Cell, Vol. 82, 495–506, August 11, 1995, Copyright © 1995 by Cell Press

Identification of a Novel Nuclear Pore-Associated Protein as a Functional Target of the HIV-1 Rev Protein in Yeast

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Summary

The HIV-1 Rev protein increases the cytoplasmic levels of unspliced and singly spliced target transcripts in metazoan systems. Based on experiments that indicate a similar function of Rev in the yeast S. cerevisiae, we have identified a yeast protein that interacts with the effector domain of Rev. The protein, Rip1p, is a novel small nucleoporin-like protein, some of which is associated with nuclear pores. Its closest known yeast relative is a nuclear pore component also implicated in mRNA transport from nucleus to cytoplasm. Analysis of strains that overexpress Rip1p or that are deleted for the *RIP1* gene show that Rip1p is important for the effect of Rev on gene expression, indicating that the physical interaction is of functional significance in vivo. The results suggest that Rev directly promotes the cytoplasmic transport of suitable transcripts by targeting them to the nuclear pore.

Introduction

The human immunodeficiency virus type 1 (HIV-1) Rev protein is a critical regulator of the viral life cycle and causes a switch from the early phase to the late phase of gene expression. In the early phase, products from fully spliced RNAs, including Rev, are synthesized. In the late phase, Rev function results in the appearance of unspliced or partially spliced viral transcripts in the cytoplasm, where they encode the viral structural proteins Gag, Pol, and Env (Feinberg et al., 1986; Sodroski et al., 1986). The hallmark of Rev activity and that of similar proteins from related retroviruses (such as Rex from human T cell leukemia virus type I [HTLV-I]) is therefore the export of suitable target transcripts from the nucleus to the cytoplasm. In many experimental systems, this relocalization is also accompanied by a reduction in the cytoplasmic levels of fully spliced viral RNAs (Emerman et al., 1989; Felber et al., 1989; Malim et al., 1989b; Malim and Cullen, 1993).

These observations have generated two hypotheses to explain Rev activity: either Rev inhibits some aspect of pre-mRNA splicing, which leads indirectly to enhanced pre-mRNA export, or Rev directly promotes pre-mRNA export, which has an indirect negative effect on pre-mRNA splicing (Cullen and Malim, 1991). Although both explanations are still possible (and are not necessarily mutually exclusive), recent experiments suggest that the direct mode of action of Rev is more likely to involve RNA export than pre-mRNA splicing for the following reasons. First, in certain experimental systems such as T lymphocytes, Rev-dependent relocalization of RNA is not accompanied by a reduction in the cytoplasmic levels of fully spliced mRNAs (Malim and Cullen, 1993). Second, although largely nuclear in mammalian cells, Rev is a shuttling protein and accompanies Rev response element (RRE)-containing RNAs to the cytoplasm (Kalland et al., 1994; Meyer and Malim, 1994). Third, recent experiments in the oocyte system indicate that Rev can promote export of target transcripts that are not recognized by the pre-mRNA splicing machinery (Fischer et al., 1994). Although these conclusions favor RNA transport as the cellular process targeted by Rev, more definitive conclusions require structure–function studies on the direct biochemical activities of Rev.

Mutagenesis and domain swap experiments have shown that the 116 amino acids of Rev contain at least two domains necessary for RNA export activity. The basic arginine-rich domain near the amino terminus determines RNA target selectivity by binding to the RRE, a highly structured 240 base RNA sequence located within the viral *env* intron (Hadzopoulou-Cladaras et al., 1989; Daly et al., 1989; Zapp and Green, 1989; Kjems et al., 1991a). This domain also contributes to Rev oligomerization as well as to Rev nuclear localization (Malim et al., 1989a; Hope et al., 1990; Zapp et al., 1991). The small (~10 amino acid) leucine-rich effector domain is also necessary for pre-mRNA export (Malim et al., 1991). Unlike mutations in the arginine-rich domain, however, mutations in the effector domain do not interfere with RNA binding, Rev oligomerization, or Rev subcellular localization (Malim et al., 1989a, 1991; Mermer et al., 1990; Zapp et al., 1991; Hope et al., 1992). Therefore, the effector domain has no direct connection to known biochemical processes. If we assume that Rev functions directly to promote RNA export, the identification of factors contacted by the effector domain might illuminate cellular strategies used to export RNA from the nucleus to the cytoplasm.

To identify such factors and to study Rev function, we recently established a Rev-dependent system in the yeast Saccharomyces cerevisiae (Stutz and Rosbash, 1994). Because reliable nuclear–cytoplasmic fractionation is difficult in yeast, the effect of Rev on pre-mRNA localization was indirectly monitored by examining the expression of RRE-containing transcripts encoding a functional *CUP1* gene product. Cup1p is a copper chelator and allows cells expressing *CUP1* to grow on copper-containing media in a dose-dependent manner (Hamer et al., 1985; Lesser and Guthrie, 1993). Two key reporter gene constructs, pre-mRNA coding sequence-*CUP1*-RRE (PC–CUP–RRE) and mRNA coding sequence-*CUP1*-RRE (MC–CUP–RRE), contain an inefficiently spliced 65 bp synthetic intron at the 5′ end of the *CUP1* coding sequence (Legrain and Rosbash, 1989; Stutz and Rosbash, 1994; Figure 1). In the PC–CUP–RRE construct, the intron sequence is

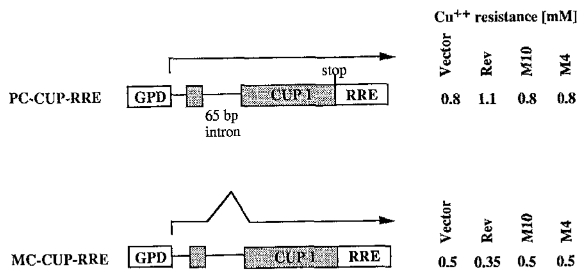


Figure 1. Schematic Drawing of the *CUP1* Reporter Constructs

Each *CUP1* transcription unit is driven by the strong GPD constitutive promoter (open box) (Guthrie and Fink, 1991). The transcription initiation site corresponds to the start of the arrow. The *CUP1* coding region is shown as a stippled box. In both constructs, a 65 bp inefficiently spliced intron interrupts the *CUP1* coding region after the third codon; the intron contains consensus 5′ splice site, branchpoint, and 3′ splice site sequences (Legrain and Rosbash, 1989). Both constructs encode at their 3′ end, after the *CUP1* stop codon, a 450 base sequence containing the complete RRE. The transcripts terminate beyond the RRE within the phosphoglycerate kinase (*PGK*) terminator (not shown; Guthrie and Fink, 1991). In the PC-CUP-RRE construct, Cup1p is encoded only from the pre-mRNA; in the MC-CUP-RRE construct, Cup1p is encoded from the spliced RNA only. The copper resistance conferred by these constructs to the yeast host strain in the presence of wild-type or mutant Rev-expressing plasmids is indicated on the right and summarized from Stutz and Rosbash (1994). The PC-CUP-RRE control strain (vector) grows up to 0.8 mM copper owing to some escape of the inefficiently spliced PC-CUP-RRE pre-mRNA into the cytoplasm.

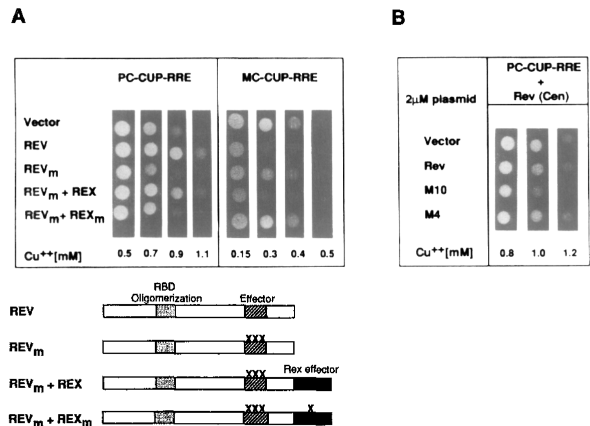


Figure 2. Rex Is Functional in Yeast and Rev M10 Has a Dominant Negative Phenotype

(A) The Rex effector domain can replace a nonfunctional Rev effector domain. Four different Rev or Rev–Rex fusion constructs (*TRP1/ARS1-CEN4*) were introduced into a yeast strain containing either the PC-CUP-RRE or the MC-CUP-RRE reporter plasmid (*LEU2/2μm*) to examine the effects of Rev or Rev–Rex fusions on pre-mRNA export or mRNA expression, respectively. The copper resistance of the double transformants was examined on Leu^- Trp^- plates containing increasing concentrations of copper. The mutant Rev construct contains three amino acid changes in the effector domain (LCC81,85,89FKW); in the mutant Rev (Rev_m) plus Rex construct, a Rex sequence encoding amino acids 75–96 (Rex effector domain) was fused to mutant Rev; in mutant Rev plus mutant Rex (Rex_m), an additional mutation (L90K) was introduced into the Rex effector domain. These three Rev and Rev–Rex constructs were a gift of T. Hope (A. A. Beeche et al., personal communication).

(B) The M10 effector domain mutation has a dominant negative phenotype. Wild-type, M10, and M4 mutant Rev constructs were expressed from a high copy number plasmid (*TRP1/2μm*) in a yeast strain transformed with the PC-CUP-RRE reporter construct (*LEU2/2μm*) and a low copy number wild-type Rev plasmid (*URA3/ARS1-CEN4*). M10 contains the effector domain mutation LE78,79DL and M4 contains the RBD/oligomerization domain mutation YSN23,25,26DDL. The growth of the triple transformants was examined on Ura^- Leu^- Trp^- plates in the presence of increasing copper concentrations.

in-frame with the *CUP1* coding sequence whereas the spliced mRNA contains early stop codons, preventing *CUP1* expression from mRNA. For this construct, the ability to grow in the presence of copper is the result of pre-mRNA translation and is taken as a measure of pre-mRNA export. In the MC-CUP-RRE construct, Cup1p is encoded only by the spliced mRNA as the *CUP1* coding sequence in the intron-containing transcript is out-of-frame. In this case, copper resistance reflects mRNA levels and is a function of splicing efficiency. Rev had a positive effect on pre-mRNA-derived gene expression, as evidenced by the enhanced copper resistance of the PC-CUP-RRE strain. MC-CUP-RRE expression, in contrast, was decreased by Rev. In both cases, the Rev effects were RRE dependent. Moreover, a mutant in the RNA-binding domain (RBD)/oligomerization domain (M4) of Rev and a mutant in the effector domain (M10) of Rev had no detectable activity (Figure 1; Stutz and Rosbash, 1994), suggesting that the mode of action of Rev in yeast might be similar to that in mammalian systems (Malim et al., 1989a).

To confirm and extend this connection, we report here additional experiments indicating that the function of Rev in yeast is indeed closely related to its activity in mammalian cells. Based on this conclusion, we identified in a two-hybrid screen (Fields and Song, 1989) a yeast gene whose product interacts with Rev, Rex, and the effector domain of Rev alone. This gene, *RIP1*, is inessential and encodes a novel small FG repeat-containing protein. Its closest known yeast relative is nucleoporin Rat7p/Nup159p, implicated in the export of polymerase II transcripts. Analysis of strains that overexpress Rip1p or that are deleted for the *RIP1* gene indicates that the physical interaction is of significance to Rev function in vivo. The experiments identify a cellular protein that interacts with the Rev effector domain and suggest that this domain interacts directly with selected nucleoporins to promote RNA export.

Results

Rev Functions in Yeast as in Mammalian Systems

To confirm and extend the similarity between Rev function in yeast and mammalian cells, we carried out a number of additional experiments in yeast. First, we assayed the biological activity of the HTLV-I Rex protein. Rex also contains an RBD/oligomerization domain and a leucine-rich effector domain (Hanly et al., 1989). Moreover, these two functional domains are interchangeable with those from Rev (Rimsky et al., 1988, 1989; Hope et al., 1991; Weichselbraun et al., 1992), indicating that these two viral proteins promote pre-mRNA export through similar mechanisms. Rev–Rex chimeric constructs were used to test whether the effector domain of Rex is functional in yeast (Figure 2A). A sequence encoding the wild-type Rex ef-

fector domain (amino acids 75–96) or a mutant Rex (L90K) effector domain was fused to the 3′ end of a mutant Rev sequence encoding a nonfunctional effector domain. The effect of these fusion constructs on pre-mRNA or mRNA expression was assayed in yeast strains containing the PC–CUP–RRE or the MC–CUP–RRE reporter construct, respectively (Figures 1 and 2A). Copper resistance of the PC–CUP–RRE strain was increased in the presence of wild-type Rev, but not mutant Rev. Addition of the wild-type Rex effector domain rescued the mutant Rev phenotype and allowed the yeast reporter strain to grow as in the presence of wild-type Rev. This positive effect on growth was eliminated by the Rex effector domain mutation L90K, which abolishes the activity of Rex in mammalian cells (T. Hope, personal communication). As predicted (Stutz and Rosbash, 1994), the opposite result was obtained in the MC–CUP–RRE reporter strain. Rev reduced the copper resistance of this strain, owing to the negative effect of Rev on mRNA expression; this effect was eliminated by mutant Rev and restored by fusion of the wild-type Rex effector domain, but it was not restored by fusion of the mutant Rex effector domain (Figure 2A). The results indicate that Rev and Rex have similar modes of action in yeast as in metazoan systems.

We then verified that the classic M10 effector domain mutant, but not the M4 RBD/oligomerization domain mutant, has a dominant negative effect in yeast (Malim et al., 1989a). In mammalian cells, M10 Rev inhibits wild-type Rev function by competing for RRE-containing target transcripts; M4 Rev is ineffective because it is an oligomerization mutant that competes poorly for stable RNA binding. Wild-type or mutant Rev proteins were expressed from a high copy number plasmid in a strain containing the PC–CUP–RRE reporter and wild-type Rev expressed from a low copy number plasmid. This strain grows up to 1.1–1.2 mM copper, and the addition of a high copy number plasmid expressing wild-type Rev had no further positive effect on PC–CUP–RRE expression. However, as in mammalian systems, addition of a high copy number plasmid expressing M10 Rev had a dominant negative effect and interfered with the positive effect of wild-type Rev on pre-mRNA export. This resulted in a decreased copper resistance of the reporter strain, which stopped growing at 1 mM copper; overexpression of M4 Rev was without effect, and the strain grew up to 1.1–1.2 mM copper, as in the presence of overexpressed wild-type Rev or vector (Figure 2B). The overexpressed proteins accumulated to similar levels as examined by Western blot analysis (data not shown).

As a third indication that the mode of action of Rev in yeast is similar to that in mammalian systems, the effects of 14 mutants that span the entire effector domain were examined (Figure 3). The choice of these Rev mutants was dictated by the fact that they had already been examined in COS cells (Malim et al., 1991). The 14 yeast expression plasmids were introduced into the PC–CUP–RRE-containing reporter strain, and the results were compared with the previously described negative and positive controls. In the absence of Rev or in the presence of the M10 or M4 mutant Rev protein, copper resistance is at 0.8 mM; in the presence of wild-type Rev, copper resistance is increased to 1.1–1.2 mM (see Figure 1; Stutz and Rosbash, 1994).

Most of the mutants had the same effects in yeast as in COS cells (Figure 3). Mutants M16, M20, M23, and M24 were indistinguishable from wild-type Rev. Mutants M10, M21, M22, and M27–M29 had strong negative effects on Rev function in yeast, as they lowered copper resistance to 0.8 mM, indistinguishable from the negative control. The M18 mutant, which has an intermediate phenotype in COS cells, also had an intermediate copper resistance (1 mM) in yeast. Only three mutants (M17, M19, and M25, indicated by an asterisk in Figure 3) more severely affected the function of Rev in yeast than in mammalian cells, and no mutant had a stronger effect in COS cells than in yeast. The more severe effect in yeast of the three Rev mutations might be related to the relatively weak effect of wild-type Rev in this organism. In the main, the analysis of these 14 Rev mutants suggests that the effector domain of Rev

	S (67)	A	E	P	V	P	L	Q	L	P	P	(L)	E	R	(L)	T	(L)	D	C	N	E	D	C	G	T	S (92)	COS CELLS ACTIVITY gTAT	YEAST PC-CUP-RRE Copper resistance [mM]
WT Rev																											+ +	1.1
M10												D	L														-	0.8
M16					D	L																					+ +	1.1
M17							D	L																			+ +	0.8 *
M18									D	L																	+	1.0
M19											D																+ +	0.8 *
M20													D	L													+ +	1.1
M21															D	L											-	0.8
M22																	D	L									-	0.8
M23																		E	D	L							+ +	1.1
M24																				K	D	L					+ +	1.1
M25																						E	D	L			+ +	0.8 *
M27												A															-	0.8
M28															A												-	0.8
M29																	A										-	0.8

Figure 3. Rev Effector Domain Mutations and Their Effects in COS Cells and Yeast

The indicated Rev effector domain mutations and their effects on the activity of Rev in COS cells have been published previously (Malim et al., 1991). The three critical leucines are circled. gTAT activity scores the ability of various Rev mutants to induce expression of the 72 amino acid form of TAT. The effects of the same mutations on the Rev response in yeast were analyzed by transforming the various Rev-expressing plasmids (*TRP1/ARS1–CEN4*) into a yeast strain containing the PC–CUP–RRE reporter construct (*LEU2*/2μm) (see Figure 1). The copper resistance of the double transformants was determined by growth on selective plates containing increasing copper concentrations. See text for an explanation of the asterisk. WT, wild type.

undergoes similar interactions in yeast and in mammalian systems.

Identification of a Yeast Protein That Interacts with Rev in the Two-Hybrid System

To find the yeast targets of the effector domain of Rev, we screened for Rev-interacting proteins (RIPs) with the yeast two-hybrid system and a yeast genomic library. In an initial screen with wild-type Rev, we identified a large number of proteins that apparently interacted with the arginine-rich RBD of Rev. To eliminate this source of background, we used the Rev mutant MB3 as the bait in the two-hybrid screen. In the MB3 mutant, Arg-41 to Arg-43 are replaced by three glycines; this mutation has no effect on the effector domain activity of Rev (Hope et al., 1990; McDonald et al., 1992).

A number of strong MB3 RIPs were identified and tested for an interaction with M10 Rev. Only one (Rip1p1) failed to show a detectable interaction with M10 Rev. The interaction of the Rip1p1 fusion (encoding amino acids 148–275 of Rip1p; see below) with additional bait constructs was then tested (Figure 4).

Interestingly, Rip1p1 also interacted well with a 30 amino acid segment of Rev spanning just the effector domain (Figure 4). This interaction was sensitive to mutations M21 and M22, which replace critical effector domain leucines with aspartic acids and which abolish Rev function in vivo (see Figure 3). Importantly, Rip1p1 also interacted with Rex (Figure 4). The Rip1p1 and Rip1p fusions (encoding the complete Rip1p, amino acids 1–430; see below) showed similar series of interactions with the panel of bait fusions. However, the interaction strengths appear lower with the total protein. This is likely due to a lower steady-state level of the complete Rip1p fusion compared with Rip1p1, as assayed by Western blotting (data not shown).

The Rev wild-type "bait"/Rev wild-type "prey" combination examines Rev dimerization (Figure 4). This homotypic interaction requires an intact RBD/oligomerization domain but is insensitive to the M10 effector domain mutation (Zapp et al., 1991). The strong interaction between Rev M10 and Rev wild type serves as a positive indicator of Rev M10 bait expression, consistent with Western blot data (not shown). The data indicate that Rip1p1 and Rip1p interact with the effector domain of Rev.

Sequence Analysis of the *RIP1* Gene

The Rip1p1 two-hybrid clone contained a 375 bp EcoRI insert, which was sequenced and also used as a probe to clone the complete *RIP1* gene from a yeast genomic library. The open reading frame is uninterrupted and encodes a 430 amino acid protein with three features characteristic of yeast nucleoporin proteins (Dingwall, 1993; Rout and Wente, 1994; Figure 5). First, there is a domain containing 27 striking XXFG tetrapeptide repeats. Second, there is a high frequency of serines, threonines, and asparagines in the small spacer regions between the repeats. Third, the repeat domain is lacking acidic residues. The Rip1p1 polypeptide contains eight XXFG repeats expressed in-frame with the acidic domain present on the

A

BAIT \ PREY	RIP1p1 (aa 148-275)	RIP1 (aa1-430)	Rev wt
Rev wt	++++	+++	++++
Rev M10	-	-	++++
Rev MB3	+++	++	-
Effector wt	++++	+++	-
Effector M21	-	-	-
Effector M22	-	-	-
Rex wt	++++	+++	-

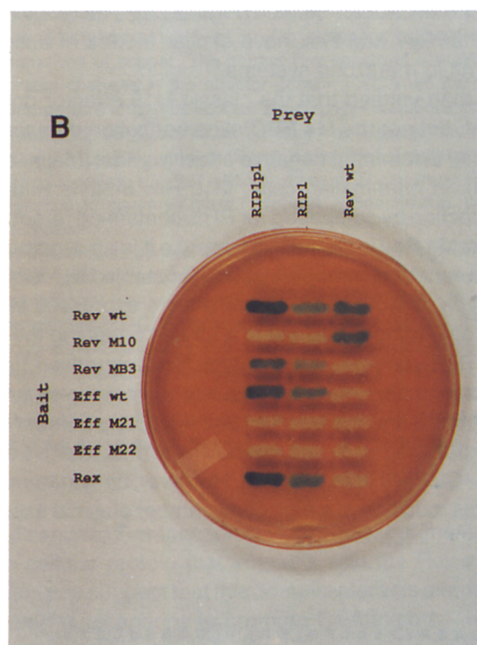


Figure 4. Rev–Rip1p Two-Hybrid Interactions

(A) Summary table of two-hybrid interactions between Rev or Rex bait constructs and Rip1p or Rev prey constructs. The bait constructs express LexA fused to Rev wild type (wt) (amino acids 1–116), Rev M10 (amino acids 1–116, LE78,79DL), Rev MB3 (amino acids 1–116, R41–43G), Rev effector wild type (amino acids 65–95), Rev effector M21 (amino acids 65–95, LT81,82DL), Rev effector M22 (amino acids 65–95, LD83,84DL), and Rex wild type (amino acids 1–189). Each bait construct was tested for interaction with three prey plasmids: *RIP1p1* (the clone isolated in the screen and encoding amino acids 148–275 of Rip1p); *RIP1* (encoding total Rip1p), and Rev wild type. The different bait/prey interactions were analyzed in diploids using a mating assay (see Experimental Procedures). The number of pluses corresponds to blue color intensity on an X-Gal indicator plate as a result of *lacZ* gene activation (shown in [B]).

(B) Analysis of the diploid strains described above on Ura$^-$ His$^-$ Trp$^-$ plus galactose and sucrose plus X-Gal indicator plates.

1		M	
2	SA**FG**	NPFTSGAKPNLSNTSGINPFTNNAASTNNMGG	
38	SA**FG**	R	
43	PS**FG**	TANTMTGGTTT	
58	SA**FG**	M	
63	PQ**FG**	TNTGNTGNTSI	
78	SA**FG**	NTSNAAKP	
90	SA**FG**	A	
95	PA**FG**	SSAPINVNPPSTT	
112	SA**FG**	A	
117	PS**FG**	S	
122	TG**FG**	AMAATS	
132	NP**FG**	KSPGSMG	
143	SA**FG**	Q	
148	PA**FG**	ANKTAIPSSSVSNSNN	
168	SA**FG**	AASNTPLTTT	
182	SP**FG**	SLQQNASQNASSTS	
200	SA**FG**	K	RIP1p1
205	PT**FG**	AATNTQ	
215	SP**FG**	TIQNTSTSSGTGV	
232	SP**FG**	T**FG**TNSNNKSPFSNLQSGAGAGS	
259	SP**FG**	TTTSKANNNNNVGS	
277	SA**FG**	TTNNQSPFSGGSG	
294	GT**FG**	SASNLNKNTNGNFQ	
312	SS**FG**	NKG	
319	FS**FG**	ITPQNDANQVSQSN	
337	PS**FG**	QTMPNTDPNISLKSNGNA	
359	TS**FG**	**FG**	
365		QQQMNATNVNANTATGKIRFVQGLSSEK	
393		DGILELADLAEETLKIFRANKFELGLVPD	
422		IPPPPALVA	

Figure 5. Amino Acid Sequence of Rip1p

The higly conserved FG residues of the degenerate XXFG tetrapeptide motif are shown in bold. The frequent serine, threonine, and asparagine residues are underlined. The boxed region (Rip1p1) corresponds to the portion of the protein encoded by the clone isolated in the two-hybrid screen.

prey expression vector (Figure 5). This suggests that the repeat domain is important for the interaction of Rip1p with Rev.

Rip1p Localizes to Nuclear Pores

To determine Rip1p localization, we raised an anti-Rip1p rabbit polyclonal antibody, which recognizes Rip1p on Western blots of yeast extracts (data not shown). In a wild-type strain, we observed punctate perinuclear staining typical of nuclear pores as well as some nucleoplasmic staining (Figure 6A, left); in the *RIP1* knockout (ΔRIP1) strain (see below), only the diffuse nucleoplasmic staining remained (Figure 6B, left). The results indicate that some nucleoplasmic staining is due to cross-reacting nuclear antigens. As a control for the integrity of the nuclear pores in the ΔRIP1 strain, the protein was stained with the monoclonal antibody (MAb 414). This antibody recognizes several yeast nuclear pore proteins (Aris and Blobel, 1989) and gives indistinguishable punctate perinuclear staining in both the wild-type and ΔRIP1 strains (Figures 6A and 6B, right). We also examined the nup133⁻ strain (Figure 6C, left). Deletion of the *NUP133* gene does not affect growth at 25°C, but nup133⁻ cells display clustering of nuclear pore complexes at one or a few sites on the nuclear envelope (Doye et al., 1994). When stained with MAb 414, the nup133⁻ cells show a crescent-shaped signal or a small number of intense spots at the nuclear periphery (Figure 6C, right). With the anti-Rip1p antibody, a qualitatively similar signal was observed (Figure 6C, left), but it was weaker and somewhat obscured by nucleoplasmic staining. We conclude that Rip1p is indeed a nuclear pore–associated protein, but we cannot exclude the possibility that some Rip1 protein is also nucleoplasmic.

Rip1p Overexpression and *RIP1* Gene Disruption Reduce the Rev Response

If Rip1p is important to Rev function in vivo, overexpression of Rip1p might influence the effects of Rev on gene expression. To test this prediction, we transformed yeast strains expressing wild-type Rev and the PC–CUP–RRE (or MC–CUP–RRE) reporter construct with a high copy number plasmid expressing total Rip1p or the fragment Rip1p2 (amino acids 120–229) from the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. The strains were also transformed with a high copy number plasmid expressing Rip1p under the control of its own promoter, and growth of these triple transformants was compared at increasing copper concentrations (Figure 7A). Overexpression of Rip1p2 or Rip1p decreased the copper resistance of the PC–CUP–RRE reporter strain, indicating that excess Rip1p2 or Rip1p reduced the positive effect of Rev on pre-mRNA export. Similarly, overexpression of Rip1p2 or Rip1p reduced the negative effect of Rev on mRNA expression, as it increased the copper resistance of the MC–CUP–RRE/Rev-containing strain. Significant overexpression was apparently required for the effects, as expression of Rip1p from its own promoter had no effect and was comparable to addition of the vector control plasmid in both reporter systems (Figure 7A). The observed changes in copper resistance were also strictly dependent on the presence of Rev, since expression of the same constructs in a strain containing a control vector instead of the Rev-expressing plasmid had no effect on the copper growth phenotypes (data not shown). There were no growth effects of these plasmids, either alone or in combination, in the absence of copper. The results indicate that an interaction between Rev and Rip1p can interfere with Rev function (see Discussion).

The *RIP1* gene was deleted by replacing the entire coding region with the *URA3* gene, which was subsequently eliminated by growth on 5-fluoroorotic acid (see Experimental Procedures; Alani et al., 1987). The ΔRIP1 strain grew indistinguishably from the parent strain at three different temperatures, indicating that this gene is inessential and that its absence is without effect under these standard growth conditions. To assay the activity of Rev in the absence of *RIP1*, we transformed the ΔRIP1 strain with yeast expression plasmids producing either wild-type Rev, M10 mutant Rev, or no protein at all (vector control), as well as with the PC–CUP–RRE or the MC–CUP–RRE reporter constructs. Growth of the double transformants was examined at increasing copper concentrations and compared with growth of the isogenic control strain (Figure 7B). The absence of the *RIP1* gene reduced the copper resistance of the PC–CUP–RRE/Rev strain (best seen at 1.1 mM

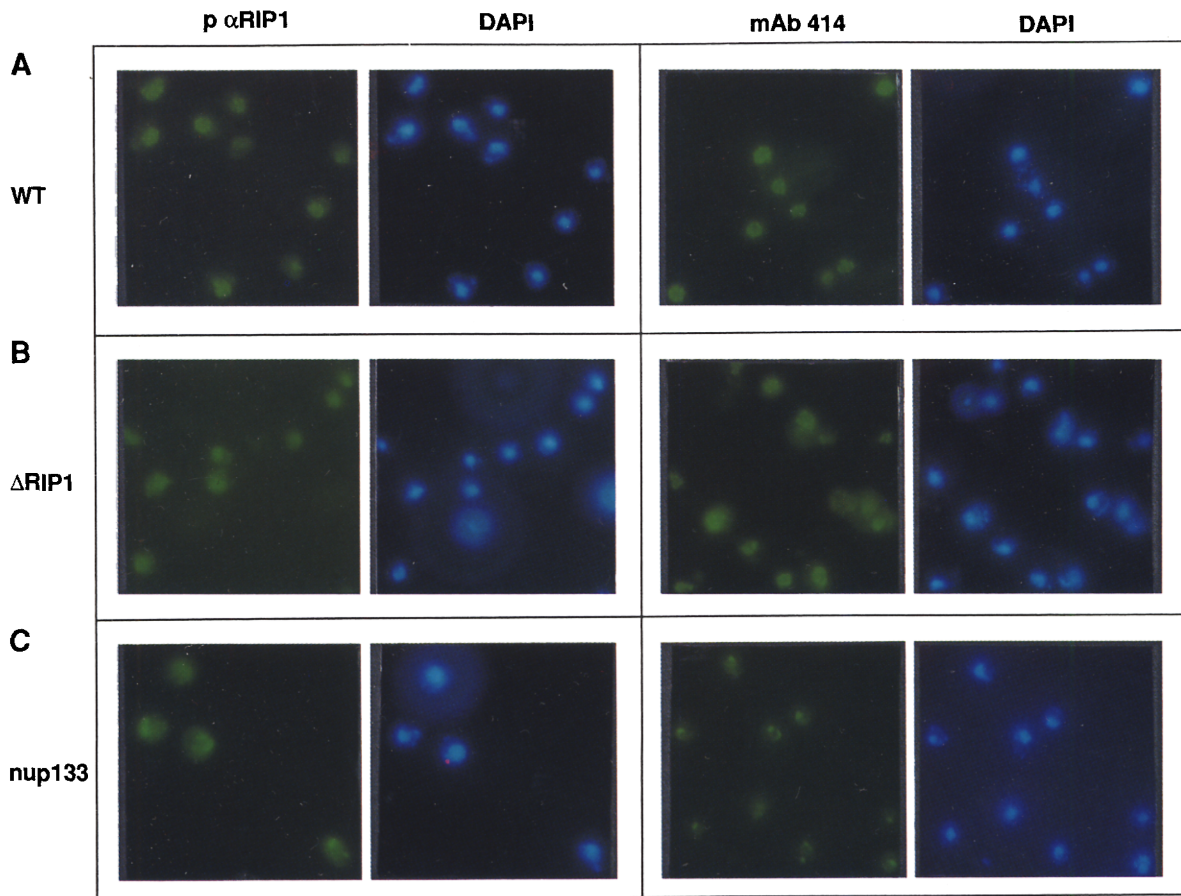


Figure 6. Punctate Nuclear Rim Staining of Yeast Cells by a Polyclonal Anti-Rip1p Antibody as Detected by Indirect Immunofluorescence Microscopy
Wild-type (WT) (A), ΔRIP1 (B), and nup133⁻ (C) (Doye et al., 1994) yeast cells were stained with a rabbit polyclonal anti-Rip1p antibody (p αRip1) (left) or with MAb 414 (right). To identify the position of the nucleus, the same cells stained with DAPI are shown next to each picture. We were not able to generate a *nup133⁻*/ΔRIP1 double mutant strain, probably because these mutants are synthetic lethals.

copper), indicating a decrease in the positive effect of Rev on pre-mRNA expression. Similarly, the absence of the *RIP1* gene increased the copper resistance of the MC–CUP–RRE/Rev strain (best seen at 0.4 mM copper), indicating a decrease in the negative effect of Rev on mRNA expression. These changes in copper resistance were directly related to Rev function, since the copper growth phenotypes of the reporter strains expressing M10 Rev or no Rev (vector control) were unaffected by the absence of *RIP1* (Figure 7B). The data show that the *RIP1* gene contributes to Rev function.

Rev Interacts with Several Yeast Nucleoporins in the Two-Hybrid System

Although the ΔRIP1 strain manifested substantially less Rev activity than the control wild-type strain, Rev still increased pre-mRNA expression and decreased mRNA expression in this strain. We speculated that the residual activity of Rev might be due to interactions with other FG repeat–containing proteins. This notion is also based on experiments demonstrating redundant functions shared by other yeast nucleoporin genes (see Discussion). Because a number of these genes are essential, it is difficult to assay directly their effects on Rev activity. Therefore, the repeat regions of five yeast nucleoporins, Nup159p (Gorsch et al., 1995), Nsp1p (Nehrbass et al., 1990), Nup1p (Davis and Fink, 1990; Bogerd et al., 1994), Nup100p (Wente et al., 1992), and Nup116p (Wimmer et al., 1992; Wente and Blobel, 1993), were tested for Rev interaction in the yeast two-hybrid assay. Based on the consensus sequence of their repeats, Nsp1p and Nup1p are two members of the XFXFG nucleoporin family, whereas Nup100p and Nup116p are two members of the GLFG family. The consensus XXFG present in the recently identified Nup159p (Gorsch et al., 1995) most closely resembles the Rip1p repeat. A portion encoding between 200 and 300 amino acids of the repeat region (comparable to the Rip1p1 fragment) from each of these five nucleoporins was cloned into the two-hybrid prey vector, transformed into yeast, and mated with strains of opposite mating type expressing the wild-type Rev bait or six other bait constructs (Figure 8; see also Figure 4).

The data show that Rev interacts with the repeat regions from at least two other nucleoporins, Nup159p and Nup100p (Figure 8). Although these interactions are weaker than those between Rev and Rip1p1, the Nup159p

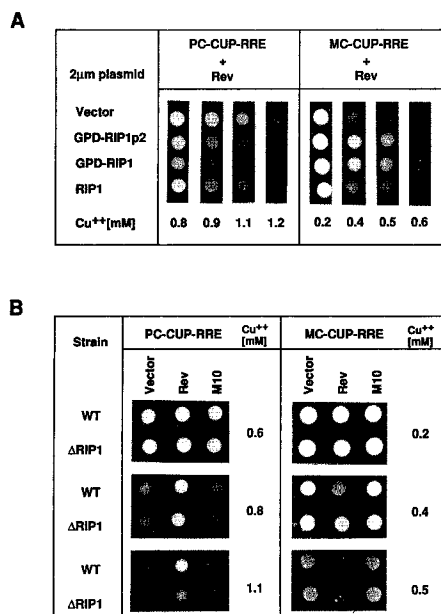


Figure 7. Rip1p Overexpression and *RIP1* Knockout

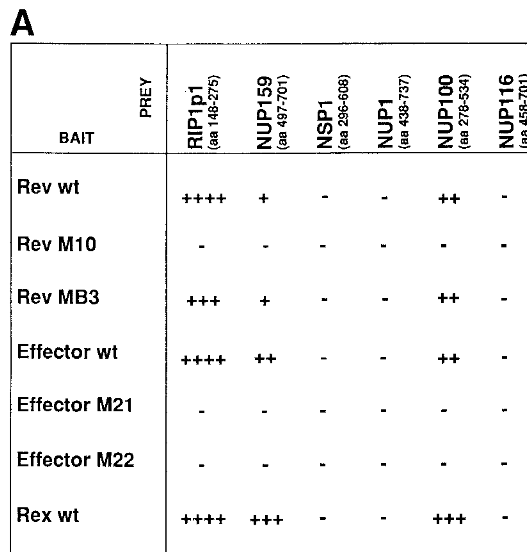
(A) Rip1p overexpression interferes with the activity of Rev. High copy number plasmids (*LEU2*/2µm) expressing Rip1p sequences were introduced into yeast strains expressing wild-type Rev (*TRP1/ARS1–CEN4*) and either the PC–CUP–RRE or the MC–CUP–RRE reporter construct (*URA3*/2µm). The overexpressing plasmids contained a portion of *RIP1* encoding amino acids 120–229 (GPD–Rip1p2) or total *RIP1* encoding amino acids 1–430 (GPD–Rip1p) under the control of the strong GPD promoter (Guthrie and Fink, 1991) or total Rip1p driven by its own promoter (*RIP1*); a plasmid without insert was used as control (vector). The growth of the triple transformants was examined on plates containing increasing copper concentrations.

(B) *RIP1* knockout reduces the Rev response. The wild-type (WT) and the *RIP1* knockout (ΔRIP1) strains were transformed with the PC–CUP–RRE or the MC–CUP–RRE reporter constructs (*LEU2*/2µm) as well as with the wild-type Rev– or the M10 Rev–expressing plasmid or vector alone (*TRP1/ARS1–CEN4*). The growth of each double transformant was examined on selective plates containing increasing copper concentrations.

and Nup100p repeat regions interact with each bait in a way qualitatively comparable to Rip1p1; they also show specific interactions with the effector domains of Rev and Rex. This is consistent with the notion that Rev might undergo functionally significant interactions with a number of repeat-containing nucleoporins, thereby explaining the residual Rev activity in the ΔRIP1 strain.

Discussion

Consistent with recent experiments indicating that Rev has a direct effect on transport (Fischer et al., 1994), we report here a functionally relevant interaction between the effector domain of Rev and Rip1p, a novel candidate nucleoporin. The *RIP1* gene was identified in a two-hybrid screen for yeast proteins that interact appropriately with the effector domain of Rev. The search was predicated on the conclusion that the general mode of action of Rev and the specific role of its effector domain are similar in yeast and in mammalian systems (Figures 2 and 3; Malim et al., 1989a; Stutz and Rosbash, 1994). As previously discussed (Stutz and Rosbash, 1994), the effect of Rev in yeast is modest as compared with mammalian systems. This might reflect the relatively high background of the yeast assay system (substantial cytoplasmic transport of pre-mRNA even in the absence of Rev) or weak conserva-



A

BAIT \ PREY	RIP1p1 (aa 148-275)	NUP159 (aa 497-701)	NSP1 (aa 296-608)	NUP1 (aa 438-737)	NUP100 (aa 278-534)	NUP116 (aa 458-701)
Rev wt	++++	+	-	-	++	-
Rev M10	-	-	-	-	-	-
Rev MB3	+++	+	-	-	++	-
Effector wt	++++	++	-	-	++	-
Effector M21	-	-	-	-	-	-
Effector M22	-	-	-	-	-	-
Rex wt	++++	+++	-	-	+++	-

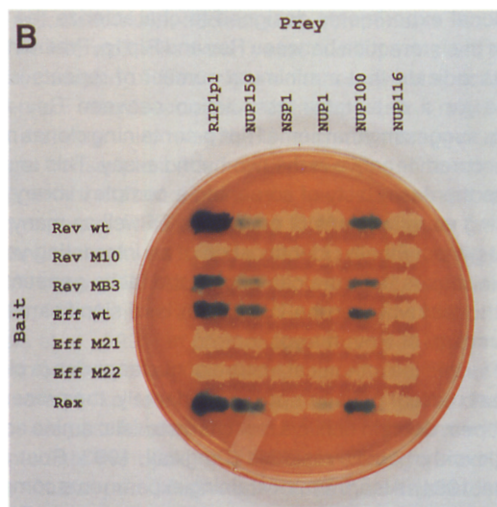


Figure 8. Rev–Nucleoporin Two-Hybrid Interactions

(A) Rev interacts with nucleoporin repeat domains. Summary table of two-hybrid interactions between Rev or Rex and nucleoporin repeat domains. The interactions were examined in diploid strains using a mating assay as already described (see Figure 4; see Experimental Procedures). The bait constructs are the same as those described in Figure 4A. The portion of the nucleoporin encoded by each prey construct is indicated at the top. The number of XXFG repeats encoded by the Rip1p1 and Nup159p fusions is 8 and 17, respectively. The number of XFXFG repeats encoded by the Nsp1p and Nup1p fusions is 14 and 11, respectively. The number of GLFG repeats encoded by the Nup100p and Nup116p fusions is 15 and 10, respectively. wt, wild type.

(B) Analysis of the diploid strains described above on Ura^- His^- Trp^- plus galactose and sucrose plus X-Gal indicator plates.

tion of the relevant components between yeast and mammals. Nonetheless, the effects of Rip1p overexpression or of the *RIP1* deletion on Rev function are substantial (Figure 7). Based on comparisons of copper resistance and *CUP1* mRNA (or pre-mRNA) levels (Stutz and Rosbash, 1994), we estimate that ~50% of the activity of Rev has been eliminated by Rip1p overexpression or by the *RIP1* gene deletion. This implies that Rip1p is a major component of the cellular machinery with which Rev interacts to promote RNA export.

Searching for RIPs with a yeast genomic library has a number of advantages over using a human cDNA library. The size of the yeast genome means that a relatively small number of transformants is sufficient to screen the entire protein coding capacity of the organism. Moreover, every coding region is present at the same frequency, independent of its abundance in the mRNA population; in contrast, mammalian cDNA libraries require huge numbers of clones to include rare cDNAs with a reasonable probability. The issue of clone frequency is heightened by some additional experiments designed to characterize the nature of the interaction between Rev and Rip1p. Preliminary results indicate that a minimum number of repeats is required for a detectable association between Rev and Rip1p, suggesting that some Rip1p-containing clones may have scored negative in the two-hybrid assay. This underscores the desirability of screening a complex library expressing many versions of each gene as well as many different genes. Finally, identification of an interacting yeast protein means that one can exploit the facile genetics of yeast to test whether the interaction is of significance to the function of Rev in vivo.

Rip1p has the canonical feature of nucleoporins (a class of yeast nuclear pore components), namely, the presence of a repeat domain rich in a few characteristic amino acids and devoid of acidic residues (Dingwall, 1993; Rout and Wente, 1994). Also, immunostaining experiments comparing wild-type and ΔRIP1 strains indicate that some Rip1p localizes to the nuclear pores (Figure 6). Based on the short repeated sequences, these nucleoporins have been subdivided into two families. Nup1p (Bogerd et al., 1994), Nup2p (Loeb et al., 1993), and Nsp1p (Nehrbass et al., 1990) belong to the XFXFG family, whereas Nup49p (Wimmer et al., 1992), Nup100p (Wente et al., 1992), Nup145p (Fabre et al., 1994; Wente and Blobel, 1994), and Nup116p (Wimmer et al., 1992; Wente and Blobel, 1993) form the GLFG family. Rip1p appears to fall into a third category, since most of its repeats are either SAFG, SPFG, or PSFG, with a consensus repeat of XXFG. The closest mammalian relative of Rip1p identified in the database is the mammalian nucleoporin CAN1 (von Lindern et al., 1992; Kraemer et al., 1994); the CAN1 carboxy-terminal repeat region is 30% identical to the Rip1p repeat region. In the same search, the closest yeast relative corresponded to Rat7p/Nup159p, which was identified in a screen for mutants impaired in RNA export and which localizes to the nuclear pore complex (Gorsch et al., 1995). Rat7p/Nup159p also contains repeats of the XXFG type in its central region, which shows 30% identity to the Rip1p repeat region. Noteworthy is the fact that Rip1p is relatively small compared with most known nucleoporins; in addition to its amino-terminal repeat domain, Rip1p only contains a short (66 amino acids) unique carboxy-terminal sequence.

An association with the nuclear pore complex would explain the Rip1p overexpression results (Figure 7A). Although expression of the Rip1p1 fragment presumably interferes with endogenous Rip1p function and thereby inhibits Rev activity, one might have anticipated that overexpression of the complete protein would enhance Rev function (or perhaps have no effect). The observed inhibitory effect is explained by proposing that some stoichiometric quantity of Rip1p is normally assembled into the pore complex. The overexpressed protein would therefore be mislocalized and would compete with endogenous nuclear pore–limited Rip1p for binding to the effector domain of Rev.

It is interesting and perhaps significant that a temperature-sensitive mutant in Rat7p/Nup159p has an even more rapid and selective effect on nuclear export of poly(A) RNA than conditional mutants of other nucleoporins that have also been proposed to play a role in RNA export (Fabre et al., 1994). As previously discussed (Elliott et al., 1994; Izaurralde and Mattaj, 1995; Gorsch et al., 1995), these observations make Rat7p/Nup159p the best candidate nucleoporin for having a specific role in promoting RNA export. Taken together with the effects of Rip1p on Rev activity, these observations suggest that these two proteins may be members of a third family of nucleoporins specialized for the transport of RNA from the nucleus to the cytoplasm. Whether these yeast proteins function to transport selective RNA substrates remains to be determined.

Rev also interacts with the repeat regions of Rat7p/Nup159p and Nup100p. These interactions raise the general issues of how to interpret differences in the interaction of Rev with different nucleoporins in the two-hybrid assay, namely, the quantitative differences among the three positive proteins and, most importantly, the qualitative differences between these three proteins and the negative nucleoporins (Figure 8). We have not employed different two-hybrid systems, and it is possible that the negative proteins will be positive with a more sensitive assay. In addition, differences between different proteins (e.g., the difference between the positive Rip1p1 and the negative Nup116p) may not reflect comparable differences in interaction strength, as low protein levels were observed by Western blotting in several cases (data not shown). Finally, we have some indication that the number of nucleoporin repeats present in the expressed fusion protein is important for the interaction with the effector domain of Rev (data not shown). As a smaller number of repeats was present in the negative Nup116p, Nsp1p, and Nup1p fusion proteins than in the positive Nup159p and Nup100p proteins (Figure 8), an interaction with the negative proteins might have been detected had we assayed a larger number of nucleoporin repeats. These considerations not-

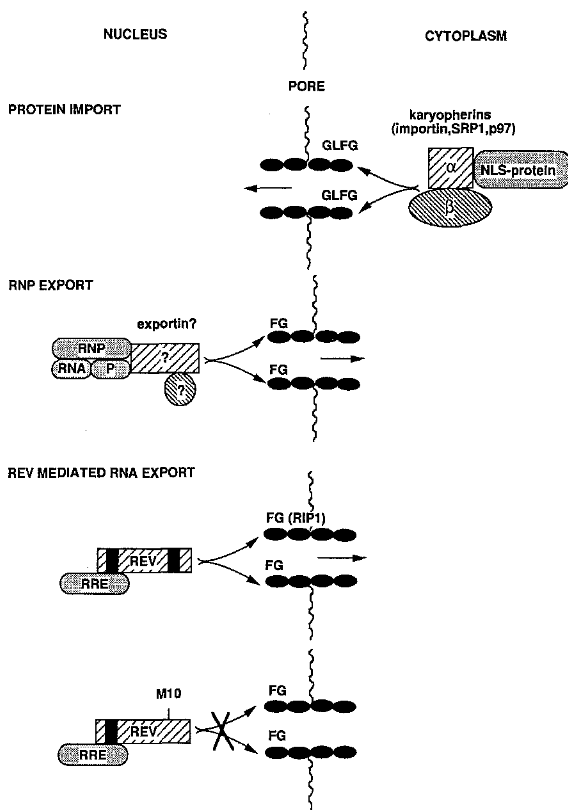


Figure 9. Model for Mode of Action of Rev

Protein import is currently modeled as occuring in two steps: docking of the nuclear localization signal (NLS)-containing substrate to the nuclear membrane followed by translocation of the substrate through the nuclear pore (Radu et al., 1995b). A cytoplasmic complex made of karyopherins α and β (importin, *SRP1*, and p97; Gorlich et al., 1994; Radu et al., 1995b; Weis et al., 1995) mediates the docking of nuclear localization signal-containing protein substrates to the nuclear pore by interacting with nucleoporin repeat motifs present on the cytoplasmic side of the nuclear membrane. We speculate that nuclear export of RNPs, RNA, or protein functions similarly with a comparable nuclear activity (exportin) mediating the docking of substrates to the nuclear pore through an interaction with FG repeats present on the nucleoplasmic side; the role of Rev might be to mimic such an activity by mediating the docking of RRE-containing transcripts to the pore by interaction with FG-containing nucleoporins, thereby initiating the translocation process.

withstanding, Rip1p is the strongest RIP we have detected, and the genetic data indicate that the interaction is biologically significant.

Whether additional nucleoporins like Nup159p and Nup100p also contribute to the biological activity of Rev in yeast remains to be determined. However, a functional interaction between Rev and these other proteins is reasonable, if not expected. There are genetic experiments indicating that nuclear pore components are functionally redundant (Fabre et al., 1994; Wente and Blobel, 1994). The *RIP1* gene is inessential and therefore probably contributes to nuclear pore function in a manner that overlaps that of other proteins. Most importantly, Rev still showed substantial biological activity in the ΔRIP1 strain (Figure 7B), indicating that the effector domain interacts with other cellular components. We therefore expect that more than one FG–containing protein will be relevant to the biological activity of Rev.

Although there are now strong indications that the primary function of Rev is to promote nuclear transport (Malim and Cullen, 1993; Fischer et al., 1994; Brighty and Rosenberg, 1994; Nasioulas et al., 1994), an independent or related role in splicing inhibition is not excluded (Lu et al., 1990; Chang and Sharp, 1989; Kjems et al., 1991b; Luo et al., 1994). In the yeast system, there are two splicing mutants that are epistatic to the effect of Rev on RNA transport (Stutz and Rosbash, 1994). This suggests that transport across the nuclear membrane cannot override certain associations between pre-mRNA and splicing components, and this view is consistent with the fact that the natural targets of Rev are inefficient splicing substrates. An association between Rev and pore components suggests that spliceosome disassembly might be accelerated at the nuclear pore. This would provide Rev with a role in splicing inhibition, but only indirectly through its association with nuclear pore components.

The interaction between yeast nucleoporins and the effector domain of Rev might enhance some specific biochemical step associated with transport, or it might more generally serve to raise the local concentration or deliver target transcripts to the nuclear envelope. The latter view suggests that Rev might play a nuclear role in promoting the export of RRE-containing RNAs similar to the cytoplasmic role played by the α and β karyopherins (importin, *SRP1*, and p97; Moore and Blobel, 1992; Gorlich et al., 1994; Moroianu et al., 1995; Radu et al., 1995a, 1995b; Weis et al., 1995) in promoting the nuclear import of nuclear localization signal–containing proteins (Figure 9). As we cannot exclude the fact that some Rip1p is nucleoplasmic, it is possible that Rip1p functions in concert with Rev to mediate the docking of RRE-containing transcripts to the nuclear pore. Further progress in understanding the mode of action of Rev should continue to illuminate the rather poorly understood process of RNA transport.

Experimental Procedures

Plasmid Constructions

The PC–CUP–RRE (*LEU2*/2μm) and MC–CUP–RRE (*LEU2*/2μm) reporter constructs have been described previously (Stutz and Rosbash, 1994). *URA3*/2μm versions of the same reporter constructs were used in the Rip1p overexpression experiment.

The Rev-expressing plasmids were obtained by PCR amplification of wild-type or mutant Rev sequences (Malim et al., 1989a) with two oligo primers containing BclI sites. The PCR fragments were cloned into the BamHI site behind the GPD promoter of the yeast *TRP1*/2μm expression vector pG1 (Guthrie and Fink, 1991) or a centromeric version thereof, as described earlier (Stutz and Rosbash, 1994), to create pG1Rev and pG1Rev/ARS1–CEN4, respectively. The Rev–Rex chimeric constructs were a gift from T. Hope and were subcloned into the BamHI site of pG1TRP1/ARS1–CEN4 after PCR amplification with primers introducing BclI sites.

The wild-type Rev construct (*URA3/ARS1–CEN4*) used in the dominant negative experiment was obtained by transferring the wild-type Rev expression cassette from pG1Rev into YCP50; the wild-type Rev cassette was purified from pG1Rev by digestion with XbaI and HindIII and cloned into the HindIII site of YCP50 using HindIII linkers.

To overexpress Rip1p from the strong constitutive GPD promoter,

a 330 bp fragment encoding amino acids 121–229 (Rip1p2) or a 1290 bp fragment encoding the whole 430 amino acid protein (Rip1p) were PCR amplified. The 5′ and 3′ primers contained a BamHI and a XhoI site, respectively, and the PCR products were cloned into the expression vector *FS3* (*LEU2*/2μm) cut with BamHI and SalI. *FS3* was obtained by transferring, from the pG1 vector (Guthrie and Fink, 1991), a HindIII–XbaI fragment containing the GPD promoter, the polycloning site, and the *PGK* terminator into the BamHI site of vector *JH21* (*LEU2*/2μm) by using BglII linkers. The *RIP1* genomic clone present on a *LEU2*/2μm plasmid was used to overexpress the whole protein from its own promoter.

Two-Hybrid Vectors and Strains

The following bait constructs were all made as LexA fusions into the EcoRI and SalI sites of pLexA (amino acids 1–202) plus PL (*HIS3*/2μm) (Ruden et al., 1991; Gyuris et al., 1993): Rev wild type (amino acids 1–116, total protein), Rev MB3 (amino acids 1–116, R41–43G), Rev M10 (amino acids 1–116, LE78,79DL), effector wild type (Rev amino acids 65–95), effector M21 (Rev amino acids 65–95, LT81, 82DL), effector M22 (Rev amino acids 65–95, LD83,84DL), and Rex wild type (amino acids 1–189, total protein). The prey constructs used for the analysis of specific interactions were made by PCR amplification from cloned or genomic DNA and EcoRI–XhoI insertion into pJG4-5 (*TRP1*/2μm) (Gyuris et al., 1993) and are as follows: Rip1p1 (amino acids 148–271), Rip1p (amino acids 1–430), Rev wild type (amino acids 1–116), Nup159p (amino acids 497–701), Nsp1p (amino acids 296–608), Nup1p (amino acids 438–737), Nup100p (amino acids 278–534), and Nup116p (amino acids 458–701).

The strain EGY48 (*MATa, trp1, ura3, LEU2::plexop6-LEU2*) contains both the integrated *LEU2* reporter LexA-op-LEU2 (Gyuris et al., 1993) and the *lacZ* reporter pSH18-34 on a *URA3*/2μm plasmid, a gift of R. Finley and R. Brent. RFY206 (*MATa, his3, leu2, ura3, trp1, lys2*), a gift from R. Finley, also contains the *lacZ* reporter pSH18-34.

Yeast Two-Hybrid Screen

The yeast two-hybrid system used in the screen was provided and used as described by Gyuris et al. (1993) with the following modifications. The prey, a yeast genomic library made by cloning AluI and HaeIII partially digested DNA into the EcoRI site downstream of the activation domain in pJG4-5, was transformed (Ito et al., 1983) into strain EGY48 containing the Rev MB3 bait construct. Transformants (4.6×10^4) were selected on Ura⁻ His⁻ Trp⁻ plus glucose (2%) medium. The colonies were scraped in TE buffer and resuspended in one pellet volume of 65% glycerol, 0.1 M $MgSO_4$, 25 mM Tris (pH 8.0) and stored in 200 μl aliquots at −80°C. The titer of the library was determined on Ura⁻ His⁻ Trp⁻ plus galactose (3%) and sucrose (1%) medium; following a 4 hr galactose induction in liquid, 5×10^7 colonies were grown on five standard 150 mm × 15 mm Ura⁻ His⁻ Trp⁻ Leu⁻ plus galactose (3%) and sucrose (1%) plates: ~1000 Leu⁺ colonies appeared; 162 of these colonies were tested, 156 of which were positive for galactose-dependent blue color (*lacZ* induction) on Ura⁻ His⁻ Trp⁻ plus galactose (3%) and sucrose (1%) plus X-Gal indicator plates. pJG4-5 plasmids were rescued and analyzed for insert size by both PCR (using primers designed on either side of the EcoRI cloning junction) and EcoRI restriction analysis. The candidate prey plasmids containing inserts were further characterized in a mating assay (described below).

Two-Hybrid Subscreen and Mating Assay

A mating assay was used as a secondary screen to eliminate candidates not specifically interacting with the effector domain of Rev. Prey candidates were transformed into EGY48, selected on Ura⁻ Trp⁻ medium, and mated to strain RFY206 containing the following panel of bait constructs described above (all were LexA fusions): Rev wild type, Rev M10, Rev MB3, effector wild type, effector M21, effector M22, Rex wild type, and LexA DNA-binding domain alone. Diploids were selected by growth on Ura⁻ His⁻ Trp⁻ medium and tested for galactose-dependent activation of the reporter genes. The criteria for a RIP (positive interaction with Rev wild type and effector wild type, while negative with Rev M10 or effector M21 or M22) were met by one candidate, Rip1p.

Genomic Clone and Sequencing Analysis

Two primers in the pJG4-5 vector near the 5′ and 3′ ends of the EcoRI insert were designed and used for the first round of sequencing. The complete *RIP1* gene was isolated from a YEp13 yeast genomic library (Lagosky et al., 1987) by colony hybridization, probed with the random-primed labeled *RIP1* EcoRI fragment purified from the two-hybrid clone (Maniatis et al., 1982). Automated sequencing of the genomic clone was facilitated by using primers designed internally to the known *RIP1* sequence. The program BLAST was used when comparing the DNA and predicted protein sequences to the GenBank and EMBL databases.

Yeast Strains and Copper Growth Assay

The copper growth assay and the wild-type copper-sensitive strain Y59ΔCUP1 (*MATa, leu2-112, ura3-52, trp-289, arg4, ade2, ΔCUP1*) have been described previously (Stutz and Rosbash, 1994).

The *RIP1* knockout strain (Y59ΔCUPΔRIP1) was generated from the haploid Y59ΔCUP1 strain. The complete *RIP1* coding region (from the AvaI site 20 nt upstream of the ATG to the StuI site 40 nt before the TAG stop codon) was deleted by homologous recombination using a construct that contained the HisG–*URA3*–HisG 3.8 kb fragment (Alani et al., 1987) flanked by 0.7 and 0.5 kb of *RIP1* 5′ and 3′ flanking sequences. The whole insert was released from pBluescript II KS by XhoI and NotI double digestion before transformation. *RIP1* gene disruption was verified by Southern blot analysis, and uracil auxotrophy of the ΔRIP1 strain was recovered by growth on 5-fluoroorotic acid (Alani et al., 1987).

Immunofluorescence

The above-described wild-type and ΔRIP1 or the temperature-sensitive nup133⁻ (Doye et al., 1994) yeast cells were grown to early log phase at 30°C or 25°C, respectively, fixed, and processed for immunofluorescence essentially as described previously (Wente et al., 1992); the fixation was done for 8 min; pictures were taken with a Zeiss Axiophot microscope. The rabbit polyclonal anti-Rip1p antibody was raised by injection of a GST–Rip1p fusion protein encoding amino acids 120–229 of Rip1p and was used at a 1:500 dilution (AGMED). The secondary antibody was a goat anti-rabbit IgG–FITC conjugate (dilution of 1:80; Sigma ImmunoChemicals). To stain for nucleoporins, we used MAb 414 (Aris and Blobel, 1989; BABCO) at a dilution of 1:100 followed by FITC-conjugated goat anti-mouse IgG (dilution of 1:100; Jackson Immunoresearch Laboratories).

Acknowledgments

Correspondence should be addressed to M. R. We thank Brian Cullen and Tom Hope for mutant *rev* and *rex* cDNAs. We thank Ed Dougherty for help in preparing the figures, Lise-Anne Monaghan for secreterial assistance, and members of the Rosbash lab and Laura Davis for helpful comments.

Received June 4, 1995; revised July 10, 1995.

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GenBank Accession Number

The accession number for the sequence reported in this paper is U30614.