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The NES–Crm1p export pathway is not a major mRNA export route in *Saccharomyces cerevisiae*

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Nuclear export signal (NES)-containing proteins are recognized by the NES receptor CRM1/Crm1p (also called exportin 1/Xpo1p). In vertebrates and *Schizosaccharomyces pombe*, the toxin leptomycin B (LMB) inhibits CRM1-mediated export by interacting directly with CRM1 and disrupting the trimeric Ran-GTP–CRM1–NES export complex. In *Saccharomyces cerevisiae*, LMB is not toxic and is apparently unable to interact with Crm1p. A second difference between the systems is that LMB has no effect on mRNA export in vertebrate systems, whereas there is evidence that *S.cerevisiae* Crm1p plays a role in mRNA export. Here we show that a single amino acid change converts *S.cerevisiae* Crm1p from being LMB insensitive to fully LMB sensitive, indicating that Crm1p is the only relevant LMB target. This new strain has no phenotype, but LMB has a rapid and potent inhibitory effect on NES-mediated export. *In situ* hybridization assays show that LMB also causes nuclear accumulation of poly(A)$^+$ RNA but with a significant delay compared with the effect on NES-mediated export. Biochemical assays indicate little or no LMB effect on cytoplasmic protein synthesis, indicating that the NES–Crm1p pathway is not a major mRNA export route in *S.cerevisiae*. We conclude that Crm1p structure and function is conserved from *S.cerevisiae* to man.

Keywords: CRM1/leptomycin B/mRNA export/nuclear export signal/*Saccharomyces cerevisiae*

Introduction

In eukaryotic cells, there is a continual exchange of proteins and macromolecules between the nucleus and the cytoplasm. This occurs via nuclear pore complexes (NPCs), large proteinaceous structures that span the nuclear envelope. Transport between the two compartments is a signal-mediated process: localization signals within protein cargos are recognized by cellular receptors, which mediate either nuclear export or import (for review see Mattaj and Englmeier, 1998). The receptor family members (importin/exportin-β proteins) share the functional characteristic of binding to phenylalanine-glycine (FG)-repeat-containing NPC proteins as well as to the small GTPase Ran. A unique characteristic of the Ran-GTPase system is the compartmentalization of the Ran regulatory proteins, which produces a gradient of the nucleotide-bound state of Ran: Ran-GDP is mostly cytoplasmic and Ran-GTP is mostly nuclear. This gradient is apparently responsible for the directionality of transport between the nucleus and the cytoplasm (Izaurralde *et al.*, 1997). Importin–cargo interactions are disrupted by Ran-GTP, which enables the release of import cargos specifically in the nucleus; exportin–cargo interactions depend on Ran-GTP to form a trimeric exportin–cargo–Ran-GTP complex, which specifically dissociates in the cytoplasm upon GTP hydrolysis.

The foundation for much of our knowledge of nuclear import comes from studies of the 'classical' nuclear localization signal (NLS) pathway (Görlich, 1998). The short, positively charged NLS is recognized in the cytoplasm by the importin-α/β heterodimer. The importin α-subunit is responsible for NLS recognition and binding, whereas the β-subunit mediates import and nuclear release through interactions with the NPC and Ran-GTP. Database searches subsequently revealed a family of proteins related to importin-β (Fornerod *et al.*, 1997b; Görlich *et al.*, 1997). As some of these family members were characterized, novel nuclear import pathways were discovered, none of which appear to utilize an importin-α subunit equivalent. These import receptors each appear to recognize and import unique NLSs, although the reverse is not the case, e.g. ribosomal protein NLSs are apparently recognized by several importin-β family members (Jakel and Görlich, 1998).

Much of our understanding of nuclear export first came from studies of the HIV-1 protein Rev (Pollard and Malim, 1998). The role of this protein in the viral life-cycle is to recognize and promote the export of unspliced and partially spliced viral RNAs. Rev is the first protein in which a nuclear export signal (NES) was identified (Fischer *et al.*, 1995; Wen *et al.*, 1995). Studies of the Rev NES led to the identification of the first export receptor, the importin/exportin-β family member CRM1/Crm1p (also called exportin 1/Xpo1p; Fornerod *et al.*, 1997a; Fukuda *et al.*, 1997a; Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). Its discovery revealed that the same protein family is capable of both import and export. CRM1/Crm1p has now been identified as the NES receptor for a variety of cellular proteins, which include transcription factors, kinases and the cytoskeletal component actin (Fukuda *et al.*, 1997b; Engel *et al.*, 1998; Wada *et al.*, 1998; Yan *et al.*, 1998; Taagepera *et al.*, 1999). The short hydrophobic NES is evolutionarily conserved, as Rev functions in a variety of organisms. Moreover, Rev-like NESs have been identified in cellular proteins from many species, including the simple yeast *Saccharomyces cerevisiae*.

Our studies have focused on the yeast NES receptor Crm1p and its connection to the *Streptomyces* metabolite leptomycin B (LMB). LMB is a known fungicide and anti-tumor agent (Hamamoto *et al.*, 1983, 1985; Yoshida *et al.*, 1990) and was first connected to Crm1p through a

genetic selection in the yeast *Schizosaccharomyces pombe*, which identified mutations in the gene *CRM1* as the cause of increased LMB resistance (Nishi *et al.*, 1994). Crm1p was known to negatively regulate the *S.pombe* transcription factor Pap1; this inhibition of Pap1p-regulated gene expression is relieved by LMB (Nishi *et al.*, 1994). Taken together, these results suggested that LMB inhibits Crm1p function, thereby blocking its regulation of Pap1p. A connection between CRM1 and NES export signals first came from studies in mammalian cells, where LMB was shown to inhibit Rev NES-mediated export (Wolff *et al.*, 1997). It has now been shown that LMB is indeed a potent inhibitor of NES-mediated transport, by binding directly to mammalian and *S.pombe* Crm1p proteins and disrupting the trimeric Ran-GTP–CRM1–NES complex (Fornerod *et al.*, 1997a; Askjaer *et al.*, 1998). This explains the previous results with Pap1p and more recently with the *S.cerevisiae* homolog Yap1p: both proteins have a NES and are constitutively exported to the cytoplasm by Crm1p, which inhibits their transcription factor activity (Toone *et al.*, 1998; Yan *et al.*, 1998); LMB relieves this inhibition by directly inhibiting Crm1p function. In *S.cerevisiae*, mutations in CRM1 have been shown to inhibit NES-mediated export (Neville *et al.*, 1997; Stade *et al.*, 1997). But in contrast to vertebrates and *S.pombe*, LMB is not toxic to *S.cerevisiae* (Hamamoto *et al.*, 1983).

Saccharomyces cerevisiae Crm1p appears to differ more generally from CRM1 in other organisms. In higher eukaryotes, competition experiments showed that Rev and cellular mRNA export pathways are non-overlapping. Furthermore, LMB has no effect on mRNA transport in mammalian cells and in the *Xenopus* oocyte system (Fornerod *et al.*, 1997a). However, an *S.cerevisiae* temperature-sensitive *crm1* strain rapidly accumulates poly(A)$^+$ RNA in the nucleus at the restrictive temperature, suggesting that Crm1p contributes to mRNA export in this species (Stade *et al.*, 1997).

To explore these differences in Crm1p structure and function between *S.cerevisiae* and other species, we have analyzed *S.cerevisiae*/*S.pombe* Crm1p chimeras and localized the region of LMB sensitivity to 117 amino acids. Within this region, a single Thr to Cys change at position 539 (T539C) is sufficient to render *S.cerevisiae* Crm1p fully LMB sensitive, with an affinity for the drug comparable with that of *S.pombe* Crm1p. In an *S.cerevisiae* strain harboring this mutation in CRM1, LMB has a rapid and dramatic effect on NES-mediated transport, similar to what has been observed in other systems. We then utilized this LMB-sensitive strain to investigate the role of Crm1p in *S.cerevisiae* mRNA transport. A variety of approaches indicate that LMB has only a marginal effect on mRNA export in this strain. Taken together, our results suggest that the major mRNA export pathway(s) in *S.cerevisiae* is not Crm1p or NES mediated. We conclude that Crm1p structure and function is conserved from *S.cerevisiae* to man.

Results

Defining the leptomycin B-sensitive region of Crm1p

To study species-specific features of Crm1p proteins, we compared Crm1p from the distantly related yeasts

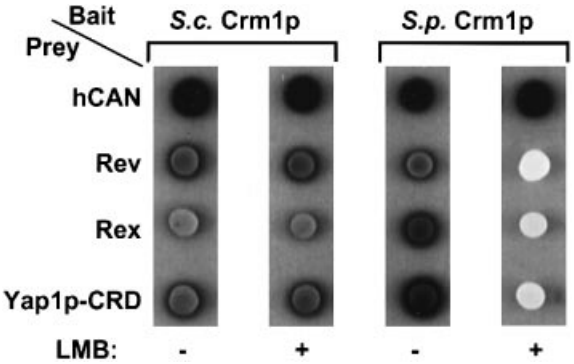


Fig. 1. The *S.pombe* Crm1p–NES two-hybrid interactions are specifically disrupted in the presence of LMB. Yeast two-hybrid interactions between *S.cerevisiae* (S.c.) and *S.pombe* (S.p.) Crm1p baits and either the NES-containing preys [Yap1p-cysteine rich domain (CRD; amino acids 532–650), Rev and Rex] or the FG-repeat-containing prey hCAN were analyzed on X-Gal indicator plates containing 0 (–) or 100 ng/ml (+) LMB.

S.cerevisiae and *S.pombe* in our two-hybrid system (Figure 1). Both *S.pombe* and *S.cerevisiae* Crm1p interact with a phenylalanine-glycine (FG)-repeat-containing protein of the NPC (hCAN) as well as with NES-containing proteins that function in both mammals (Rev, Rex) and *S.cerevisiae* (Yap1p). Because *S.cerevisiae* and *S.pombe* show differential growth sensitivity to LMB, we assayed the drug in the two-hybrid system. The interactions between *S.pombe* Crm1p and the NES-containing proteins are LMB sensitive, whereas the *S.cerevisiae* Crm1p–NES interactions are resistant. Notably, the interactions between the FG-repeat-containing protein CAN and both Crm1p proteins are insensitive to LMB; this is true for all other tested Crm1p–FG-repeat interactions (Figure 1; data not shown). Thus, LMB is able to penetrate *S.cerevisiae* cells, but the drug has no effect on the *S.cerevisiae* Crm1p–NES interaction.

Since *S.pombe* LMB sensitivity maps to Crm1p, the observed species difference is presumably due to structural variations between the respective Crm1p proteins. To investigate this possibility, we assayed four different chimeras containing the *S.cerevisiae* Crm1p sequence as the N-terminal portion and the *S.pombe* Crm1p as the C-terminal portion of the fusion proteins (CP1, CP2, CP3 and CP4). Chimeric junctions were focused around the region of Crm1p that contains a previously identified *S.pombe* double mutation, which causes increased LMB resistance (Figure 2A and B; Nishi *et al.*, 1994). All four chimeric proteins interact with NES-containing proteins in the two-hybrid assay; however, only the CP1–NES interaction is LMB sensitive (Figure 2B). Thus, the region of *S.pombe* Crm1p between junctions 1 and 2 is necessary for LMB sensitivity.

To create a *S.cerevisiae* strain that is LMB sensitive, we made a vector, pDC1, for the expression of Crm1p proteins (see Materials and methods). Although *S.cerevisiae* Crm1p expression from this construct complemented a lethal *CRM1* deletion (Δ*CRM1*), *S.pombe* Crm1p and the CP1 chimera were both unable to rescue viability (data not shown). To make a functional chimera and to refine our mapping of the LMB-sensitive region of Crm1p, we constructed a fusion gene that replaced an internal portion of *S.cerevisiae* Crm1p with the corresponding

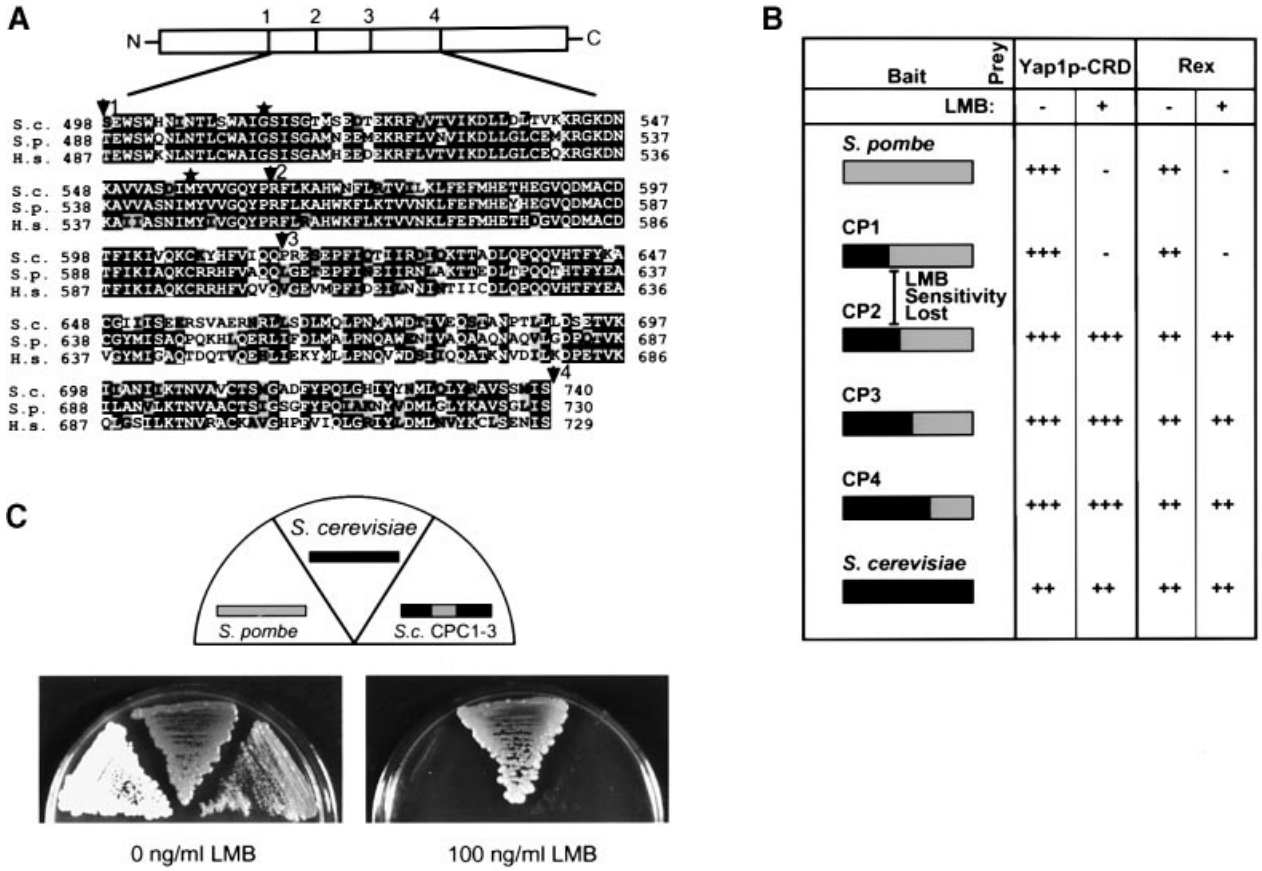


Fig. 2. *Saccharomyces cerevisiae*/*S.pombe* Crm1p chimeras reveal the LMB-sensitive region of *S.pombe* Crm1p. (**A**) Amino acid alignment of Crm1p proteins from *S.cerevisiae* (S.c.), *S.pombe* (S.p.) and human (H.s.). Shown are the positions of the chimera junctions (▼) and the positions of the increased LMB resistance double mutation identified in *S.pombe* (★). (**B**) Chimeric Crm1p–NES two-hybrid interactions. Crm1p chimeric baits (N-terminal *S.cerevisiae*/C-terminal *S.pombe)* have chimeric junctions 1 (CP1), 2 (CP2), 3 (CP3) or 4 (CP4) (see A) and are fused in-frame to the LexA DNA-binding domain. The chimera as well as full-length *S.cerevisiae* and *S.pombe* Crm1p bait interactions with the NES-containing preys Yap1p-CRD and Rex were analyzed. Shown is a summary table of these interactions on X-Gal indicator plates containing either 0 (–) or 100 ng/ml (+) LMB. (**C**) The *CPC1-3* chimera defines a region of *S.pombe* Crm1p sufficient to confer LMB sensitivity. *CPC1-3* contains *S.pombe CRM1* sequence between chimeric junctions 1 and 3 (see A). Constructs expressing either Cpc1-3p or wild-type *S.cerevisiae* Crm1p proteins under the control of the *S.cerevisiae CRM1* promoter were used to rescue a *S.cerevisiae CRM1* deletion strain. A wild-type *S.pombe* strain as well as the rescued strains were assayed for LMB sensitivity by growth on YEPD plates containing either 0 or 100 ng/ml LMB.

region (amino acids 488–604) of *S.pombe* Crm1p (*CPC1-3*). Expression of Cpc1-3p rescued Δ*CRM1*, but the strain is growth-impaired at all temperatures tested and has many phenotypes that closely mirror those seen after LMB treatment of *S.pombe* (Figure 2C, data not shown). This suggests that Cpc1-3p is only marginally functional. Importantly, the *CPC1-3* strain is sensitive to LMB, which restricted the relevant LMB-sensitive region of *S.pombe* Crm1p to 117 amino acids (Figure 2C).

A single amino acid substitution makes S.cerevisiae Crm1p fully LMB sensitive

Crm1p is a highly conserved protein, and this LMB-sensitive region contains the highest extent of conservation. As a result, only a few residues differ between LMB-sensitive (*S.pombe* and human) and LMB-resistant (*S.cerevisiae*) Crm1p proteins in this region (Figure 3A). We focused on Thr539, which is a Cys in both *S.pombe* and human but a Ser in the LMB-resistant yeast *Candida albicans* (D.Sanglard, unpublished communication). To test the contribution of this residue to LMB sensitivity, we made a *S.cerevisiae CRM1* gene containing the single point mutation Thr539→Cys (*CRM1T539C*). When expressed in the *S.cerevisiae CRM1* deletion strain, Crm1T539Cp rescues growth with no apparent phenotype, and the resulting strain is fully LMB sensitive (Figure 3B). To show further the importance of Cys539, we changed this residue of the *CPC1-3* chimera to a threonine. The resulting *CPC1-3C539T* strain is LMB resistant but is still growth-impaired like the *CPC1-3* strain (Figure 3C). Taken together, our results show that Cys539 is critical for LMB sensitivity and strongly suggest an essential role for Cys539 in LMB binding. We have also tested the nearby Ser509 residue, which is a Cys in both *S.pombe* and humans (Figure 3A); changing Ser509 to a Cys in *S.cerevisiae* Crm1p had no effect on LMB resistance (data not shown).

It was shown that LMB interacts directly with both *S.pombe* and human Crm1p, whereas *S.cerevisiae* Crm1p has no apparent affinity for LMB (Fornerod *et al.*, 1997a). To verify that the LMB sensitivity of the *CRM1T539C* strain is due to a change in LMB binding, we adopted the gel shift assay utilized by Fornerod *et al.* (1997a) (Figure 3D and E). As shown previously,

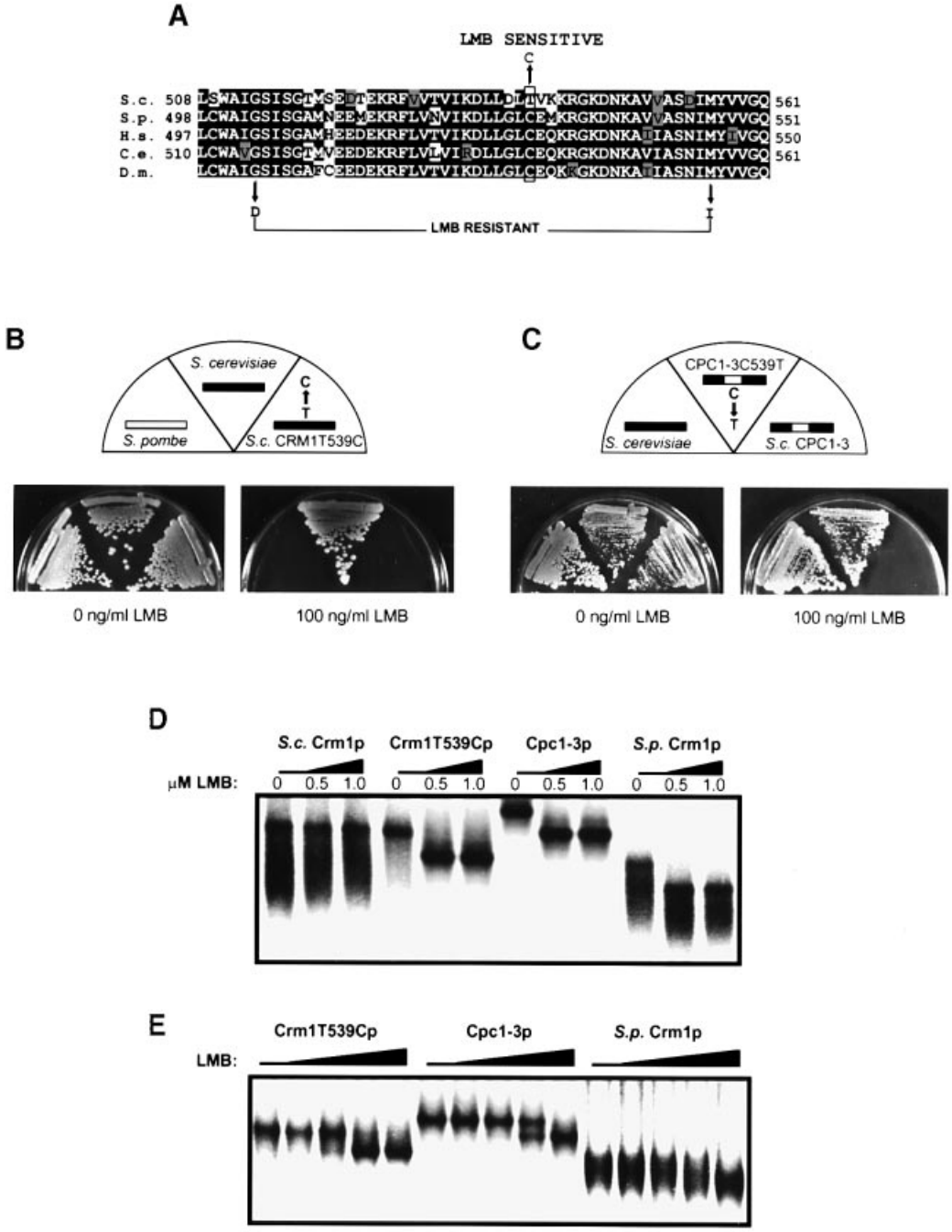


Fig. 3. A single amino acid change renders *S.cerevisiae* Crm1p LMB sensitive. (**A**) amino acid alignment of Crm1p from *S.cerevisiae* (S.c.), *S.pombe* (S.p.), human (H.s.), *Caenorhabditis elegans* (C.e.) and *Drosophila melanogaster* (D.m.) within the most highly conserved portion of the protein, containing the LMB-resistant double mutation identified in *S.pombe*. Boxed is the position corresponding to Thr539 in *S.cerevisiae* Crm1p which was changed by site-directed mutagenesis to a Cys residue to confer LMB sensitivity. Accession numbers for C.e. and D.m. *CRM1* are U64855 and AC004423, respectively. (**B**) A wild-type *S.pombe* strain and a *S.cerevisiae CRM1* deletion strain rescued with either pDC-*CRM1* or pDC-*CRM1T539C* were tested for LMB sensitivity by growth on YEPD plates containing either 0 or 100 ng/ml LMB. (**C**) The C539→T mutation in Cpc1-3p confers LMB resistance. The *S.cerevisiae CRM1* deletion strain rescued with either pDC-*CRM1*, pDC-*CPC1-3C539T* or pDC-*CPC1-3* wase tested for LMB sensitivity by growth on YEPD plates containing either 0 or 100 ng/ml LMB. (**D**) and (**E**) LMB is able to interact with *S.cerevisiae* Crm1T539Cp *in vitro*. *In vitro* translated, ^{35}S-labeled *S.cerevisiae* (S.c.), Crm1T59Cp, Cpc1-3p and *S.pombe* (S.p.) Crm1p proteins were incubated with 0, 0.5, 1.0 μM LMB (1 μM LMB = 500 ng/ml) (D) or 0, 15, 33.3, 66.7, 125 nM LMB (E) for 45 min at 30°C, and analyzed by non-denaturing PAGE.

S.cerevisiae Crm1p is unaffected by LMB, even at elevated concentrations (1 μM; Figure 3D). In contrast, LMB interacts with Crm1T539Cp as well as with the Cpc1-3p chimera. A more careful titration of the LMB concentration shows that the affinity of Crm1T539Cp for LMB is comparable with that of the *S.pombe* protein (Figure 3E). We conclude that the Cys539 residue is essential for Crm1p binding to LMB. Furthermore, the results suggest that this residue may be interacting directly with the drug (see Discussion).

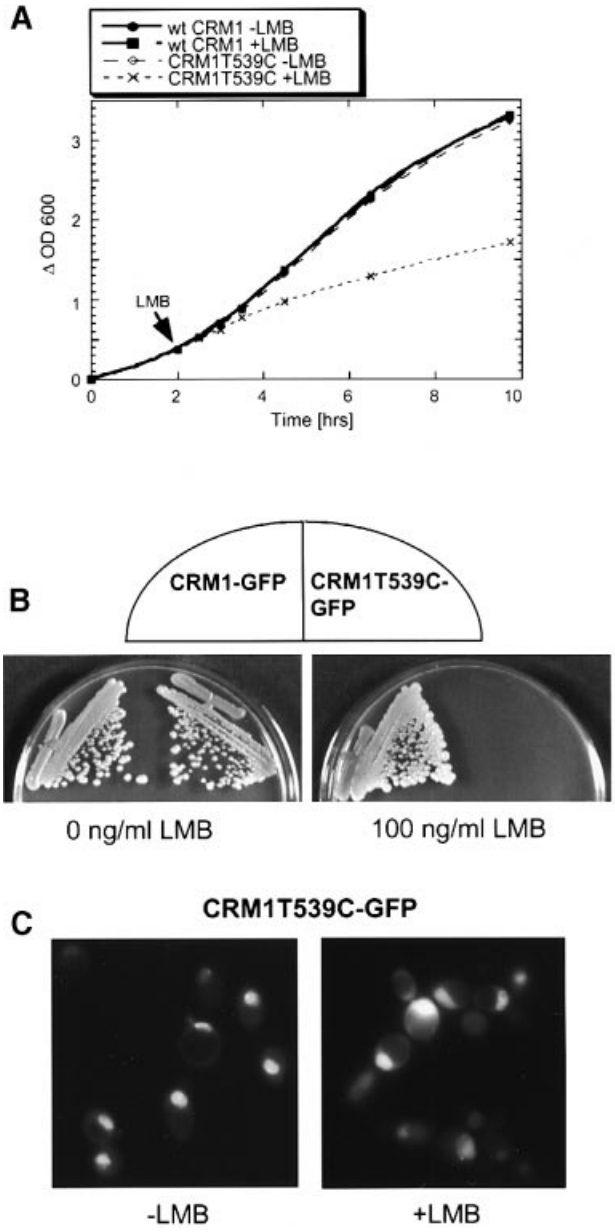


Fig. 4. *In vivo* analysis of the *S.cerevisiae* Crm1T539Cp-expressing strain. (**A**) The T539→C mutation in *S.cerevisiae* Crm1p does not affect cell growth. Overnight cultures of the *CRM1* deletion strain carrying pDC-*CRM1* or pDC-*CRM1T539C* were diluted and grown at 30°C until early log phase. At the indicated times cultures were split and were either treated with 100 ng/ml LMB or remained untreated and grown at 30°C. The increase in the optical density (Δ*OD*) at 600 nm is plotted against time. The arrow indicates the time at which LMB was added. (B) and (C) LMB treatment does not mislocalize Crm1p–GFP. The *S.cerevisiae CRM1* deletion strain rescued with either the wild-type Crm1p–GFP or Crm1T539Cp–GFP fusion proteins were tested for LMB sensitivity by growth on YEPD plates containing either 0 or 100 ng/ml LMB (**B**). The subcellular localization of Crm1T539Cp–GFP was analyzed in early log phase cells grown at 30°C in either the absence (–LMB) or presence (+LMB) of 100 ng/ml LMB for 60 min (**C**).

In vivo analysis of Crm1T539Cp-expressing cells

The T539C mutation has no obvious growth defects at all temperatures tested, suggesting that Crm1T539Cp functions with an efficiency comparable with that of wild-type *S.cerevisiae* Crm1p (Figure 4A; data not shown). Furthermore, growth of a merodiploid strain expressing

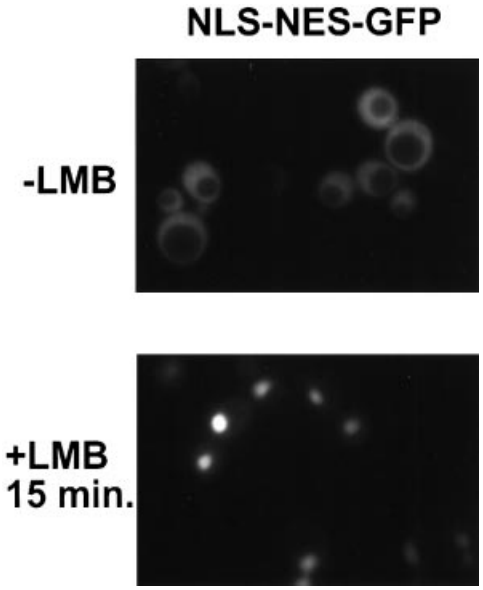


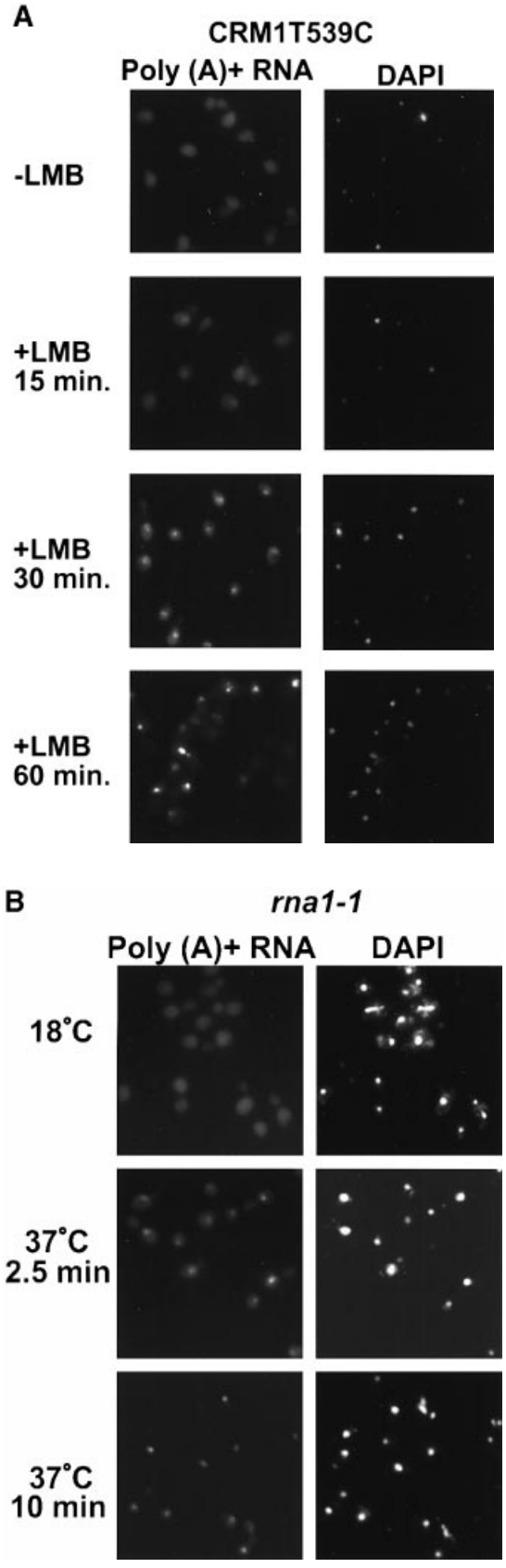
Fig. 5. LMB rapidly inhibits NES-mediated transport in *S.cerevisiae*. Crm1T539Cp-expressing cells were transformed with the NLS–NES–GFP_2 visual reporter (pPS1372, kindly provided by P.Silver). Cells were grown to early log phase and incubated for 15 min at 30°C in either the absence (–LMB) or presence (+LMB) of 100 ng/ml LMB.

both wild-type Crm1p and Crm1T539Cp is unaffected by the presence of LMB (data not shown), indicating that the phenotypes induced by LMB inactivation of Crm1p are recessive. To analyze the effects of LMB on the intracellular localization of Crm1p, the T539C mutation was introduced into a Crm1p–GFP expression construct (kindly provided by K.Weis). The modified fusion gene is able to rescue Δ*CRM1* and results in an LMB-sensitive strain (Figure 4B). Prior to LMB addition, Crm1T539Cp–GFP localization was indistinguishable from that of wild-type Crm1p–GFP: predominantly nuclear, a distinct signal at the nuclear envelope and a faint cytoplasmic signal (Figure 4C). Sixty minutes after LMB addition, the localization of Crm1T539Cp–GFP was almost identical and showed only a small increase in cytoplasmic signal. This suggests that LMB-induced inactivation of Crm1p does not target it for degradation and that the localization of Crm1p does not depend on the integrity of the NES–RanGTP–Crm1p complex. The result also distinguishes LMB from the temperature-sensitive *CRM1* mutant *xpo1-1*, which causes the protein to accumulate in nuclear spots at the restrictive temperature (Stade *et al.*, 1997).

LMB inhibition of NES-mediated export

Next we assayed the effect of LMB on NES-mediated transport in the *CRM1T539C* strain. To this end, we utilized a GFP visual reporter protein containing both a NLS and a NES: NLS–NES–GFP_2 (Taura *et al.*, 1998). This fusion protein is normally localized to the cytoplasm as well as the nucleus (Figure 5). After LMB addition, the reporter was rapidly restricted to the nucleus. After only 5 min, relocalization was readily apparent as an increase in nuclear signal and a decrease in cytoplasmic signal; this pattern became more pronounced with time (15 min is shown in Figure 5). At later time points (60 min), the cytoplasmic signal again increases, presumably due to a secondary block in NLS import (data not shown).

The wild-type Crm1p-expressing strain with the NLS–NES–GFP_2 reporter did not show any relocalization after LMB addition (data not shown). We conclude that LMB causes a rapid and potent block to NES export in *S.cerevisiae* as in other organisms.



Nuclear poly(A)$^+$ RNA accumulation in LMB-treated cells

We then tested the effect of LMB on poly(A)$^+$ RNA export by *in situ* hybridization with an oligo(dT) probe (Figure 6A). In wild-type control cells, poly(A)$^+$ RNA was diffusely localized throughout the cytoplasm and unaffected by LMB addition (data not shown). When LMB was added to the *CRM1T539C* LMB-sensitive strain, a faint nuclear signal was first detected after 15 min with no apparent decrease in cytoplasmic signal. The nuclear signal increased in intensity upon continued LMB exposure: bright nuclear spots were apparent after 30 min and were even more intense after 60 min. The cytoplasmic signal decreased continually between 15, 30 and 60 min. Interestingly, the observed effect of LMB on poly(A)$^+$ RNA nuclear accumulation appeared after the block to NES-mediated transport (Figure 5), which was already prominent at 5 min and almost maximal at 15 min.

To evaluate temporal aspects of the *in situ* assay, we examined the temperature-sensitive *rna1-1* strain (Rna1p is the Ran-GAP in *S.cerevisiae*). This mutant accumulates poly(A)$^+$ RNA in the nucleus at the non-permissive temperature, presumably by inhibiting Ran-dependent export (Amberg *et al.*, 1989; Forrester *et al.*, 1992). Mutant effects are apparently absent at the permissive temperature, and a large effect is already manifest at 78 s after a shift to the restrictive temperature (Forrester *et al.*, 1992). By *in situ* hybridization, a prominent poly(A)$^+$ RNA nuclear signal was visible after only 150 s at 37°C (the earliest time point assayed; Figure 6B). The nuclear signal continued to increase in intensity until 10 min, when a significant decrease in cytoplasmic signal was also apparent. Importantly, the LMB-mediated nuclear poly(A)$^+$ RNA signal was weaker at 15 min than the *rna1-1* nuclear signal at 2.5 min. Taken together with the comparably rapid LMB inhibition of NES-mediated export, the data suggest that Crm1p is not a major, direct exporter of most poly(A)$^+$ RNAs in *S.cerevisiae*.

In vivo protein labeling in the presence of LMB

If Crm1p plays a major role in *S.cerevisiae* mRNA export, we reasoned that cytoplasmic mRNAs should decrease after LMB exposure, as nuclear RNAs would be unable to replenish the cytoplasmic pool. We therefore decided to complement *in situ* hybridization with a different assay that measures cytoplasmic mRNA. This was indirectly visualized by the incorporation of [^{35}S]methionine into newly translated proteins

Fig. 6. LMB induces poly(A)$^+$ RNA nuclear accumulation in *S.cerevisiae*. (**A**) LMB mediates poly(A)$^+$ RNA accumulation in *CRM1T539C*. *CRM1T539C* cells were grown to log phase at 30°C (–LMB), LMB (100 ng/ml) was added and growth continued at 30°C for the specified amounts of time prior to fixation. Poly(A)$^+$ RNA was analyzed by *in situ* hybridization using an oligo(dT) probe. Nuclear DNA was stained with DAPI. (**B**) *In situ* hybridization rapidly detects poly(A)$^+$ RNA accumulation in *rna1-1*. *rna1-1* cells were grown to log phase at 18°C and rapidly shifted to 37°C. Cells were fixed at the indicated times; poly(A)$^+$ RNA and nuclear DNA were analyzed as described in (A).

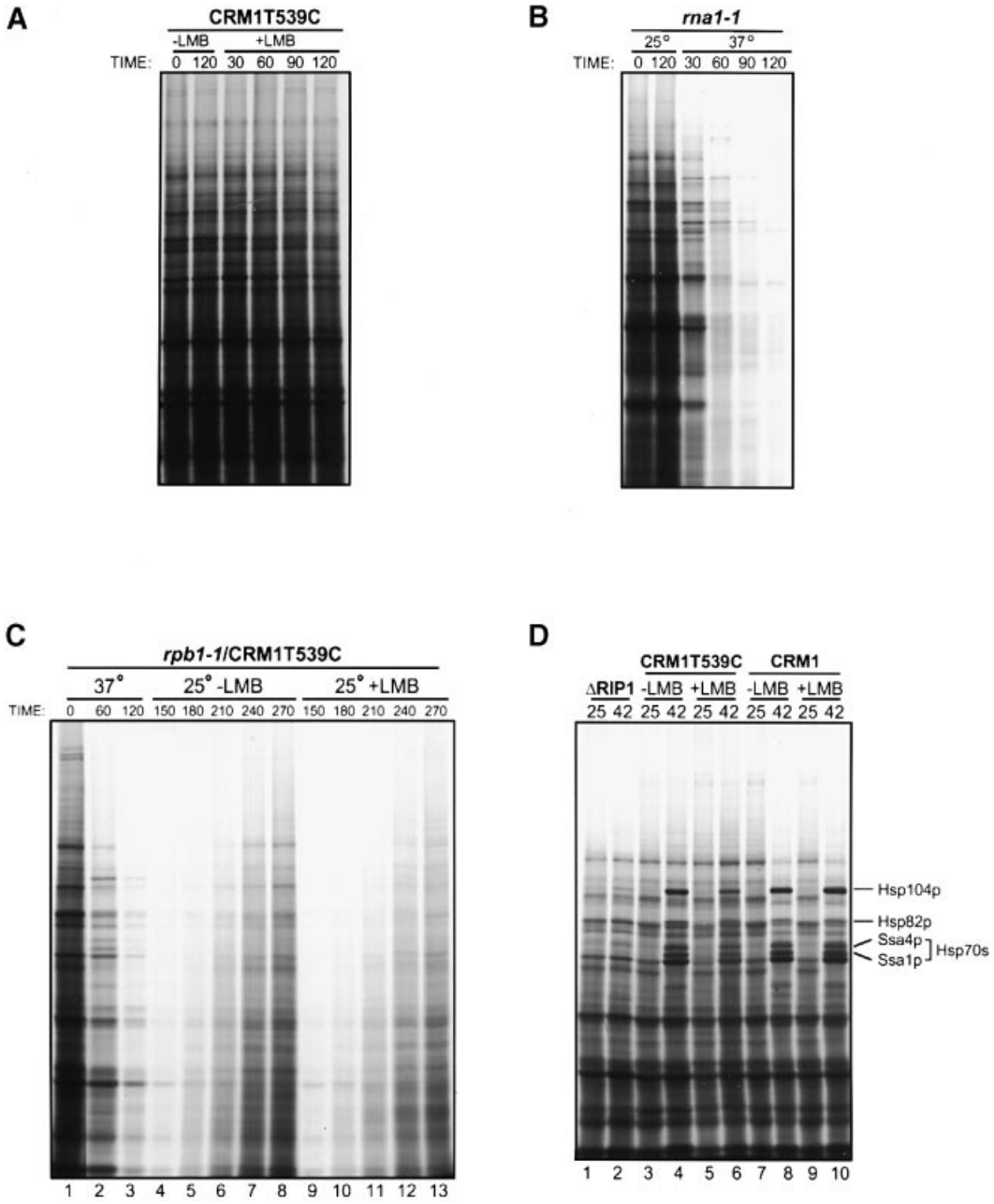


Fig. 7. Analysis of protein synthesis in the presence of LMB. (**A**) Analysis of protein synthesis in the *CRM1T539C* strain in the presence of LMB. Cultures of *CRM1T539C* cells were grown at 30°C in either the absence or presence of 100 ng/ml LMB for the indicated times and subsequently labeled for 5 min with [^{35}S]methionine. Total proteins were fractionated by 8% SDS–PAGE and autoradiographed. (**B**) Analysis of protein synthesis in the *rna1-1* strain at 37°C. *rna1-1* cells were shifted from 25°C to 37°C for the indicated times prior to labeling for 5 min as described in (A) at the appropriate temperature. Total proteins were fractionated by 7.5% SDS–PAGE and autoradiographed. (**C**) Translation of newly synthesized mRNAs in the presence of LMB. A *rpb1-1/CRM1T539C* double mutant strain was generated as described in Materials and methods. The *rpb1-1* mutant phenotype was induced by shifting cells from 25°C to 37°C for 2 h at which time the cultures were shifted back to 25°C and allowed to recover in either the absence or presence of 100 ng/ml LMB. Proteins were labeled at the indicated times as in (A). (**D**) Heat shock protein synthesis is partially inhibited by LMB. Heat shock protein synthesis was induced in a strain deleted for *RIP1/NUP42* (ΔRIP1), a strain expressing Crm1T539Cp (*CRM1T539C*) and a wild-type Crm1p-expressing strain (*CRM1*). Cells were either left untreated (–LMB) or treated with 100 ng/ml LMB (+LMB) and grown for 90 min prior to heat shock treatment at 42°C. The positions of the high-molecular-weight heat shock proteins (Hsp104p, Hsp82p and the Hsp70 proteins Ssa1p and Ssa4p; Lindquist and Craig, 1988) are indicated.

(Figure 7A). *Saccharomyces cerevisiae* cells expressing Crm1T539Cp were grown at 30°C in the absence or presence of LMB for the indicated times prior to a brief exposure to [^{35}S]methionine. Labeled proteins were subjected to SDS–PAGE and autoradiographed.

Two hours after LMB addition, no significant decrease in cytoplasmic mRNA translation was apparent. The same result was obtained when cells were grown at 37°C, and only minor effects were seen 180 min after LMB addition (data not shown). This incubation time greatly exceeds the time sufficient for a strong block to NES-mediated transport (described previously in Figure 5), indicating that the major mRNA export pathway(s) in *S.cerevisiae* is not NES–Crm1p mediated.

In contrast, the same assay shows a decrease in cytoplasmic mRNA translation at 37°C in a *rna1-1* strain (Figure 7B), consistent with a strong block to mRNA export in this mutant.

To address further the effects of LMB on mRNA export we generated a double mutant strain containing both the temperature-sensitive RNA polymerase II *rpb1-1* mutation (Nonet *et al.*, 1987) as well as the LMB-sensitive *CRM1T539C* mutation (*rpb1-1*/*CRM1T539C*). As expected, this strain is LMB sensitive and transcription is inhibited at 37°C (data not shown; Figure 7C). As the temperature-sensitive inhibition of transcription is reversible (Figure 7C, lanes 1–8), we were able to assay the effects of LMB treatment on newly synthesized mRNAs. To this end the double mutant strain was shifted to 37°C for 2 h to deplete the cytoplasmic mRNA pool and then shifted back to 25°C for 2.5 h in either the absence or presence of LMB (Figure 7C).

As expected, translation decreases dramatically after 120 min at 37°C (Figure 7C, lane 3); this decrease continues for at least 30 min after cells are shifted back to 25°C (Figure 7C, lanes 4 and 9). Translation then increases during the next 30 min at 25°C, in both the absence and presence of LMB (180 min; 60 min after LMB addition; Figure 7C, lanes 5 and 10), indicating a recovery of mRNA transcription, export and translation. The protein synthesis increase continued during the prolonged incubation at 25°C; even 2.5 h after the shift back to 25°C and LMB addition (270 min; Figure 7C, lanes 8 and 13), cytoplasmic mRNA abundance continued to increase in the presence of LMB as well as in its absence. Protein labeling in LMB was only slightly lower than in untreated cells (at most a 2-fold decrease), reflecting rather minor effects of LMB on some aspect(s) of strain recovery. In summary, inactivation of the NES–Crm1p export pathway has no major effect on transcription, transport or translation of cellular mRNAs in *S.cerevisiae*.

Heat shock protein synthesis

Previous results suggested that the apparently unique mRNA export pathway utilized by heat shock mRNAs at 42°C overlaps with the Rev-mediated export pathway in *S.cerevisiae* (Saavedra *et al.*, 1997). As we now know that the Rev export pathway is Crm1p mediated, we assayed the LMB effects on heat shock mRNA export with the protein labeling assay (Saavedra *et al.*, 1996, 1997; Stutz *et al.*, 1997). As shown previously, the absence of the nucleoporin Rip1p/NUP42 (ΔRIP1) selectively inhibits heat shock mRNA export, indicated here by the strong reduction in heat shock protein synthesis at 42°C (Figure 7D, compare lanes 2 and 8). Crm1T539Cp and Crm1p-expressing strains were grown in either the absence or presence of LMB for 90 min at 25°C and then incubated at 25°C or 42°C for a further 10 min, followed by a 20 min labeling pulse with [^{35}S]methionine at the same temperature. LMB reduced heat shock protein synthesis in the *CRM1T539C* strain (Figure 7D, lane 6) compared with control incubations (Figure 7D, lanes 4, 8 and 10). But the reduction is less severe than the dramatic drop in the Δ*RIP1* strain (Figure 7D, lanes 6 and 2). The decreased levels of heat shock protein synthesis in LMB-treated *CRM1T539C* cells suggest that Crm1p, like Rip1p, may indeed play some modest role in the export of heat shock mRNAs (see Discussion).

Discussion

The major mRNA export pathway in S.cerevisiae is not NES–Crm1p mediated

Crm1p plays an important role in NES-mediated protein export from the nucleus, as indicated by congruent results in a number of systems (Fornerod *et al.*, 1997a; Fukuda *et al.*, 1997a; Ossareh-Nazari *et al.*, 1997). It can also be considered an RNA exporter: Rev, the founding NES family member, exports viral RNAs via the CRM1 pathway (Stutz and Rosbash, 1998). In *Xenopus* oocytes, CRM1 also mediates snRNA export, presumably via an NES-containing protein (Fornerod *et al.*, 1997a). But in mammalian cells as well as in oocytes, there is no apparent role of CRM1 in generic cellular poly(A)$^+$ RNA export: mRNA export is LMB resistant and is not able to compete with Rev-mediated export (Fornerod *et al.*, 1997a; Wolff *et al.*, 1997; Otero *et al.*, 1998). In *S.cerevisiae*, however, a temperature-sensitive mutant of *CRM1*, *xpo1-1*, shows a block in poly(A)$^+$ RNA export, apparent as a strong nuclear signal within 15 min after the shift to the non-permissive temperature (Stade *et al.*, 1997). This strain also shows the expected nuclear accumulation of an NLS–NES–GFP reporter (Stade *et al.*, 1997).

In this report, we create and characterize an LMB-sensitive *S.cerevisiae* strain. We show that LMB blocks NES-mediated nuclear export very rapidly in this strain, as an effect is easily detectable at 5 min; the block must occur even more quickly, as visualization depends on the return of a detectable fraction of the GFP reporter protein to the nucleus. The poly(A)$^+$ RNA accumulation defect in these cells is only barely visible 15 min after LMB addition. This relatively slow effect is not due to a limitation in the sensitivity of the *in situ* assay, as there is significant accumulation of nuclear poly(A)$^+$ RNA signal in the *rna1-1* strain already 2.5 min after the temperature shift. We conclude that the blocks in NES-mediated export and poly(A)$^+$ RNA accumulation are temporally uncoupled in our new LMB-sensitive strain. Furthermore, we suspect that the more rapid poly(A)$^+$ RNA accumulation in *xpo1-1* does not differentiate it from the LMB effects, as the temperature-sensitive strain shows mutant phenotypes at the permissive temperature, including partial inhibition of NES export (data not shown; Stade *et al.*, 1997). To address further the contribution of Crm1p to mRNA export, we assayed protein synthesis. The results indicate that cytoplasmic mRNA abundance is unaffected even after 2 hrs of LMB incubation, suggesting that the export of most cellular mRNAs is not NES–Crm1p mediated. In a double mutant strain that inactivates transcription at 37°C and is LMB sensitive, protein synthesis recovery at 25°C is completely dependent on RNA export from the nucleus. After the shift to the permissive temperature, the difference between protein synthesis in the absence or presence of LMB is modest (2-fold at most), even at times well after potent inhibition of NES-mediated export (Figure 5). Although the NES-mediated export block uses a different assay in which the reporter is presynthesized, the protein synthesis assays indicate only minor LMB-mediated changes, even at times

when cell growth is clearly affected (Figure 4A). Indeed, these changes may even be due to the LMB-mediated growth rate decrease. Other LMB-mediated phenotypes can also be observed at these times, for example enlarged and misshapen nuclei (data not shown).

The differences between the poly(A)$^+$ RNA *in situ* hybridization assay (nuclear accumulation after 60 min) and the *in vivo* protein labeling assays (no decrease after 120 min) may be interpreted in several ways. The nuclear signal may reflect a small increase in the nuclear population of poly(A)$^+$ RNAs, due either to stabilization or to a decrease in an export step, which does not effect the overall mRNA throughput. A different interpretation posits a change in polyadenylation, for example an increase in overall tail length or an accumulation of nuclear poly(A) fragments that have been removed from primary transcripts. The decrease in the cytoplasmic *in situ* signal can be interpreted in similar ways; it may even reflect changes in RNP composition. These possibilities underscore the ambiguities associated with the *in situ* hybridization assay; without complementary approaches, it is impossible to interpret unambiguously the changes in signal. Although the protein synthesis assays are also indirect, they are interpretable only with great difficulty if the major mRNA export pathway is NES and Crm1p mediated.

A previous study indicated an overlap of the heat shock mRNA export pathway with the Rev-mediated pathway (Saavedra *et al.*, 1997), suggesting a role for Crm1p in heat shock mRNA export. But an earlier study by this same group provided evidence that the heat shock mRNA pathway functions independently of the Ran-GTPase system, indicating the contrary, namely, a mechanism distinct from that of Crm1p (Saavedra *et al.*, 1996). Although the LMB-mediated decrease in heat shock mRNA translation at 42°C is consistent with some role of Crm1p, the persistent translation of heat shock mRNAs even after 90 min of LMB treatment also implies a more modest conclusion: that heat shock mRNA export is partly but not solely mediated by a NES–Crm1p-dependent pathway. It is equally possible, however, that heat shock mRNA export is unaffected by LMB and that the observed decrease in protein synthesis is due to inhibition of other aspects of the heat shock response, e.g. transcription or translation. Further studies are necessary to determine the role of Crm1p, if any, in heat shock mRNA export.

In conclusion, our results indicate that Crm1p is not the major mRNA export receptor in *S.cerevisiae*. This is also supported by our previous work characterizing viable mutant *crm1* alleles, which inhibit NES-mediated export with no apparent poly(A)$^+$ RNA accumulation (Neville *et al.*, 1997). If there is any effect of LMB on mRNA export, it is likely to be a secondary and indirect consequence of the NES export block. One possibility is relocalization to the nucleus of the proposed NES-containing Ran regulators, Rna1p and Yrb1p (Feng *et al.*, 1999; E.Hurt, unpublished communication); indeed, experiments in *Xenopus* oocytes have shown that nuclear injection of these proteins inhibits mRNA export, presumably by collapsing the Ran-GTP gradient (Izaurralde *et al.*, 1997). It is important to note that our results do not exclude the export of a small, special fraction of yeast mRNA via the NES–Crm1p pathway. However, we do not favor this possibility, because of the relatively slow increase in nuclear poly(A)$^+$ RNA after LMB addition. We also cannot exclude the possibility that Crm1p exports poly(A)$^+$ RNA directly but via a non-canonical pathway, e.g. that Crm1p recognizes novel export signals in an LMB-insensitive manner; competition experiments suggest that this is not the case in higher eukaryotes (Fischer *et al.*, 1995; Bogerd *et al.*, 1998). As the conditional loss of other transport receptors in the importin/exportin-β family also causes a block to poly(A)$^+$ RNA export (Seedorf and Silver, 1997), these receptors or other members of this family may be more directly linked to poly(A)$^+$ RNA export.

What is the nature of the Crm1p–LMB interaction?

Our results also bear on the issue of Crm1p structure as well as LMB specificity. It is remarkable that a single amino acid substitution converts *S.cerevisiae* Crm1p from completely LMB resistant to fully LMB sensitive, with no obvious effect on strain viability or growth rate. Moreover, *S.pombe* Crm1p and *S.cerevisiae* Crm1T539Cp have similar affinities for LMB (Figure 3E). The previously identified *S.pombe* double mutation decreases but does not eliminate LMB sensitivity (Nishi *et al.*, 1994); this strain is much more LMB sensitive than the completely insensitive wild-type *S.cerevisiae* and the *CPC1-3C539T* strains. The data indicate that Crm1p is the only essential LMB target within the cell and that C539 must play a critical role in a favorable Crm1p structure. Previous work has suggested that the lactone moiety of LMB is indispensable for a stable interaction with Crm1p (Kudo *et al.*, 1998). The lactone group presents an ideal substrate for nucleophilic attack by cysteine in a Michael-type 1,4 addition reaction (Bergmann *et al.*, 1959). These speculations suggest that the structure of the Crm1p-LMB interaction as well as its direct impact on the ternary Crm1p–Ran–NES complex will be interesting.

Materials and methods

Plasmid constructions

Multiple gene-specific primers were designed with appropriate restriction enzyme sites and used to generate the following constructs, details of which may be obtained from the corresponding author. pDC1 was created by cloning the following PCR-generated fragments into pRS315 digested with *Xba*I/*Xho*I: a *Xba*I–*Bam*HI fragment encoding the 500 nucleotides upstream of the *S.cerevisiae CRM1*; a *Bam*HI–*Sal*I fragment encoding the 500 nucleotides downstream of the *CRM1* coding region, generated using a 5′ primer containing both *Bam*HI and *Xho*I cloning sites. pDC-*CRM1* was created by cloning a *Bam*HI–*Xho*I *S.cerevisiae CRM1*-HA fragment generated by PCR using the *CRM1*-HA template pLDB396 (described in Yan *et al.*, 1998) and 5′ primer MN-119 (5′-GGGG*GGATCC*ACTAGCAATAACAAA**ATG**-GAAGGAATTTTGGATTT-3′). pDC-*CPC1-3* was generated by cloning the following PCR-generated fragments into pDC1 digested with *Bam*HI and *Xho*I: *S.cerevisiae CRM1* amino acids1–497 digested with *Bam*HI and *Kpn*I; *S.pombe CRM1* amino acids 488–604 digested with *Kpn*I and *Sac*II; *S.cerevisiae CRM1* amino acids 615–end digested with *Sac*II and *Xho*I. pDC-*CRM1T539C*, p*CRM1T539C*-GFP and pDC-*CPC1-3C539T* were made by site-directed mutagenesis using the QuikChange^{TM} Site-Directed Mutagenesis Kit from Stratagene; the concentration of the plasmid templates (pDC-*CRM1*, pKW470 and pDC-*CPC1-3*, respectively) was 50 ng/μl. All mutations were confirmed by sequence analysis.

Strains

The *CRM1(*MNY7), *CRM1T539C* (MNY8), *CPC1-3(*MNY9) and *CPC1-3T539C* (MNY10) strains were created by transforming either pDC-*CRM1*, pDC-*CRM1T539C*, pDC-*CPC1-3* or pDC-*CPC1-3T539C*

(all *LEU2*/*CEN*) into LLY1044 (*MAT***a** Δ*CRM1*::KAN^r *leu2*$^-$ *his3*$^-$ *trp1*$^-$ *ura3*$^-$<*CRM1*-HA@NsiI *URA3 CEN*>) kindly provided by L.Davis; transformants were subsequently grown on 5-FOA Leu$^-$ plates. *CRM1*–GFP (MNY11) and *CRM1T539C*–GFP (MNY12) were created by transforming pKW470 or p*CRM1T539C*-GFP (both *HIS3*) into LLY1044; transformants were subsequently grown on 5-FOA His$^-$ plates. The strain *rpb1-1*/*CRM1T539C* (MNY13) was generated by mating *rpb1-1* (*MAT*α *leu2*$^-$ *ura3*$^-$ *Gal*$^+$; generously provided by P.Silver) to MNY8; diploids were selected on Leu$^-$ Trp$^-$ media and subsequently sporulated on KAc media. Dissected spores were tested for KAN^r, temperature sensitivity, growth on Leu$^-$ media and LMB sensitivity. The wild-type *S.pombe* strain (*h*$^-$) was provided by J.Haber.

Yeast two-hybrid constructs/assay

The *S.pombe* bait construct was generated by PCR amplifying *S.pombe CRM1* from *S.pombe* genomic DNA template (generously provided by T.Enoch) and digesting with *Eco*RI/*Sal*I; this fragment was then cloned into pEG202 digested with *Eco*RI and *Xho*I creating pEG202-S.p.*CRM1*. *Saccharomyces cerevisiae*/*S.pombe* chimeras were all cloned into pEG202 digested with *Eco*RI and *Xho*I using the following combinations of PCR-generated fragments: *S.cerevisiae* amino acids 1–497 as an *Eco*RI–*Kpn*I fragment and *S.pombe* amino acids 488–end as a *Kpn*I–*Sal*I fragment creating pEG202-CP1; *S.cerevisiae* amino acids 1–563 as an *Eco*RI–*Sac*II fragment and *S.pombe* amino acids 554–end as a *Sac*II–*Sal*I fragment creating pEG202-CP2; *S.cerevisiae* amino acids 1–616 as an *Eco*RI–*Sac*II fragment and *S.pombe* amino acids 607–end as a *Sac*II–*Sal*I fragment creating pEG202-CP3; *S.cerevisiae* amino acids 1–740 as an *Eco*RI–*Bgl*II fragment and *S.pombe* amino acids 731–end as a *Bgl*II–*Sal*I fragment creating pEG202-CP4. The *S.cerevisiae* Crm1p bait construct has been described previously (Stade *et al.*, 1997). The Yap1p-CRD prey construct is as described in Yan *et al.* (1998). The Rev and Rex bait constructs have been described elsewhere (Neville *et al.*, 1997).

Yeast two-hybrid interaction assays were carried out as described in Neville *et al*. (1997). LMB sensitivity was assayed by the addition of 100 ng/ml LMB to the Ura$^-$ His$^-$ Trp$^-$ + X-gal + 2% galactose/1% raffinose media used to visualize the β-galactosidase reporter gene activation.

In situ hybridization assay

In situ hybridization to detect poly(A)$^+$ RNA was performed as described previously (Neville *et al*., 1997); the labeled oligo(dT) probe was used at 500 ng/ml. The temperature-sensitive strain *rna1-1* (a gift from P.Silver) was grown to mid-log phase at 18°C and an equal volume of 56°C media was added to immediately shift the cells to 37°C. Cultures were grown at 37°C for the indicated times (see Figure 4D) prior to fixation. *CRM1T539C* and *CRM1* strains were grown at 30°C to mid-log phase, LMB was added to 100 ng/ml and growth was continued at 30°C for the indicated times prior to fixation (Figure 4E).

Gel mobility shift assay

Gel mobility shift assays were performed as described in Fornerod *et al*. (1997a). The following templates were used for *in vitro* transcription and translation reactions: T7-hCRM1 (Fornerod *et al.*, 1997b); *S.cerevisiae CRM1*, *CRM1T539C*, *CPC1-3* and *S.pombe CRM1* were PCR amplified using plasmid DNA template. PCR was for 30 cycles using Advantage cDNA PCR kit (Clontech). PCR products were phenol–chloroform extracted and 2-propanol-precipitated before use. Transcription/translation reactions were diluted 20× in PBS/8.7% glycerol and reconcentrated in microcon concentrators (Ambicon, molecular weight cut-off 30 kDa) to original volume.

In vivo protein labeling

The yeast strains were grown overnight to early log phase in complete media. Cultures were washed in water and resuspended in complete medium lacking methionine and grown at the appropriate temperature for 3 h. At t_0, cultures were split: for temperature-sensitive strains half was shifted to 37°C, while half remained at 25°C; for LMB treatments half was treated with 100 ng/ml LMB, while the other half was left untreated. At each time point 1 ml of culture was removed and mixed with 50 µCi of trans ^{35}S-label (1191 Ci/mmol, 11.02 mCi/ml, ICN Pharmaceuticals, Inc.) for 5 min at the appropriate temperature. Protein labeling was stopped by centrifugation at 4°C, removal of media and immediate freezing on dry ice. Samples were resuspended in 30 µl of 2× SDS sample buffer by vortexing, boiled for 10 min and spun in a microfuge for 5 min prior to fractionation on either a 7.5 or 8% SDS–polyacrylamide gel. Gels were dried and autoradiographed.

Heat shock *in vivo* protein labeling was performed as described in Stutz *et al.* (1997) with the following modifications: cultures were grown overnight at 25°C to early log phase in complete media. Cultures were washed in water and resuspended in complete medium lacking methionine and allowed to continue to grow at 25°C for 2 h. LMB was added to 100 ng/ml and cultures were grown for 90 min before heat shock treatment. Cells were collected by centrifugation, resuspended in 0.5–1 ml of media lacking methionine containing 100 ng/ml LMB when appropriate. 0.2 ml of cell suspension was rapidly mixed with one volume of medium lacking methionine preheated to 59°C and incubated at 42°C. The 25°C control samples were mixed with one volume of medium at 25°C. After 10 min, the samples were mixed with 50 µCi of Trans ^{35}S-label and incubated for another 20 min at the same temperature. Protein labeling was stopped as described above and samples were fractionated on a 7.5% SDS–polyacrylamide gel.

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