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A Nuclear Surveillance Pathway for mRNAs with Defective Polyadenylation¶

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The *pap1-5* mutation in poly(A) polymerase causes rapid depletion of mRNAs at restrictive temperatures. Residual mRNAs are polyadenylated, indicating that Pap1-5p retains at least partial activity. In *pap1-5* strains lacking Rrp6p, a nucleus-specific component of the exosome complex of 3′-5′ exonucleases, accumulation of poly(A)$^+$ mRNA was largely restored and growth was improved. The catalytically inactive mutant Rrp6-1p did not increase growth of the *pap1-5* strain and conferred much less mRNA stabilization than *rrp6*Δ. This may indicate that the major function of Rrp6p is in RNA surveillance. Inactivation of core exosome components, Rrp41p and Mtr3p, or the nuclear RNA helicase Mtr4p gave different phenotypes, with accumulation of deadenylated and 3′-truncated mRNAs. We speculate that slowed mRNA polyadenylation in the *pap1-5* strain is detected by a surveillance activity of Rrp6p, triggering rapid deadenylation and exosome-mediated degradation. In wild-type strains, assembly of the cleavage and polyadenylation complex might be suboptimal at cryptic polyadenylation sites, causing slowed polyadenylation.

The exosome is a complex of 3′-5′exonucleases that is conserved in eukaryotes (31) and archaea (25). In yeast, nuclear and cytoplasmic forms of the exosome share 10 components. All of these proteins are essential for viability and have sequence homology to known 3′-5′ exoribonucleases, and several have been shown to function as ribonucleases in vitro. Genetic depletion or mutation of any of these proteins results in very similar defects in RNA maturation and degradation (2), and for convenience they are often referred to as the "core" exosome components (reviewed in references 12, 32, and 48). In addition, the cytoplasmic complex is associated with the GTPase Ski7p (3, 49), while the nuclear complex is associated with an additional exonuclease, Rrp6p (2, 11), and a nucleic acid binding protein, Lrp1p/Rrp47p (30, 35).

Ski7p functions together with the core exosome in cytoplasmic mRNA turnover and RNA surveillance pathways (3, 49). In contrast, the functions of Rrp6p and Lrp1p/Rrp47p are distinct from those of the core components of the exosome during nuclear 3′ processing of several small stable RNAs, including the 5.8S rRNA (1, 2, 9, 30, 35, 47). In these cases, Rrp6p specifically processes RNA intermediates that are generated by the activity of the core exosome.

In eukaryotic mRNAs, the 3′ poly(A) tail plays key roles in translation, mRNA stability, and, at least in some cases, nuclear export. The poly(A) tail is added to the 3′ ends of mRNAs by poly(A) polymerase, Pap1p in yeast (34), within a large processing complex in a reaction that is normally coupled to cotranscriptional mRNA cleavage and transcription termination (6, 53; reviewed in references 27 and 37). In some strains with defects in pre-mRNA cleavage, long 3′-extended transcripts that are rapidly degraded by the nuclear exosome are generated (42). In certain cases, subsequent polyadenylation that is uncoupled to pre-mRNA cleavage can apparently generate functional mRNAs from pre-mRNAs that have been 3′ processed by the exosome. Rrp6p is not required for the initial processing of the 3′-extended transcripts (42). However, in strains defective in mRNA cleavage due to the *rna14-1* mutation (28, 29), Rrp6p plays a distinct role in pre-mRNA degradation following initial processing by the exosome, apparently antagonizing polyadenylation. However, recombinant Rrp6p was reported to show no preference for poly(A)$^+$ RNAs in vitro (11), so any direct role in deadenylation is unlikely to result from the intrinsic specificity of the Rrp6p exonuclease activity. A different role for RNA polyadenylation in stimulating nuclear RNA degradation by the exosome has been described recently (21, 22, 50, 52). This involves a distinct nuclear poly(A) polymerase, Trf4p (22, 39, 50, 52).

A previous analysis identified the *rrp6-1* point mutation, which alters a key residue in the catalytic region of Rrp6p, as a suppressor of the temperature-sensitive (TS) lethal mutation *pap1-1* (9, 11, 34, 36). This suggested that Rrp6p, and perhaps the nuclear exosome, plays a role in degrading mRNAs that have failed to undergo polyadenylation. Consistent with this model, Rrp6p was required to restrict mRNAs synthesized in *pap1-1* strains to a nuclear region that was proposed to lie close to the site of transcription (18).

To better define the role of the exosome in the degradation of mRNAs with defects in polyadenylation, we examined poly(A) tail length and mRNA degradation in strains carrying a collection of reported TS lethal alleles of *PAP1* (28). In each

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mutant, mRNA levels were rapidly reduced after transfer to nonpermissive temperature. However, in *pap1-5*, but not *pap1-2*, strains, the reduced level of residual mRNAs appeared to be substantially polyadenylated at the nonpermissive temperature. Further analyses led to the conclusion that the deficiency in mRNAs in the *pap1-5* strain is not due to the inability to synthesize poly(A) tails per se but to an RNA surveillance pathway that triggers nuclear deadenylation and exosome-mediated degradation of the newly synthesized pre-mRNAs.

MATERIALS AND METHODS

Strains, media, and yeast genetics. Strains were grown in YPD medium, containing 2% peptone, 1% yeast extract, and 2% glucose, or YPGal, containing 2% peptone, 1% yeast extract, and 2% galactose. Transformation was performed as described previously (17), except that 6% dimethyl sulfoxide was added prior to heat shock and the final pellet was resuspended in 0.15 M NaCl. For the strains of *Saccharomyces cerevisiae* used in this study, see Table S1 in the supplemental material. To make strain YCA42 (*pap1-5*/*rrp6*Δ), the *RRP6* open reading frame was replaced by *Kluyveromyces lactis URA3* in strain *pap1-5* by using primers 5′RRP6::URA (849) (see Table S2 in the supplemental material for the sequence) and 3′RRP6::URA (850). Transformants were selected for Ura^+ prototrophy and analyzed by Northern blotting for 5.8S rRNA processing defects. To make strains YCT56 (*pap1-5*/*GAL*::*rrp41*) and YCT59 (*pap1-5*/*rrp6*Δ/*GAL*::*rrp41*), the HIS3-GAL10-ProtA-RRP41 cassette was amplified by PCR from strain P118 with primers RRP41-1 (842) and RRP41-2 (843) and transformed into strains *pap1-5* and YCA42. Correct gene deletion was confirmed by analysis of the 5.8S rRNA processing defect. Strain YCT83 (*pap1-5*/*mtr3-1*) was obtained by sporulation of diploids resulting from crossing *pap1-5* with YCT73. The KAN-GAL-3HA-MTR4 construct was generated from strain YCBA81 by one-step PCR (22a) in strain *pap1-5* with primers MTR4-F4 (991) and MTR4-R3 (992). Transformants were selected for kanamycin resistance and analyzed by Northern blotting for 5.8S rRNA processing defects. One transformant, YCT109 (*pap1-5*/*GAL*::*MTR4*), was selected. To make strains YCT68 (*ski7*Δ) and YCT71 (*pap1-5*/*ski7*Δ), the *KAN*::*ski7* cassette was amplified by PCR from strain Y01852 (EUROSCARF) with primers SKI7-1 (993) and SKI7-2 (994) and transformed into D270 and *pap1-5*, respectively. Correct integration was confirmed by PCR. To make strains YLM122 (*ccr4*Δ) and YLM124 (*pap1-5*/*ccr4*Δ), the *KAN*::*ccr4* cassette was amplified by PCR from strain Y00387 (EUROSCARF) with primers CCR4-1 (1101) and CCR4-2 (1102) and transformed into D270 and *pap1-5*, respectively; correct integration was confirmed by PCR. To make strains YLM127 (*pan2*Δ) and YLM129 (*pap1-5*/*pan2*Δ), the *KAN*::*pan2* cassette was amplified by PCR from strain Y04461 (EUROSCARF) with primers PAN2-1 (1104) and PAN2-2 (1105) and transformed into D270 and *pap1-5*, respectively; correct integration was confirmed by PCR. Strain YLM121 (*pap1-5*/*rrp6-1*) was obtained by sporulation of diploids resulting from crossing *pap1-5* with a strain carrying the *rrp6-1* allele (11).

RNA extraction and analysis. RNA extractions were performed as described previously (41). Seven micrograms of total RNA was analyzed for each sample. Small RNAs were separated on a 6% acrylamide gel containing 8.3 M urea and transferred to a Hybond N^+ membrane by electrotransfer. High-molecular-weight RNAs were analyzed on 1.2% agarose gels and transferred by capillary elution. For the oligonucleotides used, see Table S2 in the supplemental material.

For poly(A) tail length analysis of mRNA, 7 μg of total RNA was digested with 10 μg of RNase A and 250 units of RNase T1 in 10 mM Tris, pH 8, 300 mM NaCl. The digestion was stopped by adding 10 mM EDTA, 0.25% sodium dodecyl sulfate, 25 μg/ml proteinase K, and 0.5 mg/ml glycogen. Samples were precipitated, and then 3′ end labeling of the poly(A) tails was carried out overnight at 4°C with 10 μCi [^{32}P]pCp (cytidine-3′,5′-bisphosphate) and 40 units of T4 RNA ligase in 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 3.3 mM dithiothreitol, 10 μg/ml bovine serum albumin, and 10% dimethyl sulfoxide. Samples were then phenol-chloroform extracted and precipitated, and electrophoretic separation was analyzed on a 12% acrylamide-8 M urea gel. For total poly(A) tail analysis, 7 μg of total RNA was extensively hydrolyzed with RNase A and RNase T1. Following ethanol precipitation to remove free nucleotides, residual poly(A) tracts were 3′ end labeled with [^{32}P]pCp and RNA ligase and resolved on a 12% polyacrylamide gel containing 8 M urea. Similar results were obtained with two independent experiments.

RNase H treatment. Deadenylation was performed essentially as described previously (33). Samples (20 μg) of RNA were annealed with 400 ng oligo(dT) at 68°C for 10 min and digested with 1.5 U RNase H at 30°C for 1 h.

RESULTS

In *pap1-5* mutant strains, poly(A)$^+$ mRNAs are degraded by the nuclear exosome. The *pap1-2* and *pap1-5* mutations each result in tight TS lethality at 37°C, but they have little effect on growth at 23°C (28). Both alleles have multiple mutations, and it is not clear which of them give rise to the TS phenotype. The lengths of the poly(A) tracts present in total RNA were assessed by 3′ labeling with [^{32}P]pCp following digestion with RNase A and RNase T1 (see Materials and Methods). During growth at 23°C, little difference was seen in the average poly(A) tail length between the *pap1-2* and *pap1-5* strains, both of which showed maximal poly(A) tail lengths around 20 nucleotides shorter than that of the wild type (Fig. 1A). Following transfer to 37°C for 30 min, the *pap1-2* strain retained only low levels of poly(A) (Fig. 1A, lane 5). In contrast, *pap1-5* strains retained substantial poly(A) tracts (Fig. 1A, lane 6). The signal strength was reduced at 37°C relative to 23°C, consistent with overall loss of mRNAs, but the maximal tail length distribution was not greatly shortened (see Fig. S1 in the supplemental material for PhosphorImager quantification of these data). Similar poly(A) length distribution was seen even after 90 min at 37°C (data not shown). This result would be consistent with synthesis of a reduced level of poly(A)$^+$ RNAs, which continue to be normally deadenylated in the cytoplasm. We conclude that the *pap1-5* strain, but not the *pap1-2* strain, retains significant polyadenylation activity at 37°C. This suggested that lethality in the *pap1-5* strain did not result simply from an inability to generate poly(A) tails.

The *pap1-2* and *pap1-5* alleles were combined with deletion of the *RRP6* gene. RNAs from the single- and double-mutant strains were analyzed by Northern blotting 2 h after transfer to 37°C (Fig. 1B). Increased *CYH2* and *ACT1* mRNA levels were seen for the *pap1-5 rrp6*Δ strain relative to the *pap1-5* single mutant. In contrast, no increases were seen in the *pap1-2 rrp6*Δ strain relative to *pap1-2* alone. Quantification is shown for *CYH2* transcript and is standardized relative to scR1 RNA, a component of the cytoplasmic signal recognition particle.

These observations suggested a role for the nuclear exosome in the degradation of newly synthesized poly(A)$^+$ pre-mRNAs in the *pap1-5* mutant strain. To confirm the nuclear localization of this degradation, the *pap1-5* allele was also combined with a deletion of the gene encoding the cytoplasmic exosome component Ski7p, which is required for 3′ degradation of cytoplasmic mRNAs (3, 5, 45, 49). No clear mRNA stabilization was conferred by the absence of Ski7p, and no truncated RNA species were observed (Fig. 1C).

The loss of mRNA from the *pap1-5* strain was assessed during a time course following transfer to 37°C. Several mRNAs tested (*ACT1*, *CYH2*, *RPL25*, and *MFA2* and *RPL30*, *RPS26a*, and *CYC1*) (Fig. 2A and B, lanes 8 to 14, and data not shown) were all progressively depleted at 37°C in the *pap1-5* strain, indicating that accumulation of new mRNA was inhibited. However, even at late time points, residual mRNAs were present in the *pap1-5* strain, indicating a reduced level of

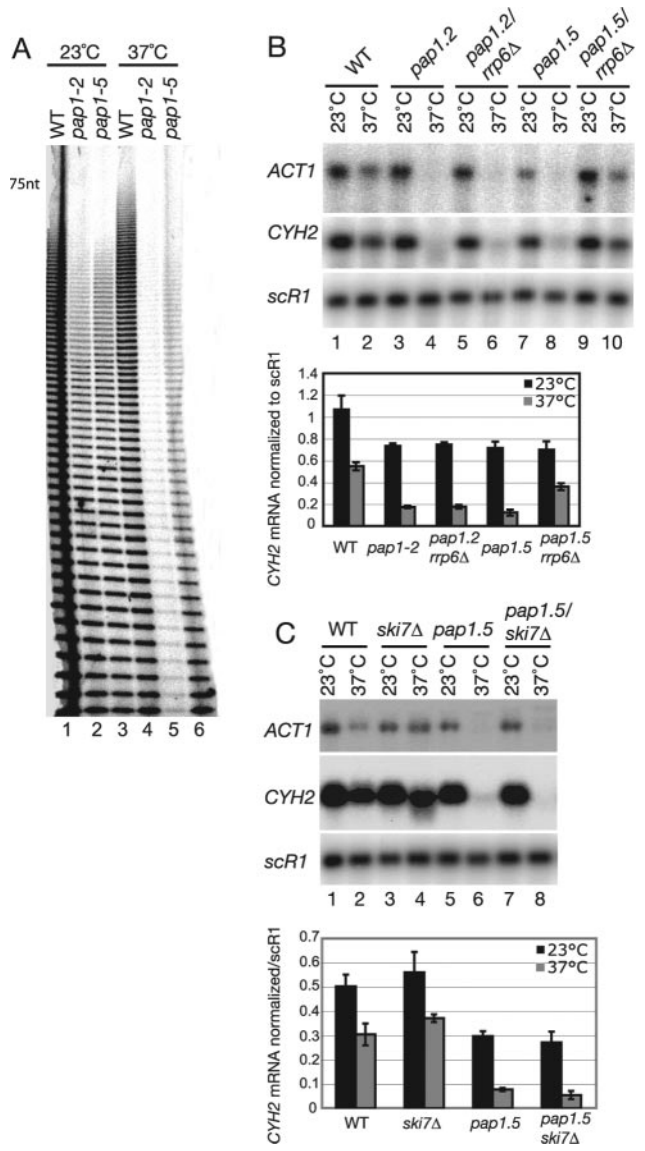

FIG. 1. The *pap1-5*, but not *pap1-2*, mutation allows poly(A) synthesis at the nonpermissive temperature and is suppressed by loss of Rrp6p. (A) Poly(A) tail length analysis of mRNAs from strains carrying the *pap1-2* and *pap1-5* mutations. Poly(A) tracts present in 7 μg of total RNA were labeled and analyzed on a 12% acrylamide-8 M urea gel. The size marker was tRNA from end-labeled total RNA. WT, wild type. (B and C) Northern blot analyses. The strains indicated were grown on glucose medium at 23°C (23°C lanes) and then shifted to 37°C for 2 h (37°C lanes). For each lane, 7 μg of total RNA was separated on a 1.2% agarose-formaldehyde gel and analyzed by Northern hybridization using the probes indicated on the left. The graphs show mean values ± standard deviations for the *CYH2* transcript, obtained from PhosphorImager quantification of three independent experiments and normalized to the scR1 loading control.

ongoing mRNA synthesis. Quantification is shown for the *CYH2* transcript, standardized to scR1 RNA.

This conclusion was greatly strengthened by analysis of the *pap1-5 rrp6*Δ strain (Fig. 2A and B, lanes 15 to 21). For all mRNAs tested, synthesis in the *pap1-5* strain at 37°C was substantially increased by the absence of Rrp6p. The *rrp6*Δ mutation alone did not strongly affect these mRNA species (data not shown).

The *pap1-5* mutation was also combined with *GAL*::*RRP41* to allow depletion of the core exosome component Rrp41p. In *pap-1-5* strains depleted of Rrp41p, a substantially different phenotype was observed (Fig. 2A and B, lanes 22 to 29). For most mRNAs tested, truncated RNA species were observed that migrated at positions below the size range of mRNAs in the wild type. Such truncated RNAs are not seen in strains lacking only Rrp41p (data not shown; see references 8 and 42), showing that they are a consequence of some defect in mRNA synthesis in the *pap1-5* strain. An exception was the *ACT1* (actin) mRNA, for which truncated RNAs were not detected by Northern hybridization of the full-length mRNA but were observed following truncation by RNase H cleavage (data not shown). A 3′-extended RNA species was visible in strains lacking Rrp41p. This RNA was also detected with a probe to the *ACT1* 3′ flanking sequence (data not shown). An increased level of this RNA species was previously observed in the *pap1-1* strain (26).

In the *pap1-5 GAL*::*RRP41* strain, very rapid loss of many mRNAs was seen after transfer to 37°C. This effect was not dependent on the *pap1-5* mutation and was also seen in *PAP1*$^+$ strains depleted of core exosome components (data not shown) or the exosome cofactor Mtr4p (see Fig. 4). In some preparations, a substantial decline in mRNA levels was also seen in wild-type strains. The mechanism underlying these precipitous reductions in cytoplasmic mRNA levels is still unclear. The mRNAs detected at later time points are presumably synthesized de novo at the nonpermissive temperature, confirming the continued synthesis of poly(A)$^+$ RNAs.

The *pap1-5* strains were also tested for decay of heat shock mRNAs, for which a pseudo-pulse-chase analysis can be performed by induction at 42°C, followed by incubation at 37°C (Fig. 2C). *SSA3* showed robust induction in the *pap1-5* strain (Fig. 2C, lanes 7 to 12). The absence of Rrp6p from the *pap1-5* strain increased the expression of *SSA3* (Fig. 2C, lanes 13 to 18). Truncated forms of *SSA3* were also evident in the *pap1-5* strain depleted of Rrp41p (Fig. 2C, lanes 19 to 24).

These results indicated that the exosome components Rrp6p and Rrp41p play distinct roles during mRNA degradation in the *pap1-5* strain. To determine whether Rrp6p acts prior to Rrp41p and the core exosome, we constructed a *pap1-5* strain that lacked Rrp6p and could be depleted of Rrp41p (Fig. 2A and B, lanes 30 to 37, and Fig. 2C, lanes 25 to 30). The absence of Rrp6p suppressed accumulation of the truncated RNA species, which were seen in the *pap1-5* strain depleted of Rrp41p alone, for most mRNAs tested, *CYH2* and *SSA3* (Fig. 2A to C) and *CYC1* and *RPL30* (data not shown). In the experiment shown in Fig. 2, the absence of Rrp6p plus Rrp41p from *pap1-5* had little effect on the *RPL25* mRNAs relative to depletion of Rrp41p alone (Fig. 2B). However, loss of the truncated *RPL25* species was evident in other experiments; the reason for this variability is still unclear.

It is notable that the reduction in mRNA levels in *pap1-5* mutant strains, and the degree of restoration in *rrp6*Δ mutants, showed substantial variation for different mRNA species. Heterogeneity has been seen in the nuclear degradation of unspliced pre-mRNAs (8). As in the cytoplasm, mRNA degradation in the nucleus apparently proceeds at species-specific rates

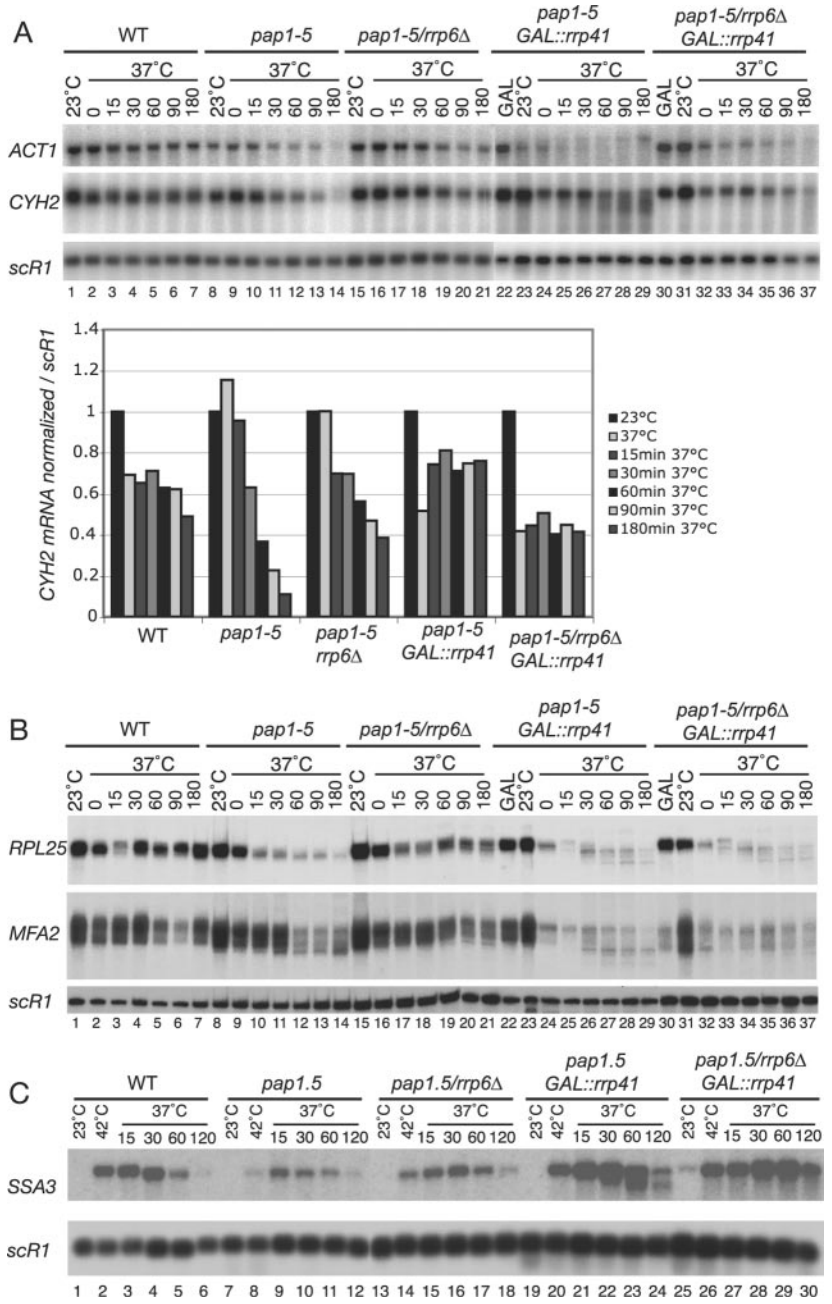

FIG. 2. Rrp6p acts prior to Rrp41p in the same degradation pathway. (A and B) Total RNA was extracted from the wild-type (WT), *pap1-5*, and *pap1-5*/*rrp6*Δ strains grown on glucose medium at 23°C and after shift to 37°C for the times indicated. Strains *pap1-5*/*GAL*::*rrp41* and *pap1-5*/*rrp6*Δ/*GAL*::*rrp41* were pregrown in galactose medium at 23°C (GAL lanes), transferred to glucose medium at 23°C for 20 h (23°C lanes), and then shifted to 37°C for the times indicated. Northern blot analysis was performed on 7 μg of total RNA separated on a 6% acrylamide-8.3 M urea gel (A) or a 1.2% agarose-formaldehyde gel (B). The graph shows levels of the *CYH2* transcripts obtained by PhosphorImager quantification of the data presented in panel A normalized to the scR1 loading control. Values obtained at 23°C were arbitrarily set as 1. (C) Northern blot of heat shock-inducible mRNA. The wild-type, *pap1-5*, and *pap1-5*/*rrp6*Δ strains were pregrown on glucose medium at 23°C. The *pap1-5*/*GAL*::*rrp41* and *pap1-5*/*rrp6*Δ/*GAL*::*rrp41* strains were pregrown in galactose medium at 23°C and transferred to glucose medium at 23°C for 20 h (23°C lanes). All strains were then shifted to 42°C for 15 min (42°C lanes), followed by transfer to 37°C for the times indicated.

and shows variations in response to mutations in the degradation machinery. This presumably reflects differences in RNP structure.

mRNAs present in the *pap1-5* strain at nonpermissive temperature are polyadenylated. To assess the polyadenylation states of mRNAs present in the *pap1-5* strains at 37°C, deadenylation was performed in vitro using RNase H and oligo(dT) (Fig. 3A; quantification is shown for the *RPL25* and *RPL30* transcripts in Fig. 3B). Deadenylation of the wild-type samples resulted in increased in-gel mobility and more coherent RNA distribution for the *RPL25*, *RPL30*, and *MFA2* mRNAs, as expected. This was also the case for RNA in the *pap1-5 rrp6*Δ

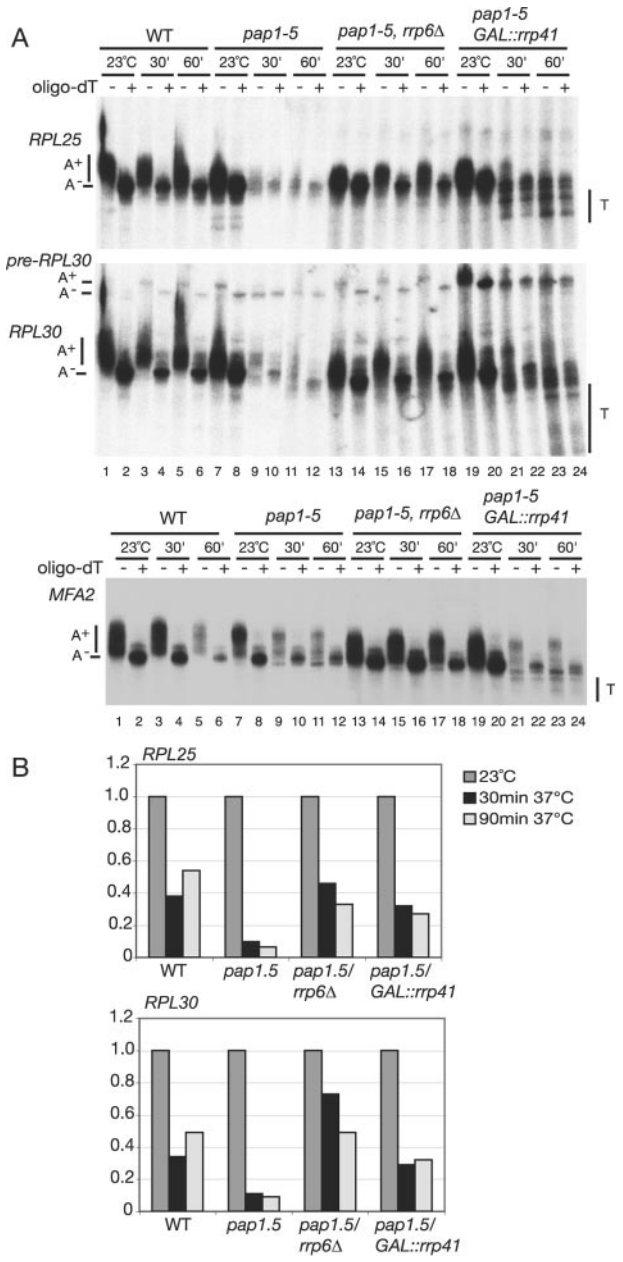

FIG. 3. Specific mRNAs in the *pap1-5* strain are polyadenylated. (A) Total RNA was extracted from the wild-type (WT), *pap1-5*, and *pap1-5*/*rrp6*Δ strains and grown in glucose medium for 30 min after transfer to 37°C. The *pap1-5*/*GAL*::*rrp41* strain was pregrown in galactose medium at 23°C, transferred to glucose medium at 23°C for 20 h, and then shifted to 37°C for 30 min. Samples were treated with RNase H plus oligo(dT) (+ lanes) and compared with untreated samples (− lanes). Samples were separated on a 6% acrylamide-8.3 M urea gel, transferred to nylon, and hybridized with *RPL25*, *RPL30*, and *MFA2* probes. T, deadenylated and truncated species. (B) PhosphorImager quantification of data from panel A. Deadenylated *RPL25* and *RPL30* mRNA (oligo-dT + lanes) was quantified using a PhosphorImager and standardized to scR1 RNA.

strain at nonpermissive temperature, confirming that polyadenylated mRNAs continue to be synthesized. In the *pap1-5* single mutant, residual *RPL30* and *MFA2* were still polyadenylated at the nonpermissive temperature, but this was less clear for *RPL25*. The *pap1-5* strain depleted of Rrp41p showed little accumulation of poly(A)$^+$ RNA relative to the *pap1-5* single mutant but accumulated deadenylated and truncated species (Fig. 3A).

The size heterogeneity of the mRNA populations complicates quantification of their abundance. We therefore compared the signals obtained for the deadenylated RNAs in Fig. 3A. *RPL25* and *RPL30* mRNA levels were quantified after deadenylation and standardized relative to scR1 RNA. Quantification (Fig. 3B) showed that even in the wild type, total levels of *RPL25* and *RPL30* mRNAs were reduced following transfer to 37°C. This reduction was substantially greater in the *pap1-5* single mutants but was largely suppressed in the absence of Rrp6p or following depletion of Rrp41p.

We conclude that in the *pap1-5* strain these mRNAs largely undergo Rrp6p-dependent deadenylation followed by Rrp41p-dependent degradation. This suggests that the drastic reductions in mRNA levels seen in the *pap1-5* strain following transfer to 37°C are not primarily due to an inability to synthesize poly(A)$^+$ mRNAs. Rather, the newly synthesized mRNAs are rapidly identified by an RNA surveillance mechanism that requires Rrp6p.

Degradation following deadenylation requires Mtr3p and Mtr4p. To confirm that the phenotypes seen on depletion of Rrp41p were due to defects in the function of the nuclear exosome, the *pap1-5* allele was combined with a TS lethal mutation in the core exosome component Mtr3p and with the *GAL*::*MTR4* allele, which allows genetic depletion of Mtr4p/Dob1p (15), a putative RNA helicase and cofactor for the nuclear exosome (Fig. 4). In the *pap1-5 mtr3-1* double-mutant strain, loss of the polyadenylated mRNA after transfer to 37°C was accompanied by accumulation of deadenylated and truncated mRNAs (Fig. 4, lanes 22 to 28). Quantification is shown for the *CYH2* mRNA and is standardized relative to scR1 RNA.

In the Mtr4p-depleted strains, the *RPL30* and *RPL25* mRNAs were very rapidly lost following transfer to 37°C. In the *pap1-5* strain depleted of Mtr4p, the appearance of de novo-synthesized mRNAs that were deadenylated and truncated was seen at later time points (Fig. 4, lanes 37 to 44). This phenotype closely resembles that seen in the *pap1-5* strain depleted of Rrp41p (Fig. 2).

We conclude that in *pap1-5* strains newly synthesized poly(A)$^+$ mRNAs are rapidly deadenylated, followed by 3′→5′ degradation by the nuclear exosome, acting together with its cofactor Mtr4p.

Deadenylation does not require the catalytic activity of Rrp6p. In vitro analyses have shown that the residue altered in the *rp6-1* allele is critically required for catalysis, and the mutant protein is therefore unlikely to show exonuclease activity in vivo (11, 36). To determine whether Rrp6p is directly responsible for mRNA deadenylation in the *pap1-5* strain, a *pap1-5 rrp6-1* strain was constructed. The levels of the *RPL30* and *RPS26a* mRNAs were mildly elevated in the *pap1-5 rrp6-1* strain relative to *pap1-5* alone, but *rrp6-1* had much less effect than *rrp6*Δ (Fig. 5A and B) and other mRNAs. The exonuclease activity of Rrp6p may participate in deadenylation but is apparently not required for degradation to occur.

In growth tests in liquid culture, the absence of Rrp6p partially suppressed the growth defect of strains carrying *pap1-5* at

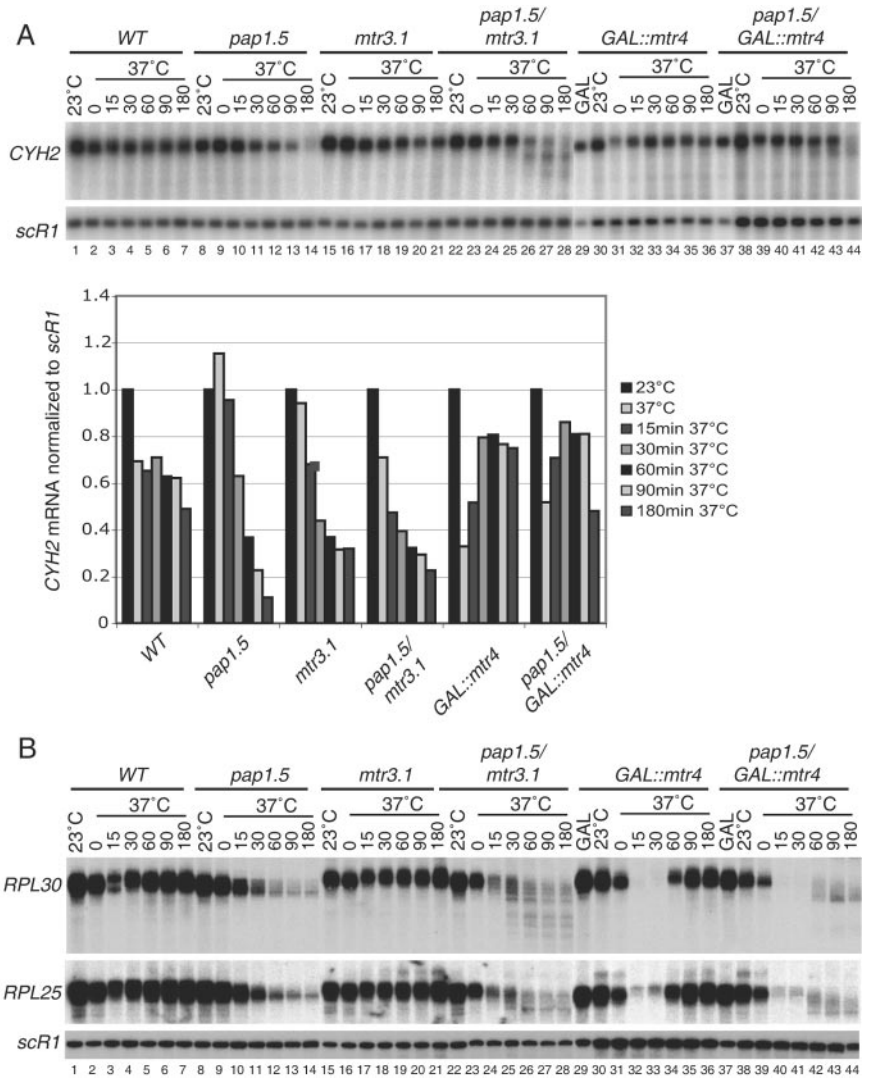

FIG. 4. 3′ degradation requires Mtr3p and Mtr4p. (A and B) Total RNA was extracted from the wild-type (WT), *pap1-5*, *mtr3-1*, and *pap1-5*/*mtr3-1* strains grown on glucose medium at 23°C and after shift to 37°C for the times indicated. Strains *GAL*::*mtr4* and *pap1-5*/*GAL*::*mtr4* were pregrown in galactose medium at 23°C (GAL lanes), transferred to glucose medium at 23°C for 20 h (23°C lanes), and then shifted to 37°C for the times indicated. Northern blot analysis was performed on 7 μg of total RNA separated on a 6% acrylamide-8.3 M urea gel (A) or a 1.2% agarose gel (B). The graph shows levels of the *CYH2* transcripts obtained by PhosphorImager quantification of the data presented in panel A, normalized to the scR1 loading control. Values obtained at 23°C were arbitrarily set as 1.

either 34°C or 37°C (shown for 37°C in Fig. 6). The *rrp6*Δ single-mutant strain is impaired in growth, but despite this, the *rrp6*Δ *pap1-5* strain clearly grew better than the *pap1-5* single mutant. In contrast, growth of the *rrp6-1 pap1-5* strain was indistinguishable from that of the strain with *pap1-5* alone, consistent with the low level of suppression seen in Northern analyses. These results suggest that Rrp6p has an RNA surveillance function that is distinct from its exonuclease activities and is required to identify mRNAs synthesized in the *pap1-5* strain as being defective and to target them for degradation.

Two deadenylase complexes characterized in yeast are the Ccr4p-Caf1p-Not complex, which is probably the major cytoplasmic deadenylase, and Pan2p-Pan3p, which has been implicated in nuclear poly(A) length control (7, 10, 14, 43, 44). To assess their participation in deadenylation, *ccr4*Δ and *pan2*Δ mutations were each combined with *pap1-5*. Neither mutation conferred significant mRNA stabilization in the *pap1-5* strain (see Fig. S2 and S3 in the supplemental material). Consistent with this, neither double mutant showed increased growth in liquid culture relative to *pap1-5* alone (data not shown). The combination of *pan2*Δ with *rrp6*Δ and *pap1-5* in a triple-mutant strain failed to increase mRNA synthesis or growth relative to the *rrp6*Δ *pap1-5* double mutant (data not shown; see Fig. S2 in the supplemental material).

DISCUSSION

Nuclear mRNAs are initially deadenylated and then degraded by the exosome. We had anticipated that mRNAs synthesized at the nonpermissive temperature in strains with de-

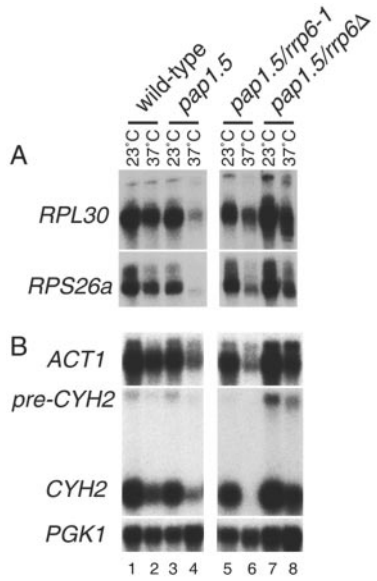

FIG. 5. mRNA levels in *pap1-5* strains lacking the exonuclease activity of Rrp6p. RNA was extracted from the strains indicated growing at 23°C and 1 h after transfer to 37°C. (A) RNA separated on 8% polyacrylamide-urea gel. (B) RNA separated on 1.2% agarose gels.

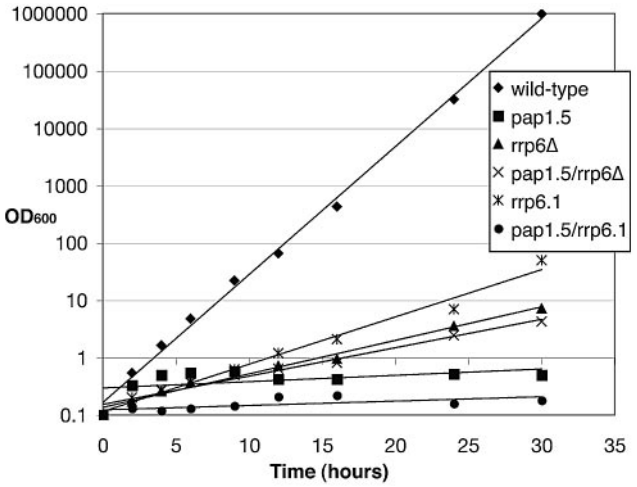

FIG. 6. Growth curves obtained following transfer to 37°C. Cells were pregrown in rich YPD medium at 23°C and transferred to 37°C at time zero. The cells were maintained in exponential growth by addition of prewarmed medium. OD_{600}, optical density at 600 nm.

fects in poly(A) polymerase would predominantly lack poly(A) tails. However, the analysis of total poly(A) RNA and individual mRNAs indicates that the TS lethal *pap1-5* strain retains substantial polyadenylation activity in vivo at the restrictive temperature. The loss of mRNAs from *pap1-5* strains is apparently due to the targeting of newly synthesized, polyadenylated mRNAs for nuclear degradation. The mRNAs are initially deadenylated by an activity that requires the nucleus-specific exosome component Rrp6p and then 3′ degraded by the core exosome, acting together with the nuclear RNA helicase Mtr4p (Fig. 7). In contrast, the *pap1-2* allele retained little poly(A)$^+$ RNA at the nonpermissive temperature, and mRNA synthesis was not clearly restored by loss of Rrp6p. While we cannot exclude the possibility that poly(A) tails detected in the *pap1* strains at nonpermissive temperature are synthesized by the poly(A) polymerase activity of Trf4p (22, 39, 50, 52), the allele specificity makes this less likely.

Deadenylation of poly(A)$^+$ mRNAs synthesized in the *pap1-5* strain at 37°C required the nuclear exosome component Rrp6p. This contains an exonuclease domain that is related to *Escherichia coli* RNase D and shows 3′-exonuclease activity in vitro (11, 36), suggesting that it might act directly as the deadenylase. However, previous analyses reported that recombinant Rrp6p shows no preference for poly(A)$^+$ RNAs in vitro (11), so any specific role in deadenylation was unlikely to result from its intrinsic activity. The *rrp6-1* point mutation is strongly predicted to inactivate the in vivo exonuclease activity of Rrp6p and phenocopies *rrp6*Δ for defects in stable RNA synthesis (9, 11, 36). However, the presence of the *rrp6-1* mutation conferred little suppression of mRNA synthesis in *pap1-5* strains and did not lead to detectable accumulation of degradation intermediates. Moreover, unlike *rrp6*Δ, the *rrp6-1* mutation conferred no growth suppression in *pap1-5* strains. This indicates that while Rrp6p is required for surveillance of the mRNAs synthesized in the *pap1-5* strain, its exonuclease activity is not required for their degradation. Rrp6p is comprised of two distinct domains, with an amino-terminal exonuclease domain and a C-terminal HRDC (helicase and RNase D C-terminal) domain. The HRDC domain has been proposed to play a regulatory role in Rrp6p function (36) and is likely to have nucleic acid binding activity (20). It is therefore possible that the HRDC domain specifically functions in surveillance in the *pap1-5* strain. Since Rrp6p is apparently not required for mRNA deadenylation, we tested two other characterized yeast deadenylases, Ccr4p and Pan2p (7, 10, 44). However, the decline in the levels of most mRNAs tested following transfer to 37°C was indistinguishable in *pap1-5* strains and *pap1-5 ccr4*Δ or *pap1-5 pan2*Δ double mutants. Moreover, mRNA levels in the *pap1-5 rrp6*Δ *pan2*Δ triple mutant were not different from those in the *pap1-5 rrp6*Δ double-mutant strain. Consistent with this observation, *pan2*Δ also conferred no growth increase in the *pap1-5* strain.

This indicates that Ccr4p and Pan2p are not individually responsible for nuclear deadenylation in the *pap1-5* background. It may be that once an mRNA has been targeted for degradation in an Rrp6p-dependent process, multiple proteins can participate in the deadenylation. During 3′ maturation of the yeast 5.8S rRNA, several 3′ exonucleases participate in the final trimming (9, 31, 46), and this is also the case for many RNA-processing and degradation steps in bacteria (24).

The features that make nuclear pre-mRNAs targets for degradation in *pap1-5* strains have not yet been established. Candidates for features that might be recognized include defects in the structure of the cleavage and polyadenylation machinery, the presence of shortened poly(A) tails, and a reduced rate of polyadenylation. We favor the last possibility and speculate that assembly of the cleavage and polyadenylation machinery at suboptimal, and therefore potentially inappropriate, sites may be correlated with a lower rate of poly(A) addition and/or reduced processivity of the reaction. The low sequence complexity of polyadenylation sites suggests that many potential

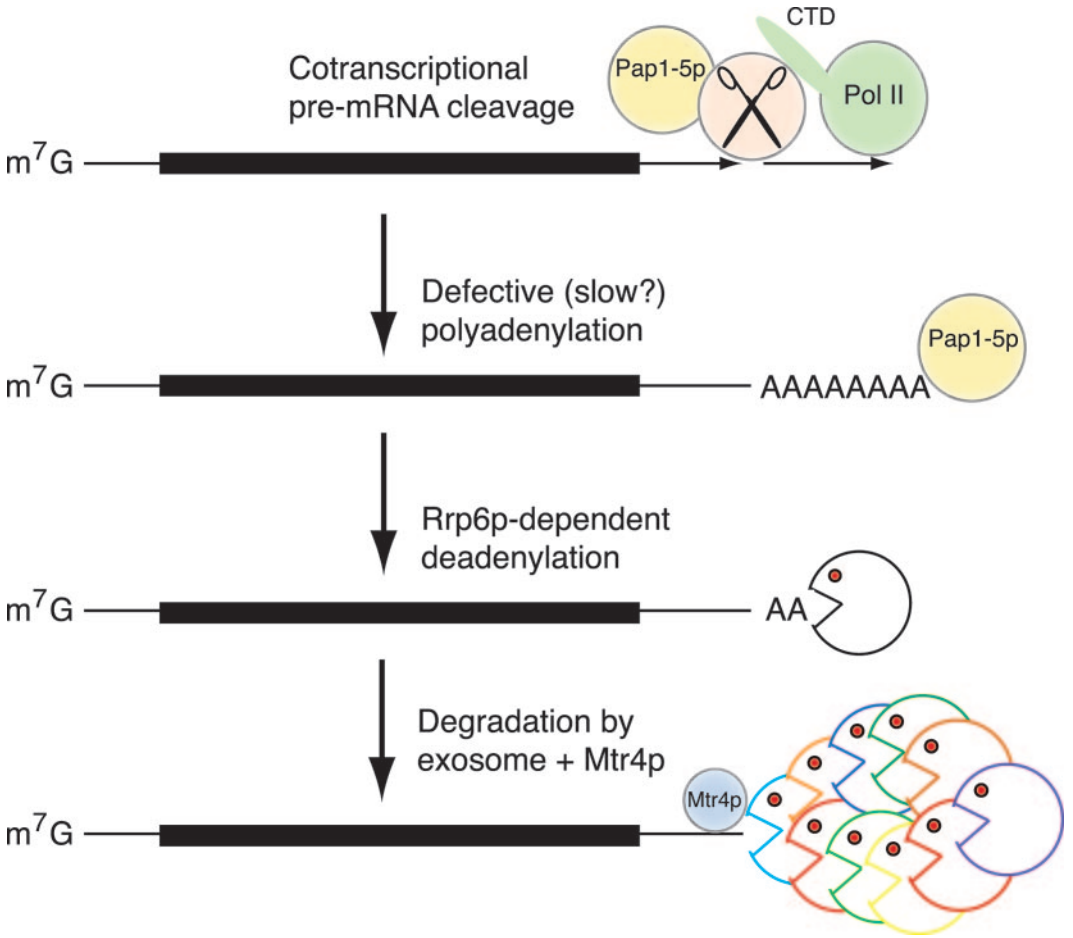

FIG. 7. Model for the degradation of pre-mRNAs in *pap1-5* strains. Several pre-mRNA 3′ cleavage and polyadenylation factors bind to the C-terminal domain (CTD) of RNA polymerase II prior to recognition of the target site on the nascent RNA transcript (reviewed in references 19 and 38). In the strains expressing the partially defective Pap1-5p, cleavage and polyadenylation still occur, but pre-mRNA surveillance is triggered. We speculate that this is a consequence of slowed polyadenylation. Deadenylation of the pre-mRNA requires the nucleus-specific exonuclease Rrp6p. Subsequent degradation requires the nuclear exosome complex indicated by the symbols on the right, and the putative RNA helicase Mtr4p.

cryptic sites exist. Glutathione *S*-transferase-tagged Rrp6p has been reported to coprecipitate with Pap1p from cell lysates (11), indicating that they can physically interact. It is conceivable that prolonged association of Pap1p with the pre-mRNA, due to slowed polyadenylation, might be sufficient to recruit Rrp6p and the exosome.

In multicellular organisms, regulated and alternative poly(A) site choice has been reported and can have important developmental consequences (4, 13, 16, 23, 40). In such cases, the nuclear RNA surveillance pathway we report here may be important in determining the relative levels of the mRNAs synthesized.

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