The Nuclear Export Signal of Splicing Factor Uap56p Interacts with Nuclear Pore-associated Protein Rae1p for mRNA Export in Schizosaccharomyces pombe*

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Mammalian UAP56 or its homolog Sub2p in Saccharomyces cerevisiae are members of the ATP-dependent RNA helicase family and are required for splicing and nuclear export of mRNA. Previously we showed that in Schizosaccharomyces pombe Uap56p is critical for mRNA export. It links the mRNA adapter Mlo3p, a homolog of Yra1p in S. cerevisiae or Aly in mammals, to nuclear pore-associated mRNA export factor Rae1p. In this study we show that, in contrast to S. cerevisiae, Uap56p in S. pombe is not required for pre-mRNA splicing. The putative RNA helicase function of Uap56p is not required for mRNA export. However, the RNA-binding motif of Uap56p is critical for nuclear export of mRNA. Within Uap56p we identified nuclear import and export signals that may allow it to shuttle between the nucleus and the cytoplasm. We found that Uap56p interacts with Rae1p directly via its nuclear export signal, and this interaction is critical for the nuclear export activity of Uap56p as well as for exporting mRNA. RNA binding and the ability to shuttle between the nucleus and cytoplasm are important features of mRNA export carriers such as HIV-Rev. Our results suggest that Uap56p could function similarly as an export carrier of mRNA in S. pombe.

Mammalian UAP56 (Saccharomyces cerevisiae Sub2p) and its functional homologs belong to the conserved DECD box class of ATP-dependent RNA helicases that play important functional roles in multiple aspects of DNA and RNA metabolism (1). They are essential in a number of organisms, including yeast, nematode, and fruit fly (2). UAP56/Sub2p is directly involved in pre-messenger RNA splicing and nuclear export of messenger RNAs in S. cerevisiae and in metazoan cells (3). Recently, in Schizosaccharomyces pombe, we found that uap56 is an essential gene for growth and that Uap56p is critical for mRNA export (4). Mammalian UAP56 functions in both ATP-independent and ATP-dependent steps of the spliceosome assembly process (5). Uap56p homologs typically contain a characteristic ATP-binding site, a catalytic DECD box, and an RNA interaction motif. Mutations within the ATP-binding pocket or the catalytic DECD box residues abolished the splicing functions of UAP56 (5). The enzymatic activity of Uap56p homologs has not been directly linked to mRNA export.

In S. cerevisiae, Sub2p was proposed to function in dissociating an intron branch point-binding protein (BBP) 4 from pre-mRNAs (6). Sub2p is also critical for the export of mRNAs of both intron-containing and intron-less genes (7, 8). It is known to be recruited to genes by Hpr1p, a component of the TFO complex (9). TFO is a transcription-elongation complex, and in yeast, in addition to Hpr1p, it contains three other nonessential proteins, Mft1p, Tho2p, and Tph2p (10). Once recruited, the major function of Sub2p in mRNA export is the recruitment of mRNA adapter Yra1p to elongating transcripts (8). Sub2p and Yra1p together with TFO form the TREX complex that was suggested to physically link the transcription apparatus to mRNA export steps (7, 11). Although the recruitment of Yra1p to intron-containing messages depended on Sub2p, its recruitment to intron-less genes was found to be unaffected in a sub2-85 mutant strain. These results imply a second function of Sub2p unrelated to Yra1p recruitment (8). Both UAP56 and Sub2p directly bind Aly/Yra1p in vitro (7, 12). In a competitive binding experiment, mRNA export carrier Mex67p could displace Sub2p from Yra1p (7). It was suggested that removal of Sub2p from Yra1p in the nucleus allows Mex67p to target mature mRNPs to the nuclear pore complex (NPC). Because Mex67p is not essential in wild type S. pombe cells for mRNA export, these results led us to propose that Uap56p was not removed from mRNPs, rather it played a critical role in NPC targeting and the export of mRNAs.

S. pombe Uap56p was originally identified as a suppressor of a cold-sensitive S. cerevisiae strain (Δnam8 prp40Ha) (13). Recently, we found that it is physically and functionally linked to two mRNA export factors as follows: Rae1p, an NPC-associated essential mRNA export factor, and Mlo3p, an S. pombe homolog of Yra1p/Aly (4). Mlo3p and Rae1p do not interact with each other directly, but they could be linked via Uap56p in a ternary protein complex in vitro (4). Another mRNA export factor Dss1p could similarly link Mlo3p and Rae1p in vitro. Based on biochemical and
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\textit{in vivo} experiments, we proposed that Uap56p and Dss1p-mediated links are critical for targeting mature mRNPs to the NPC (4).

In this study, we further explored Uap56p functions in mRNA export. Our results indicate that Uap56p does not function as an RNA-dependent helicase in mRNA export. However, its ability to bind RNA is important for exporting mRNA. We found a functionally important NES within Uap56p that mediates direct interactions with Rae1p. Loss of the NES function abrogates Rae1p interaction as well as ability to export mRNA. Taken together, these properties are consistent with Uap56p functioning as an export carrier of mRNA in \textit{S. pombe}.

**EXPERIMENTAL PROCEDURES**

Strains and Culture—Basic cell culture techniques used in this study were as described previously (14, 15). The construction of the null strain of \textit{uap56} was as described (4). The strains used in this study were as follows: wild type cells \textit{h}− \textit{leu1-32 ura4-D18, h}− \textit{∆uap56/pREP42x-uap56, h}− \textit{prp2-l} (16), and \textit{h}− \textit{uap184-1 rae1-167/pREP81X-rae1} (17).

Plasmid Constructions—The cloning sequence for \textit{uap56} was amplified from the genomic DNA and inserted into pREP42x vector (18, 19). To get a genomic construct, the gene for \textit{uap56} along with its promoter and 3′-untranslated region were inserted into an \textit{S. pombe} vector pRL1 that carried the auxotrophic marker \textit{LEU2} gene of \textit{S. cerevisiae}. This plasmid was further used to make mutations within the \textit{uap56} coding sequences. Site-directed mutagenesis was carried out to introduce \textit{GK}→\textit{AD} dec to \textit{DEAD}, \textit{DECD} to \textit{EED}, \textit{Q297R F320A}, or \textit{R382AR385AR388A} (RNA binding domain) mutations using the QuickChange site-directed mutagenesis kit from Stratagene. Plasmids for bacterial expression of N-terminal GST fusions of proteins were made using pGEX5X-3 vector.

Spot Assay for Growth—Growth conditions used have been described previously (4). Briefly (OD = 0.5), cells were serially diluted and plated on YEAA or EMM media. Growth was monitored after 3 days at 30 °C.

In Situ Hybridization—\textit{In situ} hybridization method used was described previously (20). Oligo(dT)\textsubscript{70} carrying an \textit{α}-digoxigenin at its 3′-end was used as the hybridization probe, and rhodamine-anti-digoxigenin was used for detecting the hybridization signal by using fluorescence microscopy. DAPI was used to stain DNA in the nucleus.

Expression and Purification of Recombinant Proteins—All proteins were expressed and purified from \textit{Escherichia coli} strain BL21 using the standard purification protocols. For GST and GST fusion proteins, GSH-Sepharose beads from Amersham Biosciences were used. Proteins were eluted using 250 mM glutathione. Eluted proteins were dialyzed with the universal binding buffer (20 mM Hepes-KOH, pH 7.0, 100 mM K\textit{OAc}, 2 mM Mg\textit{OAc}\textsubscript{2}, 1 mM dithiothreitol, 0.1% Tween 20, and 10% glycerol) (21). For binding reactions, GST and GST fusion proteins were bound to GSH-Sepharose and incubated with \textit{3}\textsuperscript{25}S-Rae1p protein. The labeled Rae1p protein was synthesized using \textit{in vitro} coupled transcription translation rabbit reticulocyte kit from Promega. Binding reactions were performed in the universal binding buffer (21). Bound proteins were separated on 4–12% NuPAGE gels (Invitrogen) and transferred to polyvinylidene difluoride membrane, and the membrane was then exposed to x-ray film. To detect the amount of Sepharose-bound proteins used in the binding reactions, polyclonal antibodies raised in rabbits against Uap56p were used. Standard chemiluminescent detection methods (PerkinElmer Life Sciences) were used to detect the proteins.

RT-PCR—Total RNA was extracted from \textit{S. pombe} wild type and \textit{uap56} mutant cells using a Qiagen RNA extraction kit. \textit{β}-Tubulin cDNA synthesis was performed using the Titan one-step RT-PCR kit supplied by Roche Applied Science. The products were analyzed on agarose gels.

CHIP—Chromatin immunoprecipitation (CHIP) assays were performed by following published methods using polyclonal antibodies against Uap56p and Rae1p (22, 23). Chromatin DNA was sonicated to 0.5–1 kb size fragments and then immunoprecipitated. Gene-specific PCR primers for different regions of \textit{β}-actin gene were used to amplify the precipitated DNA. For control amplification, primer sets for a nontranscribed region was included in each PCR. The sequence of the primer set was 5′-CAACAGGGCCTATAATAA-3′ and 5′-CAGATAGCTTGGATAGATATG-3′. [\textit{α}-\textit{32P}]\textit{dCTP} was added to the PCR. The amplified products were separated by electrophoresis on 6% polyacrylamide gel and quantified using the PhosphorImager.

\textit{S. pombe} NES Assay—The construction of the \textit{S. pombe} vector, pAG177, for \textit{in vivo} nuclear export assay has been described previously (24). The details of this assay have been briefly described under “Results.”

\textit{HeLa} Cell Nuclear Export Assay—Nuclear export assay in \textit{HeLa} cells was performed according to the method described previously (25, 26). DNA sequence coding for specific NES segments of interest was fused to glucocorticoid receptor (Gr) and GFP DNA sequences in Gr-GFP vector. The details of the export assay are described in the figure legends.

**RESULTS**

\textit{S. pombe} \textit{Uap56p} Is Not an Essential Splicing Factor—\textit{S. pombe} \textit{uap56} is an essential gene for growth (4). A null strain of \textit{uap56} (\textit{Δuap56/pREP42x-uap56}) was kept viable by expressing Uap56p from a thiamine-repressible \textit{nmt1} promoter in pREP42x vector by growing cells in the absence of B1 (henceforth referred to as −B1 condition). Growth in [\textit{α}−\textit{32P}]\textit{dCTP} presence of B1 (referred to as +B1 condition) results in depletion of Uap56p and inhibits growth. As a putative RNA-helicase, Uap56p contains a characteristic ATP-binding motif AKSGMGKT, the conserved catalytic DECX box, and the RNA-binding motif shown in Fig. 1A (5, 27). When the GTK motif was mutated to GNT in the ATP binding domain, both the ATPase activity and splicing were inhibited in \textit{S. cerevisiae} \textit{spu2} mutant cells (5). We made mutations within the ATP binding domain of Uap56p (\textit{GK} to \textit{DA}) to determine the role of these mutations in splicing and mRNA export in \textit{S. pombe}.

When the DECX box of mammalian \textit{Uap56} or \textit{S. cerevisiae} \textit{Sub2p} was mutated to DEAD or EED, its RNA helicase and splicing functions were inhibited (5). The conserved cystine and aspartic acid residues are located at the catalytic core and are thought to stabilize the SAT loop (28). To test the role of these mutations in splicing and mRNA export, we introduced these mutations in \textit{uap56}.

Previous biochemical work in \textit{S. cerevisiae} \textit{eIF4A} showed a single arginine to alanine mutation within the RNA binding domain was sufficient to drastically reduce cross-linking of...
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Intact RNA-binding Motif by Uap56p Is Required for mRNA Export—*S. pombe* cells expressing the ATP-binding, the DEC box, or the RNA-binding mutant Uap56p proteins are viable. We wanted to know if these mutant cells could support nuclear export of mRNA. As expected, wild type and Uap56p cells expressing Uap56p had normal mRNA export (Fig. 1D, panels a and b), and cells depleted for Uap56p accumulated poly(A)+ RNA in the nucleus (Fig. 1D, panel c). Surprisingly, however, Uap56p cells expressing the ATP-binding mutant protein, G100D/K101A, had no detectable accumulation of poly(A)+ RNA in the nucleus (Fig. 1D, panel d), demonstrating that presumptive loss of ATP binding did not affect the ability of Uap56p to export mRNA.

We also found that Uap56p cells expressing Uap56p with DEAD or the EEAD mutations did not inhibit nuclear export of mRNA (Fig. 1D, panels e and f). Because helicase activity presumably requires intact catalytic residues as well as an ability to bind and hydrolyze ATP, these results indicate that putative enzymatic functions of Uap56p are not essential for driving mRNA export in *S. pombe*. In contrast, we found extensive nuclear poly(A)+ RNA accumulation in the cells expressing the RNA-binding mutant protein (Fig. 1D, panel g). Taken together, these results indicate that Uap56p functions in mRNA export presumably as a component of the nuclear export complex rather than as an enzyme. The functional requirement of RNA binding further suggests that Uap56p may directly interact with mRNAs for mediating their export.

Uap56p May Shuttle between the Nucleus and the Cytoplasm—In human and yeast cell, Uap56p/Sub2p is stably localized in the nucleus. Uap56p/Sub2p is recruited to the transcripts during elongation and is thought to be released from the mRNP complex before the complex is targeted to the NPC (2). Our previous results suggest that Uap56p is likely targeted to the NPC (4). We wanted to know if Uap56p can exit the nucleus. GFP fused to full-length Uap56p at the C terminus localized to the nucleus (4) (reproduced here in Fig. 2B, panel a). First, we determined the region of Uap56p that contains the nuclear import signal. Different segments of Uap56p were fused to GFP at the C terminus, and their cellular localization was determined (Fig. 2, A and B). We found that GFP fusion of the N-terminal half (1–250 aa) of Uap56p localized to the nucleus (Fig. 2B, panel c). In contrast, the C-terminal half (251–434 aa) showed a diffused localization (Fig. 2B, panel b). Within the N-terminal half of Uap56p, we found that a GFP fusion of residues 1–216 aa, 1–100 aa, and 101–250 aa were diffused, whereas that of 50–250 aa was nuclear but less than that observed for the 1–250-aa fragment (N) (Fig. 2B, panels d–g). Based on these analyses, the N terminus of Uap56p appears to contain a bipartite nuclear import signal, located between 1–100 and 216–250 aa.

We next explored if Uap56p contains an NES by using a previously described nuclear export assay in *S. pombe*. The construction of the *S. pombe* vector, pAG177, for *in vivo* nuclear export assay has been described previously (24). pAG177 con-
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A

Uap56p

\[
\begin{array}{c}
\text{ATP-binding} \\
\text{95 DA 103} \\
\text{AKSGMGKT} \\
\text{204 EEAD DEC 207} \\
\text{Q297R} \\
\text{F320A} \\
\text{HRVGRAGRFG} \\
\text{A A A 390} \\
\text{RNA-binding}
\end{array}
\]

B

GK100-101DA

DEAD

EEAD

R382A R385A R388A

wild type

\(\Delta uap56/pREP42X-uap56\) -B1

\(\Delta uap56/pREP42X-uap56\) +B1

C

\(\Delta uap56/pREP42X-uap56\)  

\(prp2-1\) 

\(27^\circ C, 37^\circ C, 3\text{hr}\)

D

wild type  

\(-B1\)  

\(+B1\)  

GK100-101DA  

DEAD  

EEAD  

R382A R385A R388A

Poly(A) RNA

DAPI
tains RNA-binding motifs (RRM) of crp79 fused to GFP at the C
terminus. To identify an NES within Uap56, DNA fragments for various portions of the coding sequences were inserted between the RRM and the GFP encoding sequences creating RRM-NES-GFP fusions. Plasmids expressing the RRM-NES-
GFP fusions were transformed into rae1-167 uap56-184/1-
pREP81X-rae1 SL27 double mutant. The RRM-GFP (pAG177)
control fusion protein is cytoplasmic when Rae1p is expressed
(−B1) in the double mutant (Fig. 2D, panel a). Under synthetic
lethal conditions when Rae1p is depleted, mRNA accumulates
in the nucleus and retains the fusion protein (Fig. 2D, panel b).
However, in the presence of NES the fusion protein can exit
the nucleus showing a diffused localization of the fusion protein
throughout the cell, indicative of a functional NES (25).

Fig. 2C shows a schematic diagram of the fragments used in
the nuclear export assay. By using this assay, we found that a
region between 250 and 434 aa (Fig. 2D, panels c and d) of
Uap56p but not 1–250 aa (data not shown) was capable of
exporting the RRM-NES-GFP fusion in SL27 cells under syn-
thetic lethal conditions. But the fusion protein relocated to the
nucleus in the double mutant following inactivation of Rael-167p at 35 °C for 30 min (Fig. 2D, compare panels c, d, and e).
These data suggest that under synthetic lethal conditions the
fusion protein was not immobilized in the cytoplasm by some
retention mechanism. By deletion analyses, the NES activity
was mapped between 250 and 350 aa (Fig. 2C) that caused partial loss of the nuclear export
activity (Fig. 2D, panels g and h). Thus, we conclude
that the full NES is contained within the 250–350-aa region.
We next made several mutations within the NES (250–350 aa)
to identify residues that are critical for mediating nuclear
export of the fusion protein. Among these residues we identi-
cified two single mutations Q297R and F320A within the 100-aa
region (Fig. 2C) that caused partial loss of the nuclear export
activity (data not shown). When these two mutations were
combined, there was a significant loss of nuclear export of the
fusion protein as judged by the nuclear localization of the RRM-
NES (Q297R/F320A)-GFP fusion (Fig. 2D, panel i).

To further test whether the NES property is evolutionarily
conserved, we used a HeLa cell-based nuclear export assay. For
this assay, as described previously (24, 25), the test export
sequence was inserted between DNA fragments encoding Gr
and GFP, and its ability to export the fusion was tested. As
a positive control, nuclear export activity from Rev-NES was
used, whereas Gr-GFP was used as a negative control (Fig. 2E).
By using this assay we confirmed that the 250–350 aa of
Uap56p was able to export a Gr-GFP fusion from the HeLa cell
nucleus (Fig. 2E). The same sequence carrying the Q297R/
F320A double mutations was unable to export Gr-GFP under
the conditions of the export assay (Fig. 2E). Thus, Uap56p con-
tains a conserved nuclear export signal within the C-terminal
half between the 250- and 350-aa region. Taken together,
Uap56p carries a nuclear import and a conserved nuclear
export signal raising the possibility that it shuttles between
the two cellular compartments.

Mutations within the NES Region Inhibit Growth and mRNA
Export—We next tested the role of the NES in mRNA export
and growth. Cells expressing the NES mutant Uap56p grew
slower than ∆uap56 cells expressing the wild type Uap56p (Fig. 3A). Therefore, mutations within the NES affected
cellular growth.

In parallel we tested the level of mRNA export in the strains
expressing mutant Uap56p (Fig. 3B, panels c–e). The presence
of Uap56p Q297R led to some loss of function as ∆uap56 cells
expressing the mutant protein accumulated poly(A)^+ RNA
in the nucleus (Fig. 3B, panel c). In the case of Uap56p F320A,
there was a distinct pattern of poly(A)^+ RNA at the nuclear
pore suggesting a loss of Uap56p function affecting movement
of mRNA at the NPC (Fig. 3B, panel d and inset). When Q297R
and F320A mutations were combined in Uap56p, the double
mutant accumulated mRNA in the nucleus (Fig. 3A, panel e).
These results suggest that the functional integrity of NES
is important for Uap56p to export mRNA.

NES Region of Uap56p Binds Rae1p—We previously showed
that Rae1p and Uap56p interact with each other (24). To analyze
which region of Uap56p interacts with Rae1p, we made GST
fusions of full-length, N-terminal (1–252 aa) and C-terminal
halves (252–434 aa) of Uap56p. We then tested their ability
to interact with ^35S-Rae1p made in rabbit reticulocyte
extract. As expected, GST-Uap56p was able to interact with
Rae1p, whereas GST alone could not (Fig. 3C, top, compare
lanes 3 and 2). Furthermore, the C-terminal half, but not the
N-terminal half, was able to bind ^35S-Rae1p under the same
experimental condition (Fig. 3C, top, compare lanes 5 and 4).
Because the C-terminal region contains the NES, we tested
GST fusion of 250–350 aa (GST-NES) of Uap56p for its abil-
ity to bind Rae1p. We found that GST-NES fusion was able to
retain Rae1p efficiently (Fig. 3C, lane 6). We then tested the
effect of the NES mutations on Rae1p interaction. A GST-
NES fusion carrying the double mutation Q297R/F320A did
not interact with ^35S-Rae1p (Fig. 3C, lane 7). GST fusion of a
full-length Uap56p with these two mutations was also unable
to bind ^35S-Rae1p (Fig. 3C, top, lane 8). These results suggest
that the NES is the only region of Uap56p that interacts with
Rae1p. It is likely that a loss of this biochemical interaction is
the basis for loss of mRNA export function of Uap56p.

Uap56p Is Recruited Early in mRNA Export—We used
immunoprecipitation of chromatin DNA by anti-Uap56p anti-
body in the wild type strain to test whether Uap56p is recruited
to genes. PCR amplification of different regions of β-actin gene

FIGURE 1. Splicing and poly(A)^+ RNA localization in uap56 mutant cells. A, a schematic diagram of Uap56p and the location of different mutation sites used in this study. B, growth comparisons of different Uap56p cells expressing Uap56p mutants with wild type cells are as indicated. C, total RNA was extracted from ∆uap56/pREP42X-uap56 cells in the presence and absence of B1 (+B1 and −B1), prp2-1 mutant cells, and ∆uap56 cells expressing the mutant Uap56p proteins, Uap56p (G100D/K101A-GK100–101D), Uap56p (DEAD), Uap56p (EED), or Uap56p RNA-binding mutant. Lanes 1 and 10 are the marker lanes. The β-tubulin cDNA is as indicated for ∆uap56/pREP42X-uap56 cells in the absence and presence of B1 (lanes 2 and 3), prp2-1 cells at 27 and 37 °C (lanes 4 and 5), uap56 (G100D/K101A), uap56 (DEAD), uap56 (EED), and uap56 RNA-binding mutant cells (lanes 6–9). D, poly(A)^+ RNA localization in wild type cells (panel a), ∆uap56/pREP42X-uap56 cells in the absence and presence of B1 (panels b and c), uap56 (G100D/K101A-GK100–101D) (panel d), uap56 (DEAD) (panel e), uap56 (EED) (panel f), and uap56 (RNA-binding) (panel g) mutant cells. The corresponding lower panels (panels h–n) show DAPI staining of the DNA.
associates with the genes. Uap56p-Rae1p interaction is therefore likely a later step in mRNA export.

Uap56p was also recruited to genes. We performed immunoprecipitation experiments as above and found that in wild type protein, i.e. less at the promoter (1.9-fold over the control) and more toward the promoter (2.9-fold), middle (3.9-fold), and 3'-end (4.2-fold) of β-actin gene (Fig. 4, B, panel b, and D). These results indicate that RNA binding was not a critical factor in the initial recruitment of Uap56p. This is not surprising, however, because Uap56p, like Sub2p, may be recruited by multiple mechanisms, including protein-mediated interactions with THO components such as Hpr1p (8). In addition, our results suggest that interaction between Uap56p and RNA takes place after the initial recruitment step.

**Rae1p Is Not Recruited to Genes**—Rae1p interacts with Dss1p and Uap56p, both of which appear to be recruited to genes (see Ref. 4 and the results above). We wanted to test whether Rae1p was also recruited to genes. We performed immunoprecipitation experiments with wild type cells by anti-Rae1p antibody and tested for its enrichment on β-actin gene by using similar experimental conditions as before. Rae1p did not associate with any region of β-actin gene significantly. (Fig. 4, B, panel c, and D). These results therefore suggest that Rae1p may not be associated with the genes. Uap56p-Rae1p interaction is therefore likely a later step in mRNA export.

**DISCUSSION**

In this study we show that Uap56p is not essential for splicing in *S. pombe*, and its mRNA export functions do not require a functional ATP-binding pocket or catalytic residues of the DECD box. In contrast, RNA binding is critical for Uap56p to function in mRNA export. We provide evidence that Uap56p has import as well as NES. Thus, Uap56p may be able to shuttle between the nucleus and the cytoplasm. Uap56p physically interacts with Rae1p via the NES. This interaction is critical for both its nuclear export activity as well as mRNA export function. These results suggest Uap56p functions nonenzymatically in mRNA export presumably as part of the mRNP-export complex.

**S. pombe Uap56p May Function as an mRNA Carrier**—Based on the Rev paradigm, an ability to bind RNA and the possession of a NES are considered critical attributes in a protein to potentially function as an RNA carrier (2). Export carriers are also known to shuttle between the two cellular compartments. Our results suggest that Uap56p possesses all of these attributes. Mutational analyses of Uap56p revealed that the RNA-binding motif was functionally important for Uap56p in mRNA export. In RNA-helicase E1F4A, these mutations were shown to abolish RNA binding (30). We found that RNA binding was not important for the recruitment of Uap56p to genes. At this time it is not clear at what step of mRNA export RNA binding becomes critical for function of Uap56p. If Uap56p functions as a carrier, functional interaction with RNA can take place during or after splicing of the messages.

We found a 100-amino acid sequence at the C-terminal half of Uap56p that contains a functional nuclear export signal that is also conserved in human cells. Nonclassical import sequences were located in the N-terminal half of Uap56p. These results are consistent with Uap56p shuttling between the two cellular compartments. Notably the *Chironomus tentans* homolog of Uap56p, HEL, was shown to co-migrate with exporting mRNPs and was seen to be released within the nuclear pores prior to the release of Aly (31). Taken together, our results suggest that Uap56p likely functions as an mRNA export carrier in *S. pombe*.

How does *S. pombe* Uap56p function in mRNA export? We found that in *S. pombe* Uap56p was recruited to genes early. Based on biochemical interaction studies, we previously demonstrated the formation of an Mlo3p-Uap56p-Rae1p complex. These results suggested that Uap56p may link Mlo3p to Rae1p (4). In this study we show that Uap56p interacts with Rae1p via (promoter, 5'-end, middle region, and 3'-end) was used to determine Uap56p recruitment (Fig. 4A). For control, a nontranscribed region from *S. pombe* genome was co-amplified after immunoprecipitation (see figure legends and “Experimental Procedures” for details). Using [32P]dCTP, quantitative PCR was performed to amplify different regions of the β-actin gene as well as the nontranscribed control region from the immunoprecipitated extract by the anti-Uap56p antibody or pre-bleed sera (–IP). For quantitative measurement, the amount of PCR product obtained from the actin gene was normalized against the control region and the pre-bleed samples (Fig. 4C). Uap56p appeared to be enriched progressively from the promoter to the 3’-end region of β-actin gene. Average enrichment was 1.4-fold at the promoter, 2.1-fold at the 5’-end, 3.2-fold at the middle portion, and 5.9-fold at the 3’-end, respectively (Fig. 4, B, panel a, and D, bars 1–4). These results together suggest that Uap56p associates with the β-actin gene, and its recruitment is biased toward the 3’-end of the gene, consistent with its association with elongating transcripts. We conclude that Uap56p, like Sub2p, is recruited during early mRNA export steps.

We next wanted to test whether the RNA-binding mutant could be recruited to genes. We used Δuap56 cells expressing the triple mutant Uap56p (R382A/R385A/R388A) in similar immunoprecipitation experiments as above and found that the recruitment pattern of the mutant Uap56p was similar to the wild type protein, i.e. less at the promoter (1.9-fold over the control) and more toward the promoter (2.9-fold), middle (3.9-fold), and 3’-end (4.2-fold) of β-actin gene (Fig. 4, B, panel a, and D). These results indicate that RNA binding was not a critical factor in the initial recruitment of Uap56p. This is not surprising, however, because Uap56p, like Sub2p, may be recruited by multiple mechanisms, including protein-mediated interactions with THO components such as Hpr1p (8). In addition, our results suggest that interaction between Uap56p and RNA takes place after the initial recruitment step.

*Fig. 2. Characterization of the import and export signals of Uap56p.* A, schematic diagram of Uap56p and its different coding fragments fused to GFP. The expression of the fusion proteins was driven by uap56 genomic promoter. The GFP localizations of the fusion proteins are shown as N for nuclear and D for diffused. B, localization of Uap56p and its fragments fused to GFP in *S. pombe* cells. FL stands for full-length Uap56-GFP (panel a), C-terminal half (251–434 aa-GFP) (panel b), N-terminal half (1–250 aa-GFP) (panel c), 1–216 aa-GFP (panel d), 50–250 aa-GFP (panel e), 1–100 aa-GFP (panel f) and 101–250 aa GFP (panel g). Their corresponding DAPI panels are shown (panels h–n). C, schematic diagram showing the C-terminal fragments of Uap56p fused to GFP used in D and E. Asterisk denotes the site of mutations. D, localization of RRM-GFP fusion proteins pAG177-GFP and various RRM-NES-GFP fusions expressed in *nup184-1 rae1-167/pREP81X-rae1* double mutant cells in the presence (+B1) or absence (−B1) of thiamine. RRM-GFP localization in −B1 and +B1 (panels a and b) and various RRM-NES-GFP fusions in the absence (panel c) or presence of +B1 for 20 h at permissive temperature (panel d) and at the restrictive temperature of 35 °C for 30 min after the cells were grown in presence of B1 for 20 h (panel e) are shown. Panels f–i correspond to different fragments used to map the NES region within the C terminus of Uap56p. Their corresponding DAPI panels are shown below (panels j–o). E, regions of NES or its mutants as indicated in the figure were expressed in HeLa cells as Gr-GFP chimeric proteins. The localization of the fusion proteins under different treatment conditions is indicated. No treatment, the fusion protein is largely cytoplasmic. Import, cells treated with 2 μm corticosteroid for 30 min at 37 °C, and the fusion protein accumulates in the nucleus. Export, cells were washed following treatment with the hormone with PBS to remove the hormone; 30 min of incubation was performed at 37 °C in the presence of cycloheximide to inhibit new protein synthesis. Gr-GFP was used as negative control and Gr-Rev-NES-GFP as positive control.
the NES, and this interaction is vital for the function of Uap56p in mRNA export. Unlike human RAE1, S. pombe Rae1p is mostly localized at the NPC and is thought to function at the nuclear pore (4). We also found that Rae1p is not actively recruited to genes. It is reasonable to hypothesize that Uap56p-Rae1p interaction takes place at the nuclear pore where Rae1p is bound to Nup98p of the NPC. Uap56p could be involved with targeting and export of mature mRNP to the NPC via its interaction with Rae1p.

Role of Rae1p in mRNA Export—Mex67p/TAP is a major mRNA export carrier in most eukaryotic cells. S. pombe Δmex67 strain is viable and exhibits no mRNA export defect indicating that it is functionally redundant in wild type cells. But a combination of rae1-167 ts mutation with Δmex67 resulted in synthetic lethality, suggesting a functional relationship between Rae1p and Mex67p (32). Our working hypothesis is that Rae1p functions at the nuclear pore for the assembly and disassembly of mRNP complexes. The physical interactions of Rae1p with Dss1p shown previously and its interactions with the Uap56p NES region described in this study provide new clues about how it may function during mRNA export. Based on whether Rae1p is static or dynamic at the NPC, we can envi-
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was shown to be asymmetrically disposed within the NPC in electron microscopic studies (33). Irrespective of the mode of action of Rae1p, it appears to play a direct and critical role in mRNP targeting and export in S. pombe.

By combining functions of Uap56p and Dss1p in S. pombe, a picture emerges regarding mRNP targeting in S. pombe. Interactions between Rae1p and Mlo3p are mediated by Uap56p and Dss1p (4). In vivo and in vitro, Dss1p and Uap56p are both able to link the two proteins. Based on growth and the level of mRNA accumulation in the nucleus in the Δdss1 and uap56 NES mutant strains, it appears that individually each interaction is sufficient for growth but not for efficient mRNA export. It is likely that simultaneous interactions with both proteins are required for productive association between mRNPs and NPC leading to efficient export of mRNAs.

Uap56p and Splicing in S. pombe—In S. cerevisiae, Sub2p is involved in an ATP-dependent early spliceosome assembly step where, together with DEX(H/D) protein Prp5p, it promotes an exchange of binding partners at the pre-mRNA branch site (5, 6). Branch point-binding protein initially binds the branch site. Mud2p is thought to stabilize the binding of BBP to the branch point. Sub2p may displace Mud2p, thereby destabilizing binding of BBP to the branch point (6). The removal of BBP allows the formation of a short duplex between pre-mRNA and U2 small nuclear RNP. This proposed function is deemed essential because Sub2p was no longer essential in a Δmud2

REFERENCES

1. de la Cruz, J., Kressler, D., and Linder, P. (1999) Trends Biochem. Sci. 24, 192–198
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2. Cullen, B. R. (2003) J. Cell Sci. 116, 587–597
3. Reed, R., and Hurt, E. (2002) Cell 108, 523–531
4. Thakurta, A. G., Gopal, G., Yoon, J. H., Kozak, L., and Dhar, R. (2005) EMBO J. 24, 2512–2523
5. Zhang, M., and Green, M. R. (2001) Genes Dev. 15, 30–35
6. Kistler, A. L., and Guthrie, C. (2001) Genes Dev. 15, 42–49
7. Strasser, K., and Hurt, E. (2001) Nature 413, 648–652
8. Lei, E. P., and Silver, P. A. (2002) Genes Dev. 16, 2761–2766
9. Zenklusen, D., Vinciguerra, P., Wyss, J. C., and Stutz, F. (2002) Mol. Cell. Biol. 22, 8241–8253
10. Jimeno, S., Rondon, A. G., Luna, R., and Aguilera, A. (2002) EMBO J. 21, 3526–3535
11. Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A. G., Aguilera, A., Struhl, K., Reed, R., and Hurt, E. (2002) Nature 417, 304–308
12. Zhou, Z., Luo, M. J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000) Nature 407, 401–405
13. Libri, D., Graziani, N., Saguez, C., and Boulay, J. (2001) Genes Dev. 15, 36–41
14. Alfa, C., Fantes, P., Hyams, J., Mcleod, M., and Warbrick, E. (1993) Experiments with Fission Yeast, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
15. Moreno, S., Klar, A., and Nurse, P. (1991) Methods Enzymol. 194, 795–823
16. Kaufer, N. F., and Potashkin, J. (2000) Nucleic Acids Res. 28, 3003–3010
17. Whalen, W. A., Yoon, J. H., Shen, R., and Dhar, R. (1999) Genetics 152, 827–838
18. Maundrell, K. (1993) Gene (Amst.) 123, 127–130
19. Forsburg, S. (1993) Nucleic Acids Res. 21, 2955–2956
20. Amberg, D. C., Goldstein, A. L., and Cole, C. N. (1992) Genes Dev. 6, 1173–1189
21. Kunzler, M., and Hurt, E. C. (1998) FEBS Lett. 433, 185–190
22. Partridge, J. F., Borgstrom, B., and Allshire, R. C. (2000) Genes Dev. 14, 783–791
23. Noma, K., Allis, C. D., and Grewal, S. I. S. (2001) Science 293, 1150–1155
24. Thakurta, A. G., Whalen, W. A., Yoon, J. H., Bharathi, A., Kozak, L., Whiteford, C., Love, D. C., Hanover, J. A., and Dhar, R. (2002) Mol. Biol. Cell 13, 2571–2584
25. Love, D. C., Sweitzer, T. D., and Hanover, J. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10608–10613
26. Thakurta, A. G., Gopal, G., Yoon, J. H., Saha, T., and Dhar, R. (2004) J. Biol. Chem. 279, 17434–17442
27. Schmid, S. R., and Linder, P. (1991) Mol. Cell. Biol. 11, 3463–3471
28. Shi, H., Cordin, O., Minder, C. M., Linder, P., and Xu, R. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17628–17633
29. Kozak, L., Gopal, G., Yoon, J. H., Sauna, Z. E., Ambudkar, S. V., Thakurta, A. G., and Dhar, R. (2002) J. Biol. Chem. 277, 33580–33589
30. Pause, A., Methot, N., and Sonenberg, N. (1993) Mol. Cell. Biol. 13, 6789–6798
31. Kiesler, E., Miralles, F., and Visa, N. (2002) Curr. Biol. 12, 859–862
32. Yoon, J. H., Love, D. C., Guhathakurta, A., Hanover, J. A., and Dhar, R. (2000) Mol. Cell. Biol. 20, 8767–8782
33. Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000) J. Cell Biol. 48, 635–651