The EMBO Journal Vol.18 No.20 pp.5778–5788, 1999

Rat8p/Dbp5p is a shuttling transport factor that interacts with Rat7p/Nup159p and Gle1p and suppresses the mRNA export defect of *xpo1-1* cells

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In a screen for temperature-sensitive mutants of *Saccharomyces cerevisiae* defective for mRNA export, we previously identified the essential DEAD-box protein Dbp5p/Rat8p and the nucleoporin Rat7p/Nup159p. Both are essential for mRNA export. Here we report that Dbp5p and Rat7p interact through their N-terminal domains. Deletion of this portion of Rat7p (Rat7pΔN) results in strong defects in mRNA export and eliminates association of Dbp5p with nuclear pores. Overexpression of Dbp5p completely suppressed the growth and mRNA export defects of *rat7ΔN* cells and resulted in weaker suppression in cells carrying *rat7-1* or the *rss1-37* allele of *GLE1*. Dbp5p interacts with Gle1p independently of the N-terminus of Dbp5p. Dbp5p shuttles between nucleus and cytoplasm in an Xpo1p-dependent manner. It accumulates in nuclei of *xpo1-1* cells and in cells with mutations affecting Mex67p (*mex67-5*), Gsp1p (Ran) or Ran effectors. Overexpression of Dbp5p prevents nuclear accumulation of mRNA in *xpo1-1* cells, but does not restore growth, suggesting that the RNA export defect of *xpo1-1* cells may be indirect. In a screen for high-copy suppressors of the *rat8-2* allele of *DBP5*, we identified YMR255w, now called *GFD1*. Gfd1p is not essential, interacts with Gle1p and Rip1p/Nup42p, and is found in the cytoplasm and at the nuclear rim.

Keywords: DEAD-box proteins/mRNA export/nucleocytoplasmic shuttling/RAT8/YMR255w-GFD1

Introduction

Transport of macromolecules between nucleus and cytoplasm is an essential process in eukaryotic cells (for review see Corbett and Silver, 1997; Ohno *et al.*, 1998) and takes place through nuclear pore complexes (NPCs). NPCs are large proteinaceous complexes embedded in the nuclear envelope and contain (in yeast) ~30 proteins called nucleoporins. Through a genetic approach, we determined that several nucleoporins function specifically in mRNA export (Gorsch *et al.*, 1995; Heath *et al.*, 1995; Li *et al.*, 1995; Goldstein *et al.*, 1996; Dockendorff *et al.*, 1997). Transport also requires soluble receptors, which recognize signals in molecules to be transported, as well as the activities of Ran (Gsp1p in yeast), a small ras-like GTPase, and its effectors (Rna1p and Prp20p). These components function to ensure that cargoes are transported only in the proper direction (for review see Görlich, 1998). The known receptors are all related to importin β (Kap95p/Rsl1p in yeast). They share a Ran-binding domain and interact with the FG repeat regions of nucleoporins. Ran regulates the assembly and disassembly of complexes between transport receptors and their cargoes.

The export of mRNA to the cytoplasm appears to be considerably more complex than transport of smaller cargoes, including proteins and tRNAs (for review see Stutz and Rosbash, 1998). mRNAs are exported as mRNP particles, which contain mRNA and several RNA-binding proteins. These proteins may function to package the mRNA in an exportable configuration and may also directly mediate interactions with other transport factors (for review see Nakielny and Dreyfuss, 1997).

Several additional proteins required for mRNA export have been identified through genetic and biochemical approaches; most are highly conserved between yeast and metazoans. These include Mex67p (TAP in human cells) (Segref *et al.*, 1997), Gle1p/Rss1p/Brr3p (hGLE1 in human cells) (Del Priore *et al.*, 1996; Murphy and Wente, 1996; Noble and Guthrie, 1996a; Watkins *et al.*, 1998), Gle2p (Rae1p in *Schizosaccharomyces pombe*) (Brown *et al.*, 1995; Murphy *et al.*, 1996) and Mtr2p (Santos-Rosa *et al.*, 1998; Katahira *et al.*, 1999). All are essential, and yeast strains carrying temperature-sensitive (ts) mutations in any of these genes show rapid and strong defects in mRNA export. Interestingly, none of these factors is required for protein transport. Transport receptors and Ran/Gsp1p shuttle continuously between nucleus and cytoplasm. Mex67p and Mtr2p have also been shown to shuttle (Segref *et al.*, 1997; Santos-Rosa *et al.*, 1998), as do several of the RNA-binding proteins that exit the nucleus as part of the mRNP. The specific functions and mechanisms of action of these export factors have not yet been determined.

The only export receptor of the importin β family that shows defects in mRNA export when mutated is Crm1p/Xpo1p. Xpo1p mediates the export of proteins containing Leu-rich nuclear export signals (LR-NES) (Stade *et al.*, 1997). Although poly(A)$^+$ RNA accumulates in nuclei of *xpo1-1* cells at 37°C, it is not known whether this reflects a direct or indirect role for Xpo1p in mRNA export. The finding that protein synthesis continues at a normal level when cells carrying a leptomycin B-sensitive allele of *XPO1* are treated with leptomycin B is consistent with the idea that mRNA export continues even when Xpo1p is not functional (Neville and Rosbash, 1999).

The same genetic screen we used to identify RNA export-specific nucleoporins also identified a DEAD-box protein, Dbp5p/Rat8p (Snay-Hodge *et al.*, 1998). DEAD-box proteins are thought to use the energy derived from

ATP hydrolysis to unwind RNA secondary structure and to alter RNA–protein interactions. Multiple DEAD-box proteins are found in all organisms from bacteria to mammals. Approximately 28 have been identified in yeast. Biochemical and genetic studies indicate that DEAD-box proteins play an essential role in most steps of RNA metabolism from synthesis to turnover (for review see de la Cruz *et al.*, 1999).

Dbp5p is essential for mRNA export. In strains carrying ts alleles of *DBP5*, poly(A)$^+$ RNA accumulates in nuclei of all cells rapidly (<10 min) and synchronously upon a shift to 37°C (Snay-Hodge *et al.*, 1998; Tseng *et al.*, 1998). Dbp5p is very abundant, has ATP-binding and ATPase activities, and also shows RNA-unwinding activity but only when assayed in the presence of a yeast cell extract (Tseng *et al.*, 1998). Most Dbp5p is cytoplasmic, but a fraction associates with NPCs (Snay-Hodge *et al.*, 1998). The N-terminal region (amino acids 9–79) of Dbp5p is not essential (Snay-Hodge *et al.*, 1998).

In this paper, we show that Dbp5p and Rat7p/Nup159p interact and this association requires their N-termini. Rat7p is one of several nucleoporins essential for mRNA export and is not required for transport of proteins or export of tRNA (Sarkar and Hopper, 1998) and small ribosomal subunits (Moy and Silver, 1999). We also show that Dbp5p interacts with the export factor Gle1p, shown previously to be associated with NPCs (Del Priore *et al.*, 1996; Murphy and Wente, 1996).

In addition, we report that Dbp5p shuttles between nucleus and cytoplasm. Dbp5p accumulates in nuclei of *xpo1-1* cells, and also in cells carrying mutant alleles of *MEX67*, *RNA1*, *PRP20* or *GSP1* (Ran). Overexpression of Dbp5p prevents mRNA accumulation in *xpo1-1* cells shifted to 37°C. This supports the idea that Xpo1p may not play an essential role in mRNA export. Finally, we have conducted a screen to identify high-copy suppressors of the *rat8-2* allele of *DBP5*. Through this we identified YMR255w, which encodes a non-essential 21 kDa protein of unknown function, located in the cytoplasm with some accumulation at the nuclear rim, a pattern similar to that of Dbp5p.

Results

Overexpression of Dbp5p suppresses the growth and export defects of rat7 mutant strains

We are interested in defining the specific functions that Dbp5p and Rat7p play in mRNA export. In the experiments presented here, we used the three mutant alleles of *RAT7* described in earlier publications. One is the original *rat7-1* allele, which encodes a protein truncated within its C-terminal third, removing one of two adjacent coiled-coil regions (Gorsch *et al.*, 1995). *rat7ΔR* and *rat7ΔN* encode proteins lacking the central repeat domain or the N-terminal third of Rat7p, respectively (Del Priore *et al.*, 1997). The central repeat domain is not essential whereas growth becomes temperature sensitive when the N-terminus is deleted (Del Priore *et al.*, 1997). There is greater accumulation of poly(A)$^+$ mRNA in nuclei of *rat7ΔN* cells at 23°C than in *rat7-1* cells, and a complete export block at 37°C. Rat7-1p is lost from NPCs at 37°C, but Rat7pΔN is not.

We tested whether overexpression of Dbp5p would affect the growth and mRNA export defects of *rat7* mutants. As a control, we overexpressed Gle1p, which we identified previously as a high-copy suppressor of *rat7-1* (Del Priore *et al.*, 1996). Compared with Gle1p, overexpressed Dbp5p was a better suppressor of the 37°C growth defect of *rat7-1* cells (Figure 1). Dbp5p enhanced mRNA export in *rat7-1* cells (Figure 2; compare B and H), but nuclear accumulation of poly(A)$^+$ RNA was still readily detected, similar to the effect of overexpression of Gle1p on mRNA export (Del Priore *et al.*, 1996). Overexpressed Dbp5p (Figure 1), but not Gle1p (data not shown), was also able to suppress the growth defect of *rat7ΔN* cells at 37°C (Figure 1) and completely prevented accumulation of poly(A)$^+$ RNA in *rat7ΔN* nuclei at both 23 and 37°C (Figure 2I and J). In addition, Dbp5p was able to suppress the growth defect of *rss1-37* cells (Figure 1), but Gle1p could not suppress the *rat8*-2 growth defect (data not shown). Modestly-reduced nuclear and increased cytoplasmic levels of poly(A)$^+$ RNA were observed when Dbp5p was overexpressed in *rss1-37* cells (Figure 2, compare F and L). The 2μ plasmids used for these studies result in an ~8-fold increase in Dbp5p (Snay-Hodge *et al.*, 1998) and an ~5-fold increase in Gle1p levels (Del Priore *et al.*, 1996).

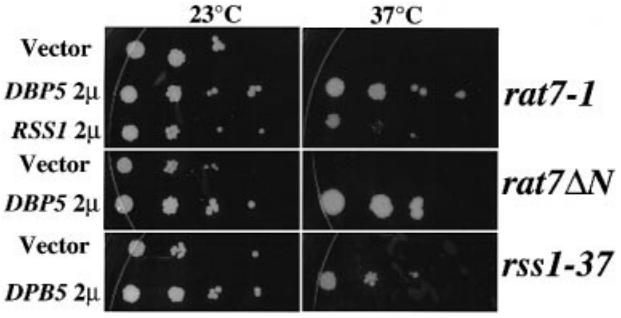

Fig. 1. Suppression of ts growth defects by overexpression of Dbp5p. Yeast strains carrying the *rat7-1*, *rat7ΔN* or *rss1-37* alleles were transformed with high-copy (2μ) plasmids expressing Dbp5p or Gle1p. As a control, the same strains were transformed with the vector. Strains were grown on selective media, diluted to OD_{600} of 0.05, and 1:5 serial dilutions were spotted onto plates, which were incubated for 4 days at the temperatures indicated.

Dbp5p interacts with the N-terminal domain of Rat7p

These results suggested that Dbp5p might interact physically with Rat7p. Dbp5p was able to pull down wild-type Rat7p (Figure 3A, lane 1) and Rat7pΔR (Figure 3B, lane 4), but not Rat7pΔN (Figure 3B, lane 2). Rat7p is unstable under the extraction conditions used, explaining the breakdown products seen in this experiment. Dbp5p also showed a strong interaction with the N-terminal third of Rat7p when the latter was expressed as a separate polypeptide (Figure 3C, lane 3). In an attempt to determine which portions of Dbp5p interact with Rat7p, we tested two N-terminally-deleted mutants. A greatly reduced interaction was seen when amino acids 9–79 of Dbp5p were deleted (Figure 3A, lane 2) even though this allele supports growth without detectable mRNA-export defects (Snay-Hodge *et al.*, 1998). No interaction was detected between Rat7p and Dbp5pΔ9-122 (Figure 3A, lane 3); Dbp5pΔ9-122 does not support growth. Western blot analyses demonstrated that the Dbp5pΔ9-79 and Dbp5pΔ9-122

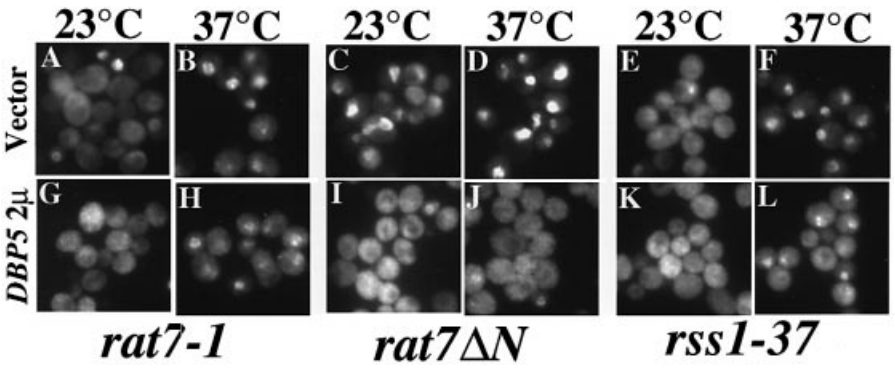

Fig. 2. Over-expression of Dbp5p suppresses the mRNA export defect of *rat7ΔN* cells. *rat7-1*, *rat7ΔN* and *rss1-37* cells were transformed either with a high-copy (2μ) plasmid expressing Dbp5p or with empty vector. Cells were analyzed by *in situ* hybridization using an oligo$(dT)_{50}$ probe.

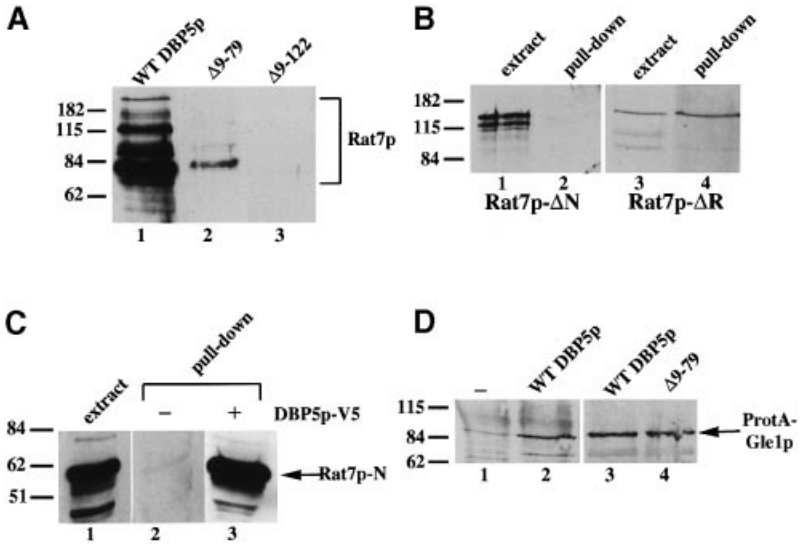

Fig. 3. Western analysis of proteins pulled down with Dbp5p-His. (**A**) Strains harboring His-tagged wild-type (WT) or N-terminally deleted Dbp5p were subjected to pull-down and the eluted proteins analyzed with an anti-Rat7pR antibody. Rat7p is highly susceptible to degradation under the extraction conditions used (Gorsch *et al.*, 1995). (**B**) His-tagged wild-type Dbp5p was expressed in strains containing deletion alleles of *RAT7*. Bound proteins were analyzed as in (A), except that an anti-Rat7pN antibody was used for lanes 3 and 4. (**C**) His-tagged Dbp5p and Rat7pN were co-expressed in a wild-type strain and subjected to pull-down analysis as in (A), except that the anti-Rat7pN antibody was used. (**D**) His-tagged Dbp5p, either wild-type or N-terminally deleted, was expressed in strains containing ProtA–Gle1p and subjected to pull-down analysis. An anti-protein A antibody was used to detect ProtA–Gle1p.

proteins were present at high levels in the extracts used (data not shown). We were unable to co-immunoprecipitate Rat7p with only the N-terminal portion of Dbp5p (aa1–122) (data not shown), indicating that this region is necessary but not sufficient for the interaction between Rat7p and Dbp5p.

Additional biochemical analyses also indicated that Dbp5p and Gle1p interact with one another (Figure 3D, lane 2). Dbp5pΔ9-79 and wild-type Dbp5p interacted equally well with Gle1p (Figure 3D, compare lanes 3 and 4). However, little or no interaction was seen between Gle1p and Dbp5pΔ9-122 (data not shown). It is not possible from these studies to determine if the interactions are direct or mediated by other proteins.

Two-hybrid analyses were also used to examine these interactions. The data summarized in Table I indicate a strong interaction of the N-terminal third of Rat7p with Dbp5p, a somewhat weaker interaction with Dbp5pΔ9-79, and little or no interaction with Dbp5pΔ9-122. No interactions were seen between Dbp5p and any of the other nucleoporins tested. Dbp5p could not interact with the central repeat region of Rat7p (Table I), but a strong two-hybrid interaction was seen between this region of Rat7p and Xpo1p. Equally strong interactions were seen between Xpo1p and the repeat regions of Nup100p and Rip1p/Nup42p, as well as human CAN/NUP214 and NUP98, consistent with earlier results of others (Neville *et al.*, 1997). It was not possible to isolate cells expressing full-length Gle1p in a two hybrid construct (F.Stutz, personal communication) so we used a construct expressing only the C-terminal half (257–538), missing the non-essential N-terminal region and the essential coiled-coil domain (Del Priore *et al.*, 1996). This portion of Gle1p interacted strongly with Dbp5p. We conclude that Dbp5p can interact with the N-terminal part of Rat7p and with the C-terminal half of Gle1p.

Expression of high levels of Rat7p fragments reduces growth and mRNA export

Next we investigated the effects on growth and mRNA export of inducing high-level expression of either the N-terminal region or the central repeat domain of Rat7p,

Table I. Yeast two-hybrid interactions between Dbp5p, nucleoporins and transport factors

β-galactosidase activity[a]

Prey	Bait						
	Dbp5p WT	Dbp5p (Δ9-79)	Dbp5p (Δ9-122)	Rat7p (1–500)	Xpo1p WT	Gle1p (257–538)	Gfd1p WT
Dbp5p WT	–	–	–	++	–	+++	+
Rat7p repeats (497–701) XXFG	–				+++	+	–
Rat7p N-terminus (1–500)	+++	++	–				–
Nup1p N-terminus (1–488)						–	–
CAN/NUP214 repeats (1691–1894) XXFG	–	–	–	–	+++	+++	–
Rip1p repeats (121–230) XXFG	–	–	–	–	+++	+	+++
Nup100p repeats (278–539) GLFG	–	–	–	–	+	–	–
Human NUP98 repeats (41–515) GLFG	–	–	–	–	++	++	–
Nup1p repeats (438–737) XFXFG	–	–	–	–	–	–	–
Gle1p (257–538)	–	–	–	–	–	–	+
Gfd1p	+	–	–	–	–	+++	–

[a]β-galactosidase production was measured in diploid strains obtained by mating haploids containing bait and prey constructs. Three distinct types of nucleoporin repeats (XXFG, GLFG, XFXFG) were used along with Xpo1p and the N-terminal non-repeat region of Nup1p as controls to verify the specificity of the interactions detected. The positive control (a LexA DNA-binding domain fused to a *GAL4* activation domain) gave 6000–8000 Miller units (Miller, 1972) while the negative controls (empty prey vector pJG4-5 along with various baits) gave 50–180 Miller units.
Key: +++, >6000 Miller units; ++, 4000–6000 Miller units; +, 200–4000 Miller units; –, <200 Miller units.

using the inducible *GAL1* promoter. The Western blot in Figure 4A shows that the levels of these fragments were very low (Rat7pN) or nil (Rat7pR) in the absence of galactose. After galactose induction, both Rat7p fragments accumulated to very high levels compared with the level of endogenous Rat7p. After 36 h in galactose, Rat7pN caused a modest accumulation of poly(A)$^+$ RNA in nuclei (Figure 4B, compare panels A and C), but no defects in growth. This minor defect was suppressed by overexpression of Dbp5p in these cells (Figure 4B, panel E).

In contrast, Rat7pR impaired growth of wild-type cells at 23 and 30°C (Figure 4C), whereas normal growth was seen at 37°C. However, mRNA export was unaffected by high-level expression of Rat7R (data not shown). Overexpression of Dbp5p had no effect on the growth inhibition caused by high-level expression of Rat7pR, whereas expression of Xpo1p from a high-copy plasmid suppressed this growth defect at 30°C, but not at 23°C (Figure 4C). The most likely explanation for this is that the loss of the dominant-negative effect of Rat7pR at 37°C reflects an interaction between Rat7pR and Xpo1p that is weaker than that which occurs during the probable interactions of the full-length proteins at the NPC. Taken together, these data are consistent with overexpressed Rat7pN titrating Dbp5p and overexpressed Rat7pR titrating Xpo1p.

The N-terminal domain of Rat7p is essential for association of Dbp5p with NPCs

In earlier studies we demonstrated that Dbp5p was located primarily in the cytoplasm with a fraction associated with NPCs (Snay-Hodge *et al.*, 1998). The data described above suggest that the N-terminal portion of Rat7p is important for this interaction. Therefore, we examined the location of a fully functional Dbp5p–GFP (green fluorescent protein) fusion in *rat7ΔN* cells as well as in *nup82Δ108* cells, in which Rat7p dissociates from NPCs when cells are shifted to 37°C (Belgareh *et al.*, 1998). At 23°C Dbp5p–GFP associated with NPCs as well as wild-type Dbp5p in *nup82Δ108* cells (Figure 5A). As reported previously, Dbp5p associated with clustered NPCs in the *rat2-1* strain (Snay-Hodge *et al.*, 1998). In contrast, in *rat7ΔN* cells, Dbp5p was not detected at the nuclear rim at either 23 or 37°C. Dbp5p could be detected at the nuclear rim in *rat7-1* cells incubated at 23°C, but the intensity of the signal was much lower than for other strains. Upon shifting cells to 37°C, we observed no change in the intensity of nuclear rim-associated Dbp5p–GFP in wild-type or *rat2-1* cells. Rat7p dissociated from NPCs in both *rat7-1* and *nup82Δ108* cells shifted to 37°C. These data indicate that the N-terminal portion of Rat7p is essential for association of Dbp5p with NPCs.

Dbp5p shuttles between nucleus and cytoplasm in an Xpo1p-dependent manner

Shuttling across the nuclear envelope is a property of many factors required for mRNA export, including transport receptors and Gsp1p (Ran). To determine whether Dbp5p shuttles, we examined the location of Dbp5p–GFP in *xpo1-1* cells. Dbp5p was detected primarily in nuclei at 23°C, but a cytoplasmic signal was clearly evident. Following a shift to 37°C, Dbp5p was detected only in nuclei (Figure 5B), a condition where Xpo1p-dependent protein export is blocked. This indicates that Dbp5p is a shuttling protein. When cells were returned to 23°C, most of Dbp5p–GFP was exported from nuclei within 1 h (data not shown).

Dbp5p accumulates in nuclei in several strains with ts defects in nuclear transport

The behavior of Dbp5p in *xpo1-1* cells led us to analyze the distribution of Dbp5p in cells carrying mutations affecting other soluble mRNA transport factors. At 37°C, strong nuclear accumulation of Dbp5p was seen in cells that harbor mutant alleles of the components of the Ran–GTPase system (*rna1-1*, *prp20-1* or *gsp1-1*) (Figure 5B). Note that a cytoplasmic signal for Dbp5p can be seen in all of these strains except *rna1-1*. Dbp5p accumulated in nuclei of *yrb2Δ* cells shifted to 16°C for 1 h (data not shown). Yrb2p is one of the few transport factors identified

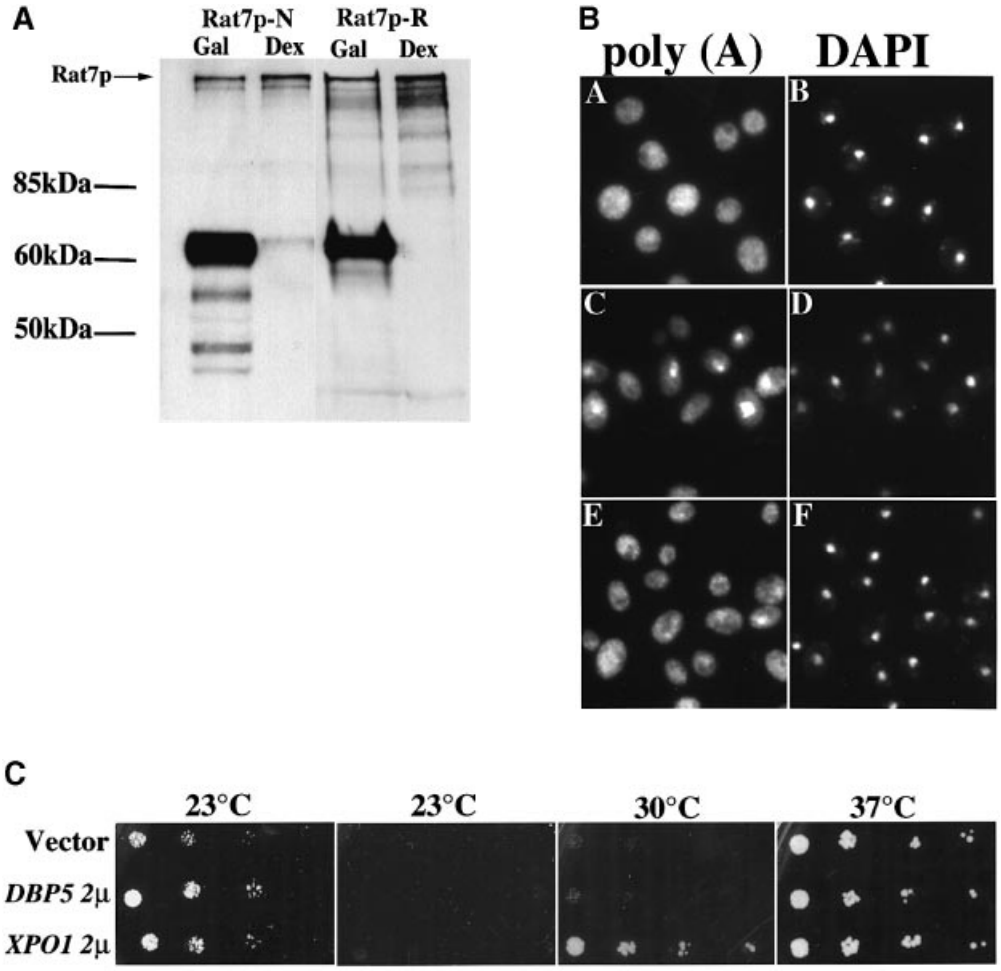

Fig. 4. Expression of high levels of Rat7pN or Rat7pR in wild-type cells. (**A**) Extracts were prepared from cells expressing either Rat7pN (aa1–456) or Rat7pR (aa457–899) under an inducible *GAL* promoter. Extracts made either prior to (right lanes) or following induction with 2% galactose for 1 h (left lanes) were analyzed by Western blotting. The position within the gel of the endogenous wild-type Rat7p is indicated. (**B**) The effect of high-level expression of Rat7pN on mRNA export was determined by *in situ* hybridization (A, C, E); panels B, D and F show DAPI staining of the same cells as are shown in panels A,C and E, respectively. Panels A and B, cells grown in dextrose; panels C and D, cells grown for 36 h on 2% galactose; panels E and F, cells containing a high-copy *DBP5* plasmid, shifted to 2% galactose for 36 h. (**C**) Inhibition of growth of wild-type cells by Rat7pR and its suppression by high-copy *XPO1*. Serial dilutions (1:5) of wild-type cells carrying a 2μ plasmid containing either no insert (vector), *DBP5* or *XPO1* were plated on dextrose (left panel) or galactose (three right panels) and incubated at the temperatures shown for 3 days.

that is important for protein export (Noguchi *et al.*, 1997; Taura *et al.*, 1998); cells with a disruption of *YRB2* are cold-sensitive for both growth and protein export. Interestingly, mutation of *MEX67* (*mex67-5*) also caused nuclear accumulation of Dbp5p, whereas mutation of *GLE1* (the *rss1*-37 allele) or *GLE2* (data not shown) did not. To assess the specificity of these effects, we examined the location of Dbp5p in other mutant strains. Dbp5p was distributed normally in strains carrying mutations affecting several nucleoporins required for mRNA export (*RAT7/NUP159*, *RAT2/NUP120*, *RAT3/NUP133*, *RAT9/NUP85* and *NUP145/RAT10*), in two strains with mutations of protein synthesis initiation factors (*tif1-1* and *prt1-1*) and in strains disrupted for two non-essential importin β family members (*msn5Δ* or *sxm1Δ*). Tif1p encodes the DEAD-box protein eIF4A; Prt1p is a component of eIF3.

Overexpression of Dbp5p prevents accumulation of poly(A)$^+$ RNA in xpo1-1 cells

When *xpo1-1* cells are shifted to 37°C, poly(A)$^+$ RNA accumulates in nuclei (Stade *et al.*, 1997; Figure 6A). Although overexpression of Dbp5p could not suppress the growth defect of *xpo1-1* cells (data not shown), it completely prevented accumulation of poly(A)$^+$ RNA in these cells (Figure 6A, compare panels C and G). We localized overexpressed Dbp5p in *xpo1-1* cells, and observed cytoplasmic Dbp5p both at 23°C and following a shift to 37°C (Figure 6B). At 23°C, all cells showed primarily cytoplasmic Dbp5p, with little or no nuclear signal; in addition, over-expression of Dbp5p enhanced the growth of *xpo1-1* cells at 23°C. Following the shift to 37°C, all cells showed both nuclear and cytoplasmic Dbp5p. Cytoplasmic Dbp5p was most abundant in cells expressing the highest levels of Dbp5p. This suggests that Dbp5p acts within the cytoplasm to prevent an mRNA export block in these cells. To make certain that mRNA continued to be produced and exported following the shift to 37°C, we induced expression from a *GAL* promoter and analyzed RNA accumulation by Northern blot analysis. No RNA was produced when cells were grown on dextrose or raffinose; transcription was induced by a shift to 2% galactose, and resulted in identical levels of mRNA in

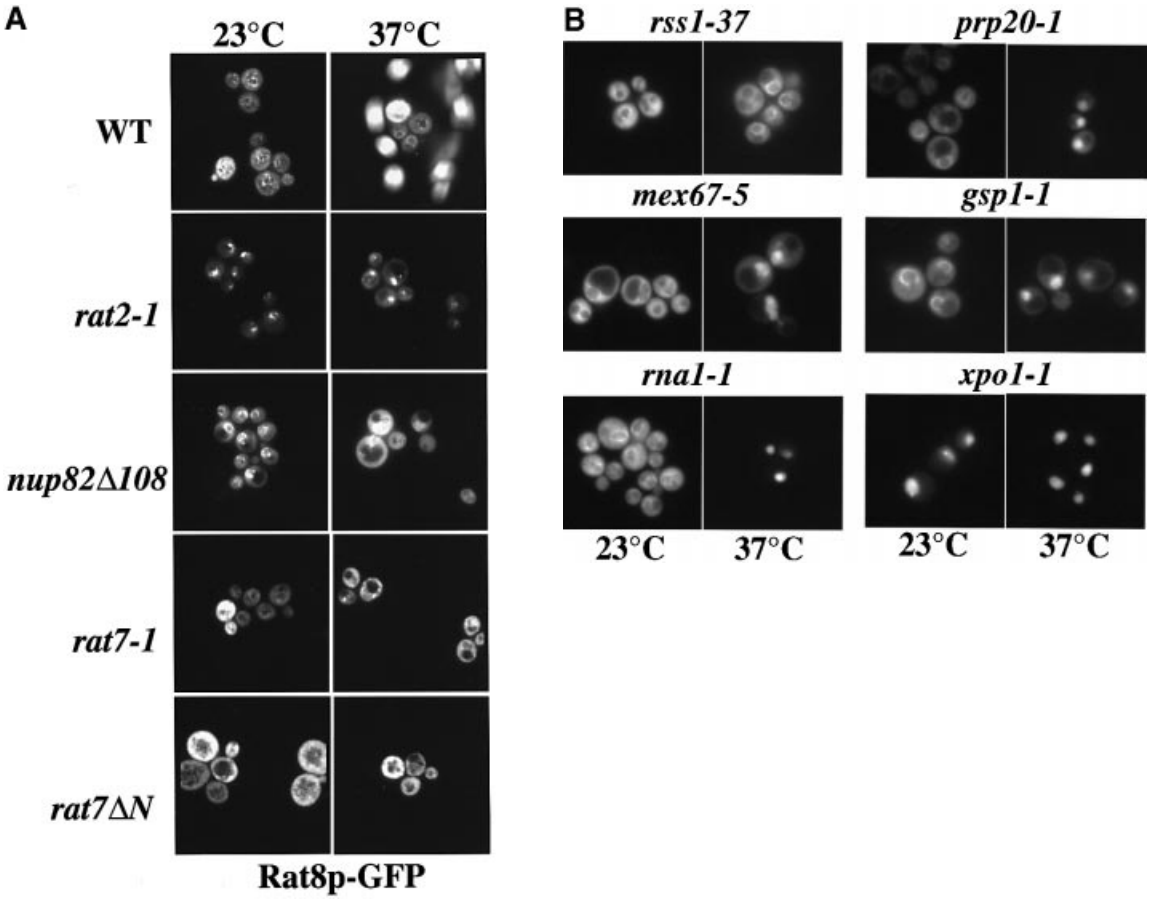

Fig. 5. Localization of Dbp5p in strains carrying mutations affecting mRNA export. Dbp5p–GFP was observed in living cells by spotting overnight cultures onto coverslips coated with 1% polylysine. Strains were shifted to 37°C for 30–150 min (based on the times previously determined to be necessary for maximal mRNA export defects to occur), then spotted onto prewarmed coverslips and slides and photographed using either a Zeiss Axioplan B (**A**) or Axiophot (**B**), both equipped with a cooled CCD camera. In (A) a *z*-series was taken of each strain with 0.2 μm increments, deconvolved using OpenLab software (Improvision, Conventry, UK) and then 3D rendered. In (B) single slices that included the nucleus were photographed, and no deconvolution was performed.

wild-type and *xpo1-1* cells after either 15 or 60 min in galactose, with or without the high-copy *DBP5* plasmid (data not shown). Taken together, these observations suggest that either Xpo1p does not play an essential direct role in mRNA export or high-level expression of Dbp5p restores an mRNA export function of Xpo1-1p without suppressing its protein export defect.

Screen for high-copy suppressor of the rat8-2 allele of DBP5

To identify proteins that might interact with Dbp5p, we conducted a screen for high-copy suppressors of the *rat8-2* allele. Our previous studies indicated that cells carrying the *rat8-2* allele are only moderately temperature sensitive. On a *CEN* plasmid, *rat8-2* supports growth at 34°C and below, but not at 37°C (Snay-Hodge *et al.*, 1998) while this allele is temperature sensitive above 30°C when integrated in place of *DBP5*.

We screened for high-copy suppressors at 34°C in cells carrying an integrated *rat8-2* allele (see Materials and methods). Open reading frame (ORF) YMR255w was identified many times in this screen. It is able to suppress the growth defect of the *rat8-2* and *rss1-37* strains at 34°C but not at higher temperatures (Figure 7A). The 21.5 kDa protein encoded by YMR255w is non-essential, contains a putative coiled-coil domain, and is very basic (pI 9.95) (Nobrega *et al.*, 1998). By two-hybrid analysis, this protein interacts strongly with Gle1p and Rip1p, and less strongly with Dbp5p (Table I). We saw no interaction between Gfd1p and either N-terminal truncation of Dbp5p. V5 epitope-tagged Gfd1p was localized to the cytoplasm and the nuclear rim (Figure 7B). This is a pattern similar to that of Dbp5p, though the nuclear rim staining is less distinct for Gfd1p–V5. Details on the isolation and further characterization of this suppressor will be described in a separate paper (H.V.Colot, P.Stafford, C.A.Hodge and C.N.Cole, in preparation). We have named this gene *GFD1* (good for Dbp5p). The same protein has been identified by another laboratory as a suppressor of a *GLE1* mutation (Strahm *et al.*, 1999).

Discussion

Dbp5p is a shuttling nuclear transport factor

Many proteins that participate in nuclear transport shuttle across the nuclear envelope. This includes Gsp1p, all known transport receptors and some of the soluble factors required for mRNA export (e.g. Mex67p, Mtr2p). In this paper, we demonstrate that Dbp5p is a shuttling transport factor, based on its reversible nuclear accumulation in

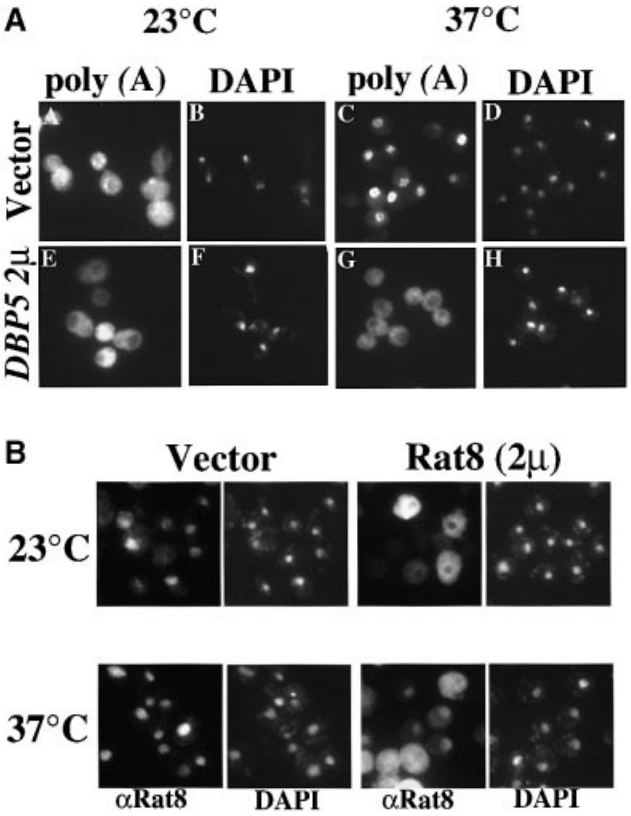

Fig. 6. (**A**) Overexpression of Dbp5p prevents *xpo1-1* cells from developing an mRNA export defect. *In situ* hybridization (A, C, E, G) was performed to detect poly(A)+ mRNA in *xpo1-1* cells carrying either an empty 2μ vector (A–D), or the same vector expressing Dbp5p (E–H). Cells were stained with DAPI to indicate the location of the nucleus. (**B**) Localization of Dbp5p expressed from a high-copy plasmid. Indirect immunofluorescence was performed on *xpo1-1* cells overexpressing Dbp5p, or transformed with a control 2μ vector, at both 23 and 37°C.

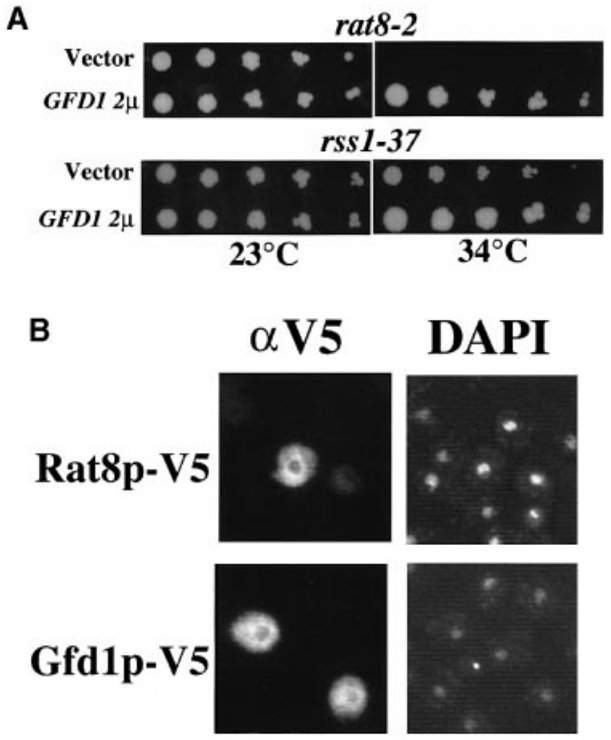

Fig. 7. GFD1 is a high-copy suppressor of rat8-2 and rss1-37. (**A**) Growth of CSY550 (with the *rat8-2* allele integrated at the *DBP5* locus) and SPY23(with the *rss1-37* allele integrated at the *GLE1* locus), with and without a 2μ plasmid containing YMR255w/GFD1. (**B**) Localization of V5-tagged Gfd1p and V5-tagged Dbp5p in wild-type cells by indirect immunofluorescence using an anti-V5 antibody.

xpo1-1 cells. Overexpression of Dbp5p completely prevented accumulation of poly(A)$^+$ RNA in *xpo1-1* nuclei (Figure 6) under conditions where transcription of a galactose-regulated reporter gene was unchanged. At least two possible scenarios can explain this observation. Perhaps Xpo1p does not play an essential major role in mRNA export. In this case, our data suggest that the primary function of Xpo1p for mRNA export may be to maintain Dbp5p outside the nucleus. Alternatively, Xpo1p and Dbp5p might interact during export. In the *xpo1-1* strain, this complex could be less stable. Overexpression of Dbp5p could act to stabilize this complex and maintain Xpo1p's activity in mRNA export. Note that overexpression of Dbp5p does not overcome the protein export defect of *xpo1-1* cells.

These data are consistent with Xpo1p exporting Dbp5p from the nucleus. However, we have not detected an interaction between Dbp5p and Xpo1p in the two-hybrid system. One possibility is that Dbp5p interacts weakly with Xpo1p. Alternatively, another protein might bridge the interaction between Dbp5p and Xpo1p. This could be a novel protein or a previously identified soluble transport factor, such as Gle1p or Mex67p. Although we detected interactions between Gle1p and Dbp5p, we saw no two-hybrid interaction between Dbp5p and Mex67p. Possibly, these proteins interact only at NPCs. Dbp5p does not appear to contain a LR-NES, but since only a limited number of LR-NESs have been defined, the spectrum of sequences able to serve as NESs is not known. Dbp5p could also interact with Xpo1p at a site different from the LR-NES binding site.

Dbp5p accumulated in the nuclei of several strains with mutations affecting proteins involved in nuclear transport, including components of the Ran/Gsp1p system (Gsp1p, Rna1p and Prp20p), which are essential for nuclear import and nuclear export. Interestingly, it appears that Dbp5p could enter, but not exit, the nucleus in strains with mutations affecting the Ran–GTPase system. Xpo1p and Yrb2p are the only proteins known whose mutation affects protein export but not protein import (Noguchi *et al.*, 1997; Stade *et al.*, 1997; Taura *et al.*, 1998). Since maintenance of Dbp5p in the cytoplasm appears to be essential for mRNA export, any mutation affecting protein export would be expected to result in mislocalization of Dbp5p.

All DEAD-box proteins contain a putative ATP-binding motif, and several have been shown to hydrolyze ATP. In addition, *in vitro* assays have shown that many DEAD-box proteins have RNA unwinding activity. The protein synthesis initiation factor eIF4A (Tif1p and Tif2p in *Saccharomyces cerevisiae*) is among the most thoroughly studied DEAD-box proteins (Pause and Sonenberg, 1992; Pause *et al.*, 1993). Although eIF4A shows modest RNA unwinding activity alone, this activity is dramatically stimulated by eIF4B, an RNA binding protein (Rozen *et al.*, 1990). Similarly, other proteins may be required to mediate the interaction of Dbp5p with RNA.

Gfd1p, a non-essential coiled-coil protein, was identified as a high-copy suppressor of the *rat8-2* allele. Nothing is known about its function. Since it is not essential, it cannot be an eIF4B-like co-factor for Dbp5p. One possibility is that Gfd1p enhances the activity of Dbp5p. Alternatively, Gfd1p could aid in the associations among various transport factors and nucleoporins, as suggested by the two-hybrid interaction observed between Gfd1p and Rip1p. Further studies will be needed to define the mechanism of suppression by Gfd1p as well as its normal role in the cell.

What is the biochemical function of Dbp5p?

Most if not all DEAD-box proteins are thought to have RNA unwinding activity, but this has been shown for only a limited number of these proteins. Dbp5p shows this activity when a yeast extract is present, but not as a purified protein (Tseng *et al.*, 1998). DEAD-box proteins are also likely to mediate the remodeling of RNA–protein complexes. An important step in mRNA export is the removal of hnRNP proteins that accompany the mRNA during export and then shuttle back to the nucleus. We hypothesize that during mRNA export, Dbp5p plays an active role in removing hnRNP proteins from mRNA.

The very large Balbiani rings seen in *Chironomus tentans* are exported as linear hnRNPs and extend simultaneously into both nuclear and cytoplasmic compartments. Even average-sized mRNAs (1000–2000 nucleotides) are much larger than small RNAs (tRNAs are $<$100 nucleotides) and almost all proteins. One possibility is that the ATPase activity of Dbp5p is harnessed during export both to remove bound proteins and, at the same time, to move the mRNP out of the nucleus. This would make mRNA export mechanistically distinct from the transport of proteins and small RNAs in requiring ATP hydrolysis for the actual work of transport.

While our data and those of others support an essential role for Dbp5p at the cytoplasmic face of the NPC, a nuclear function has not yet been defined. It would not be surprising if RNA export required DEAD-box proteins on both sides of the NPC. Since Dbp5p was not detected at the nuclear face of the NPC (Strahm *et al.*, 1999), any association of Dbp5p with the nuclear face must be brief but could still be functionally important. The possibility that mRNA export is essential for Dbp5p export is ruled out since we saw no accumulation of Dbp5p in nuclei of *rss1-37* cells, which have a very strong and rapid mRNA export defect. Dbp5p does not accumulate in nuclei in any strains with mutations affecting nucleoporins. Perhaps Dbp5p is exported in association with Mex67p. Mex67p and Dbp5p (along with other proteins) might associate with the hnRNP in the nucleus. During transport, the hnRNP would encounter Rat7p on the cytoplasmic side of the NPC. Interaction of the hnRNP with Rat7p through Dbp5p could signal arrival on the cytoplasmic side and activate Dbp5p to participate in disassembly of the hnRNP complex.

We tested whether Dbp5p would accumulate in nuclei when synthesis of mRNA was prevented, either by using the *rpb1-1* mutation or by inhibiting transcription with thioleutin. A slight accumulation of Dbp5p in the nucleus was seen following thioleutin treatment, but none in the *rpb1-1* strain (C.A.Hodge and C.N.Cole, unpublished results). This suggests that Dbp5p might be able to exit the nucleus in two ways, either as part of an hnRNP or not associated with poly(A)$^+$ RNA.

Interactions of Dbp5p with NPCs

The experiments presented above indicate that Dbp5p interacts with NPCs through the N-terminal third of Rat7p. This interaction was ablated when aa9–122, but not 9–79, were deleted from Dbp5p, or when the N-terminal third of Rat7p was absent (Rat7pΔN). Consistent with this, Dbp5p was lost from NPCs in *rat7-1* and *nup82Δ108* cells shifted to 37°C. Rat7p and Nup82p interact with each other through their C-terminal coiled-coil domains, which are truncated in these mutant alleles. In both of these strains, Rat7p dissociates from NPCs when cells are shifted to 37°C (Belgareh *et al.*, 1998).

Dbp5p overexpression suppresses the growth and mRNA export defects of *rat7ΔN* cells. This could occur in at least two ways: at high concentration, Dbp5p might be able to perform its essential functions when not directly associated with NPCs. Alternatively, Dbp5p might interact weakly (below the level of detection by fluorescence microscopy) with NPCs in the absence of the N-terminal domain of Rat7p. Overexpression of Dbp5p might increase the amount of Dbp5p associated with NPCs, even though binding would be much weaker without the N-terminus of Rat7p. We have been unable to determine whether overexpression of Dbp5p results in association of Dbp5p with NPCs of rat7ΔN cells because of the very high level of cytoplasmic Dbp5p under these conditions. Interestingly, mutation of *GLE1* (*rss1-37*) also results in reduced amounts of Dbp5p at the nuclear rim (Strahm *et al.*, 1999) and high-copy Dbp5p is a weak suppressor of the *rss1-37* mutation. We hypothesize that Dbp5p's interactions with NPCs involve contacts with both nucleoporins and NPC-associated transport factors (e.g. Gle1p). Our findings also indicate that the Rat7p–Dbp5p interaction is important specifically for mRNA export since export of rRNA and tRNA is unaffected by the *rat7-1* mutation (Sarkar and Hopper, 1998; Moy and Silver, 1999).

Structural and functional domains of Rat7p/Nup159p

Our studies provide evidence that Rat7p contains an N-terminal region that performs a functional role in mRNA export by providing a site for interaction of Dbp5p with NPCs. When the N-terminus of Rat7p was absent, an interaction between Dbp5p and NPCs was undetectable, and mRNA export was very inefficient at 23°C and blocked at 37°C (Del Priore *et al.*, 1997). Our finding that Dbp5p overexpression completely suppressed the mRNA export and growth defects of *rat7ΔN* cells suggests that the N-terminus of Rat7p plays no structural role in NPCs. In fact, *rat7ΔN* cells grow better than wild-type cells when Dbp5p is overexpressed (C.A.Hodge and C.N.Cole, unpublished results). This suggests that interaction with Dbp5p may be the primary function of the N-terminus of Rat7p. In contrast, it appears that the C-terminal region of Rat7p performs primarily a structural role within the NPC. Association of Rat7p with NPCs requires the coiled-coil domain, and is temperature-sensitive when this domain is truncated (Gorsch *et al.*, 1995). Furthermore, overexpression of Dbp5p was only weakly able to suppress

Table II. Yeast strains and plasmids

Strain name	Genotype	Source
CCY1	*MATα ura3-52 leu2Δ1 his3Δ200 prp20-1*	P.Silver laboratory
CHY149	*MATα ura3-52 leu2Δ1 trp1Δ63 RAT7ΔR*	this study
CHY173	*MATα RAT7::HIS3 his3Δ200 ura3-52 leu2Δ1 rat7ΔN*	this study
CSY550	*MAT***a** *leu2Δ1 trp1Δ63 ura3-52 rat8-2*	Snay-Hodge *et al*. (1998)
CSY570	*MATα his3Δ200 ura3-52 leu2Δ1* pCS835	Snay-Hodge *et al.* (1998)
CSY571	*MATα rat2-1 his3Δ200 ura3-52 leu2Δ1*	Snay-Hodge *et al.* (1998)
DAr1-1	*MAT***a** *ura3-52 leu2-3 112 his7 ade2-1 rna1-1*	D.Amberg/C.N.Cole laboratory
FY86	*MATα ura3-52, leu2Δ1, his3Δ200*	Winston *et al.* (1995)
LGY101	*MATα his3Δ200 ura3-52 leu2Δ rat7-1*	Gorsch *et al.* (1995)
nup82Δ108	*MAT***a** *his3Δ200 leu2-3 112 lys2-801 trp1-1 ura3-52 NUP82::HIS3 (pnup82Δ108 LEU2 CEN)*	Hurwitz and Blobel (1995)
OHY068	*MAT***a** *ura3-52 his3Δ200 leu2-3 trp1-63 GSP1::HIS3 GSP2::HIS3 (*pDW36 *gsp1-1*)	C.Hammell and Wong *et al.* (1997)
SPY23	*MATα ura3-52, leu2Δ1, his3Δ 200 rss1-37*	this study
xpo1-1	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3 XPO1::LEU2* pKW457	Stade *et al*. (1997)
Y-881	*MATα ade2 his3 leu2 trp1 ura3 MEX67::HIS3 (*pUN100 *mex67-5*)	Segref *et al*. (1997)
Plasmids		
pCS833	3.5 *Bam*HI–*Kpn*I fragment from pCS831 subcloned into YEplac181 (*LEU2* 2μ)	
pCS834	3.5 *Bam*HI–*Kpn*I fragment from pCS835 subcloned into YCplac33 (*URA3 CEN*)	
pCS835	GFP–S65T subcloned into the *Eag*I site within pCS831	
pCS7N	PCR fragment encoding aa1–456 of Rat7p subcloned into pPS311	
pCSΔ9-79GFP	*CEN LEU2* plasmid encoding Dbp5pΔ9-79 with GFP inserted after aa 8.	
pCSX195	3.8 kb *Sph*I–*Sac*I fragment of pKW446 subcloned into YEplac195 (*URA3* 2μ)	
pCS7R	PCR fragment encoding aa457–899 of Rat7p subcloned into pPS311	
pKW446	*XPO1* cloned into pRS313 (*LEU2 CEN*)	Stade *et al*. (1997)
pKW457	*xpo1-1* cloned into pRS313 (*LEU2 CEN*)	Stade *et al*. (1997)
pRAT830	3.5kb *Stu*I fragment from pRAT8.26 subcloned into YEplac195	Murphy and Wente (1996)
pVDP7	*RSS1/GLE1* subcloned into YEplac181	
Gle1-PA	fusion of protein A from *Staphylococcus aureus* to N-terminus of *GLE1* under control of *NUP116* promoter, in pRS314 (*TRP1 CEN*)	
pHVm5	PCR fragment with ORF and flanking regions of *GFD1* subcloned into the *Eag*I site of YEp24 (*URA3* 2μ)	

the transport defect of *rat7-1* cells. Since overexpressed Dbp5p was unable to suppress any defects of *nup82Δ108* cells, the Nup82p coiled-coil domain must perform additional roles beyond anchoring Rat7p in the NPC.

Interaction of Dbp5p with transport factors

Both pull-down and two-hybrid analyses demonstrated that Dbp5p also interacts with Gle1p. Gle1p was identified earlier as a high-copy suppressor of the *rat7-1* mutation (Del Priore *et al.*, 1996), through a synthetic lethal screen starting with a disruption of the GLFG-repeat nucleoporin *NUP100* (*GLE1*) (Murphy and Wente, 1996), and by screening cold-sensitive mutants for mRNA export defects (*BRR3)* (Noble and Guthrie, 1996). *GLE1* was also identified in a synthetic lethal screen beginning with a strain containing a disruption of *RIP1/NUP42* (Stutz *et al.*, 1997). Strong defects in mRNA export occur in strains carrying ts alleles of *GLE1*, but its precise function is not known. Although it contains sequences able to function as a nuclear export signal when fused to a reporter protein (Murphy and Wente, 1996), Gle1p has never been detected in the nucleus and does not accumulate there in *xpo1-1* cells (Stade *et al.*, 1997) or under any other conditions tested. It is located primarily at the nuclear rim, but some is cytoplasmic. Its interaction with Dbp5p suggests that these two proteins interact, most likely at the NPC, possibly via Rat7p.

Rat7p also serves as a docking site for Mex67p, which interacts directly with both Mtr2p and RNA (Segref *et al.*, 1997; Santos-Rosa *et al.*, 1998). Strains with mutations in *mex67* or *mtr2* are defective for mRNA export (Santos-Rosa *et al.*, 1998). Dbp5p was unable to suppress *mex67-5* (our unpublished results) whereas high-level expression of Mtr2p suppresses *mex67-5* (Santos-Rosa *et al.*, 1998). An attractive model is that Rat7p serves as a platform on which Dbp5p, Gle1p, Mex67p and Mtr2p (and perhaps other factors) interact to mediate RNA export.

Materials and methods

Yeast strains and genetic methods

Yeast strains and plasmids used in this study are listed in Table II. Strains were grown using standard methods. For growth assays, strains were grown overnight in selective media and diluted to an A_{600} of 0.05. Serial dilutions (1:5) were spotted onto selective plates and incubated at various temperatures for 3–6 days.

Plasmids

To place the N-terminal third (1–456) and the central repeat region (457–899) of Rat7p under *GAL* control, the portions of the *RAT7* gene encoding these fragments were amplified using PCR such that they could then be subcloned into the *LEU2 CEN* plasmid, pPS311 (a galactose-inducible expression plasmid obtained from P.Silver, Harvard Medical School). The resulting plasmids (pCS7N and pCS7R) were transformed into wild-type cells (FY86).

To prepare a plasmid expressing Dbp5pΔ9-79–GFP, we used PCR to amplify the GFP sequences from pCS835. The GFP PCR product was inserted into the *Eag*I site of a construct where the sequences encoding Dbp5pΔ9-79 had been deleted, and the *Eag*I site was retained. This inserts GFP following aa8 of Dbp5p.

Plasmids expressing Gfd1p tagged with both V5 and His_6 and Dbp5p tagged in the same way were purchased from Invitrogen (Carlsbad, CA). The ORFs encoding these proteins had been cloned into pYES2/GS (*URA3* 2μ), which places them under control of a *GAL* promoter. For high-copy expression, the ORF and flanking regions of *GFD1* were PCR-amplified from a high-copy library clone using primers containing

flanking *Eag*I sites, and cloned into the *Eag*I site of YEp24 to generate pHVm5. Fragments encompassing the deletions of aa9–79 and 9–122 of Dbp5p were used to replace the corresponding fragment in the plasmid encoding tagged Dbp5p. A ProtA-Gle1p plasmid was obtained from S.Wente (Washington University, St Louis, MO).

In situ hybridization, indirect immunofluorescence and localization of GFP fusion proteins in living yeast cells

The *in situ* hybridization assay, using an oligo$(dT)_{50}$ probe coupled to digoxigenin, was performed as described previously (Amberg *et al.*, 1992; Gorsch *et al.*, 1995). To localize Dbp5p–GFP in living cells, cells were grown in selective medium and then spotted onto glass coverslips coated with 1% polylysine (Sigma Chemical Co., St Louis, MO) which were then inverted onto a microscope slide and viewed with a Zeiss Axiophot T or Axioplan B microscope equipped with a cooled CCD camera. For some experiments, GFP was localized in fixed cells by using a rabbit polyclonal antibody against GFP (1:5000), a gift from Dr Pamela Silver (Harvard Medical School, Boston, MA), followed by goat anti-rabbit IgG coupled to Alexa 568 (Molecular Probes, Portland, OR). A mouse monoclonal antibody to the V5 epitope was obtained from Invitrogen and used at a 1:2000 dilution. The secondary antibody was horse anti-mouse (Vector Laboratories, Burlingame, CA) and was used at a 1:1000 dilution. Dbp5p was also localized by indirect immunofluorescence using an anti-Dbp5p antibody, as described previously (Snay-Hodge *et al.*, 1998).

Two-hybrid analyses

Yeast strains (EGY42 and EGY48) and vectors (pEG202, pJG4-5 and pSH18-34) for two-hybrid analyses were a generous gift of Dr Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA). Two-hybrid constructs were obtained by cloning PCR-derived wild-type genes or gene fragments into either bait vector pEG202 or prey vector pJG4-5. Yeast *GLE1*, *GLE2*, *NUP100*, *NUP1* and *CRM1/XPO1*, and metazoan NUP98 and CAN/NUP214 two-hybrid constructs were obtained from Dr Francoise Stutz (Institut de Microbiologie, Lausanne, Switzerland). Most have been described previously (Stutz *et al.*, 1996). Yeast strains carrying the indicated bait and prey vectors were analyzed by Western blotting to verify that approximately equivalent levels of the proteins of interest were being expressed (data not shown). Two-hybrid assays were conducted as described (Ausubel *et al.*, 1999). β-galactosidase production from a *lacZ* reporter gene was assayed in yeast strains obtained by mating appropriate bait and prey constructs. The bait constructs express LexA fusions to Dbp5p, N-terminal deletions of Dbp5p missing aa9–79 or 9–122, Rat7p N-terminal sequences (aa1–500), Crm1p/Xpo1p (aa1–1084) and Gle1p (aa257–538). The prey constructs express fusion proteins containing an SV40 NLS, the acidic B42 activation domain, and an HA tag, fused to Rip1p repeats (aa121–230), Nup1p N-terminus (aa1–488), Nup100p repeats (aa278–539), NUP98 repeats (aa41–515) or Nup1p repeats (aa438–737). The positive control for activation uses plasmid pSH17-4, in which an ADH promoter drives the expression of LexA fused to a *GAL4* activation domain. The negative control uses the empty prey vector, pJG4-5, and matings to various bait constructs. Activation of the β-galactosidase reporter was measured using the assay developed by Miller (1972). The positive control gave 6000–8000 Miller units of β-galactosidase activity and the negative controls gave 50–180 units.

Extracts and pull-down experiments

Extracts were prepared from cultures grown to an A_{600} of 0.5–0.8. Where required, genes under *GAL* control were induced overnight with 2% galactose. Spheroplasts were prepared as described previously (Newman *et al.*, 1985) and then either used directly or frozen on dry ice and stored at –80°C. One milliliter of lysis buffer (20 mM HEPES pH 7.6, 150 mM NaCl, 0.2 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.1% Thesit (Boehringer Mannheim); 0.5 mM DTT and Complete Protease Inhibitor tablets (–EDTA; Boehringer Mannheim) was added per 100 A_{600} units of original culture. The suspension was homogenized using a Dounce homogenizer for eight strokes, followed by centrifugation for 10 min at 9000 r.p.m.. The supernatant was used directly for pull-down analyses. Lysates were diluted to 2–5 mg/ml and 1 ml was incubated with 100 µl TALON beads (Clontech, Palo Alto, CA) to bind His-tagged Dbp5p. After a 1.5 h incubation with agitation at 4°C, the beads were collected by centrifugation and washed 4 times (5 min each, on ice) with 8 volumes of lysis buffer. The associated proteins were then eluted by a 5 min incubation on ice with 350 µl 0.6 M NaCl in lysis buffer. Most of the His-tagged protein remained bound to the beads under these conditions. Glycogen (5 µg) and 4 volumes of acetone were added. After 10 min at room temperature and a 10 min spin in a microcentrifuge, the pellet was dried and resuspended in 30–40 µl sample buffer for Western blot analysis. At least half of the sample was used for each lane shown. Extract lanes contained 50–100 µg of protein.

Western blotting

Samples were separated using 10% polyacrylamide–SDS gels and transferred to nitrocellulose. For Figure 4, extracts were prepared and the blots processed and probed as described previously (Del Priore, 1997). For Figure 3, extracts and pull-down samples were prepared as described above. The antibody concentrations for anti-Rat7p antibody were: 1:5000 (Figure 4) or 1:10 000 (Figure 3), 1:20 000 for anti-Rat7pR, and 1:1000 for anti-protein A (Sigma). All secondary antibodies were used at 1:10 000 and were visualized by ECL (Amersham Pharmacia).

High-copy suppressor screen

Strain CSY550 harboring the *rat8-2* mutation (Snay-Hodge *et al.*, 1998) was transformed with a YEp24-based yeast genomic library (Carlson and Botstein, 1982) and plated at 30°C. The 20 000 transformants obtained were collected and replated at 34°C. After 4 days, seven colonies had appeared. After an additional incubation overnight at room temperature, 24 additional colonies appeared. Colonies were replated on 5-FOA plates at 34°C, and the 21 whose growth depended on the *URA3* library plasmid were retested by rescuing the plasmid and retransforming it into CSY550. Concurrently, the initial transformants were also plated at 16°C and 11 candidates obtained. After sequencing and subcloning analyses, several plasmids (five from 34°C and two from 16°C) contained *GFD1*.

Acknowledgements

We thank Françoise Stutz, Pamela Silver, Susan Wente, Karsten Weis and Michael Hurwitz for strains and plasmids; Pamela Silver for antibodies against GFP; and Erica Golemis for plasmids, strains, and advice about the two-hybrid system. We also acknowledge the Fannie E.Rippel Foundation for a grant that supported equipment purchases necessary for some of the image analyses presented here, and the Englert Cell Analysis Center for assistance with some of the microscopy. We are grateful to Françoise Stutz for helpful discussions and sharing results with us prior to publication, and we thank the members of the Cole laboratory for comments on this manuscript. This research was supported by a grant from the National Institutes of General Medical Sciences, NIH (GM33998) and by a core grant (CA-16038) from the National Cancer Institute to the Norris Cotton Cancer Center. P.S. was supported by an NIH/NCI training grant (CA09658).

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Received June 21, 1999; revised August 27, 1999; accepted August 31, 1999