

Assembly of an Export-Competent mRNP Is Needed for Efficient Release of the 3'-End Processing Complex after Polyadenylation[∇]

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Before polyadenylated mRNA is exported from the nucleus, the 3'-end processing complex is removed by a poorly described mechanism. In this study, we asked whether factors involved in mRNP maturation and export are also required for disassembly of the cleavage and polyadenylation complex. An RNA immunoprecipitation assay monitoring the amount of the cleavage factor (CF) IA component Rna15p associated with poly(A)⁺ RNA reveals defective removal of Rna15p in mutants of the nuclear export receptor Mex67p as well as other factors important for assembly of an export-competent mRNP. In contrast, Rna15p is not retained in mutants of export factors that function primarily on the cytoplasmic side of the nuclear pore. Consistent with a functional interaction between Mex67p and the 3'-end processing complex, a *mex67* mutant accumulates unprocessed *SSA4* transcripts and exhibits a severe growth defect when this mutation is combined with mutation of Rna15p or another CF IA subunit, Rna14p. RNAs that become processed in a *mex67* mutant have longer poly(A) tails both in vivo and in vitro. This influence of Mex67p on 3'-end processing is conserved, as depletion of its human homolog, TAP/NXF1, triggers mRNA hyperadenylation. Our results indicate a function for nuclear mRNP assembly factors in releasing the 3'-end processing complex once polyadenylation is complete.

A significant advance in the area of eukaryotic gene expression has been the appreciation of multiple interrelationships between activities needed to get a functional mRNA to the cytoplasm for translation. Coordination occurs at the level of transcription, capping, splicing, 3'-end processing, and assembly of the mRNA into a ribonucleoprotein particle (RNP) that can be transported (for reviews, see references 2, 5, 12, 43, 51, and 55). Overseeing all of this is a nuclear mRNA surveillance mechanism which ensures that only correctly formed mRNP reaches the cytoplasm and that defective ones are degraded (for a review, see reference 70).

Processing at the mRNA 3' end is accomplished by a multi-subunit complex that recognizes signals on the nascent RNA, cleaves at the poly(A) site, and adds a poly(A) tract. The subunits of the complex are largely conserved across eukaryotes, and in the yeast *Saccharomyces cerevisiae*, this complex can be separated biochemically into three factors: cleavage and polyadenylation factor (CPF), cleavage factor (CF) IA, and Hrp1p (59). The length of the RNA poly(A) tail is restricted by the recruitment of specific poly(A)-binding proteins, such as Nab2p and Pab1p (6, 19, 22, 40, 80), and by the action of poly(A)-specific nucleases in the nucleus and the cytoplasm (59). Previous studies showed that 3'-end processing factors,

with the exception of Hrp1p, Pab1p, and Nab2p, do not shuttle in and out of the nucleus (9, 38, 48). Thus, the exported mRNP does not include CF IA and CPF components, yet the signal sequences that stably hold these factors onto the mRNA as it receives its tail are present on the final polyadenylated product. Therefore, a mechanism must exist to disrupt the processing complex upon completion of polyadenylation. The phosphatase activity of the CPF component Glc7p has been implicated in this step (32), but little else is known about how recycling of cleavage and polyadenylation factors might be accomplished.

Release of 3'-end processing factors could be coupled to the assembly of mRNA into export-competent mRNP. Like post-transcriptional processing, this is a multistep process whose goal is the acquisition of proteins that will directly interact with the nuclear pore (for reviews, see references 13, 43, 51, and 66). Early steps in this assembly are thought to include the association of mRNP maturation factors, such as the THO complex and the yeast serine/arginine-rich (SR) protein Npl3p, with the elongating transcription complex and the cotranscriptional recruitment of the RNA-binding protein Yra1p by an interaction with the 3'-end processing factor CF IA (1, 47, 49, 53, 67). Several studies suggest that the THO complex brings in Sub2p, a DECD-box RNA helicase, which then receives Yra1p from CF IA (47, 74, 75, 83). Together, THO, Sub2p, and Yra1p form the TREX (transcription and export) complex. By interaction with its Hpr1p subunit, THO also recruits Mex67p (37), which acts as a heterodimer with Mtr2p to move the mature mRNP across the nuclear pore to the cytoplasm. However, the weak interaction of Mex67p with mRNA must be stabilized by protein adaptors, such as Yra1p and Npl3p (69, 71). As Sub2p and Mex67p interact with the same region of Yra1p, a remod-

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TABLE 1. Yeast strains

Strain or mutation	Genotype	Source or reference
W303-1A	<i>MATa leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-1115</i>	R. Rothstein
W303-1B	<i>MATα leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-1115</i>	R. Rothstein
<i>mex67-5</i>	W303-1A but <i>mex67-5</i>	46
<i>mex67-6</i>	W303-1B but <i>mex67-6</i>	F. Stutz, unpublished data
<i>rna14-3</i>	W303-1B but <i>rna14-3</i>	4
<i>rna15-2</i>	W303-1B but <i>rna15-2</i>	4
<i>hpr1Δ</i>	W303-1A but <i>hpr1::HIS</i>	54
<i>tho2Δ</i>	W303-1A but <i>tho2::KAN^r</i>	54
<i>sub2-201</i>	W303-1A but <i>sub2-201</i>	68
<i>YRA1</i>	<i>MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 <pLEU2-YRA1></i>	79
<i>GFP-<i>yra1-8</i></i>	<i>MATa ade2 his3 leu2 trp1 ura3 yra1::HIS3 <pLEU2-HA-GFP-<i>yra1-8</i>></i>	79
<i>MTR2</i>	<i>MATa ade2 his3 leu2 trp1 ura3 mtr2::HIS3 <pRS316-MTR2></i>	69
<i>mtr2-26</i>	<i>MATa ade2 his3 leu2 trp1 ura3 mtr2::HIS3 <pRS315-mtr2-26></i>	69
<i>sac3Δ</i>	W303-1A but <i>sac3::KAN^r Hsp104-CacO::TRP1 LacI-GFP::HIS3</i>	77
<i>FY23</i>	<i>MATa trp1Δ63 ura3-52 leu2Δ1</i>	82
<i>rat8-2</i>	<i>FY23 but rat8-2</i>	72
<i>rat7-1</i>	<i>FY23 but rat7-1</i>	33
<i>GLE1</i>	W303-1A but <i>gle1::HIS3 <pGLE1 LEU2 CEN></i>	73
<i>gle1-37</i>	W303-1A but <i>gle1::HIS3 <pgle1-37 LEU2 CEN></i>	73
<i>NPL3</i>	<i>MATa his3 npl3::HIS3 ura3-1 lys2 leu2 ade2 <pNPL3 LEU2 CEN></i>	52
<i>npl3-3</i>	<i>MATa npl3::HIS3 his3 ura3-1 lys2 leu2 ade2 ade3 <pnp13-3 LEU2 CEN></i>	52

eling event is thought to occur on the mRNP in which Mex67p exchanges with Sub2p (74). The Sac3p-Thp1p heterodimer is an additional mRNA adaptor that interacts with both Mex67p and with the nuclear pore (16, 26). Docking of mRNP at the pore opening and a final quality control check are further facilitated by the interaction of proteins such as Yra1p and the poly(A)-binding protein Nab2p with the basket structure on the nuclear side of the pore (25, 34, 79). Other proteins acquired by the mRNP in the nucleus, such as the helicase Dbp5p/Rat8p, function on the cytoplasmic side of the pore to release the carrier proteins (3, 56, 78, 81).

Our current knowledge of mRNA export events provides a platform to explore whether the recycling of processing factors depends on a connection to the subsequent export step. In this study, we show that factors involved in mRNP assembly are needed for the efficient removal of the 3'-end processing complex from the mRNA once the poly(A) tail has reached its appropriate length. This activity may allow recycling of processing factors so that they can work on new precursors while at the same time releasing the mRNA for nuclear export.

MATERIALS AND METHODS

Yeast strains. All yeast strains used in this study are described in Table 1.

Analysis of RNAs made in vivo. RNA preparation and RNase H-Northern blotting analysis of *SSA4* RNAs were done as previously described (54). The RNase H DNA oligonucleotide probes used were SC3 (5'-GCCTGCTCCTGG GGCACC) and SC128 (5'-GATTGCTGTACATTTCCGAGC).

RNA immunoprecipitation assay. An RNA immunoprecipitation assay was developed based on previously published ones (31, 57). Yeast cells were grown in liquid YPGal medium containing 2% galactose instead of dextrose at permissive temperatures and then shifted to 37°C for 1 h. Formaldehyde was added to the culture to a final concentration of 1%, and cells were incubated at room temperature for 20 min. Glycine (300 mM) was added to quench the cross-linking reaction. A total of 10⁹ cells were collected, washed extensively, and lysed in 1 ml of FA lysis buffer (50 mM HEPES-KOH, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] [pH 7.5]) by vortexing with glass beads for a total of 16 min (30 s vortexing and 90 s on ice). After centrifugation at 14,000 rpm at 4°C for 10 min, the supernatant was incubated with protein A-agarose beads absorbed with either rabbit anti-Rna15p antiserum or preimmune serum overnight at 4°C.

The beads were successively washed for 5 min at room temperature with 1 ml of the following: FA lysis buffer containing 275 mM NaCl, FA lysis buffer containing 500 mM NaCl, FA lysis buffer, and TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). After the last wash, the beads were incubated in 200 μ l of elution buffer (50 mM Tris-HCl, 5 mM EDTA, 10 mM dithiothreitol, 1% SDS [pH 7.5]) for 1 h at 65°C to release the RNA and reverse the formaldehyde cross-linking. The supernatant was collected and combined with 150 μ l of TE buffer, which was added to the beads after the elution buffer. After extraction with phenol (pH 4.2) and precipitation with ethanol, the nucleic acids were dissolved in water and treated with DNase (RQ1 DNase; Promega) to digest any contaminating DNA. After DNase had been inactivated by heating to 65°C for 10 min, one-tenth of the immunoprecipitated RNA was amplified by radioactive RT-PCR to determine the abundance of *GAL7* and *SSA4* transcripts. The total RNA before immunoprecipitation was used as the input control. The primer for reverse transcription was oligo(dT)₁₂₋₁₈, and PCR primers were 5'-GAAGAAGCTTGCCCTCAATT AGC-3' and 5'-CAGTCTTTGTAGATAATGAATCTGACC-3' for *GAL7* and 5'-GGTACTTTTGATGTCTCTCTGCTATCC-3' and 5'-CACTGGCTCCAAT GTAGATCTAAAC for *SSA4*.

In vitro cleavage and polyadenylation assays. Yeast whole-cell extracts, preparation of radioactively labeled, precursor RNA, and the in vitro 3'-end cleavage and polyadenylation assays were carried out as previously described (17, 35, 68, 84). For immunoprecipitation of RNAs from in vitro processing reactions, the assay was performed by incubating precursor with 10 μ l of extract in a 100- μ l total reaction volume at 37°C for 20 min. The reactions were stopped by adding 900 μ l immunoprecipitation buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA [pH 7.5]), and the samples were incubated with protein A-agarose beads absorbed with either anti-Rna15 antiserum or preimmune serum as a control for 1 h at 4°C. The beads were washed three times with 1 ml of immunoprecipitation buffer and once with 1 ml of TE buffer (pH 7.5) at 4°C. After the last wash, the RNA was released by incubating the beads in 200 μ l of elution buffer (50 mM Tris-HCl, 5 mM EDTA, 10 mM dithiothreitol, 1% SDS [pH 7.5]) for 30 min at 65°C. After purification by phenol-chloroform-isoamyl alcohol extraction and concentration by ethanol precipitation, RNAs were separated on 5% denaturing polyacrylamide gel containing 8.3 M urea and visualized with a phosphorimager.

For depletion of poly(A) nuclease (PAN) from extract, the *mex67-5* strain and its isogenic wild-type (WT) control were modified to express Pan2 protein with a tandem affinity purification tag at its C terminus according to the method previously described (63). Extract from these cells (100 μ l; 500 μ g of protein) was added to 50 μ l of rabbit immunoglobulin G (IgG)-agarose beads (catalog no. A2909; Sigma) equilibrated with buffer D (20 mM Tris-HCl, 0.2 mM EDTA, 50 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 2 μ M pepstatin A, 0.6 μ M leupeptin) and then mixed for 1 h at 4°C to deplete Pan2-tandem affinity purification protein from the extract. Four microliters of supernatant was then used for processing assays or Western blot analysis using peroxidase-coupled antipeptide (P1291; Sigma).

Western blotting. Whole-cell extracts containing 10 mg total protein were denatured in SDS sample buffer at 95°C for 10 min. The proteins were fractionated by electrophoresis on 10% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Western blotting using rabbit antiserum against Rna15p or monoclonal antibody against Pta1p was performed as previously described (84). The sources of other antibodies are as follows: Rpb3 (mouse monoclonal), Neoclone (catalog no. W0012); hNXF1, a gift from J. Katahira; and hnRNP C, a gift from S. Pinol-Roma.

Human cell culture and transient transfection. Human cell lines were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and supplemented with penicillin and streptomycin (Invitrogen). Small interfering RNA (siRNA)-mediated knockdown in HEK293T cells was carried out by a double-transfection procedure. First, 3×10^5 cells were seeded in 3.5-cm wells. One day later, the cells were transfected with 45 pmol siRNA using Silentfect (Bio-Rad). After two days, the cells were retransfected with 45 pmol siRNA and 2 µg of the plasmid pEGFP-C1 (Clontech) using Lipofectamine 2000 (Invitrogen). Protein and total RNA were isolated after two additional days. Northern blot analysis and RNase H digestion were done as previously described (54).

siRNA-mediated knockdown in U2OS cells grown on coverslips (cell line 2e11; Exo1), as described in reference 7, was done with Lipofectamine Plus reagent (Invitrogen) using 45 pmol of siRNA. Concomitantly, the cells were transfected with a vector expressing HIV-Tat protein to induce expression of the integrated HIV transgene. After 24 h, the cells were subjected to RNA fluorescence in situ hybridization (FISH) analysis using a Cy3-labeled probe directed to the 24 MS2 sites in human immunodeficiency virus type 1 (HIV-1) transcripts (7). The sequences of the siRNA targets for hNXF1 and the control (TEL/AML) are TGAGCATGATTCAGAGCAA and AAGGAGAAUAGCAGAAUG CAU, respectively.

RESULTS

Rna15p is retained on polyadenylated mRNA in several mutants that cause defects in mRNA export. The interaction of the CF IA subunit Rna15p with a 3'-end processing signal upstream of the cleavage site is critical for holding the complex on the substrate during the cleavage and the poly(A) addition steps (36). Gilbert and Guthrie (32) showed that the Glc7p phosphatase acted on Npl3p and promoted export by facilitating the association of Mex67p with mRNA. The *glc7-5* mutant used in this study caused a decrease in the amount of Mex67p cross-linked to poly(A)⁺ mRNA by UV light but an increase in the cross-linking of Rna15p. However, a later study found that Glc7p activity, and a cycle of phosphorylation and dephosphorylation, is also essential for mRNA 3'-end processing (39). That study raised the possibility that the Rna15 retention observed in the *glc7-5* strain was not due to an mRNP assembly defect related to the accumulation of phosphorylated Npl3p but was due instead to modification of the 3'-end processing complex. To clarify the role of mRNP formation and export in the recycling of cleavage-polyadenylation factors, we examined Rna15p retention in several thermosensitive mutants known to accumulate poly(A)⁺ mRNA in the nucleus. These act at different stages in mRNA export and include the THO complex, Sub2p, and Sac3p, which facilitate assembly of the export-competent mRNP; the Mex67p/Mtr2p export receptor complex; the Yra1p and Npl3p adaptor proteins; and Rat7p, Dbp5p, and Gle1p, which function on the cytoplasmic side of the pore.

For this analysis, we used an Rna15p antibody to immunoprecipitate RNA from cells that had been chemically cross-linked in vivo and then measured the amount of polyadenylated RNA by oligo(dT)-primed reverse transcription followed by PCR amplification with gene-specific primers. An increase in the RT-PCR signal in mutant strains compared to the isogenic WT control would reflect the impact of the particular

mutation on the removal of 3'-end processing factors after polyadenylation. For each yeast strain examined, controls were included in which the reverse transcriptase was omitted. In addition, RT-PCR was also conducted on a portion of the input sample to verify that equivalent amounts of starting material were used in each experiment (Fig. 1A).

After cells were incubated at 37°C for 1 h, little Rna15p remained on polyadenylated *GAL7* mRNA in WT cells (Fig. 1A). In contrast, Rna15p was retained on *GAL7* mRNA in several temperature-sensitive export mutants, with a strong and reproducible increase in signals for *hpr1Δ*, *tho2Δ*, *sub2-201*, *mex67-5*, *mex67-6*, *mtr2-26*, *GFP-yra1-8*, and *sac3Δ* (Fig. 1A). This was not simply a consequence of blocking mRNA export, as Rna15p retention was not observed in samples prepared from *npl3-3*, *rat7-1*, *dbp5-2*, or *gle1-37* cells (Fig. 1A and data not shown). Elevated levels of Rna15p on polyadenylated RNA from *mex67-5* cells were also detected with a PCR amplicon targeting the *SSA4* transcript (Fig. 1B).

Because of the importance of Mex67p acquisition in moving the mRNP through the pore, the *mex67-5* and *mex67-6* alleles were chosen for subsequent experiments. Western blotting analysis showed that increased retention of Rna15p was not due to a general increase in Rna15p levels in these strains (Fig. 1C). To verify that Rna15p was a valid indicator for general 3'-end processing complex retention, we also performed the immunoprecipitation assay using a polyclonal antiserum against Pta1p, a CPF component that acts as a scaffold protein for processing machinery assembly (30). Robust association of Pta1p with polyadenylated RNA was indeed observed in the *mex67-6* mutant for both *GAL7* and *SSA4* transcripts (Fig. 1D). Similar to Rna15p, Pta1p protein levels were not altered in the *mex67-6* mutant compared to the WT (Fig. 1C). Overall, our findings indicate that certain export factors such as the TREX complex and the Mex67/Mtr2p heterodimer are required for the timely removal of the 3'-end processing machinery, as mutation of those factors causes processing proteins to be retained on polyadenylated RNA.

Mutant alleles of MEX67 exhibit RNA processing defects in vivo as well as synthetic growth defects with *rna14* and *rna15* mutants. Of the factors implicated in 3'-end processing complex disassembly by the immunoprecipitation assay of Fig. 1, the Mex67p/Mtr2p heterodimer is of particular interest, since Sac3p, Yra1p, and Hpr1p all directly contact Mex67p (reference 43 and references therein). To better understand the relationship between Mex67p and 3'-end processing, we next asked how *mex67* mutants might impact this mRNA maturation step in vivo. To this end, we first examined the effects of combining the *mex67-6* mutation with the *rna14-3* and *rna15-2* alleles, which trigger deficient mRNA 3'-end processing in vivo and in vitro (38, 58, 60). Interestingly, the *mex67-6/rna14-3* and *mex67-6/rna15-2* double mutants are inviable at 30°C, a temperature that allows growth of the individual mutants (Fig. 2A). This clear genetic interaction suggests that the defect caused by the *mex67* mutation could interfere with normal functioning of CF IA and is consistent with our recent finding that *rna14-3* and *rna15-2* show synthetic lethality with *tho2Δ* (68), a TREX mutation that also gives strong Rna15p retention (Fig. 1A).

As reported earlier, mutations in *MEX67*, *RNA14*, and *RNA15* all lead to nuclear retention of *SSA4* RNA (54). To examine possible in vivo processing problems caused by a

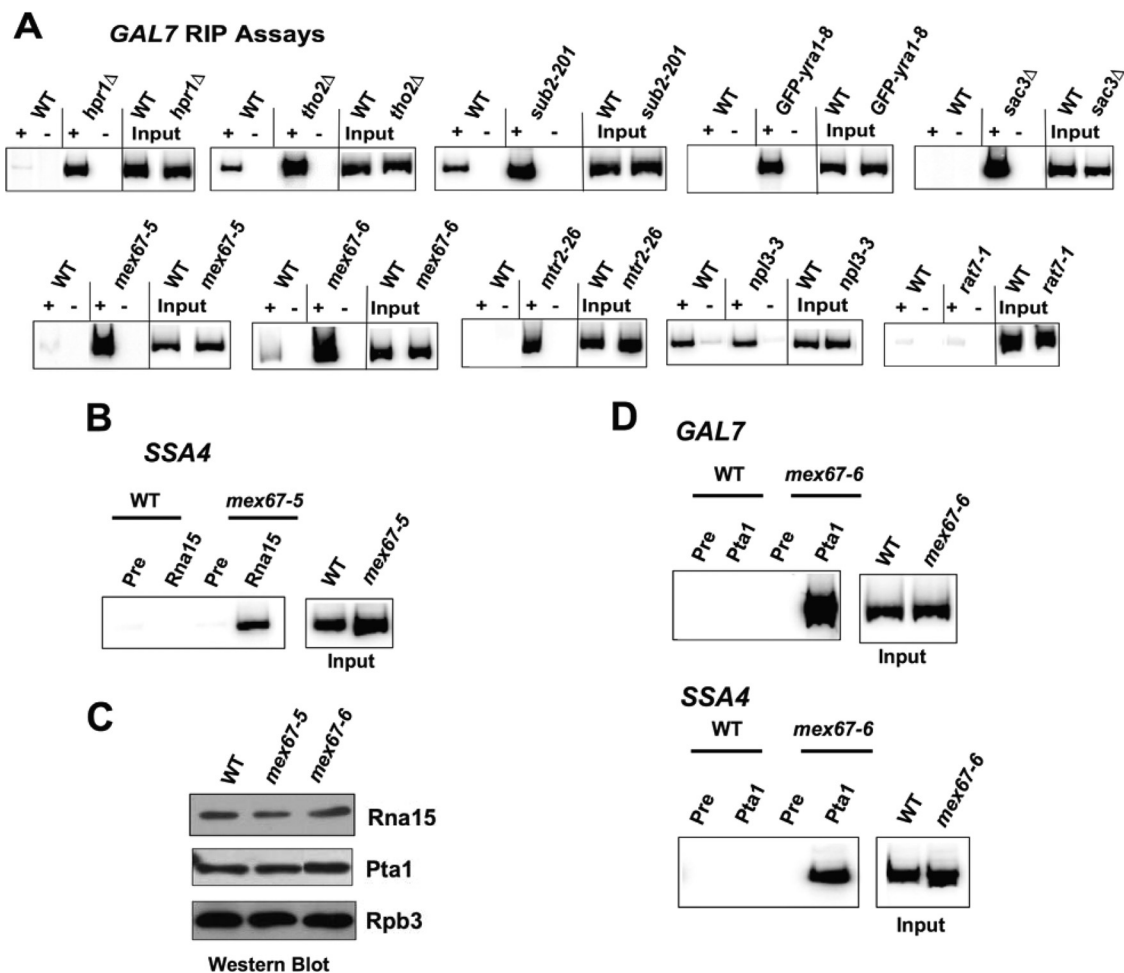


FIG. 1. Increased retention of Rna15p on polyadenylated transcripts in certain export mutants. (A) Rna15p is retained on polyadenylated *GAL7* transcript in *hpr1Δ*, *tho2Δ*, *sub2-201*, *mex67-5*, *mex67-6*, *mtr2-26*, *GFP-yra1-8*, and *sac3Δ* but not in *npl3-3* or *rat7-1* mutant cells. Cells were grown in liquid YPGal medium at 25°C and then shifted to 37°C for 1 h before formaldehyde cross-linking. Whole-cell lysates from isogenic WT or mutant strains were incubated with Rna15p antiserum to immunoprecipitate the cross-linked protein-RNA complex. The immunoprecipitated RNA was reverse transcribed with an oligo(dT) primer in the presence of reverse transcriptase (+). To control for DNA contamination, the reverse transcriptase was also omitted from some samples (-). Each lane shows the radioactive PCR signal with a primer set within the *GAL7* coding region after reverse transcription. The lanes labeled "Input" show RT-PCR amplification of input RNA from each experiment. The results for each mutant and WT control are representative of at least three independent experiments, except for *hpr1Δ*, which was analyzed twice. (B) Rna15p is retained on polyadenylated *SSA4* transcript in the *mex67-5* mutant. The RNA immunoprecipitation assay was performed as in Fig. 1A, but a primer set upstream of the *SSA4* poly(A) site was used in the PCR. Immunoprecipitation with preimmune serum (pre) is shown as a control. (C) Western blotting of cell lysates with antibody against the CF IA subunit Rna15p, the CPF subunit Pta1p, or the Rpb3p subunit of RNAPII. Extract was prepared from WT, *mex67-5*, and *mex67-6* strains after shifting to 37°C for 1 h. (D) Pta1p is retained on polyadenylated RNA in the *mex67-6* mutant. The RNA immunoprecipitation assay was performed as for panel B but with Pta1p antiserum instead of Rna15p antiserum. Pta1p levels on polyadenylated RNAs were increased for both *GAL7* (top) and *SSA4* (bottom) transcripts. Immunoprecipitation with preimmune serum (lanes marked "pre") is shown as a control.

Mex67p defect, we used an RNase H/Northern blotting strategy (Fig. 2B) to determine the status of the *SSA4* heat shock transcripts after transcription induction by a temperature shift to 37°C. This approach allows reliable detection of both adenylated and 3'-end-extended transcripts. As shown previously, *SSA4* RNA levels in WT cells are high after 15 min at 37°C and decline by 60 min. These RNAs are polyadenylated, as evidenced by their shortening when treated with RNase H and oligo(dT) (Fig. 2C, lanes 1 to 2). In contrast, poly(A)⁺ RNAs do not accumulate in *ma15-2* cells, and consistent with the processing defect of this mutant, 3'-end-extended *SSA4* RNAs are visible after 15 min at 37°C and increase at 60 min (Fig. 2C, lanes 4 to 5). The longest read-through RNAs reach

into the downstream *RTT105* gene as they are shortened by additional treatment with the oligonucleotide SC128 (Fig. 2C, lane 6). The oligonucleotide SC128 is positioned at the beginning of *RTT105* (Fig. 2B). These *SSA4* species, which are present at only low levels in WT cells, increase in abundance in the *mex67-5* and *mex67-6* mutant strains after 60 min at 37°C (Fig. 2C, lanes 7 to 12). However, in contrast to the *ma15-2* mutant, *SSA4* RNAs are polyadenylated in the *mex67* mutants at the restrictive temperature, but as previously reported (38, 41, 45), poly(A) tails are much longer than normal (Fig. 2C, compare lanes 1, 7, and 10). Treatment with oligo(dT) and RNase H confirmed that the transcript length difference is due to the poly(A) tail and not a change in the body of the RNA (Fig. 2C, lanes 8 and 11).

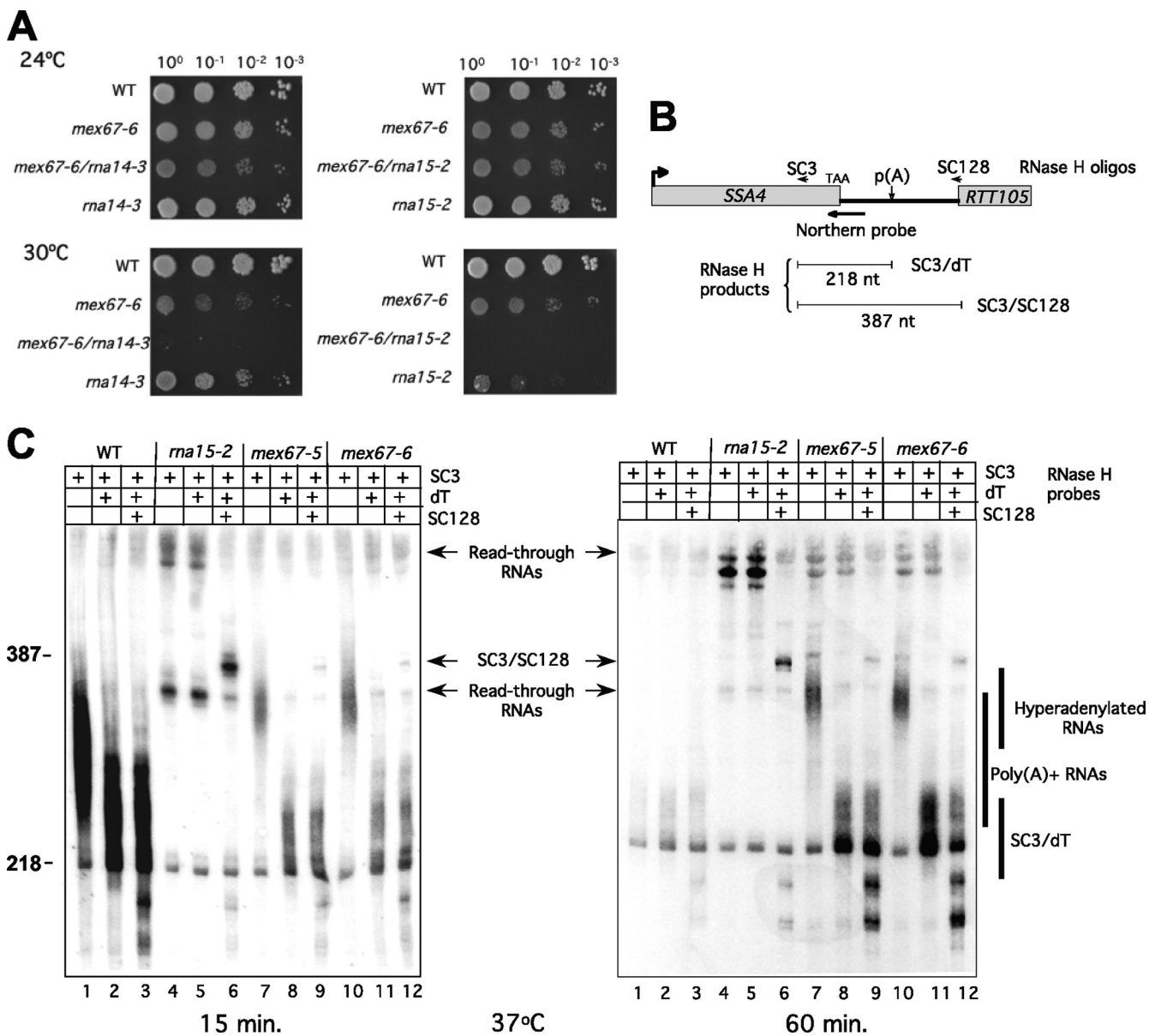


FIG. 2. Genetic interactions and shared in vivo phenotypes of *mex67* and CF IA mutants. (A) Pairing of *mex67-6* with *rna14-3* (left) or *rna15-2* (right) causes synthetic lethality at 30°C. Cells from WT, *mex67-6*, *rna15-2*, *rna14-3*, *mex67-6/rna15-2*, and *mex67-6/rna14-3* strains were grown in liquid YPD medium at 24°C, after which 10-fold serial dilutions were spotted on YPD plates and incubated for 2 to 3 days at the indicated temperatures. (B) Schematic of the RNase H/Northern blotting strategy to examine *SSA4* transcripts. Treatment with oligonucleotide SC3 and RNase H in the 3' end of the *SSA4* open reading frame shortens transcripts and increases resolution. Additional treatment with oligo(dT) shortens the poly(A) tails, resulting in a 218-nucleotide product if all poly(A) is removed. Treatment with oligonucleotide SC128 at the beginning of the downstream *RTT105* gene trims the longest read-through RNAs to 387 nucleotides. The position of the probe used for Northern blotting is also indicated. (C) Northern blot analysis of *SSA4* transcripts from WT, *rna15-2*, *mex67-5*, and *mex67-6* cells grown at 25°C and then shifted to 37°C for 15 or 60 min. RNAs were treated with the indicated oligonucleotides and RNase H. The blot was hybridized with a probe complementary to sequences upstream of the *SSA4* poly(A) site. The positions of hyperadenylated and normal-length poly(A)⁺ RNAs, poly(A)-depleted RNA (SC3/dT), and transcripts that extend beyond the *SSA4* poly(A) site (read-through RNAs) and their derivatives after treatment with oligonucleotide SC128 (SC3/SC128) are indicated.

Another frequent phenotype linked to mutations affecting mRNA export is the accumulation of specific transcripts in transcription site-associated foci detected by FISH analysis (45, 76). When FISH probes specific to sequences upstream of the *SSA4* poly(A) sites were used, these foci were clearly visible in the *mex67-5*, *mex67-6*, *rna14-3*, and *rna15-2* mutants

shifted to 37°C for 15 or 60 min but not in WT cells (Fig. 3). The composition of the dots in *rna14-3* and *rna15-2* cells includes read-through transcripts, as evident from a prominent signal using probes targeting sequences downstream of the poly(A) site (Fig. 3). A lower-level signal from 3'-end-extended *SSA4* species was detected in the *mex67-5* and *mex67-6*

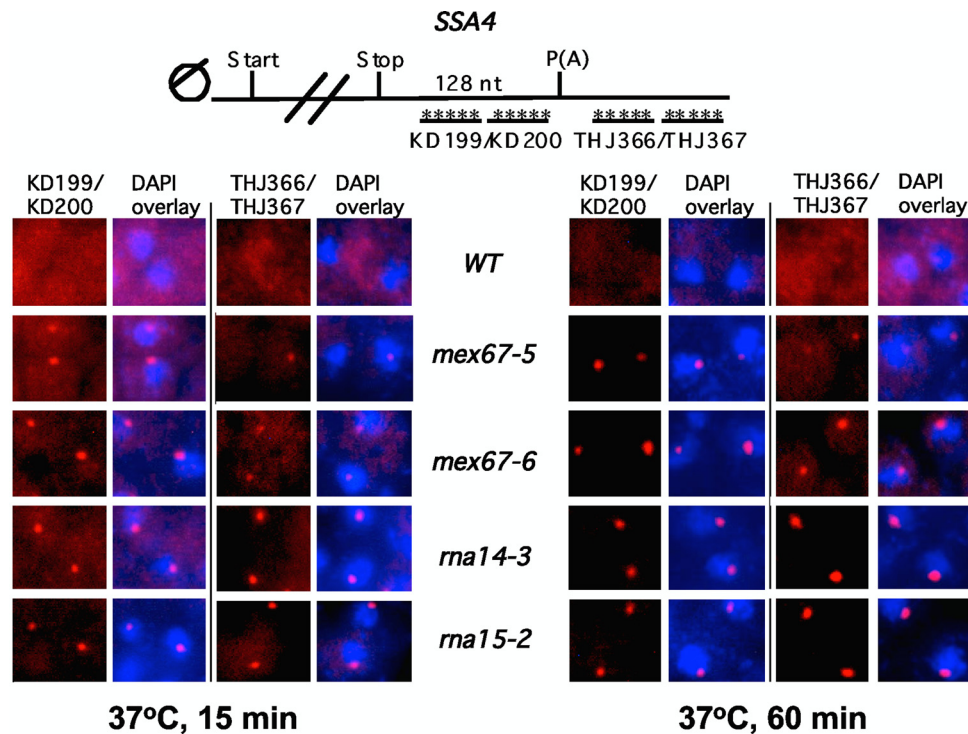


FIG. 3. Detection of *SSA4* transcripts in fixed cells by RNA FISH analysis. WT, *mex67-5*, *mex67-6*, *rna15-2*, and *rna14-3* cells were grown at 25°C and then shifted to 37°C for 15 or 60 min. The positions of the FISH probes for detection of *SSA4* RNAs containing sequence upstream (KD199/KD200) or downstream (THJ366/THJ367) of the poly(A) site are indicated in the diagram at the top. Cells were costained with DAPI (4',6-diamidino-2-phenylindole) and overlaid as indicated.

mutant strains, in agreement with the Northern blot data in Fig. 2C. Taken together, these analyses demonstrate that *mex67* and CF IA mutants manifest common phenotypes in vivo, including defects in mRNA 3'-end formation and appearance of transcripts in nuclear foci.

RNA polyadenylated in *mex67-5* extract has longer poly(A) tails. Mutations of the THO complex or Sub2p result in the rapid degradation of mRNA (44, 54, 65, 68). However, the small amount of product that escapes degradation exhibits tails longer than those formed in WT both in vivo and in vitro (65, 68). This observation, and the shared phenotype of Rna15p retention on poly(A)⁺ mRNA in vivo in THO/*sub2* and *mex67* mutants (Fig. 1A), prompted us to examine the activity of extract from the *mex67-5* mutant. Hence, processing extract was prepared from WT and *mex67-5* strains grown at 25°C and shifted to 37°C for 1 h. Extract was then incubated with radioactive *GAL7* polyadenylation precursor under conditions in which the substrate is cleaved and polyadenylated at the *GAL7* poly(A) site. The results of time course experiments are shown in Fig. 4A and B. For WT extract, the maximum amount of processing is achieved after 10 to 20 min of incubation. The *mex67-5* extract processes the substrate with WT efficiency, and in both extracts, the initial tails, which have comparable lengths at the 5-min time point, are gradually trimmed over time (Fig. 4A). However, in *mex67-5* extract, tail shortening occurs with a much slower kinetics, and even after 180 min of incubation, the tail is not reduced to the length seen in WT extract (Fig. 4B). When the processing reaction is performed at 37°C instead of 30°C, no trimming occurs in the *mex67-5* extract (Fig. 4B),

suggesting that the temperature-sensitive *mex67-5p* protein is responsible for the longer tails. From these experiments, we conclude that the in vivo phenotype of hyperadenylation is mirrored in vitro for the *mex67-5* mutant.

The PAN nuclease is thought to trim the poly(A) tails before mRNA export from the nucleus (22). To explore the role of PAN in poly(A) trimming in vitro, we integrated a tandem affinity purification epitope in frame with the *PAN2* coding sequence of the nuclease subunit and depleted the extract of PAN by incubation with IgG-bound beads. The levels of the tagged-Pan2p in WT and *mex67-5* extracts are equivalent (Fig. 4C, left), indicating that loss of PAN does not explain the longer tails of *mex67-5*. However, PAN is primarily responsible for the trimming, as its removal significantly hinders tail shortening in both extracts (Fig. 4C, right, compare lanes 1 and 2 to lanes 3 and 4). Even with PAN depletion, the poly(A) tracts formed in the *mex67-5* extract are longer than those in WT extract, suggesting that the RNPs formed on polyadenylated RNAs in mutant extract are also resistant to other 3'-5' nucleases remaining in the extract.

Retention of the 3'-end processing complex on polyadenylated RNAs in vitro, similar to what we observed in vivo (Fig. 1A), could block access of PAN to the poly(A) tail or continually replenish the tail because of the poly(A) polymerase that is part of the complex. To address this possibility, we immunoprecipitated the products of in vitro processing reactions with the Rna15p antibody. Examination of the RNAs in the input samples confirms that processing is efficient but tails are longer in the *mex67-5* extract (Fig. 4D, lanes 1 and 2). In both

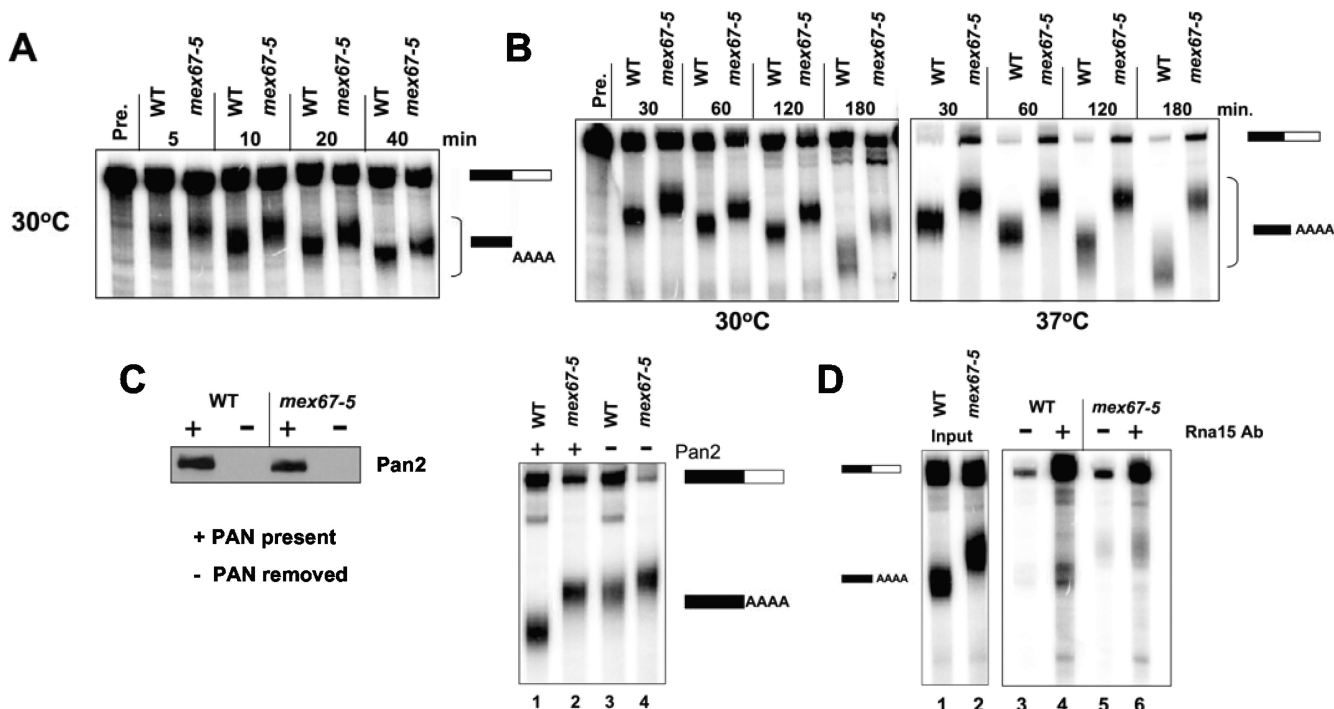


FIG. 4. Analysis of in vitro coupled cleavage and polyadenylation reactions in *mex67-5* extract. (A) Extract from *mex67-5* cells processes RNA efficiently but gives longer poly(A) tails than that of WT extract. Cells were grown in liquid YPD at 25°C and then shifted to 37°C for 1 h before extracts were prepared. Processing reactions were performed by incubation with a radioactively labeled precursor RNA containing the *GAL7* poly(A) site (Pre) at 30°C for the indicated times. (B) Tails are not shortened in *mex67-5* extract if processing is performed at 37°C instead of 30°C. The slight decrease in processing efficiency in the *mex67-5* extract at 37°C in this experiment is not reproducible. (C) The PAN nuclease trims tails synthesized in vitro. Extracts were prepared from WT and *mex67-5* cells expressing tandem affinity purification-tagged Pan2p, and PAN was removed by incubation with IgG beads. (Left) Western blotting analysis of Pan2 in mock-depleted (+) or PAN-depleted (-) extract. (Right) Processing in extracts with or without PAN. Reactions were performed for 120 min at 30°C. (D) The *mex67-5* mutation does not cause an increased association of Rna15p with RNAs that are polyadenylated in vitro. Processing reaction mixtures were incubated at 30°C for 30 min, and RNAs were immunoprecipitated using Rna15p antibody (+) or preimmune serum (-).

samples, a significant amount of unprocessed RNA is brought down with anti-Rna15p compared to aliquots treated with pre-immune serum (Fig. 4D, lanes 3 to 6), suggesting that a stable processing complex has assembled on this precursor RNA. However, only a small proportion of polyadenylated RNA is immunoprecipitated, and this is not increased in *mex67-5* relative to WT extract. Thus, the phenotype of Rna15p retention observed in vivo in this mutant is not readily replicated in vitro, and the slower trimming observed in *mex67-5* extract is more likely caused by a poorly formed mRNP containing the defective *mex67-5p* protein.

Depletion of the human Mex67p homolog TAP/NXF1 induces hyperadenylation and transcript retention phenotypes.

To assess whether phenotypes caused by loss of Mex67p function are conserved in higher eukaryotes, we depleted the human homolog of Mex67p, TAP/NXF1, using RNA interference in human HEK293 cells. TAP/NXF1 protein levels were 60 to 70% reduced, as determined by Western blotting analysis, compared to levels obtained by use of a control siRNA (Fig. 5A, bottom panels, compare lanes 1 and 2 to lane 3). Northern blotting analysis, used to detect mRNA produced from a reporter gene containing the simian virus 40 early poly(A) site, revealed significantly longer species in cells treated with TAP/NXF1 siRNA than in control cells (Fig. 5A, top, compare lanes 2 and 3). To determine if the increased

length was due to extended poly(A) tails, RNA was subjected to RNase H/oligo(dT) treatment before analysis. Reporter RNA in control and TAP/NXF1-depleted samples was reduced to the same size when oligo(dT) was used, while the length was unchanged upon incubation with a DNA oligonucleotide complementary to sequences downstream of the reporter RNA poly(A) site (Fig. 5B). These results demonstrate that depletion of TAP/NXF1 also causes RNA hyperadenylation.

To assay for transcript localization upon TAP/NXF1 knock-down, we employed a U2OS cell line stably expressing HIV-1 transcripts containing 24 MS2 sites from an approximately 70-copy gene array (7). In control cells, a Cy3-labeled MS2 RNA FISH probe detected HIV-1 transcripts in the cytoplasm as well as in a nuclear focus presumably representing nascent transcripts (Fig. 5C). In contrast, upon NXF1 depletion, the majority of the reporter mRNA remained in the nucleus (Fig. 5C), and interestingly, some cells exhibited enlarged dots reminiscent of the situation in *S. cerevisiae* (Fig. 3). Thus, at the level of transcript localization, depletion of TAP/NXF1 causes a phenotype similar to that of Mex67p inactivation.

DISCUSSION

In the highly controlled sequence of mRNA biogenesis, 3'-end processing events must be coordinated with formation of

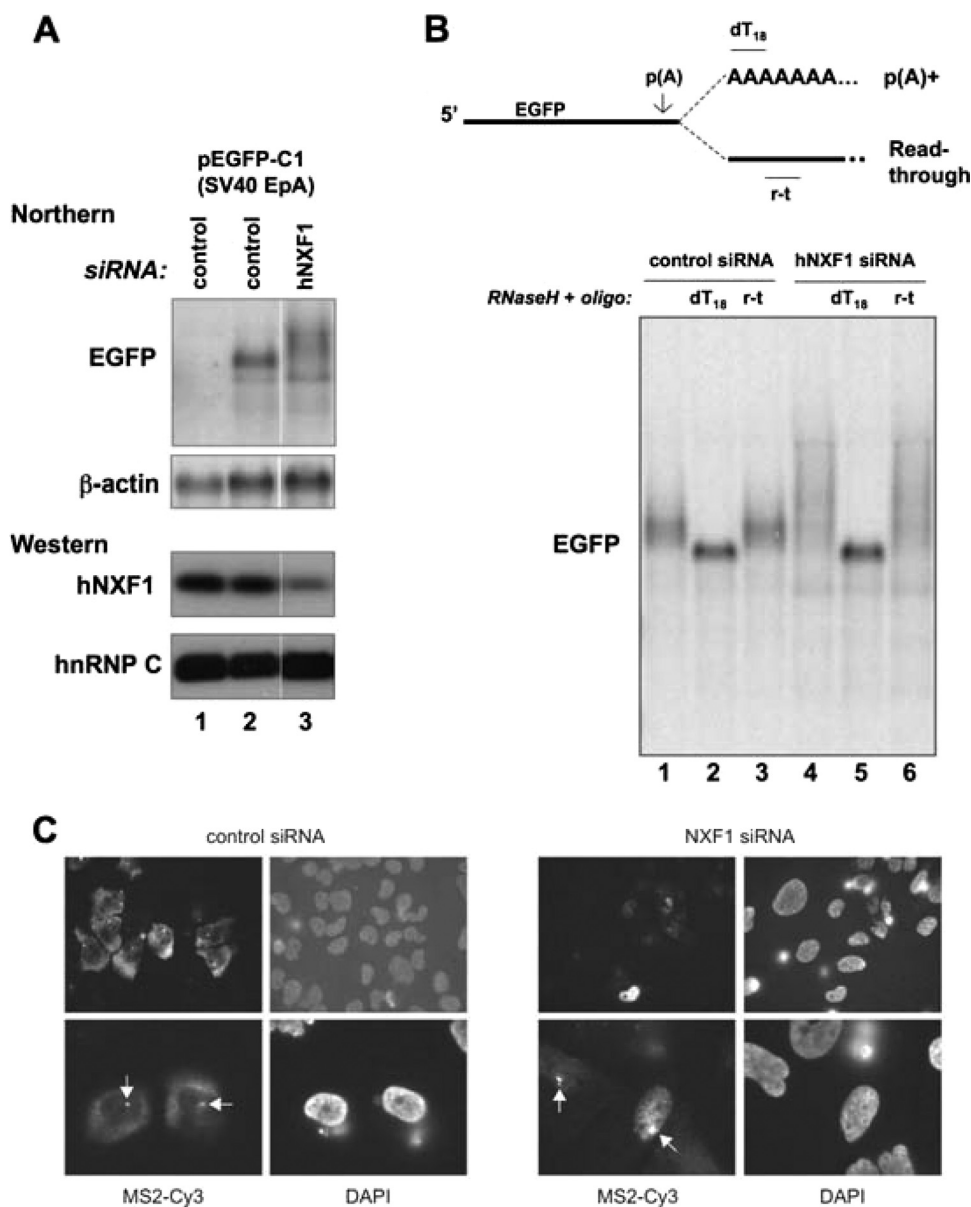


FIG. 5. The mRNA hyperadenylation phenotype is conserved in human cells depleted for NXF1/TAP. (A) Northern blotting analysis was done with total RNA isolated from HEK293T cells either mock-transfected (lane 1) or transfected with a plasmid expressing enhanced green fluorescent protein (EGFP) mRNA (lanes 2 and 3). In addition, cells were treated with either control (lanes 1 and 2) or NXF1/TAP (lane 3) siRNA. The blots were hybridized with probes directed against EGFP RNA or β -actin (control). Western blotting analysis used an antibody specific for NXF1/TAP or an antibody against hnRNP C as a loading control. Intervening lanes not relevant to this study were removed. (B) EGFP RNAs are hyperadenylated upon NXF1/TAP downregulation. RNase H/Northern blotting analysis of the 3' ends of the EGFP transcripts from panel A. Positions of oligonucleotides used for the RNase H digestions (dT₁₈ and r-t) are indicated on top of the panel. Lanes 1 and 4, no oligonucleotide; lanes 2 and 5, oligo(dT)₁₈; lanes 3 and 6, oligonucleotide complementary to sequence downstream of the poly(A) site. (C) MS2 RNA FISH of U2OS cells stably expressing HIV-1 transcripts containing 24 MS2 sites. Cells were treated with either control siRNAs or siRNAs targeting TAP/NXF1. Cells were counterstained with DAPI as indicated. Arrows indicate nuclear dots.

an export-competent mRNP. This interrelationship predicts that polyadenylation influences mRNA export efficiency, which has been supported by several findings. For example, mutations in subunits of the cleavage-polyadenylation complex, such as the Rna14p, Rna15p, and Pcf11p subunits of CF IA, and in the poly(A) polymerase itself, Pap1p, lead to a block in export of mRNAs (8, 38, 41, 54). mRNAs may simply need a poly(A) tail of sufficient length for efficient export (18, 20, 21,

27, 42), but processing factors such as Pcf11p may also help by directly recruiting the Yra1p adaptor to the nascent RNA (47).

In this study, we provide evidence that a reciprocal relationship also exists between polyadenylation and export, with export factors being required for timely release of the 3'-end processing machinery after polyadenylation of precursor. We found that mutations in the THO/Sub2 complex, Yra1p, Sac3p, and the Mex67p/Mtr2p heterodimer all resulted in the same

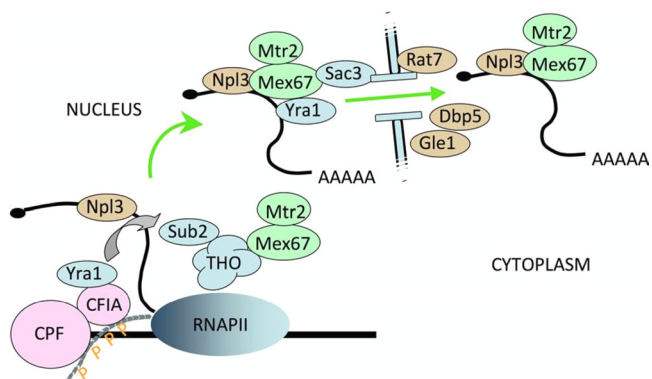


FIG. 6. Model depicting the assembly of an export-competent mRNP. As is evident from recent reviews (13, 43, 55, 67), the process of mRNA export is more complex than portrayed here, and for simplicity, only factors examined in this study are indicated. The THO complex is thought to help recruit both Sub2p and Mex67p to the transcribed gene. The 3'-end processing factors, CPF and CF IA, interact with the phosphorylated CTD of the elongating RNAP II. CF IA recruits Yra1p to the elongation complex, and perhaps concurrently with polyadenylation, a reorganization occurs in which Yra1p is transferred to Sub2p and then to the Mex67p/Mtr2p complex. Npl3p is also recruited cotranscriptionally and exits the nucleus as part of an mRNP containing Mex67p/Mtr2p and other export factors. Sac3p, as part of the TREX2 complex, interacts with Mex67p and the nuclear pore. After passage through the pore, Dbp5p, Gle1p, and Rat7p are important for remodeling the mRNP on the cytoplasmic side. In our study, mutation of the factors shown in light blue and green caused retention of the Rna15p subunit of CF IA on polyadenylated mRNA, while those indicated in brown did not.

pattern of defective release of Rna15p protein from final polyadenylated product, whereas defects in factors that act primarily after passage through the nuclear pore, such as Dbp5p, Rat7p, and Gle1p, did not cause Rna15p retention (Fig. 6). Surprisingly, the *npl3-3* mutant did not increase retention of Rna15p on *GAL7* mRNA (Fig. 1A) or on *RPS11A* or *RPS4B* mRNAs (data not shown), which associate preferentially with Npl3p in pulldown assays from cell extract (50). This mutation maps to the RNA binding domain in the N terminus of Npl3p and causes a strong mRNA export defect at 37°C without mislocalizing Npl3p to the cytoplasm (52). Thus, Npl3p, while important for suppressing 3'-end formation at cryptic sites by antagonizing the binding of CF IA (10, 11), may not be needed to disassemble the 3'-end processing complex once the RNA is polyadenylated. Alternatively, such an activity could be mediated by the C-terminal domain, whose dephosphorylation correlates with efficient Mex67p loading (32). The effect of the *npl3-3* mutation on Mex67p recruitment is not known. Nevertheless, our findings with *npl3-3* and with the export factors that act at the cytoplasmic side (Rat7p, Dbp5p, and Gle1p) reinforce the conclusion that poor release of the 3'-end processing complex is not simply a consequence of an mRNA export block but is dependent on the stage at which mRNP assembly is defective. Overall, our findings suggest that formation of an export-competent mRNP stimulates 3'-end processing by helping the factors recycle onto new precursor transcripts. This remodeling activity could also facilitate export by removing retention factors, such as CF IA and CPF, which would hold the mature mRNA in the nucleus.

Coupling of early steps in mRNP assembly with 3'-end formation. In addition to the work presented here, many other studies have indicated that the stage at which mRNP assembly and export are blocked determines the impact on 3'-end formation. For example, abnormalities in factors involved in early events of mRNP assembly, such as THO/Sub2p, are more likely to cause transcription elongation defects (14, 15, 28, 46, 62), a block in transcription in the vicinity of the poly(A) site (68), and the accumulation in this region of a large protein complex which contains processing factors (64). However, the latter phenomenon is not observed in mutants of Mex67p or Yra1p, which are involved in later assembly steps. The mechanism leading to this complex is not yet known, but it is suggestive of an accumulation of defective mRNPs at the 3' end of the gene.

THO/Sub2p defects also induce rapid 3'-5' degradation of RNA Polymerase II transcripts (65) via specific degradation of the Pap1p regulator, Fip1p, which in turn leads to slower polyadenylation (68). By allowing 3'-5' exonucleases easier access to the mRNA 3' end, this mechanism could help the cell deal with the buildup of defective mRNP. In agreement with this notion, the downregulation of polyadenylation in these mutants requires components of the nuclear surveillance apparatus, such as Rrp6p and Trf4p. Thus, for blocks in the early stage of assembly, polyadenylation is hindered by causing premature transcription termination close to the poly(A) site as well as by rapidly degrading processed RNA before it receives a protective poly(A) tail.

In THO/Sub2 mutants, some mRNAs escape rapid decay and become sequestered at or near the site of transcription (65). This tethering may be another important mechanism to prevent malformed mRNPs from reaching the cytoplasm. The intranuclear foci indicative of this retention are also seen with defects in later stages of mRNP assembly, passage through the pore, or remodeling of the mRNP on the cytoplasmic side (45, 76, 77). The findings presented here indicate that when steps before pore transit are defective, polyadenylated RNAs remain associated with the 3'-end processing complex. This prolonged association and the contacts known to exist between the 3'-end processing complex and RNA polymerase II (12) may contribute to keeping these faulty mRNPs in the nucleus until they are degraded or correctly remodeled. This link to the transcription complex or perhaps to chromatin would be maintained until detachment is facilitated by a THO-initiated remodeling step. The preferred nuclear localization of most of the cleavage-polyadenylation factors (38) suggests that they could act similarly to the RES complex, which is needed not only for efficient splicing but also to prevent nuclear leakage of unspliced pre-mRNA (23). Alternatively, or in addition, there could be a checkpoint for the presence of 3'-end processing factors at the nuclear pore, similar to the way the Mlp1 protein is thought to mediate retention of intron-containing mRNAs without affecting splicing (29). We do not see retention of Rna15p with the *rat7-1*, *dbp5-2*, or *gle1-37* strains. Thus, for mutants affecting later stages of export, other mechanisms must be operative to restrict the RNA to nuclear dots.

Defects in 3'-end processing primarily associated with the final steps of mRNP assembly. Analysis of *HSP104* RNA produced in THO/Sub2 mutants revealed that a pool of stable, processed RNAs had poly(A) tails longer than normal (65), a

phenomenon mirrored in the *in vitro* analysis of these mutants (68). Hyperadenylation, perhaps combined with slower degradation, is even more common with mutations that affect later stages of mRNP assembly and export and is often accompanied by the accumulation of transcripts extending beyond the poly(A) site (38, 41, 45; also this study). The work described here suggests a possible mechanism to explain these defects in 3'-end processing for *mex67* mutants. We propose that in these mutants, 3'-end processing factors cannot be displaced by mRNP reorganization and thus become sequestered on polyadenylated mRNA. Retention would result in fewer processing factors available to work on new transcripts, which in turn would lead to the slower processing seen *in vivo* in *mex67* mutants. In contrast to our *in vivo* data, we did not observe Rna15 retention *in vitro* or a decrease in the efficiency of processing in *mex67* extract. Furthermore, addition of recombinant Mex67p/Mtr2p heterodimer does not disturb 3'-end processing *in vitro* (data not shown), suggesting that Mex67p does not compete with Rna15p for a common binding site. It is possible that slow release of the 3'-end processing complex is manifested *in vivo* only when processing and mRNP formation are coupled to transcription, resulting in an association of CF IA and CPF with polyadenylated mRNA that is more stable than that formed in extract.

Processing *in vitro* does recapitulate the hyperadenylation phenotype seen with *mex67-5* cells *in vivo*. Tails formed in *mex67-5* extract start out with the normal length seen in WT extract and are still trimmed by the PAN nuclease, but more slowly, and they are not trimmed at all if *mex67-5p* is further damaged by heat treatment of the extract (Fig. 4A and B). This observation suggests that the signal to stop poly(A) addition is operational in *mex67-5* and that there is sufficient Nab2p and Pab1p to limit tail length. Moreover, we find no sign that after prolonged incubation in *mex67-5* mutant extract, RNAs are subjected to recleavage, which is a consequence of limited availability of Nab2p *in vitro* (80). A more likely explanation for the longer tails is that the malformed mRNP blocks recruitment or activation of PAN or its access to the 3' end. Interestingly, the PAN nuclease complex is also required for efficient release of mRNAs from sites of transcription (22). Estruch et al. (24) recently found that deletion of *PAN2* or *PAN3*, both of which encode PAN subunits, allowed the *mex67-5* strain to grow at 35°C. In line with the improved growth at higher temperatures, the export defect of *mex67-5* was dramatically relieved in the double mutants, confirming the importance of PAN function in slowing the transit of poorly formed mRNPs from the nucleus of *mex67* mutants.

Research over the last few years has documented a spectrum of ways in which defects in mRNP assembly and export negatively impact 3'-end formation: hindering transcription through the poly(A) site, a slow-down of poly(A) synthesis, rapid degradation from the 3' ends, accumulation of unprocessed transcripts, and hyperadenylation. To this list we can now add retention of processing factors on polyadenylated mRNA. Which phenotypes predominate in a particular mutant probably depends on the composition of the partially formed mRNP, and these phenotypes illustrate how the cell has integrated these two steps to maintain mRNP quality. We propose that in the normal course of mRNA synthesis, mRNP reorganization triggered by factors such as TREX and Mex67p dis-

places the processing machinery once its job is done. In agreement with such a model, a recent comprehensive analysis of maturing mRNP complexes has shown that complexes obtained by Yra1p or Sac3p affinity purification contained cleavage-polyadenylation factors, but those obtained using tagged Mex67p did not (61). The mechanism is likely to be complicated; it is probably related to the overall architecture of the mRNP as it assembles and not to the action of a single component, and it possibly involves posttranslational modifications to the processing complex that affect RNA binding or cross-factor connections. Our results showing that depletion of the human homolog of Mex67p, NXF1, also gives hyperadenylation indicate that this interplay of 3'-end processing and export is likely to be conserved. A focus of future studies will certainly be identification of important interactions involved in mRNP remodeling at the 3' end.

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