinct binding activity of the Mex67p/Mtr2p heterodimer, either with or without the Mex67p C domain, to all types of Nup repeat sequences. It is possible that Mtr2p within the Mex67p/Mtr2p complex enhances NPC binding by direct interaction with Nups. However, we assume that Mex67p significantly contributes to this NPC binding. How could the Mex67pΔC/Mtr2p core complex bind to repeat sequences of Nups? We have found that in human cells, p15, which is homologous to NTF2, forms a heterodimeric complex with TAP/Mex67p (Katahira et al., 1999). It was discussed that the NTF2-like p15 may bind to repeat sequences of Nups (Katahira et al., 1999) in a similar way as NTF2 binds to FXFG Nups, such as Nsp1p or p62 (Clarkson et al., 1996; Chaillan-Huntington et al., 2000). The crystal structure of NTF2 shows that a hydrophobic cavity is made up by several conserved hydrophobic residues (e.g., Y-18 and Y-19), which bind an exposed aromatic residue (Phe72) within RanGDP (Bullock et al., 1996). It appears that Mtr2p is not homologous to p15 and NTF2, and therefore one cannot say whether it has a NTF2-fold. Interestingly, the M domains of Mex67p and TAP are similar to p15 and NTF2 in the sense that conserved thyrosines and phenylalanines with p15/NTF2 can be aligned with corresponding residues within the Mex67p/TAP family (Hurt, E., unpublished data). Thus, the M domain of Mex67p/TAP may have an NTF2-fold with a hydrophobic cavity. Accordingly, the M domain of Mex67p may interact with repeat Nups by a mechanism that is similar to the NTF2/FXFG or NTF2/Ran interaction. Accordingly, Mtr2p, which also binds to the M domain of Mex67p, could cooperatively affect binding of Mex67p to repeat Nups.

Another possible interaction site of the Mex67p/Mtr2p complex at the nuclear pores is the Nup84p complex, which was previously shown to interact with Mtr2p in vivo (see Santos-Rosa et al., 1998). This would agree with the earlier finding that a significant fraction of Mtr2p remains NPC-associated, whereas mutated Mex67p dissociates from the pores (Santos-Rosa et al., 1998). Since no apparent affinity of Mtr2p alone to Nup repeats could be found in vitro, Mtr2p binding to Mex67p increases cooperatively the affinity of the Mex67p/Mtr2p heterodimer to different repeat Nups, such as Nup116p, Nup159p, Nsp1p, Rip1p, and possibly others. These interactions, which may be dynamic in vivo, may contribute to the localization of the Mex67p/Mtr2p complex at the pores.

Since GLFG, FXFG, or FG Nups are found at different sites within the structural framework of the NPC (Kraemer et al., 1995; Fahrenkrog et al., 1998; Stoffler et al., 1999; Strahm et al., 1999), a consecutive array of repeat Nups along the pore channel may provide transient docking sites for the shuttling Mex67p/Mtr2p complex. Accordingly, the Mex67p/Mtr2p heterodimer with bound mRNP cargo could translocate through the nuclear pores by consecutive interactions with different repeat Nups. In this sense, the Mex67p/Mtr2p complex resembles the shuttling importin/karyopherin β family members, which were also demonstrated to interact with different repeat Nups (Rexach and Blobel, 1995).

We have shown by immunogold labeling that Mex67p and Mtr2p are located at both the nuclear and cytoplasmic site of the pores (Santos-Rosa et al., 1998). The Nsp1p/Nup49p/Nup57p complex is also found at both sites of the NPC, but the Nup82p/Nup159p/Nsp1p complex, which is essentially involved in nuclear mRNA export, is located asymmetrically on the cytoplasmic site (Kraemer et al., 1995, Hurwitz et al., 1998; Stoffler et al., 1999). Interestingly, the Nup116p/Gle2p complex was recently demonstrated to associate with the Nup82p complex (Bailer et al., 2000). These findings, together with our observation that the Mex67p/Mtr2p complex exhibits a strong physical and genetic interaction with the GLFG Nup, Nup116p, could explain the crucial role of Nup116p in nuclear mRNA export (Wente and Blobel, 1993; Fabre et al., 1995).

Thus, the Nup82p/Nsp1p/Nup159p complex and Nup116p/Gle2p complex could provide GLFG, FXFG, and FG repeat docking sites for the Mex67p/Mtr2p complex during passage through the pores and before release into the cytoplasm. Interestingly, the Nup84p complex, which consists of six Nups, all of them without repeat sequences (Siniossoglou et al., 1996), is also located on the cytoplasmic site of the NPC, and genetically interacts with the Mex67p/Mtr2p complex. Based on immunogold labeling data, all members of the Nup84p complex are found at both sites of the NPC (Rout et al., 2000) and this NPC module has multiple roles: it is involved in nuclear membrane and pore biogenesis and thus has a structural role within the NPC (Siniossoglou et al., 1996, 1998); it could interact with cytoskeletal structures and their loss in mutants alters NPC distribution within the nuclear membrane (for discussion and model, see Doye et al., 1994; Stoffler et al., 1999); and it is involved in mRNA export and (for example) could play a role in binding to or release of the Mex67p/Mtr2p complex from repeat Nups. To further address the question of release factors, we plan to set up an in vitro assay to identify such putative dissociation factors by using whole cell yeast lysates or nuclear fractions as a source to remove Mex67p/Mtr2p from the different Nup repeats.

In contrast to Mex67p, its human homologue TAP can interact with repeat Nups on its own (Katahira et al., 1999; Bachi et al., 2000), suggesting that the regulation of TAP binding to repeat Nups and its dissociation may follow a different mechanism than for Mex67p. However, TAP binds to p15, which is functionally related to Mtr2p, but not homologous in its primary sequence. It remains to be shown whether the TAP/p15 complex exhibits an increased binding affinity to repeat Nups as well. Therefore, the mechanism by which the mammalian TAP/p15 heterodimer dynamically interacts with nuclear pore proteins may have evolved differently.

In conclusion, we have shown that the Mex67p/Mtr2p complex interacts directly with different repeat domains of Nups. A transient association of this shuttling mRNA exporter with the nuclear pores is crucial for the mechanism of nuclear mRNA export. Accordingly, the Mex67p/Mtr2p heterodimer could interact successively with different repeat Nups during passage through the pore channel, before it is released into the cytoplasm.

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