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RNA Degradation by the Exosome Is Promoted by a Nuclear Polyadenylation Complex

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Summary

The exosome complex of 3′–5′ exonucleases participates in RNA maturation and quality control and can rapidly degrade RNA-protein complexes in vivo. However, the purified exosome showed weak in vitro activity, indicating that rapid RNA degradation requires activating cofactors. This work identifies a nuclear polyadenylation complex containing a known exosome cofactor, the RNA helicase Mtr4p; a poly(A) polymerase, Trf4p; and a zinc knuckle protein, Air2p. In vitro, the *Trf*4p/*A*ir2p/*M*tr4p *p*olyadenylation complex (TRAMP) showed distributive RNA polyadenylation activity. The presence of the exosome suppressed poly(A) tail addition, while TRAMP stimulated exosome degradation through structured RNA substrates. In vivo analyses showed that TRAMP is required for polyadenylation and degradation of rRNA and snoRNA precursors that are characterized exosome substrates. Poly(A) tails stimulate RNA degradation in bacteria, suggesting that this is their ancestral function. We speculate that this function was maintained in eukaryotic nuclei, while cytoplasmic mRNA poly(A) tails acquired different roles in translation.

Introduction

The exosome is a multisubunit complex with ten common components that are shared between the nuclear and cytoplasmic forms of the complex, often referred to as the "core" exosome. All of these are essential for viability and have either proven or predicted 3′–5′ exonuclease activity (Allmang et al., 1999b; Mitchell et al., 1997). Around 15% of the core exosome population is associated with an additional exonuclease, Rrp6p (Allmang et al., 1999b; Briggs et al., 1998), which is restricted to the cell nucleus in yeast. Rrp6p functions together with the core exosome, but the absence of Rrp6p frequently results in phenotypes that are distinct from depletion of core exosome components, showing that their functions are substantially separable. The absence of Rrp6p is not lethal but does impair growth and leads to temperature sensitivity (Briggs et al., 1998).

The accumulation of 3′-extended and polyadenylated precursors to small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) was observed in strains lacking Rrp6p (Allmang et al., 1999a; Mitchell et al., 2003; van Hoof et al., 2000), and the polyadenylation of pre-rRNA and rRNA (Kuai et al., 2004) and a modification-defective tRNA (Kadaba et al., 2004) has been reported. Modification-defective $tRNA^{Met}$ was stabilized by a mutation in the putative poly(A) polymerase Trf4p and stabilization was also seen in strains mutant for the exosome components Rrp6p and Rrp44p/Dis3p, suggesting that polyadenylation by Trf4p exposes the tRNA to degradation by the exosome (Kadaba et al., 2004).

The purified exosome functions as a 3′–5′ exonuclease in vitro but showed only a slow distributive activity, leading to the accumulation of numerous degradation intermediates (Mitchell et al., 1997). In contrast, individual components were active in vitro as recombinant proteins. In particular, recombinant Rrp44p/Dis3p showed a highly processive activity that efficiently degraded RNA to an oligonucleotide product (Mitchell et al., 1997). This suggests that, in its default state, the exosome is largely inactive, a potentially important feature in protecting cellular RNAs against inappropriate or nonspecific degradation. It seemed clear that some activation mechanism must alter the exosome to convert the slow, distributive in vitro activity into the rapid, processive activity observed in in vivo activity.

A good candidate for an activating cofactor was a DEVH box, putative ATP-dependent RNA helicase Mtr4p/Dob1p (YJL050w) (de la Cruz et al., 1998; Huh et al., 2003; Liang et al., 1996). Mtr4p is localized to the nucleoplasm and nucleolus and functions together with the exosome in the degradation and maturation of many nuclear RNA substrates. Here we report that Mtr4p is associated with the TRAMP complex, which can activate the exosome for the degradation of RNA substrates in vitro and in vivo.

Results

Identification of the TRAMP Complex

In order to identify proteins that interact with the exosome cofactor Mtr4p, a two-hybrid analysis was performed using the entire *MTR4* open reading frame (ORF) as bait, in a fusion with the *GAL4* DNA binding domain in plasmid pB27 (Hybrigenics SA, Paris) (Fromont-Racine et al., 2000). This screen identified 22 clones containing regions of the Trf5p ORF and five clones with the Trf4p ORF. No other significant hits were found (for complete data, see Table S1 in the Supplemental Data available with this article online). Trf4p and Trf5p are related to the β nucleotidyltransferase superfamily (Aravind and Koonin, 1999). For Trf5p, the

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minimal region common to all of the clones interacting with Mtr4p is amino acids 53–199 (see Figure 1A), which lies immediately N terminal to the nucleotidyltransferase domain. Each fragment of Trf4p recovered contains a large region of the ORF, precluding more accurate mapping; however, given the high overall homology between Trf4p and Trf5p (57% identity, 70% similarity over 559 aa), it seems very likely that they share a homologous Mtr4p binding site. The central regions of the two proteins are very homologous (73% identity over 363 aa), and this homology extends into the minimal interaction region between Trf5p and Mtr4p (Figure 1A).

In other two-hybrid analyses, Air2p was identified as an ORF interacting strongly with Lsm2p (Fromont-Racine et al., 2000). Air1p and Air2p (*a*rginine methyltransferase-*i*nteracting *R*ING finger protein) are 48% identical and contain RING-type fingers or zinc knuckle domains (Inoue et al., 2000). Purification of a fusion between Air2p and a tandem affinity purification (TAP) tag failed to provide evidence for stable association with the Lsm2-8p complex, but Mtr4p and Trf4p were identified in the precipitate. This association was supported by a recent high-throughput, systematic analysis (Krogan et al., 2004).

Initial proteomic analyses used a standard TAP-purification protocol (Rigaut et al., 1999), but substantially better yields were obtained following modifications to the procedure (see Experimental Procedures). Figure 1B shows a Coomassie blue-stained gel of large-scale precipitates of TAP-tagged Air2p, Trf4p, Trf5p, and Mtr4p. Major bands were excised and identified by mass spectrometry. Air2p-TAP coprecipitated Mtr4p and Trf4p with apparent stoichiometry. Mtr4p and Air2p coprecipitated with Trf4p-TAP, again with apparent stoichiometry. In the doublet labeled as Air2p in Figure 1B, both bands were identified as Air2p. Mtr4p coprecipitated with Trf5p-TAP, but no bands corresponding to the mobility of Air1p or Air2p were observed. Finally, Trf4p, Trf5p, and Air2p coprecipitated with Mtr4p-TAP; however, in this case, the recovery of Mtr4p was clearly greater than the other proteins. The apparent stoichiometry of the complex makes it unlikely that the proteins associated only via substrate RNAs. Moreover, RNase treatment of the Trf4p-TAP complex bound to IgG prior to release by TEV cleavage did not abolish the recovery of protein bands with the gel mobility predicted for Mtr4p and Air2p (Figure S1).

To confirm these interactions and determine their salt sensitivity, Trf4p-TAP, Trf5p-TAP, and Air2p-TAP were purified following washing of the columns with 100 or 500 mM NaCl (Figure 1C). The presence of Mtr4p in the precipitate was assessed by Western blotting using anti-Mtr4p (anti-Dob1p) antibodies (de la Cruz et al., 1998). Mtr4p was coprecipitated with Trf4p-TAP, Trf5p-TAP, and Air2p-TAP in preparations washed with 100 mM NaCl but was lost following washing at 500 mM NaCl. The amount of Mtr4p coprecipitated with Trf4p was higher than with Trf5p, consistent with the estimate that Trf4p is ~3-fold more abundant than Trf5p (Ghaemmaghami et al., 2003). Ten-fold less of the Mtr4p-TAP preparation was used in Figure 1B, confirming that only a fraction of the Mtr4p population is associated with the complex, even under physiological salt conditions. Western blotting confirmed that all four TAP-tagged proteins were bound to the IgG column and cleaved by TEV protease with similar efficiency (data not shown). The nucleolar ribosome synthesis factor Nop1p (Aris and Blobel, 1988; Schimmang et al., 1989) was not detectably coprecipitated with any of the TAP-tagged proteins (Figure 1B), and Mtr4p was not detectably recovered in a mock precipitation from the nontagged control strain (P51) (data not shown).

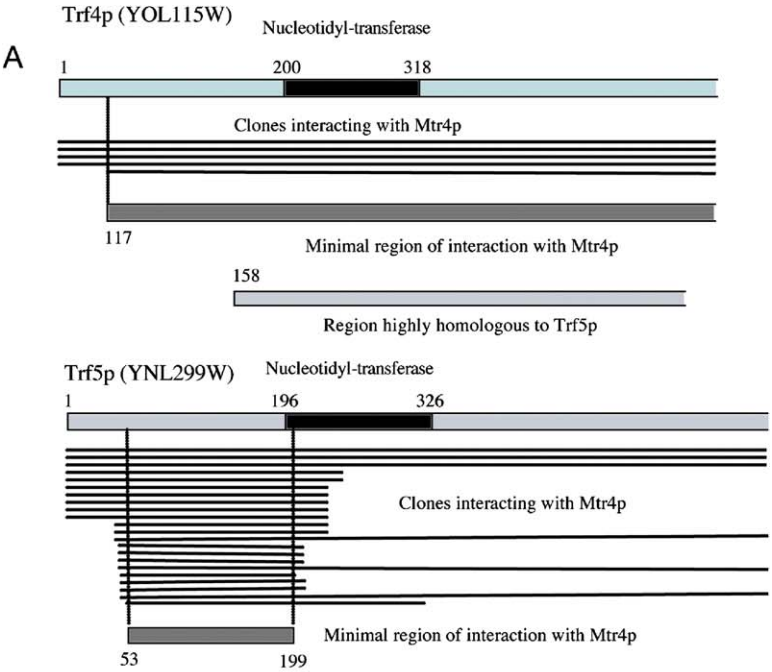
We conclude that Trf4p, Air2p, and Mtr4p form a salt labile complex, which appears to be approximately stoichiometric based on the Coomassie blue staining and will be referred to as TRAMP. Trf5p forms a distinct complex with Mtr4p but does not copurify with Air1p or Air2p. In addition to its participation in the TRAMP complex, a large pool of free Mtr4p also exists. This indicates that Mtr4p is likely to have functions that are not shared by the TRAMP complex, a conclusion that is supported by in vivo analyses (see below).

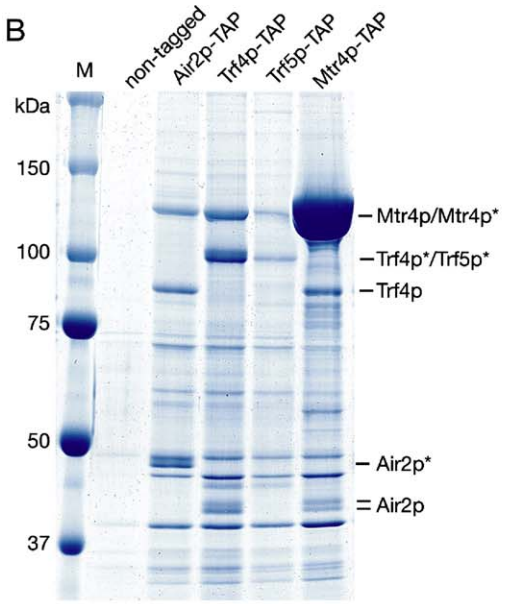
Characterization of the TRAMP Complex

The association of Mtr4p with the putative poly(A) polymerases Trf4p and Trf5p led us to test an Mtr4p-TAP precipitate for polyadenylation activity. Mtr4p-TAP purified at 150 mM NaCl, which was expected to be associated with Trf4p, Trf5p, and Air2p, was assessed for polymerase activity in the presence of 1 mM ATP, UTP, GTP, or CTP (Figure 2A) using a 37 nt RNA substrate derived from the pBS polylinker (Mitchell et al., 1997). Robust polyadenylation activity was seen with ATP, with much lower polymerase activity in the presence of GTP. With UTP, only 1–2 nt was added, and no activity was seen in the presence of CTP.

To determine whether the observed polyadenylation is due to the presence of the complex associated with Mtr4p, we compared the activity of a 150 mM salt precipitate (TRAMP samples in Figure 2B) with Mtr4p-TAP purified with washing at 500 mM salt (Mtr4p samples in Figure 2B), which dissociates Trf4p, Trf5p, and Air2p from Mtr4p. Coomassie blue staining (data not shown) demonstrated that the 500 mM salt wash did not significantly alter the recovery of Mtr4p itself. The high-salt Mtr4p preparation showed no poly(A) or poly(G) addition (Figure 2B), confirming that the polymerase activity shows salt-labile association with Mtr4p.

Poly(A) polymerase activity was then assayed using Trf4p-TAP, Trf5p-TAP, and Air2p-TAP purified following washing with 500 mM NaCl (Figure 2B). Trf4p was associated with a poly(A) polymerase activity that closely resembled the 150 mM salt preparations of Mtr4p (TRAMP lanes). In contrast, no activity was detected for Trf5p-TAP. Western blotting of the protein A tag that remained bound to the IgG column after TEV cleavage confirmed that all three TAP-tagged proteins had bound with similar efficiency and were quantitatively cleaved by TEV (data not shown). The Air2p-TAP preparation showed a lower but clearly detectable polyadenylation activity, indicating that it was still associated with the active polymerase. We conclude that the poly(A) polymerase activity present in 150 mM preparations of Mtr4p-TAP is likely to be associated with the TRAMP complex between Mtr4p, Trf4p, and Air2p, with





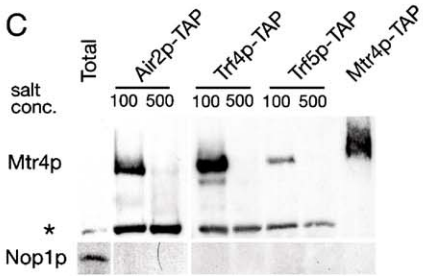


Figure 1. Mtr4p Interacts with Trf4p, Trf5p, and Air2p

(A) Regions of Trf4p and Trf5p identified in a two-hybrid screen with full-length Mtr4p as bait. See Table S1 for coordinates.

(B) Coomassie blue-stained gel, showing large scale TAP purifications. TRAMP components identified by mass spectrometry of excised bands are indicated. Proteins with altered mobility due to the presence of the tandem affinity tag are indicated with an asterisk. Other prominent bands were identified as common contaminants of TAP purifications: proteins of the large ribosomal subunit and cytoplasmic translation factors. In other analyses, the additional bands visible in the Mtr4p-TAP lane were identified as Mtr4p breakdown products.

(C) Western analysis showing the salt sensitivity of coprecipitation of Mtr4p with Air2p-TAP, Trf4p-TAP, and Trf5p-TAP. The cell lysates containing the TAP-tagged proteins indicated were bound to IgG columns and washed with either 100 mM or 500 mM NaCl, as indicated, prior to elution by TEV cleavage. Eluted proteins were analyzed by Western blotting with anti-Mtr4p and anti-Nop1p. Approximately 10-fold less of the control Mtr4p-TAP eluate was loaded than the other samples. The asterisk indicates an unidentified crossreacting protein.

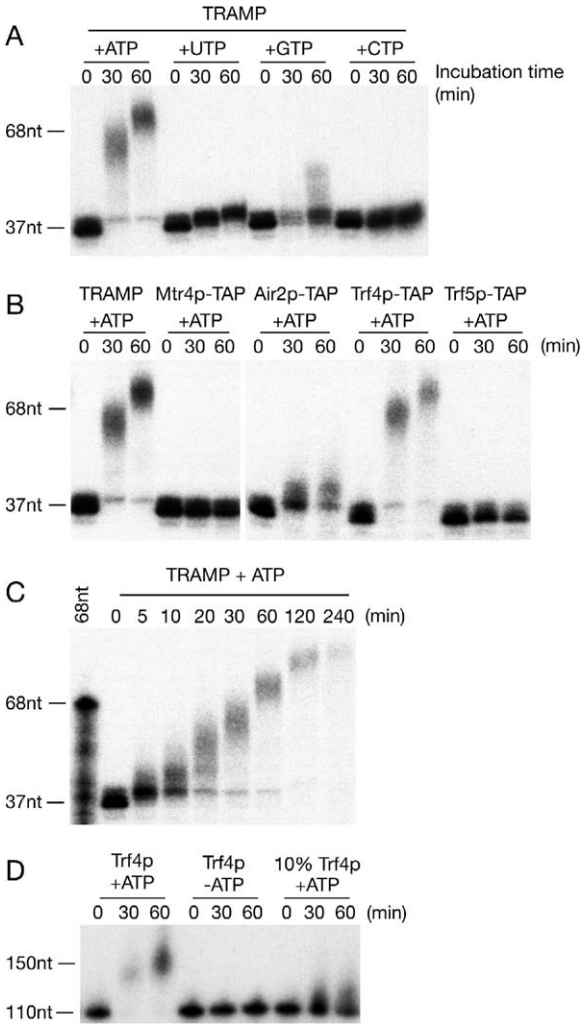


Figure 2. TRAMP Polyadenylates an RNA Substrate

TRAMP samples were copurified with Mtr4p-TAP following washing at 150 mM NaCl. Mtr4p, Trf4p, Trp5p, and Air2p samples were isolated by purification of the corresponding TAP-tagged proteins following washing at 500 mM NaCl. RNA products were separated by polyacrylamide gel electrophoresis and detected by autoradiography. Incubation times are in minutes at 30°C.

(A–C) The 37 nt RNA substrate is a 5′ end labeled, 37 nt transcript from the pBS polylinker. (A) Transcript incubated with TRAMP in the presence of 1 mM ATP, GTP, CTP, or UTP, as indicated. (B) Transcript incubated in the presence of 1 mM ATP with TRAMP copurified with Mtr4p-TAP or with the individual TAP-tagged proteins indicated separated by washing at 500 mM NaCl. (C) Longer time course of incubation in the presence of 1 mM ATP with TRAMP copurified with Mtr4p-TAP.

(D) A 5′ end-labeled 110 nt pre-tRNA transcript incubated with Trf4p-TAP in the presence and absence of ATP and with a 10-fold reduced amount of enzyme (10%) lanes. The position of migration of a 150 nt size marker is indicated.

catalysis performed by Trf4p. In contrast, Trf5p showed no in vitro activity.

To better characterize the mode of poly(A) addition, more detailed time courses were analyzed (Figure 2C). Poly(A) tails added to the 37 nt substrate by either the TRAMP complex or purified Trf4p appeared to be quite discrete in size, with an elongation rate of approximately one nucleotide per minute.

We also assayed polyadenylation on a substrate that was potentially more physiologically relevant. In vivo analyses indicated that an undermodified pre-tRNA was subject to Trf4p-dependent degradation (Kadaba et al., 2004), suggesting that an unmodified pre-tRNA transcript should be a substrate for in vitro polyadenylation and degradation. Incubation of a 110 nt substrate encoding *S. pombe* pre-tRNA^{SupSI} (Krupp et al., 1991) with TRAMP or isolated Trf4p gave a rate of poly(A) addition very similar to that seen with the 37 nt substrate (Figure 2D and data not shown). Both the synchrony and slow rate of poly(A) addition suggested a distributive mode of elongation. This was tested by using 10-fold less Trf4p in the reaction to alter the ratio between the RNA and Trf4p (10% lanes in Figure 2D). This led to only a small increase in the length of the RNA substrate, and no products were detected of the length seen at the higher Trf4p concentration. We conclude that Trf4p and the TRAMP complex show a distributive mode of poly(A) addition. Such a mode of elongation might be an important feature of a polymerase that is associated with a degradative activity, since it implies frequent dissociation from the substrate, potentially allowing access to exonucleases.

Stimulation of Exosome Activity by the TRAMP Complex

For initial analyses of in vitro activity, we used a standard TAP purification protocol (Rigaut et al., 1999). The exosome was purified using a TAP-tagged form of the core component Csl4p, following washing at 500 mM NaCl, while TRAMP was copurified with Mtr4p-TAP at 150 mM NaCl. On the 37 nt transcript (Figure 3A), the exosome alone was able to initiate degradation but was largely stalled after a few nucleotides, as previously reported (Mitchell et al., 1997). The combination of the exosome and the TRAMP complex reduced polyadenylation of the substrate but also suppressed the accumulation of the intermediates, suggesting that the processivity of the exosome had been enhanced. Some stabilization of the full-length RNA was also observed, suggesting that binding and slow poly(A) addition by TRAMP may impede initial access of the exosome to the 3′ end of the substrate.

Analyses were also preformed using the 110 nt pre-tRNA transcript. This is predicted to be structured and has a single nucleotide 3′ overhang, features that should make it relatively resistant to 3′ exonuclease digestion. The exosome or TRAMP complexes alone showed a very low level of degradation of the tRNA transcript (Figure 3B). Degradation was stimulated by the combination of the exosome with the TRAMP complex, with the appearance of an activity that converted the input RNA to a 4 nt product (Figure 3B; see Figure S2 for comparison of the reaction product to an RNA ladder). Interestingly, this activity closely resembles that previous reported for the recombinant exosome subunit Rrp44p (Mitchell et al., 1997). To confirm that degradation was stimulated by the TRAMP complex,

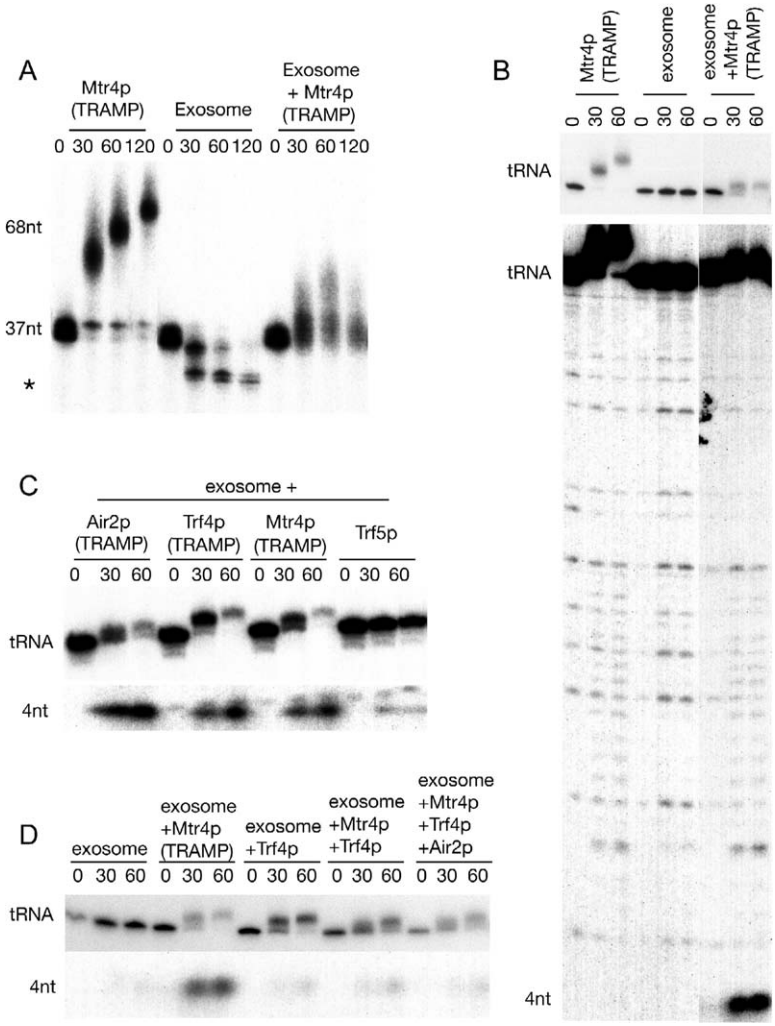


Figure 3. The TRAMP Complex Stimulates RNA Degradation by the Exosome

The exosome was copurified with Csl4p-TAP following washing with 500 mM NaCl. TRAMP was copurified with Mtr4p-TAP, Trf4p-TAP, or Air2p-TAP at 150 mM NaCl. The individual proteins were purified following dissociation of the TRAMP complex by washing with 500 mM NaCl. All reactions contain 1 mM ATP. Products were separated on 12% polyacrylamide gels. Incubation times are in minutes at 30°C.

(A) The 37 nt RNA substrate was incubated with TRAMP copurified with Mtr4p-TAP and/or the exosome. Major degradation intermediates produced by the exosome in the absence of TRAMP are indicated with an asterisk.

(B) The 110 nt pre-tRNA substrate was incubated with the exosome and/or TRAMP as indicated.

(C) Air2p-TAP, Trf4p-TAP, Trf5p-TAP, and Mtr4p-TAP were purified under conditions expected to lead to recovery of the intact TRAMP and tested for their ability to stimulate tRNA degradation by the exosome. The lower panel was exposed 4-fold longer than the upper panel.

(D) Individual components of TRAMP were separated by purification following washing at 500 mM NaCl and incubated together with the exosome complex. The lower panel was exposed 4-fold longer than the upper panel.

we compared the activity of Air2p, Trf4p, Mtr4p, and Trf5p purified under physiological conditions (Figure 3C) and following washing at 500 mM salt (Figure 3D). The exosome was activated by the TRAMP complexes copurified with Air2p, Trf4p, and Mtr4p but was not stimulated by Trf5p purified under the same conditions (Figure 3C). In contrast, the proteins purified after washing with 500 mM salt failed to stimulate degradation singly or in combination. We conclude that the intact TRAMP complex is required for degradation. The activity of the exosome, TRAMP, or exosome + TRAMP was not stimulated by the addition of 1 mM phosphate to the reaction (data not shown), a concentration that was previously shown to activate *E. coli* PNPase and the recombinant exosome subunit Rrp41p (Mitchell et al., 1997). This may indicate that a hydrolytic component of the exosome, rather than one of the phosphorolytic components, is activated by the TRAMP complex. We cannot, however, exclude the possibility that sufficient phosphate is carried over with the purified protein complexes to stimulate activity.

We also analyzed the activity of the TRAMP and exosome complexes purified under the modified conditions used to obtain the complexes visualized in Figure 1C. These preparations showed substantially higher in vitro activity (Figure 4). Both the exosome and Air2p (TRAMP) preparations were able to partially degrade the substrate, but the combination yielded a synergistic increase in degradation (Figures 4A and 4B). To assess the effects of a poly(A) tail on exosome activity, the tRNA substrate was polyadenylated by treatment with Trf4p (Figure 4D). The exosome alone was able to efficiently remove the poly(A) tail, generating a product with gel mobility that was indistinguishable from the nonpolyadenylated transcript. Addition of high-salt-purified Mtr4p failed to stimulate degradation of the deadenylated tRNA and indeed appeared to be mildly inhibitory. We conclude that the poly(A) tail can be rapidly removed by the exosome alone, but its presence does not strongly stimulate degradation of the structured tRNA region, even in the presence of the Mtr4p RNA helicase. The role of poly(A) tail addition in stimulating degradation was further assessed by analyses in the presence and absence of added ATP and in the presence of cordycepin (3′ deoxyadenosine) 5′ triphosphate, an ATP analog that cannot be extended

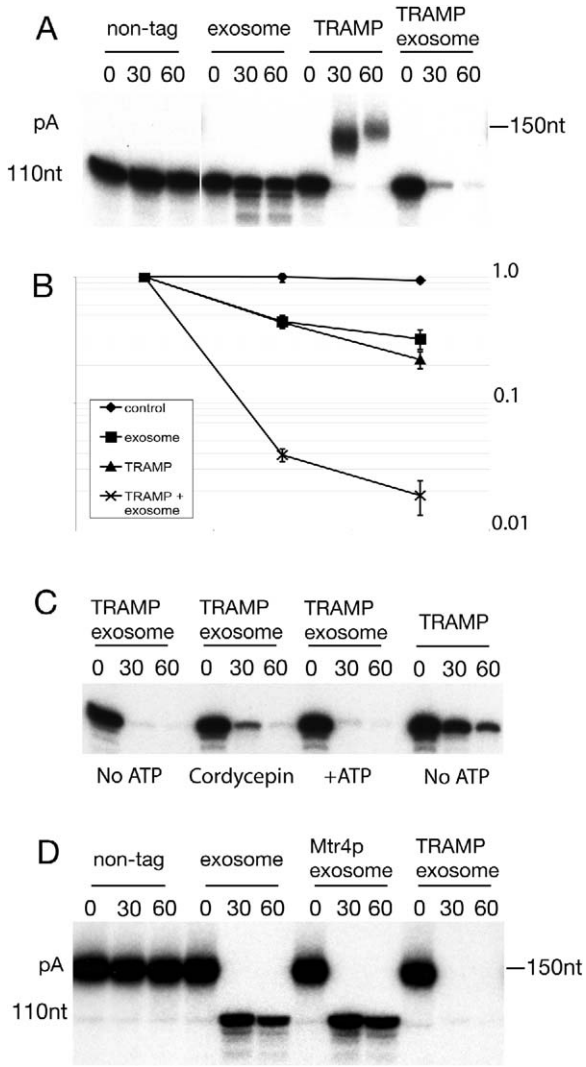


Figure 4. Stimulation of the Exosome Requires the Intact TRAMP Complex but Does Not Require ATP

TRAMP was copurified with Air2p at 100 mM NaCl, and the individual components were purified following washing with 500 mM NaCl to disrupt the TRAMP complex. The exosome was purified with Csl4p-TAP, following washing at 500 mM NaCl. The substrate is a 110 nt tRNA transcript, which is estimated to be present in approximately 10-fold molar excess over the exosome. Products were separated on 8% polyacrylamide gels.

(A) The exosome was incubated together with intact TRAMP or the individual components recovered after washing at 500 mM in the presence of 1 mM ATP.

(B) Graph showing PhosphorImager quantification of input RNA remaining with time. The data are the mean of three independent experiments. Bars show ± one standard error.

(C) The exosome was incubated with the 110 nt tRNA transcript alone or with the TRAMP complex, in the absence of added ATP (No ATP lanes), or in the presence of 1 mM ATP or 1 mM cordycepin triphosphate.

(D) The 110 nt tRNA transcript was polyadenylated by treatment with purified Trf4p-TAP. The polyadenylated RNA was then incubated with the exosome alone or in combination with purified Mtr4p or the TRAMP complex. A small amount of nonpolyadenylated input RNA can be seen in the 0 min time points, and this comigrates with the exosome product.

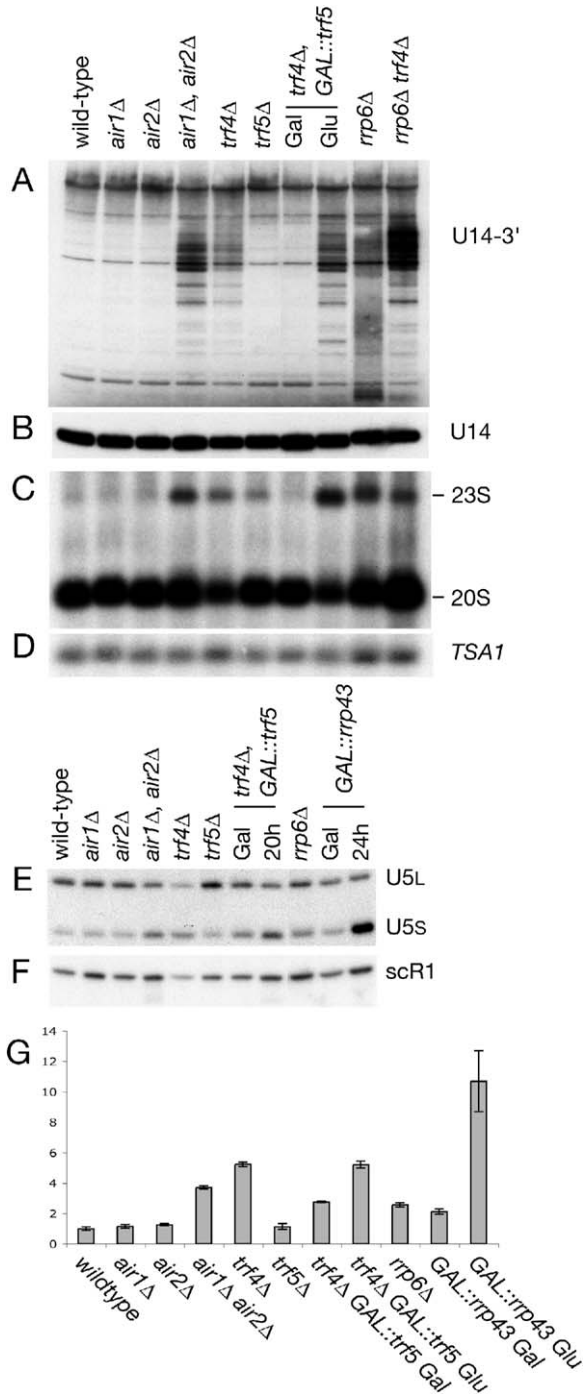
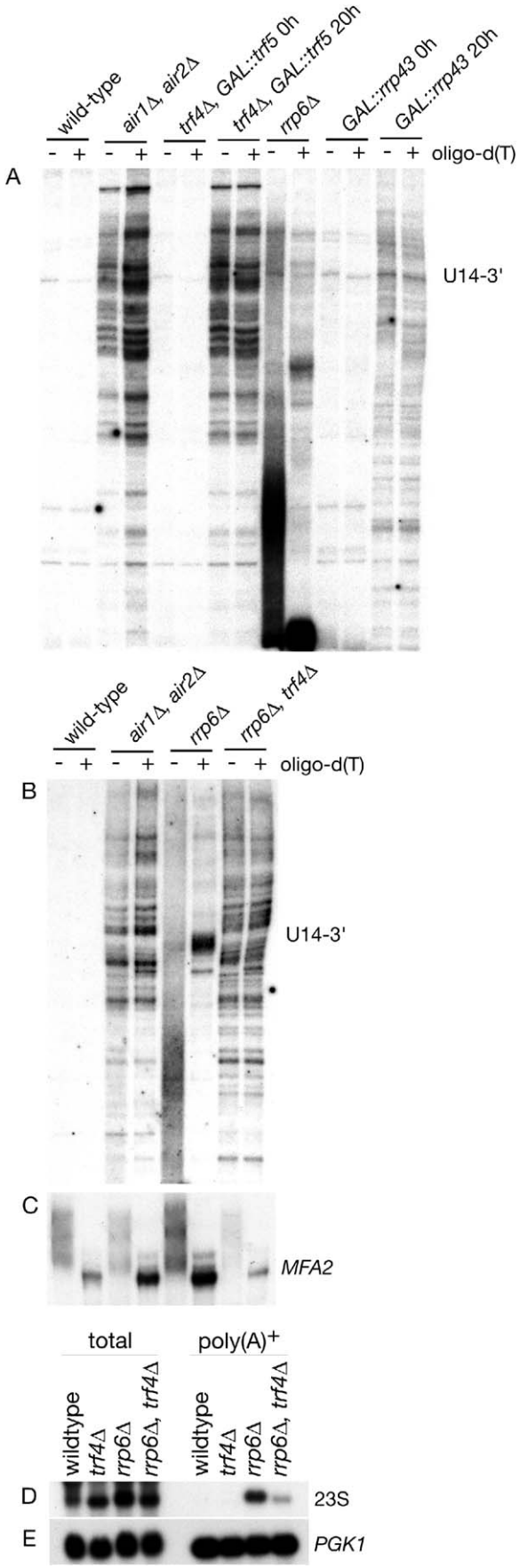


Figure 5. The TRAMP Complex Is Required for Normal RNA Processing and Degradation In Vivo

(A–F) Northern hybridization of U14 snoRNA (probe 202), pre-rRNA (probe 004), U5 snRNA (probe 244). Strains with *GAL*-regulated alleles were grown in galactose and transferred to glucose for the times indicated. All strains were grown at 25°C. The *TSA1* mRNA was used as a loading control.

(G) Quantification of alterations in U5 isoforms, with the wild-type ratio set as 1. The graph shows the mean of three independent experiments based on PhosphorImager quantification. Bars show ± one standard error.



into poly(A) chains (Figure 4C). In four independent experiments using three different TRAMP preparations, similar stimulation of degradation by TRAMP was seen in the presence or absence of added ATP. The presence of cordycepin triphosphate in place of ATP was mildly inhibitory to the reaction. We cannot exclude the possibility that a small amount of ATP is present in the purified protein complexes. However, we estimate that the RNA substrate is present in ~10-fold molar excess over the protein complexes, and it seems unlikely that this makes a significant contribution to the degradation activity.

We conclude that neither the polyadenylation activity of Trf4p nor the putative ATP-dependent RNA helicase activity of Mtr4p is required for the stimulation of exosome activity by the TRAMP complex on this naked RNA transcript in vitro. We would, however, anticipate that these activities are important for activity on the highly structured RNA-protein complexes that are the substrates for the exosome in vivo. The data would be consistent with the activation of one of the hydrolytic components of the exosome, e.g., Rrp44p, which shows highly processive, ATP-independent RNA exonuclease activity in vitro (Mitchell et al., 1997).

TRAMP Components Participate in Exosome Activity In Vivo

Strains depleted of Mtr4p show in vivo defects in the processing and degradation of nuclear RNAs substrates that resemble those of exosome mutants. To determine whether TRAMP participates in these functions, strains lacking Trf4p, Trf5p, Air1p, and Air2p were tested for defects in nuclear RNA processing and degradation (Figures 5 and 6). Trf4p and Trf5p are at least partly functionally redundant in vivo, since double mutants are synthetic lethal (Castano et al., 1996b). We therefore constructed and tested a strain in which *TRF4* was deleted and *TRF5* was placed under *GAL* regulation to allow its depletion on glucose medium. Strains lacking both Air1p and Air2p are viable but impaired in growth (Inoue et al., 2000), and we therefore constructed and tested an *air1Δ*, *air2Δ* strain.

Exosome mutants accumulate 3′-extended forms of the 5.8S rRNA as well as many snoRNAs and snRNAs and are also defective in the degradation of the aberrant 23S pre-rRNA (Allmang et al., 1999a; Allmang et al., 2000; van Hoof et al., 2000). 3′-extended forms of the U14 snoRNA (labeled U14-3′ in Figure 5A) were not seen in the wild-type, *trf5Δ*, *air1Δ*, or *air2Δ* strains but

Figure 6. Polyadenylated RNAs Are Accumulated in Strains Lacking the Exosome Component Rrp6p but Not in TRAMP Mutants

(A–C) Northern hybridization of 3′-extended U14 snoRNA (probe 202) and the *MFA2* mRNA (probe 402). Total RNA was deadenylated by treatment with RNase H and oligo(dT) prior to gel separation. Strains with *GAL*-regulated alleles were grown in galactose and transferred to glucose for the times indicated. The *rrp6Δ* strain was grown at 25°C. All strains except *GAL::rrp43* are isogenic.

(D and E) Northern hybridization of 23S pre-rRNA (probe 004) and the *PGK1* mRNA (probe 403). Poly(A)-enriched fractions were obtained by binding to oligo(dT). Five-fold more of the poly(A)+ samples was loaded.

were readily detectable in the *air1Δ, air2Δ* double mutant and in the *trf4Δ* strain. Growth of the *trf4Δ, GAL::trf5* strain on galactose medium prevented accumulation of U14-3′, indicating that Trf5p overexpression can suppress the *trf4Δ* phenotype. In contrast, depletion of Trf5p in the *trf4Δ*, *GAL::trf5* strain grown on glucose resulted in a stronger phenotype than the *trf4Δ* single mutant. In strains lacking the nuclear exosome component Rrp6p, shorter and more heterogeneous U14-3′ species were seen, whereas the *rrp6Δ*, *trf4Δ* strain resembled *trf4Δ* (see below). No alterations in the level of the mature U14 were seen in the TRAMP mutants, as previously reported for exosome mutants (Figure 5B). This indicates that the 3′-extended species probably represent RNAs that would normally have been degraded in the wild-type strain, rather than productive processing intermediates. Increases in the abundance of several small stable RNA species were previously reported in exosome mutants, suggesting that a substantial fraction of the precursor population may normally be degraded during maturation (Allmang et al., 1999a).

Accumulation of the 23S pre-rRNA was observed in *air1Δ*; *air2Δ* strains and in both the *trf4Δ* and *trf5Δ* strains but was most marked in the *trf4Δ* strain that was also depleted of Trf5p, suggesting that both Trf4p and Trf5p normally participate in pre-rRNA degradation. In the case of the U5 snRNA, exosome mutants increased the ratio of the short to long forms of the RNA, probably due to instability of the short form, which lacks a 3′ terminal stem structure. Increases of around 5-fold in the $U5_S$:$U5_L$ ratio relative to the wild-type were seen in the *air1Δ*, *air2Δ*, and *trf4Δ* strains (Figures 5D and 5F), whereas depletion of the core exosome component Rrp43p resulted in a 10-fold increase. A low but detectable level of 3′-extended 5.8S rRNA was also seen in the same strains (data not shown). This was, however, much less marked than in the exosome mutants or strains depleted of Mtr4p, indicating that TRAMP does not play a major role in 3′ maturation of the 5.8S rRNA by the exosome and Mtr4p.Together, these data suggest that the TRAMP complex may be more important for the functions of the nuclear exosome in RNA surveillance and degradation than RNA maturation.

The poly(A) status of 3′-extended pre-U14 was assessed by comparison of samples with and without deadenylation by treatment with RNase H plus oligo(dT) (Figures 6A and 6B; the resolution of the gel is much higher than that used for Figure 5). In *air1Δ*, *air2Δ*, and *trf4Δ, GAL::trf5* strains, a pattern of discrete bands was seen, which was little altered by RNase H treatment. In the *rrp6Δ* strain, the U14-3′ RNA was highly heterogeneous but collapsed into two prominent bands following deadenylation. The *GAL::rrp43* strain showed weaker accumulation of bands similar in size to those in the TRAMP mutant, some of which were altered in mobility by deadenylation. The level of accumulation in the TRAMP mutants was greater than in a strain depleted of Rrp43p, but *GAL* depletion is never complete, and this strain will retain residual exosome activity.

During the processing of the 5.8S rRNA and the degradation of 3′-extended pre-mRNAs, the role of the nuclear-specific exosome component Rrp6p is subsequent to and dependent on the activity of the "core exosome," which is comprised of the ten components common to the nuclear and cytoplasmic forms of the complex (Allmang et al., 1999b; Briggs et al., 1998; Torchet et al., 2002). This appears also to be the case for 3′-extended U14. To determine whether the activity of the TRAMP complex also lies upstream of Rrp6p, we constructed a strain lacking both Trf4p and Rrp6p (Figure 6B). In the *trf4Δ, rrp6Δ* strain, little polyadenylation was detected and the pattern of extended RNAs closely resembled that of the *air1Δ, air2Δ* mutant. Accumulation of the major RNA species seen in the *rrp6Δ* single mutant was suppressed. Analysis of the *MFA2* mRNA confirmed that the deadenylation reaction was efficient (Figure 6C).

We conclude that the TRAMP complex and the core exosome are required for the initial degradation of 3′-extended pre-U14. In the presence of functional TRAMP and core exosome complexes, the absence of Rrp6p leads to the accumulation of truncated degradation intermediates, which become polyadenylated.

We also assessed the poly(A) status of the 23S pre-rRNA by selection on an oligo(dT) column (Figure 6D). In an *rrp6Δ* strain, the 23S RNA was retained following poly(A) selection. In contrast, the 23S RNA was not detectably recovered in poly(A)-selected RNA from the *air1Δ; air2Δ* strain. In the *rrp6Δ, trf4Δ* strain, polyadenylation of the 23S RNA was largely suppressed.

We conclude that the TRAMP complex polyadenylates nuclear RNA precursors that are targeted for degradation and can stimulate the activity of the exosome during nuclear RNA surveillance.

Discussion

Activation of the Exosome

We have described the identification of the TRAMP complex, which contains the DEVH box putative ATP-dependent RNA helicase Mtr4p/Dob1p, the zinc knuckle putative RNA binding protein Air2p, and the poly(A) polymerase Trf4p. This complex can greatly stimulate RNA degradation by the exosome in vitro and is required for the degradative activity of the exosome on several of its nuclear RNA substrates in vivo.

We envisage that TRAMP can play multiple roles in stimulating RNA degradation of RNA-protein complexes in vivo (Figure 7). The putative RNA binding protein Air2p may recruit the complex to target RNAs via RNA or protein interactions (see below). This association allows the poly(A) polymerase Trf4p to add a single-stranded poly(A) tail, potentially making the RNA a better substrate for 3′ degradation. The TRAMP complex also activates a processive degradative activity of the exosome. Degradation through stable RNA and, particularly, RNA-protein structures may be facilitated by the putative helicase activity of Mtr4p. Two other DExH/D box proteins were recently reported to have "RNPase" activity (Fairman et al., 2004). Together these activities are likely to make a major contribution to the rapid degradation of structured RNAs and RNA-protein complexes in vivo.

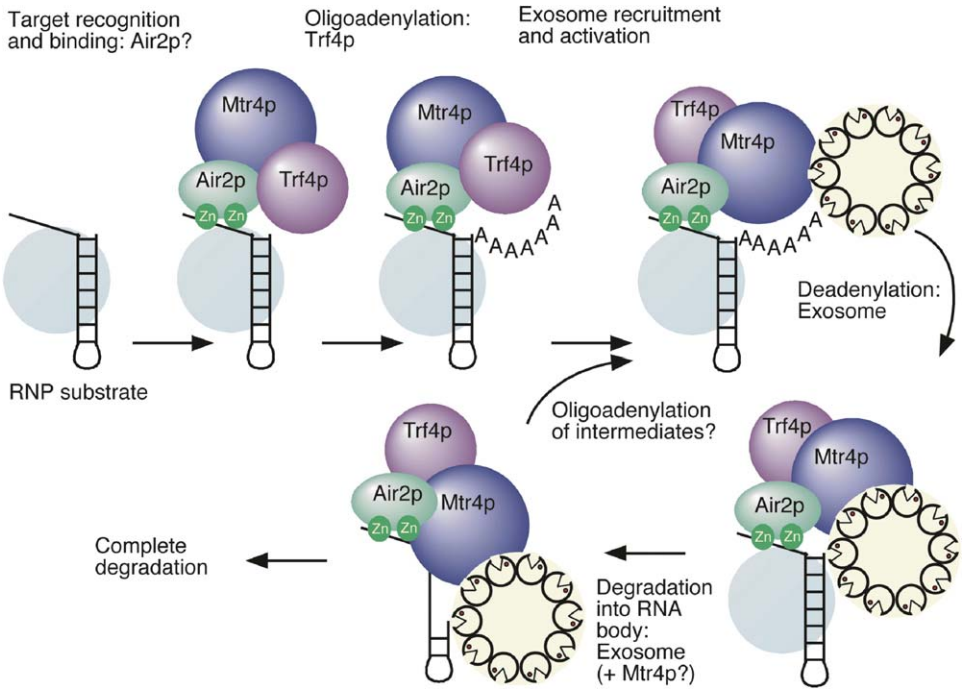


Figure 7. Model for the Roles of the TRAMP Complex in RNA Degradation

The TRAMP complex interacts with RNAs or RNP complexes, making them targets for degradation. In the case of defective pre-tRNAs, this might involve direct binding to the RNA; however, on most substrates, we envisage that this will primarily be via protein:protein interactions. In either case, the zinc finger domains of Air2p might be involved in substrate binding. The RNA is then polyadenylated by Trf4p. Exosome recruitment and activation then requires the intact TRAMP complex. The activated exosome then rapidly deadenylates the RNA and can penetrate into regions of stable structure. We anticipate that Mtr4p is important for dissociation or remodeling of stable RNP structures to allow passage of the exosome.

The TRAMP complex was able to stimulate in vitro degradation of a purified, naked RNA substrate by the exosome in the absence of added ATP, showing that polyadenylation is not essential for activated degradation. Consistent with this conclusion, a catalytically inactive *trf4* mutation is viable in combination with a *trf5Δ* deletion (Wyers et al., 2005), whereas the double deletion is lethal (Castano et al., 1996b). This shows that the in vivo function of Trf4p/Trf5p does not strictly require the polyadenylation activity. However, it seems very likely that this plays an important role in exosome activation in vivo and might also stimulate degradation by other nucleases.

The TRAMP complex appears to be very well equipped to promote RNA degradation, but just why so many activities are required in one complex is less clear. This is, however, reminiscent of the presence of multiple different types of exonuclease in the exosome itself, which contains processive hydrolytic, distributive hydrolytic, and processive phosphorolytic activities. A general explanation may be that many RNAs, particularly the spacer regions of stable RNA precursors, diverge very rapidly and evolution has therefore equipped the degradation system to deal with all eventualities.

Composition of the TRAMP Complex

The proteomic data show that Mtr4p forms separate complexes with Trf4p plus Air2p and with Trf5p. We identified Air2p but not Air1p coprecipitated with Trf4p-TAP and Mtr4p-TAP. However, the in vivo analyses strongly indicate that Air1p can functionally replace Air2p in the TRAMP complex, and it may be that Air1p is simply less abundant than Air2p.

Trf4p and Trf5p are closely related members of a large family of predicted nucleotidyltransferase that are present in all eukaryotes. These include the cytoplasmic poly(A) polymerases gld-2 in *C. elegans* and Cid13p in *S. pombe* (Kwak et al., 2004; Wang et al., 2002; Wang et al., 2000), but localization analyses show Trf4p-TAP and Trf5p-TAP to be exclusively nuclear (data not shown) (Huh et al., 2003). Trf4p purified under conditions that disrupt its association with Mtr4p showed a robust poly(A) polymerase activity in vitro on two different substrates tested, with substantial specificity for ATP incorporation. In contrast, Trf5p did not show polyadenylation activity in our in vitro assays. Trf5p contains all of the catalytic residues present in the canonical poly(A) polymerase Pap1p, and its lack of in vitro activity may be related to the failure to coprecipitate Air2p with TAP-Trf5p. Recent analyses have shown that recombinant Trf4p is active in polyadenylation only if coexpressed with Air1p or Air2p (Wyers et al., 2005). Deletion of both *TRF4* and *TRF5* is synthetic lethal (Castano et al., 1996b), and depletion of Trf5p from a *trf4Δ* strain enhanced the RNA accumulation phenotypes relative to the strain lacking only Trf4p, whereas Trf5p overexpression suppressed the *trf4Δ* phenotype.

These results strongly indicate that a Trf5p-containing complex exists in vivo, which can partially substitute for the Trf4p-containing TRAMP complex.

Previous analyses have concluded that Trf4p and Trf5p function in DNA replication and chromosome cohesion as components of DNA polymerase sigma (also referred to as Pol kappa) (Castano et al., 1996a; Castano et al., 1996b). However, a more recent report failed to reproduce the DNA polymerase activity (Saitoh et al., 2002), and the relationship between its DNA polymerase activity and the role of Trf4p in the TRAMP complex is currently unclear.

A key outstanding question is the mechanism of recognition of RNAs and RNA-protein complexes as substrates for TRAMP/exosome degradation. The canonical Pap1p family of poly(A) polymerases has an RNA recognition motif (RRM) (Wang et al., 2002), but this is absent in GLD-2 and related cytoplasmic poly(A) polymerases (Kwak et al., 2004), and no recognizable RNA binding motif is present in Trf4p or Trf5p (M. Dlakic, personal communication). RNA recognition activity is conferred on GLD-2 by association with a KH domain, RNA binding protein GLD-3 (Kwak et al., 2004; Wang et al., 2002). In the case of Trf4p, we envisage that the specificity of RNA and/or RNP recognition may be provided by the zinc knuckle protein Air2p.

In Vivo Functions of the TRAMP Complex

Previous work has established roles for the nuclear exosome and Mtr4p in the processing and/or degradation of precursors to many stable RNA species including rRNAs, snRNAs, and snoRNAs. In vivo analyses of strains lacking the other TRAMP components indicate that the complex is required for the processing and/or degradation of exosome substrates including the U14 snoRNA, the U5 snRNA, and the pre-rRNA. In the case of the 3′-extended pre-U14 snoRNA, which we examined in some detail, the TRAMP complex acts upstream of the nuclear-specific exosome component Rrp6p. During the processing of other nuclear exosome substrates, the core exosome obligatorily acts before Rrp6p, with "exonuclease handover" occurring at defined positions during maturation (Allmang et al., 1999b; Briggs et al., 1998; Torchet et al., 2002).

Strains lacking Rrp6p also accumulate polyadenylated forms of other small stable RNAs (5S rRNA, U6 snRNA, and U18 snoRNA), and this was also suppressed by deletion of *TRF4* (Wyers et al., 2005), indicating that this may be a general phenomenon.

Poly(A) addition by Trf4p has a function that is presumably very different from pre-mRNA polyadenylation by the canonical polymerase Pap1p. It seems likely that part of the functional difference arises from the speed and processivity of the polyadenylation reactions. Within the mRNA cleavage and polyadenylation complex, Pap1p is highly processive, rapidly adding around 250 adenine residues in human cells and 60–90 residues in yeast (Brown and Sachs [1998] and references therein). This processive activity presumably prevents access of the exosome to the 3′ end of the RNA until polymerization is complete and the transcript has been bound by the poly(A) binding protein Pab1p. In contrast, incubation with Trf4p led to slow polyadenylation (c1nt min^{-1} in vitro) with a distributive mode of addition, which is likely to be accompanied by frequent dissociation of the polymerase from the substrate. Consistent with this idea, the presence of the exosome suppressed accumulation of the polyadenylated substrate in vitro. We speculate that oligo(A) tails added in vivo by TRAMP are never normally extended to the length at which the poly(A) binding protein Pab1p would bind, unless exosome activity is compromised. In the absence of exosome activity, oligo(A) tails added by TRAMP may act as primers for polyadenylation by Pap1p. One possibility is that elongation of the tail to a length that allows Pab1p binding stimulates rapid polyadenylation by Pap1p.

It is conceivable that there are situations in which TRAMP can generate functional mRNA poly(A) tails, either alone or by initiating poly(A) addition by Pap1p. For example, strains carrying *rna14-1* mutations are defective for mRNA 3′ end cleavage and polyadenylation. This leads to formation of long 3′-extended transcripts that are rapidly degraded by the core exosome (Torchet et al., 2002). In *rna14-1* strains that also lack Rrp6p, a posttranscriptional polyadenylation activity generates functional mRNAs, and this is a good candidate for a TRAMP-dependent biosynthetic activity. In this context, it is noteworthy that a substantial number of sequenced human cDNAs have poly(A) tails at locations that lack the signature upstream AAUAAA sequence or close variants (see, for example, Beaudoing et al. [2000]). This suggests that polyadenylation at these sites may result from an alternative pathway.

Conclusions

The exosome is well conserved in all eukaryotes tested, and homologs of Mtr4p, Trf4/5p, and Air1/2p are each present in plant, fly, and human genomes, indicating that the pathway of RNA processing and degradation described here is likely to be evolutionarily conserved. Moreover, RNA oligoadenylation also occurs in *E. coli*, where it activates both stable RNA and mRNA degradation by 3′ exonucleases (Li et al. [1998], Li et al. [2002], Mohanty and Kushner [2002]; reviewed in Dreyfus and Regnier [2002], Kushner [2002]). We speculate that the ancestral role of poly(A) addition was the stimulation of exonuclease degradation, and this function is maintained in modern bacteria. Following the appearance of the nuclear envelope, the ancestral function of oligoadenylation in degradation was retained in the eukaryotic nucleus, while longer poly(A) tails in the cytoplasm took on distinctly different functions in mRNA stability and translation.

Experimental Procedures

Strains and Media

Strains were grown in YPD medium containing 2% peptone, 1% yeast extract, and 2% glucose or YPGal 2% containing 2% peptone, 1% yeast extract, and 2% galactose. Strains of *S. cerevisiae* used in this study are listed in Table S2. Transformation was performed as described (Gietz et al., 1995).

Two-Hybrid Analyses

The full-length ORF of YJL050w was amplified from *S. cerevisiae* genomic DNA using primers S633 and S634 (see Table S2). The PCR product was digested with Sfi1 and cloned into Sfi1-digested

vector pB27 (lexA, C-terminal fusion) supplied by Hybrigenics SA (Paris). Correct cloning was confirmed by sequencing. Two-hybrid screening of a *S. cerevisiae* genomic library was performed by Hybrigenics under the auspices of EU grant QLG2-CT-2001-01554 using techniques modified from (Fromont-Racine et al., 1997).

In Vivo Analyses

Yeast RNA extraction and Northern analysis were performed as described (Tollervey, 1987). Oligonucleotide probes used are listed in Table S3.

Deadenylation of RNA

RNA (20 μg total) was hybridized to 500 ng $(dT)_{18}$ in 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl by slow cooling from 68°C to 30°C. The mixture was adjusted to 37 mM Tris-HCl (pH 7.5), 83 mM NaCl, 6.7 mM EDTA, 1 mM DTT, and 30 μg/ml BSA and digested for 1 hr at 30°C with 1U RNase H. Samples were resolved on an 8% acrylamide urea gel. Poly(A)$^+$ RNA was isolated using an mRNA Isolation Kit System IV (Promega).

Protein Purification

Initial TAP purifications were based on the protocol of (Rigaut et al., 1999). Briefly, yeast strains were grown in 2 l YPD to OD_{600} 2.0–3.0 and harvested by centrifugation. Cells were lysed with glass beads (Ø 0.4–0.6 mm) in 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.1% Nonidet P40, and 150 mM NaCl, including a protease inhibitor cocktail (TMN buffer). The cell lysate was cleared by centrifugation at 30,000 × g for 20 min at 4°C. TAP-tagged yeast proteins were precipitated on an IgG Sepharose column. The protein extract was passed through the resin by gravity flow and washed ten times with 10 ml TMN buffer containing 150 or 500 mM NaCl, including two 5 ml washes with agitation. Proteins were recovered using TEV protease at 1:100 in TMN buffer. The residual protein A tag was recovered by washing with 3% glacial acetic acid. Samples were analyzed by Western blotting.

Following analyses aimed at optimizing TRAMP recovery, the protocol was altered. In the initial protocol, we observed that, despite the addition of 20 mM Tris at pH 7.6, the cell lysate was approximately pH 5.8. This was therefore reset to pH 7.0 by addition of 1 M Tris base prior to column binding. Western blotting showed that the coprecipitation of Mtr4p with Air2p-TAP and Trf4p-TAP was more efficient following binding at 100 mM than with 150 mM NaCl, and the lower concentration was therefore used in the improved protocol. Binding to the IgG column was performed in batch, for 4–5 hr. All preparations used 2 l of culture at OD600 ~ 2, producing ~200 μl of the final TEV fraction.

Proteins were separated by SDS-gel electrophoresis and visualized by Coomassie blue staining. Tryptic peptides from excised bands were prepared for mass spectrometry as described (Shevchenko et al., 1997). Mass spectrometry analysis was carried out on an Applied Biosystems Voyager DE-STR MALDI-TOF instrument using a α-cyano-4-hydroxycinnamic acid (CHCA) matrix. Proteins were identified using the MSDB protein database.

In Vitro Assays

Polyadenylation and RNase assays were performed in buffer containing 20 mM Tris (pH 7.6), 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 100 μg/ml BSA, 0.8 u/μl RNasin (buffer M). Nucleotides were added to 1 mM. Standard RNA polymerization and degradation reactions used 2 μl of the TRAMP and/or 4 μl of the exosome preparation in a 25 μl reaction volume. The yield of exosome and TRAMP showed some variation between preparations, but gel analyses of preparations used in one set of analyses indicated that the exosome was present at 650 pM final concentration and Mtr4p-TAP was present at 7 nM (with the TRAMP complex around 10-fold less abundant), while the RNA substrate was at 13 nM. Reactions were stopped by addition of formamide/EDTA gel loading buffer on ice. The 37 nt transcript was produced by in vitro transcription of XbaI-linearized pBS(–) using T3 RNA polymerase, and the 110 nt tRNA transcript produced by SP6 transcription of a Taq$^{\alpha}$I linearized construct containing the *S. pombe* $tRNA^{Ser}$ (*SupSI*) gene (Krupp et al., 1991). Transcripts were treated with calf intestinal phosphatase and purified on 12% or 6% polyacrylamide gels. The correct-sized RNA band was identified by UV shadowing, excised, and eluted. Eluted RNA was 5′ labeled using polynucleotide kinase and [γ-^{32}P] ATP and gel purified as above. 5′ labeled, poly(A)$^+$ RNA was prepared from prelabeled RNA by incubation in the presence of high-stringency, affinity-purified Trf4p-TAP in buffer M + 1 mM ATP. RNA was phenol/chloroform extracted, ethanol precipitated, and gel purified.

Supplemental Data

Supplemental Data include two figures and three tables and can be found with this article online at http://www.cell.com/cgi/content/full/121/5/713/DC1/.

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