Regulation of *NAB2* mRNA 3′-end formation requires the core exosome and the Trf4p component of the TRAMP complex

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ABSTRACT

The nuclear exosome functions in a variety of pathways catalyzing formation of mature RNA 3′-ends or the destruction of aberrant RNA transcripts. The RNA 3′-end formation activity of the exosome appeared restricted to small noncoding RNAs. However, the nuclear exosome controls the level of the mRNA encoding the poly(A)-binding protein Nab2p in a manner requiring an A_{26} sequence in the mRNA 3′ untranslated regions (UTR), and the activities of Nab2p and the exosome-associated exoribonuclease Rrp6p. Here we show that the A_{26} sequence inhibits normal 3′-end processing of *NAB2* mRNA in vivo and in vitro, and makes formation of the mature 3′-end dependent on trimming of the transcript by the core exosome and the Trf4p component of the TRAMP complex from a downstream site. The detection of mature, polyadenylated transcripts ending at, or within, the A_{26} sequence indicates that exosome trimming sometimes gives way to polyadenylation of the mRNA. Alternatively, Rrp6p and the TRAMP-associated Mtr4p degrade these transcripts thereby limiting the amount of Nab2p in the cell. These findings suggest that *NAB2* mRNA 3′-end formation requires the exosome and TRAMP complex, and that competition between polyadenylation and Rrp6p-dependent degradation controls the level of this mRNA.

Keywords: RNA processing; TRAMP complex; exosome; mRNA 3′-end formation

INTRODUCTION

Most eukaryotic mRNAs require cleavage and polyadenylation to form their mature 3′-ends (Edmonds 2002). Large complexes of proteins recognize sequence elements in the 3′-untranslated (UTR) regions of nascent RNA polymerase II transcripts leading to cleavage of the precursor mRNAs (pre-mRNAs), which elicits transcription termination, at least in part as a result of the action of the 5′–3′ exonuclease Rat1p/Xrn2p on the portion of the transcript still bound to the paused RNA polymerase (Proudfoot and O'Sullivan 2002; Kim et al. 2004; West et al. 2004; Bentley 2005; Buratowski 2005). The released pre-mRNA undergoes polyadenylation by a process in which specific poly(A)-binding proteins regulate the activity of poly(A)-polymerase and influence the eventual length of the poly(A) tail (Wahle 1991, 1995; Bienroth et al. 1993; Hector et al. 2002; Dheur et al. 2005). The binding of these proteins, as well as others, forms a ribonucleoprotein (RNP) complex that profoundly affects the physical stability of the mRNA in the nucleus, as well as the efficiency of its export to the cytoplasm (Jensen et al. 2003; Rodriguez et al. 2004; Fasken and Corbett 2005).

Defects in mRNA 3′-end processing result in significant defects in pre-mRNA metabolism. Mutations that inhibit cleavage and polyadenylation, either by disrupting 3′-end processing sequences or by decreasing the activity of essential 3′-end formation factors, cause a dramatic decrease in mRNA levels (Patel and Butler 1992; Proweller and Butler 1994; Das et al. 2000; Hilleren et al. 2001; Libri et al. 2002). Partial reversal of this effect by depletion of components of the nuclear exosome revealed the existence of a surveillance system that degrades improperly processed pre-mRNAs (Burkard and Butler 2000; Torchet et al. 2002; Milligan et al. 2005). In this pathway, failure to cleave and/ or polyadenylate pre-mRNAs produces an RNP recognized and degraded by the exoribonucleolytic activities of the

exosome. In some cases, where defects produce 3′ extended transcripts, it appears that release of the transcript as the exosome nears the normal 3′-end processing site allows polyadenylation and the production of functional transcripts (Torchet et al. 2002). Recent evidence showed that this process plays a role in the 3′-end formation of the *CTH2* mRNA in budding yeast (Ciais et al. 2008).

The exosome contains a nine-subunit core complex found in archael cells and in the nucleus and the cytoplasm of eukaryotic cells (Butler 2002; Houseley et al. 2006; Lorentzen and Conti 2006). This complex contains exonucleolytic activity in archaea, but not in humans or budding yeast. Instead, the ribonuclease activity of the core exosome resides in Dis3p/Rrp44p, which acts as an endonuclease and a hydrolytic exonuclease (Dziembowski et al. 2007; Greimann and Lima 2008; Schaeffer et al. 2009; Schneider et al. 2009). Two additional components, the putative helicase Dob1p/Mtr4p and the 3′–5′ exoribonuclease Rrp6p, function with the core exosome in the nucleus (Briggs et al. 1998; de la Cruz et al. 1998; Allmang et al. 1999). A variety of studies indicate that the nuclear exosome plays a role in an RNA surveillance pathway that degrades improperly processed mRNAs, rRNAs, and tRNAs, as well as normal mRNAs retained in the nucleus (Bousquet-Antonelli et al. 2000; Burkard and Butler 2000; Hilleren et al. 2001; Das et al. 2003; Fang et al. 2004; Galy et al. 2004; Kadaba et al. 2004; Kuai et al. 2004). The nuclear exosome, in cooperation with Lrp1p (Rrp47p), also plays a critical role in the 3′-end processing of 5.8S rRNA and many snRNAs and snoRNAs (Allmang et al. 1999; van Hoof et al. 2000; Mitchell et al. 2003; Peng et al. 2003; Phillips and Butler 2003).

In mammalian cells, the cytoplasmic exosome appears to function in the major mRNA degradation pathway, which contrasts with the predominant role of the 5′–3′ pathway of mRNA degradation in yeast (Wang and Kiledjian 2001; Mukherjee et al. 2002; Lejeune et al. 2003). Experiments in mammalian cells indicate that RNA-binding proteins may play a critical role in the degradation of certain normal mRNAs by the cytoplasmic exosome. Specifically, mRNAs carrying destabilizing AU-rich elements (AREs) in their 3′-UTRs require ARE-binding proteins to activate transcript degradation by the exosome (Chen et al. 2001). In some cases, the accumulation of truncated, polyadenylated mRNA intermediates after exosome depletion in mammalian cells implied a role for exosome-mediated surveillance of mRNAs (West et al. 2006).

Surveillance of mRNA 3′-end processing by the nuclear exosome implies that competition may occur between factors involved in mRNA production and degradation (Bousquet-Antonelli et al. 2000; Burkard and Butler 2000; Torchet et al. 2002; Milligan et al. 2005). Indeed, evidence suggested that this competition might act to regulate the concentration of the poly(A)-binding protein Nab2p of *Saccharomyces cerevisiae* (Roth et al. 2005). These experiments showed that the nuclear exosome limits the level of *NAB2* mRNA in a manner dependent on a sequence of 26 consecutive adenosines (A_{26}) in the *NAB2* mRNA 3′-untranslated region. The Nab2 protein also limited the amount of its own mRNA in an A_{26}- and Rrp6p-dependent manner, implying that Nab2p binds this sequence and recruits the exosome to degrade its own transcript. These experiments also provided evidence that the A_{26} sequence might inhibit normal mRNA 3′-end processing of *NAB2* mRNA, thereby exposing the transcript to surveillance by the exosome. In this study, we present evidence that Nab2p acts to repress 3′-end formation of its own mRNA leading to 3′-end formation downstream from the mature 3′-end of the mRNA. The exosome and the TRAMP complex trim this 3′ extended transcript back to the A_{26} sequence at which time Rrp6p and the RNA helicase Mtr4p accelerate degradation of the mRNA, or polyadenylation occurs to produce a stable, mature transcript. We propose that the concentration of Nab2p determines whether this novel mechanism of 3′-end formation results in polyadenylation or degradation of *NAB2* mRNA.

RESULTS

Nab2p inhibits mRNA 3′-end formation of its own mRNA

Previous experiments showed that overexpression of Nab2p caused a decrease in the level of its mature mRNA (Roth et al. 2005). This effect did not occur after replacement of the transcript's 3′-UTR-encoded A_{26} with U_{26}, suggesting that Nab2p binds the A_{26} sequence and destabilizes its own mRNA. Moreover, a loss-of-function mutation (*nab2-1*) in Nab2p caused increased levels of *NAB2* mRNA. In an effort to understand the relative contributions of mRNA degradation and 3′-end formation to *NAB2* mRNA levels, we analyzed the effect of altering the gene dosage of *NAB2* on its own expression. These experiments used a strain with a chromosomal deletion of *NAB2* and a single-copy plasmid bearing either the normal or the *nab2-1* allele of *NAB2*. The strain also contained a reporter plasmid expressing a *GFP-NAB2* fusion with the strong *CYC1* polyadenylation signal 250 nucleotides (nt) 3′ to the A_{26} sequence (Fig. 1A). This plasmid expresses two transcripts—one ending in the *NAB2* 3′-UTR at the A_{26} and the second ending at the *CYC1* poly(A) site. Previous experiments showed that loss of Rrp6p increased the level of the shorter *NAB2* 3′-UTR transcript without changing the level of the longer transcript, suggesting that Rrp6p affects the degradation, but not 3′-end formation of this mRNA (Roth et al. 2005). Here, we analyzed the effect of changing Nab2p levels on production of these two reporter mRNAs. In the presence of a normal copy of *NAB2*, this plasmid produces predominantly transcripts ending at the *CYC1* site (Fig. 1B, lane 1). We placed a stem–loop designed to block translation (Oliveira et al. 1993) upstream of the *GFP-NAB2* start

A

B
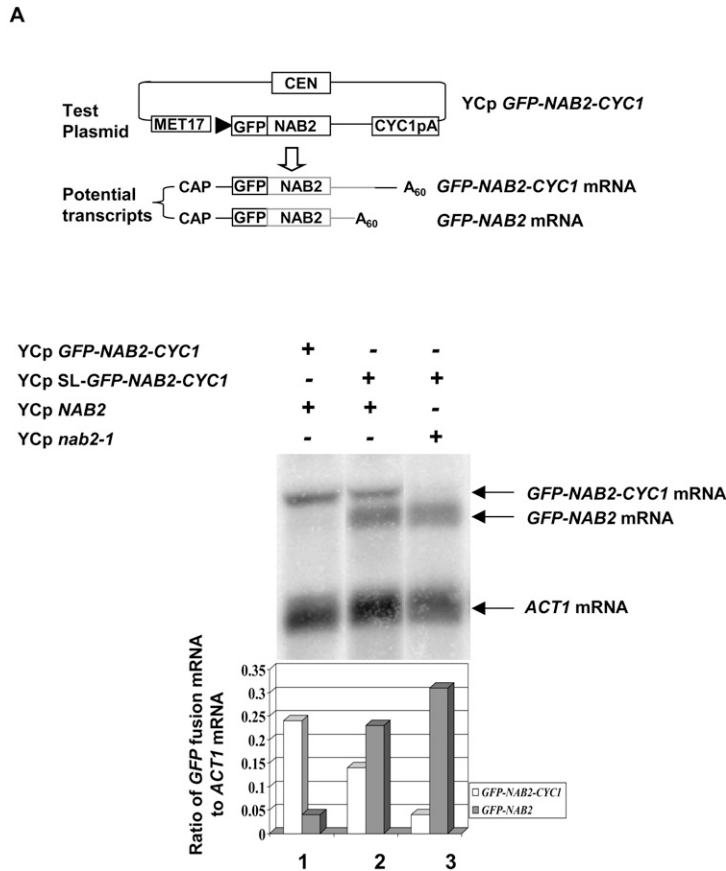
FIGURE 1. Nab2 protein inhibits 3′-end formation of its own mRNA. (*A*) Diagram of the test plasmid, which allows transcription from the *MET17* promoter of the inserted test gene fused to green fluorescent protein (GFP). CYC1pA designates the polyadenylation site of *CYC1*. The potential transcripts, distinguished by their different 3′-ends, are shown *below* the diagram of the test plasmid. (*B*) Strain YSB501 (YCp*NAB2*) or YSB502 (YCp*nab2-1*) carrying the indicated plasmids was grown in SCD–URA-LEU at 30°C; total RNA was isolated and the levels of *GFP-NAB2* transcripts determined by Northern blot analysis with an oligonucleotide probe complementary to the *GFP* portion of the mRNA. YCpSL-*GFP-NAB2-CYC1* designates the use of the reporter plasmid with the stem–loop insertion in the 5′-UTR of the reporter mRNA. The bar graph *below* reports the ratio of the *GFP*-fusion transcripts to *ACT1* mRNA and is based on two independent measurements.

the transcript ending at the *CYC1* site (Fig. 1B, lane 3). Thus, a further decrease in Nab2p levels results in nearly all of the 3′-end formation occurring at the *NAB2* site. These findings indicate that as the activity of Nab2p increases in the cell, it inhibits 3′-end formation at its own site and destabilizes its own mRNA.

The *NAB2* mRNA 3′-end maps to the A_{26} sequence in the 3′-UTR

We analyzed the *NAB2* mRNA 3′-ends using the ligation-mediated poly(A) tail assay (LM-PAT) (Salles et al. 1999). We carried out LM-PAT using mRNA-specific primers to *RPL3* (previously called *TCM1*) mRNA (as a positive control) and *NAB2* mRNA, and then separated the products by gel electrophoresis to compare the size of the *NAB2* products with that of the *RPL3* products (Fig. 2A). Previous experiments mapped the polyadenylation site of *RPL3* mRNA (Proweller and Butler 1994), and the LM-PAT procedure yielded the expected ∼375–450-bp products (Fig. 2A, lane 2). The length heterogeneity of these products reflects the known heterogeneity of mRNA poly(A) tails in yeast and confirms that the LM-PAT procedure preserves their lengths. LM-PAT using a *NAB2*-specific primer (OSB217) (Fig. 2B) produced DNA products ∼250–350 bp in length, consistent with 3′-end formation of *NAB2* mRNA near the A_{26} sequence within the 3′-UTR (Fig. 2A, lanes 3,4). The results also show increased amounts of *NAB2* cDNAs in the *rrp6*-Δ strain compared with the *RRP6* strain, consistent with the increase in *NAB2* mRNA levels in *rrp6* mutants (Fig. 2A, lane 4). LM-PAT of *NAB2* also produced several longer DNA products, but subsequent DNA sequencing did not reveal any similarity of these products to the *NAB2* locus.

The DNA sequence of several *NAB2* LM-PAT products suggested that the majority end within the A_{26} sequence and sometimes carry more than the template-encoded adenosines (data not shown). However, the presence of the A_{26} sequence could cause artifacts in this procedure, so we determined the *NAB2* mRNA 3′-end formation site by sequencing circular reverse transcription-polymerase chain reaction (cRT-PCR) products (Couttet et al. 1997; Mullen and Marzluff 2008). Total RNA samples from the *RRP6* strain YSB3001 were decapped and then circularized with

codon and found that this mutation abolished the presence of Gfp-Nab2p in the nucleus of cells bearing the plasmid (data not shown). The stem–loop mutation resulted in a sixfold increase in the level of the transcript ending at the *NAB2* site and twofold decrease in the level of the transcript ending at the *CYC1* site (Fig. 1B, lane 2). Thus, the decrease in the level of Gfp-Nab2p caused by blocking translation of the plasmid-borne gene fusion results in a significant increase in the level of stable transcript produced from the reporter plasmid and, consistent with published results (Roth et al. 2005), indicates that the normal plasmid produces Gfp-Nab2p, which functions as a negative regulator of its own expression. Expression of the stem–loop *GFP-CYC1* plasmid in a strain carrying the *nab2-1* mutation caused a 1.3-fold increase in the level of the transcript ending at the *NAB2* site, and a fourfold decrease in production of
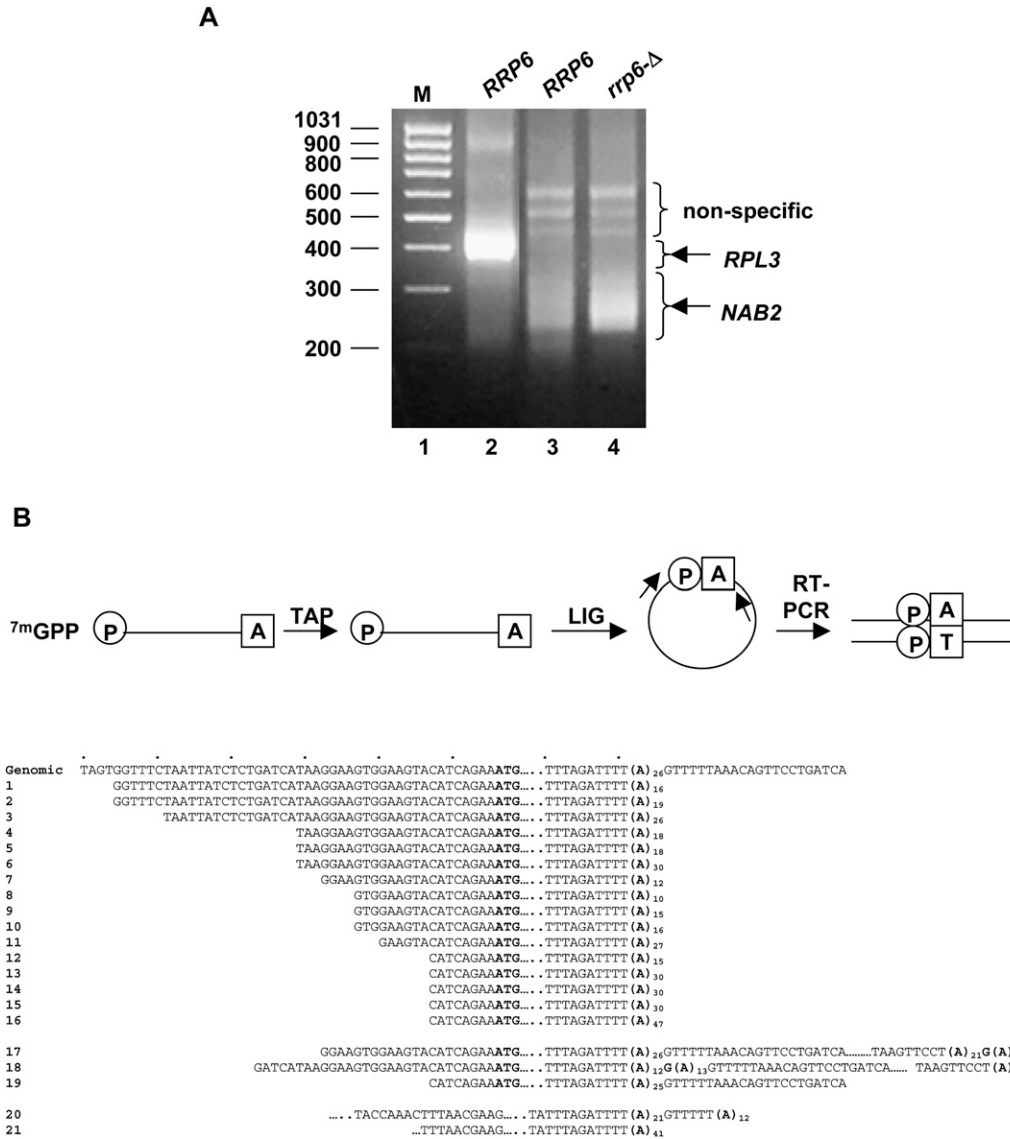
A

M   RRP6   RRP6   rrp6-Δ

1031 —
900 —
800 —
600 —
500 —   non-specific
400 —   ← RPL3
300 —
                               ← NAB2
200 —

1   2   3   4

B

⁷mGPP (P)————[A] →TAP→ (P)————[A] →LIG→ (circular) →RT-PCR→ (P)[A]/(P)[T]

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Genomic   TAGTGGTTTCTAATTATCTCTGATCATAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₂₆GTTTTTAAACAGTTCCTGATCA
1              GGTTTCTAATTATCTCTGATCATAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₆
2              GGTTTCTAATTATCTCTGATCATAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₉
3                 TAATTATCTCTGATCATAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₂₆
4                          TAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₈
5                          TAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₈
6                          TAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₃₀
7                             GGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₂
8                                GTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₀
9                                GTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₅
10                               GTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₆
11                                  GAAGTACATCAGAAATG…..TTTAGATTTT(A)₂₇
12                                       CATCAGAAATG…..TTTAGATTTT(A)₁₅
13                                       CATCAGAAATG…..TTTAGATTTT(A)₃₀
14                                       CATCAGAAATG…..TTTAGATTTT(A)₃₀
15                                       CATCAGAAATG…..TTTAGATTTT(A)₃₀
16                                       CATCAGAAATG…..TTTAGATTTT(A)₄₇

17                             GGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₂₆GTTTTTAAACAGTTCCTGATCA………TAAGTTCCT(A)₂₁G(A)₁₃
18          GATCATAAGGAAGTGGAAGTACATCAGAAATG…..TTTAGATTTT(A)₁₂G(A)₁₃GTTTTTAAACAGTTCCTGATCA……  TAAGTTCCT(A)₁₄
19                                       CATCAGAAATG…..TTTAGATTTT(A)₂₅GTTTTTAAACAGTTCCTGATCA

20                    …..TACCAAACTTTAACGAAG…..TATTTAGATTTT(A)₂₁GTTTTT(A)₁₂
21                            …TTTAACGAAG…..TATTTAGATTTT(A)₄₁
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FIGURE 2. The mature 3′-end of *NAB2* mRNA maps to the A_{26} sequence in the 3′-UTR. (*A*) Agarose gel electrophoretic analysis of the product of LM-PAT analysis of the strains with the indicated genotypes. (M) The lane with molecular size standards. (*B*) Diagram of the cRT-PCR method for a model mRNA. TAP (tobacco acid pyrophosphatase) treatment removes the cap, leaving a 5′-phosphate and the 3′-poly(A) tail, which is ligated (LIG) with RNA ligase to produce a circular RNA that is amplified by RT-PCR with (arrows) specific primers. The sequences obtained using primers to *NAB2* mRNA are shown *below* the genomic sequence with the start codon (ATG) and A_{26} sequence in bold.

RNA ligase (Fig. 2B). RT-PCR was carried out with primers complementary to regions 3′ to the ATG start codon and 5′ to the TAG stop codon of *NAB2* mRNA. The cDNAs were cloned and the sequence of the ligation junction determined to identify the 5′- and 3′-ends of the mRNAs. Of the 21 independent clones sequenced, 17 have 3′-ends corresponding to the A_{26} sequence in the *NAB2* 3′-UTR (Fig. 2B, clones 1–16,21). These clones contain eight different 5′-ends suggesting multiple transcription start sites for *NAB2*, although we do not discount the possibility that some of these represent intermediates in the 5′–3′ mRNA decay pathway. The length of the poly(A) tails in these clones ranged from 10 to 47 adenosines, suggesting the addition of some of the adenosines by a post-transcriptional mechanism.

Four clones end downstream from the A_{26} sequence: two of these end with poly(A) tails added 114 nt downstream from the beginning of the A_{26} sequence (Fig. 2B, clones 17,18). Remarkably, each of these clones clearly shows the presence of a single G within the poly(A) sequences: one within the A_{26} and one within the 3′ poly(A) tail. A third clone has an unadenylated 3′-end 21 nt downstream from the template-encoded adenosine stretch, which, in this case, is 25 adenosines long (Fig. 2B, clone 19).

Two clones have 5′-ends within the *NAB2* coding sequence (Fig. 2B, clones 20,21). One has a 41-nt poly(A) tail at the A_{26} sequence, while the second has a 12-nt poly(A) tail added 6 nt beyond an A_{21}, rather than an A_{26} sequence. The truncated 5′-ends of these transcripts suggest that they may represent intermediates in the 5′–3′ mRNA decay pathway.

Two of the sequenced clones (Fig. 2B, clones 17,21) ended downstream from the template-encoded A_{26} sequence, but contained fewer than the expected number of internal adenosines. We amplified this region by PCR from genomic DNA isolated from strain YSB3001, cloned the PCR products, and determined their sequence. Five clones contained 25 consecutive adenosines, four contained 26 adenosines, and one contained 27 adenosines. This distribution is consistent with the propensity of DNA polymerases to produce deletion mutations upon replication of repetitive sequences (Shinde et al. 2003; Garcia-Diaz and Kunkel 2006). These findings suggest that small variations in the length of the adenosine tract in the 3′-UTR of *NAB2* cDNAs may result from slippage during replication or the process of RT-PCR. Overall, the data suggest that the major 3′-end of *NAB2* mRNA maps in the A_{26} sequence, with a minor 3′-end occurring 114 nt downstream.

NAB2 mRNA 3′-end formation requires the core exosome

Previous experiments showed that the presence of 26 consecutive adenosines (A_{26}) 140 nt 3′ to the *NAB2* stop codon inhibited mRNA 3′-end formation at this site and rendered the transcript sensitive to degradation by the nuclear exosome (Roth et al. 2005). In support of the view that the A_{26} sequence inhibits normal mRNA 3′-end processing, its replacement with U_{26} resulted in efficient 3′-end formation at that site (Roth et al. 2005). Nevertheless, the presence of more than 26 adenosines at this site in cDNAs derived from mature *NAB2* mRNAs implies that polyadenylation occurs at this site, perhaps by a mechanism different from classical cleavage and polyadenylation. Since the nuclear exosome plays an A_{26}-dependent role in the regulation of *NAB2* mRNA levels, we asked whether depletion of core exosome components might alter the pattern of 3′-end formation of *NAB2* mRNA. We compared *NAB2* mRNAs produced from the chromosomal allele in cells in which the endogenous promoters for *RRP43* or

RRP46 were replaced with the $tetO_7$ promoter. Treatment of these cells with doxycycline represses expression of these genes, depletes the cells of the core exosome components Rrp43p and Rrp46p, and results in the expected defects in rRNA processing (Fang et al. 2004). Depletion of Rrp43p or Rrp46p causes a clear decrease in the mobility of *NAB2* mRNA but has no detectable effect on the mobility of *ACT1* mRNAs or *SCR1* RNA, a stable RNA polymerase III transcript (Fig. 3A). Moreover, depletion of Rrp43p or Rrp46p increases the amount of *NAB2* mRNA twofold relative to the control transcripts.

Next, we cleaved the *NAB2* transcripts with RNase H and an oligonucleotide complementary to a site approximately one-third of the way from the start to the stop codon of *NAB2* mRNA (Fig. 3B). Northern blot analysis of the products revealed that the distal product of RNase H cleavage increases by ∼100 nt after depletion of Rrp43p and Rrp46p, indicating that the increase in length results from a defect in 3′-end formation (Fig. 3B). Previous experiments showed that a temperature-sensitive mutation (*rrp4-1*) in the core exosome component Rrp4p caused an increase in *NAB2* mRNA levels, but the resolution of the transcript did not clearly indicate a change in its length (Roth et al. 2005). RNase H cleavage shows that the *rrp4-1* mutation clearly causes a defect in 3′-end formation of *NAB2* mRNA (Fig. 3C, lane 4). Interestingly, deletion of *RRP6* does not result in 3′ extended transcripts (Fig. 3C,
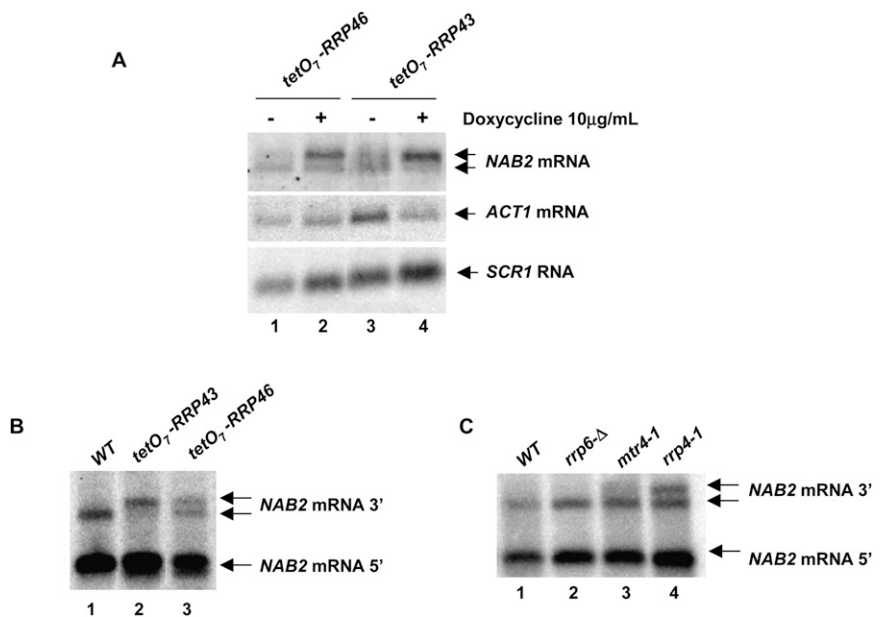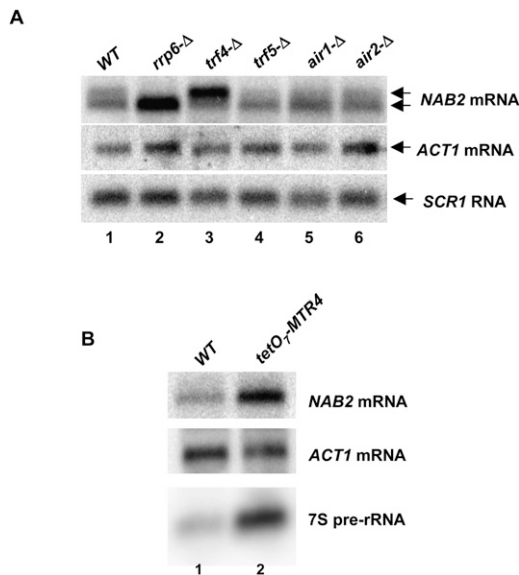

FIGURE 3. Depletion of core exosome components results in longer *NAB2* mRNAs. (*A*) YSB3002 (*tetO₇-RRP43*) or YSB3003 (*tetO₇-RRP46*) was grown in YPD at 30°C and treated (+) or not (−) with doxycycline for 8 h after which total RNA was prepared and RNA levels determined by Northern blotting. (*B,C*) Northern blot analysis of total RNA after RNase H treatment as described in Materials and Methods using OSB313 and total RNA from strains (*B*) YSB3001 (wt), YSB3002 (*tetO₇-RRP43*), or YSB3003 (*tetO₇-RRP46*); and (*C*) CWO4 (WT), YSB214 (*rrp6-Δ*), MS157-1A (*mtr4-1*), or YRP1222 (*rrp4-1*).

lane 2). These findings indicate that normal 3′-end formation of *NAB2* mRNA requires the activity of the core exosome, but not the nucleus-specific component Rrp6p. The fact that we detected no change in the length of *ACT1* mRNA indicates that this effect is specific and does not result from depletion of components of the canonical mRNA cleavage and polyadenylation system.

NAB2 mRNA 3′-end processing requires the TRAMP component Trf4p

The TRAMP complex composed of Mtr4p, Air1p or Air2p, and Trf4p or Trf5p enhances the rate of RNA degradation by the nuclear exosome (Kadaba et al. 2004; LaCava et al. 2005; Vanacova et al. 2005). Accordingly, we asked whether strains lacking TRAMP components cause defects in *NAB2* mRNA 3′-end formation. Indeed, strains lacking Trf4p produce increased amounts of the longer form of *NAB2* mRNA, without changing the amount or size of *ACT1* mRNA or *SCR1* mRNA (Fig. 4A, lane 3). In contrast, strains devoid of Trf5p, Air1p, or Air2p produce *NAB2* mRNA in amounts and lengths similar to the normal control (Fig. 4A, lanes 4–6). The *mtr4-1* mutation in the gene (*DOB1/MTR4*) encoding the putative helicase component of the TRAMP complex caused a marginal increase in the level of *NAB2* mRNA (Roth et al. 2005). RNase H cleavage of *NAB2* mRNA from an *mtr4-1* mutant showed no significant change in the length of the transcript (Fig. 3C, lane 3). To avoid potential allele-specific effects of the
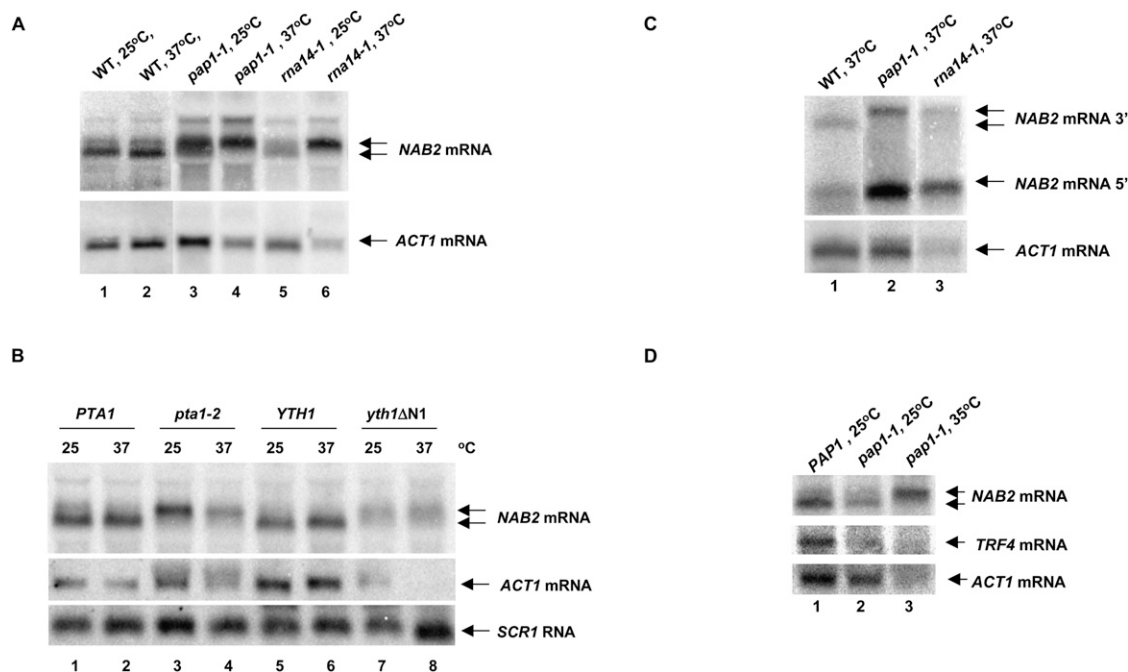
mtr4-1 mutation, we revisited the role of Mtr4p in *NAB2* expression by depleting the protein from cells in which the chromosomal gene promoter was replaced with the *tetO₇* promoter. After 5 h of treatment with doxycycline, Northern blot analysis revealed a sixfold increase in the level of 7S pre-rRNA, as expected since conversion of 7S to 5.8S rRNA requires Mtr4p and the core exosome (Fig. 4B, lane 2; de la Cruz et al. 1998). Likewise, depletion of Mtr4p results in a fourfold increase in the amount of *NAB2* mRNA. However, loss of Mtr4p activity has no detectable effect on the length of the transcript. We conclude that the Trf4p component of the TRAMP complex plays a specific role in the 3′-end formation of *NAB2* mRNA and that Mtr4p plays a role in controlling the ultimate level of the transcript.

Defects in the mRNA cleavage and polyadenylation system cause a 3′-end formation defect for *NAB2* mRNA

The requirement for TRAMP and the core exosome in *NAB2* mRNA 3′-end processing implies that defects in the classical cleavage and polyadenylation components should have no effect on the length of *NAB2* mRNA. We tested this assumption by analyzing the length of *NAB2* mRNAs in strains carrying temperature-sensitive mutations in the cleavage and polyadenylation factor (CPF) or cleavage factor IA (CFIA) complexes of the mRNA 3′-end processing apparatus. Northern blot analysis of mRNAs from strains with temperature-sensitive mutations in *PAP1*, *RNA14*, *PTA1*, and *YTH1* reveal that these defects lead to the accumulation of 3′ extended *NAB2* transcripts (Fig. 5A,B). Significantly, 3′ extended *NAB2* transcripts accumulate in the *pap1-1* mutant even at the permissive temperature (Fig. 5A, lane 3). Oligonucleotide-directed RNase H cleavage of transcripts from the *pap1-1* and *rna14-1* strains confirms that the increase in length results from the distal portion of the mRNA (Fig. 5C). These findings suggest that the CPF and CFIA mutations have effects similar to defects in TRAMP and the core exosome on *NAB2* 3′-end formation.

Three observations suggest that the effects of these mutations may affect *NAB2* 3′-end processing indirectly. First, while shift of the *pap1-1* and *rna14-1* strains to the nonpermissive temperature causes the expected decrease in *ACT1* mRNA levels, it does not lower the level of *NAB2* mRNA in the *pap1-1* strain and appears to increase the level in the *rna14-1* strain (Fig. 5A). Second, while defects in CPF and CFIA affect 3′-end processing of many mRNAs, the defects in TRAMP and the core exosome appear specific to *NAB2* mRNA (Figs. 3, 4A). Third, the *pap1-1* defect causes the accumulation of a 3′ extended *NAB2* mRNA. Since the cleavage step of mRNA 3′-end processing in yeast does not require Pap1p, 3′ extended transcripts do not accumulate in *pap1-1* mutants (Patel and Butler 1992; Proweller and Butler 1994). Instead, the levels of most mRNAs decrease

FIGURE 4. The TRAMP component Trf4p is required for normal 3′-end formation of *NAB2* mRNA. (*A*) Northern blot analysis of total RNA from strains YSB1001 (*RRP6*), YSB1005 (*rrp6-Δ*), YSB1017 (*trf4-Δ*), YSB1020 (*trf4-Δ*), YSB1060 (*air1-Δ*), and YSB1061 (*air2-Δ*). (*B*) Northern blot analysis of total RNA from strains YSB3001 (WT) or YSB3017 (*tetO₇-RRP46*) grown for 5 h in the presence of 10 μg/mL doxycycline.

FIGURE 5. Mutations in CPF and CFIA components cause *NAB2* mRNA 3′-end formation defects. (*A,B*) Northern blot analysis of total RNA isolated from strains (*A*) YSB213 (WT), YSB215 (*pap1-1*), or YSB202 (*rna14*-1); and (*B*) FY41 (*PTA1*), FY1284 (*pta1-2*), YT2 (*YTH1*), and YT3 (*yth1*ΔN1) before and after shift for 1 h from 25°C to 35°C. All strains were grown in YPD, except YT2 and YT3, which were grown in synthetic complete dextrose without uracil (Fink 1991). (*C*) Northern blot analysis of the products of RNase H cleavage of total RNA from strains with the indicated genotypes after a 1-h shift to 35°C. (*D*) Northern blot analysis of total RNA prepared from strains with the indicated genotypes before (25°C) and after shift for 1 h to 35°C.

upon inactivation of Pap1p owing to the inhibition of polyadenylation and subsequent degradation of the transcripts by the nuclear exosome (Burkard and Butler 2000). However, some poly(A)$^-$ mRNAs accumulate after inactivation of Pap1p in a *pap1-1* strain, and they end at their normal 3′-end cleavage sites (Proweller and Butler 1994).

We reasoned that if the expression of a key component of the TRAMP or core exosome complex was unusually sensitive to Pap1p activity, then a defect in Pap1p would indirectly result in defective 3′-end formation of *NAB2* mRNA. Accordingly, we analyzed the level of *TRF4* mRNA in a *pap1-1* strain since this transcript, as well as six of the 10 components of the core exosome, exhibits a relatively short half-life (5–8 min) that should result in its relatively rapid depletion upon inhibition of polyadenylation (Wang et al. 2002). Indeed, Northern blot analysis of *TRF4* mRNA levels shows that the *pap1-1* mutation results in a significant loss of *TRF4* mRNA even at the permissive temperature (Fig. 5D). We conclude that the 3′ extension of *NAB2* mRNA seen in the CPF and CFIA mutants likely reflects depletion Trf4p.
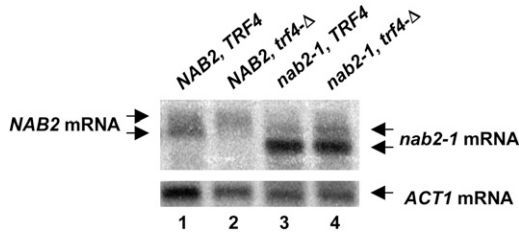
Nab2p defect bypasses the requirement for Trf4p in *NAB2* mRNA 3′-end formation

The results presented in Figure 1 indicated that decreasing Nab2p activity results in increased 3′-end formation of the

NAB2 transcript at the site within the A_{26} sequence. This suggests that Nab2p may act to repress 3′-end formation at this site, leaving the processing of the transcript dependent on the exosome and TRAMP complex. This view implies that a defect in Nab2p activity should bypass the requirement for the exosome and TRAMP in 3′-end formation of the mRNA. We tested this by asking where the 3′-end of *NAB2* mRNA lies in a strain carrying the *nab2-1* and *trf4-Δ* mutations. The results show that while the majority of *NAB2* transcripts end at the distal site in the *NAB2, trf4-Δ* strain, the majority of the transcripts end at the proximal site in the *nab2-1, trf4-Δ* strain (Fig. 6). These latter transcripts appear shorter and more abundant than normal since the *nab2-1* mutation deletes a small portion of the N terminus of Nab2p and increases the level of the transcript fourfold owing to the inability of the defective Nab2p to enhance mRNA degradation by Rrp6p (Roth et al. 2005). These findings support the idea that Nab2p represses 3′-end formation at the A_{26} sequence, thereby rendering 3′-end processing dependent on the exosome and TRAMP complex.

The *NAB2* A_{26} sequence inhibits mRNA 3′-end formation in vitro

The dependence of *NAB2* 3′-end formation on the Trf4p and the exosome and the apparent repressive effect of the

FIGURE 6. The *nab2-1* mutation bypasses the requirement for Trf4p in *NAB2* mRNA 3′-end processing. Northern blot analysis of total RNA from strains YSB501 [*nab2::HIS3* pACY717 (*NAB2 LEU2 CEN*)], YSB503 [*nab2::HIS3 trf4::KANMX* pACY717 (*NAB2 LEU2 CEN*)], YSB502 [*nab2::HIS3* pACY1152 (*nab2-1 LEU2 CEN*)], and YSB504 [*nab2::HIS3 trf4::KANMX* pACY1152 (*nab2-1 LEU2 CEN*)] grown in YPD at 30°C.

A_{26} sequence on normal 3′-end formation prompted us to analyze *NAB2* 3′-end processing in vitro. In this assay, synthetic radiolabeled RNAs are incubated in a yeast extract fraction capable of carrying out accurate 3′-end cleavage and polyadenylation (Butler et al. 1990). Figure 7 shows a diagram of the control *CYC1* substrate and the results of incubating the ^{32}P-labeled RNA in the extract. In the presence of CTP, the substrate undergoes cleavage at the polyadenylation site, but polyadenylation fails because of the lack of ATP (Fig. 7B, lane 3). In the presence of ATP, the cleavage product is polyadenylated, as expected (Fig. 7B, lane 4). A synthetic ^{32}P-labeled *NAB2* RNA containing its A_{26} sequence and 305 nt of sequence downstream undergoes cleavage and polyadenylation in the extract, but cleavage occurs ~110 nt downstream from the A_{26} sequence. In contrast, a substrate in which U_{26} replaces A_{26} undergoes cleavage and polyadenylation at the downstream site, as well as at the U_{26} sequence. This result parallels that seen when U_{26} replaces A_{26} in vivo causing all 3′-end formation to occur at the U_{26} site and making the transcript insensitive to Rrp6p (Roth et al. 2005). These findings support the conclusions that formation of the mature 3′-end of *NAB2* mRNA does not occur by canonical cleavage and polyadenylation and that the A_{26} sequence inhibits cleavage and polyadenylation at this site.

DISCUSSION

This study presents evidence indicating that normal 3′-end formation of the *NAB2* mRNA occurs by a novel mechanism requiring the action of the core exosome and the Trf4p component of the TRAMP complex. The major 3′-end of *NAB2* mRNA maps to a site at or within an A_{26} sequence in its 3′-UTR (Fig. 2). However, several observations suggest that, in the presence of excess Nab2p, this end does not form by the normal cleavage and polyadenylation process. First, Nab2p represses the usage of this site in addition to destabilizing the transcript (Fig. 1). This conclusion refines the previous model that only featured

destabilization of *NAB2* mRNA by Nab2p (Roth et al. 2005). Second, a defect in Nab2p bypasses the requirement for Trf4p in 3′-end formation (Fig. 6). Third, our findings suggest that the mature 3′-end of *NAB2* mRNA results from exosome trimming of a 3′ extended pre-mRNA, followed by polyadenylation at, or within, the A_{26} sequence.
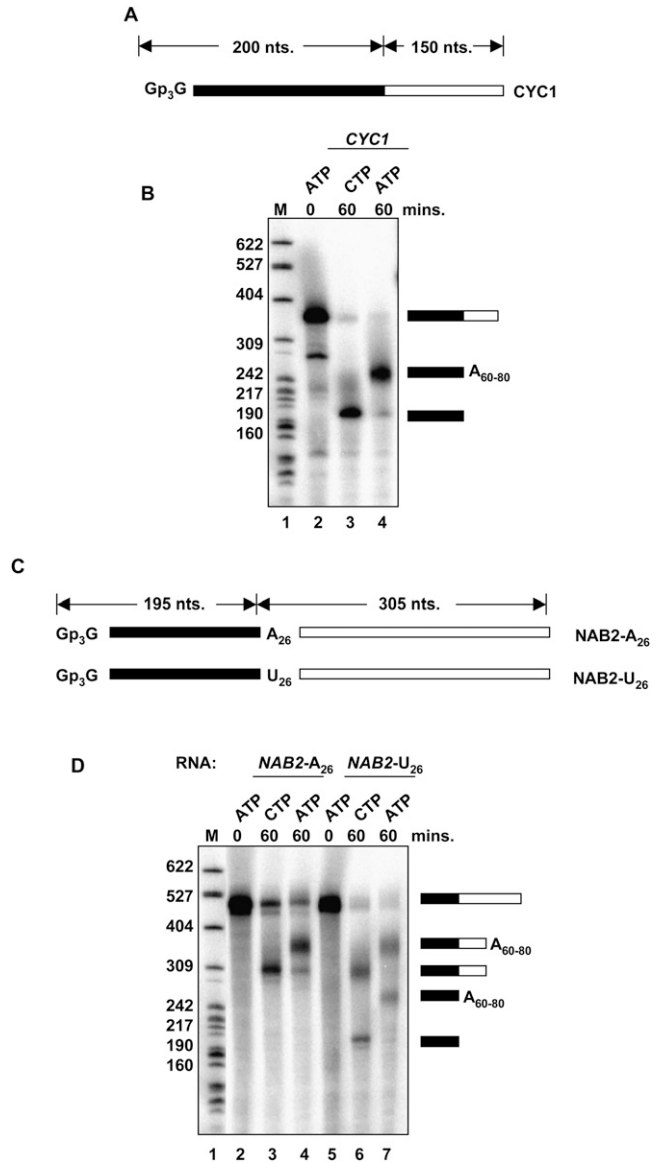
FIGURE 7. The *NAB2* A_{26} sequence inhibits mRNA 3′-end processing in vitro. Processing of synthetic ^{32}P-labeled RNAs in vitro. (*A*) Diagram of the synthetic *CYC1* RNA showing the portion (white) 5′ to the cleavage site and (black) 3′ to the cleavage site. (*B*) Processing of the *CYC1* RNA in vitro. (*Right*) The diagrams show the positions of the substrate and products of the reaction. (*Left*) The numbers refer to the positions of the length markers (M). (*C*) Diagram of the synthetic *NAB2* RNAs showing the portion (white) 5′ and (black) 3′ to the A_{26} or U_{26}. (*D*) Processing of the *CYC1* RNA in vitro. (*Right*) The diagrams show the positions of the substrate and products of the reaction. (*Left*) The numbers refer to the positions of the length markers (M).

Depletion or mutation of core exosome components or Trf4p resulted in the accumulation of *NAB2* transcripts ending 3′ of the normal site (Figs. 3–5). In all cases in which the 3′ extended transcript exists, its level exceeds that of the mature transcript found in normal cells. The fact that only mature transcripts accumulate upon depletion of Rrp6p or Mtr4p indicates that they do not play a necessary role in formation of the *NAB2* mRNA 3′-end. However, the elevated level of mature *NAB2* mRNA observed under these conditions suggests a role for Rrp6p and Mtr4p in controlling the ultimate amount of the transcript (Roth et al. 2005).

Inactivation of components of CPF and CFIA, required for cleavage and polyadenylation of mRNAs, results in 3′ extended *NAB2* mRNAs (Fig. 5). Three observations suggest that this effect does not reflect inhibition of processing at the A_{26} site of the pre-mRNA. First, inactivation of these components results in stable 3′ extended *NAB2* mRNAs, while other mRNAs are degraded by the exosome under these conditions (Fig. 5; Burkard and Butler 2000; Torchet et al. 2002; Milligan et al. 2005). Second, defects in CPF and CFIA may abrogate 3′-end trimming by the exosome indirectly by depleting Trf4p (Fig. 5). The apparent loss of Trf4p activity upon inactivation of Pap1p in a *pap1-1* strain may also explain the reported requirement for Pap1p in the polyadenylation of some noncoding RNAs (van Hoof et al. 2000; Kuai et al. 2004; Carneiro et al. 2007; Grzechnik and Kufel 2008). Third, the A_{26} site is not cleaved and polyadenylated in vitro, but is activated for this process by replacement of U_{26} for A_{26} (Fig. 7).

Based on these and other observations, we propose the following model for *NAB2* mRNA 3′-end formation. The region just 5′ of the A_{26} contains sequence elements similar to those necessary for formation of CFIA and CPF complexes (Guo and Sherman 1995; Dichtl and Keller 2001), so we suggest that the canonical 3′-end processing system recognizes the mature site, but fails to cleave because of the inhibitory effect of the A_{26} sequence, most likely because it binds Nab2p. Instead, a *NAB2* mRNA end forms downstream from the mature 3′-end of the transcript, probably at the secondary 3′-end site observed in vivo and in vitro ∼110 nt downstream from the mature 3′-end (Figs. 2, 7). Next, the core exosome and TRAMP degrade the transcript in cycles of adenylation by Trf4p and degradation by the core. When the core exosome reaches the A_{26} sequence, it dissociates and two alternative events may occur: (1) If Nab2p levels are insufficient for it to remain bound to the A_{26} sequence, polyadenylation occurs, forming the mature mRNA; (2) if Nab2p levels are high, the protein remains bound to the A_{26} sequence and recruits or activates Rrp6p to degrade the transcript with the aid of Mtr4p, and possibly the core exosome. Since core exosome and Trf4p defects trap the transcript in the longer form, our experiments cannot tell if degradation requires their activity. Nevertheless, since core exosome and Trf4p depletion elevate *NAB2* mRNA levels, we conclude that degradation of the transcript by Rrp6p and Mtr4p requires, at the very least, prior 3′-end processing by the core exosome. Clarification of the details of this model, including how the initial 3′-end of the pre-mRNA forms, requires further experimental work.

We assume that Trf4p carries out its role in the initial trimming of *NAB2* mRNA in the context of the TRAMP complex, but we have no direct evidence to support this claim. The lack of an apparent requirement for the Mtr4p in the initial trimming of the pre-mRNA might reflect the absence of this putative helicase in the complex, or a lack of a requirement for it to remove secondary structures or proteins bound to the RNA in this region. Likewise, our experiments do not indicate a requirement for Air1p or Air2p, the putative RNA binding components of the TRAMP complex. We could not isolate a viable *air1, air2* mutant, making it impossible to test if these proteins play a redundant role in formation of the *NAB2* 3′-end. Nevertheless, considering the facts that (1) the role of Trf4p in RNA degradation appears to occur in the context of the TRAMP complex; (2) the activity of Trf4p requires the activity of at least one of the Air proteins; and (3) Mtr4p plays a key role in the subsequent degradation of the *NAB2* transcript, it seems likely that TRAMP works with the core exosome to form the 3′-end of *NAB2* mRNA (Kadaba et al. 2004, 2006; LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005; Egecioglu et al. 2006; Houseley and Tollervey 2006).

These experiments suggest that the TRAMP complex and the core exosome participate in the 3′-end formation of a normal mRNA. Previous studies showed that defects in Pap1p, the mRNA poly(A) polymerase, resulted in the Rrp6p-dependent degradation of pre-mRNAs, indicating that Rrp6p plays a surveillance role that carries out the degradation of slowly polyadenylated transcripts (Burkard and Butler 2000; Milligan et al. 2005). Likewise, defects in mRNA 3′-end processing factors Rna14p and Rna15p resulted in the production of 3′ extended transcripts that underwent degradation by the exosome (Torchet et al. 2002). In some cases, it appeared that exosome trimming could stop at, or near, the poly(A) site, whereupon the transcript underwent polyadenylation (Torchet et al. 2002). The cell appears to have adopted this surveillance mechanism to form the mature 3′-end of the budding yeast *CTH2* mRNA (Ciais et al. 2008) and, in the present case, of *NAB2* mRNA. In the case of *NAB2*, the A_{26} and, perhaps, a nonproductive cleavage and polyadenylation complex just upstream, serve to elicit a transition in exosome degradation such that either polyadenylation occurs, or degradation resumes using Rrp6p and Mtr4p. In this view, trimming of the transcript into the A_{26} sequence would allow it to act as a primer for poly(A) polymerase in the stalled polyadenylation complex. In the case of *CTH2* mRNA, specific sequence elements in the pre-RNA may

bind Nrd1p and Nab3p, which then recruit the exosome to the transcript in a manner similar to that which serves to regulate the levels of *NRD1* mRNA (Arigo et al. 2006; Ciais et al. 2008). Previous studies showed that a defect in Nrd1p results in increased levels of *NAB2* mRNA, suggesting a role for Nrd1p in regulating 3′-end formation (Vasiljeva and Buratowski 2006). The *NAB2* mRNA contains several potential Nrd1p and Nab3p binding sites, but the role of these factors in *NAB2* mRNA processing remains to be addressed.

The novel mechanism of mRNA 3′-end formation proposed here for *NAB2* mRNA serves to regulate the level of Nab2p in the cell. Nab2p plays a key role in this regulation since increases in its activity decrease its mRNA levels, and loss of its function results in more *NAB2* mRNA (Roth et al. 2005). This autoregulatory circuit requires the presence of the A_{26} in the transcript's 3′-UTR, which almost certainly reflects the poly(A)-binding specificity of Nab2p (Anderson et al. 1993). Nab2p plays important roles in mRNA export and the control of poly(A) tail lengths during mRNA 3′-end processing (Green et al. 2002; Hector et al. 2002). Moreover, changes in Nab2p activity by mutation or alteration of Nab2p levels cause defects in mRNA metabolism that lead to significant growth defects in the affected cells. These observations provide a rationale for understanding the evolution of the autoregulatory mechanism that controls *NAB2* mRNA levels.

MATERIALS AND METHODS

Strains and reagents

Yeast media and experimental reagents were prepared by standard protocols (Fink 1991). Yeast strains are described in Table 1. Oligonucleotides are listed in Table 2. Chemicals were obtained from Sigma, US Biological, or Fisher.

LM-PAT and cRT-PCR analysis

The protocol for the LM-PAT was followed directly as specified in Salles and Strickland (1999). The oligonucleotide used as phosphorylated oligo(dT)$_{13}$ was OSB349. The oligonucleotide used as the oligo(dT)$_{12}$-anchor was OSB290. The 5′ *RPL3* and *NAB2* primers used to amplify the resulting LM-PAT cDNAs by PCR were OSB5 and OSB217, respectively. PCR products were cloned into the pGEM-T-Easy blue/white-screening vector (Promega) and transformed to XL1 *Escherichia coli*, and appropriate clones were selected for subsequent sequencing.

cRT-PCR was performed as described by Mullen and Marzluff (2008). Fifty micrograms of total yeast RNA was treated with 25 units of RQ1 DNase (Promega) in 50 μL of the manufacturer's buffer containing 40 units of Ribolock (Fermentas) for 20 min at 37°C. The products were extracted once with phenol:chloroform: isoamyl alcohol (50:48:2), and the RNA in the aqueous layer was precipitated with ethanol. The RNA was collected by centrifugation, dried, and resuspended in 25 μL of H$_2$O. Ten micrograms of the RNA was treated with 5 units of tobacco acid pyrophosphatase (Epicentre) in 20 μL of the manufacturer's buffer containing

TABLE 1. Yeast strains

Strain	Genotype	Reference
YSB200	MATa *ade2-1 his3-11,5 leu2-3,112 trp1-1 ura3-1*	W303a
YSB202	MATa *ade2-1 his3-11,5 leu2-3,112 trp1-1 ura3-1 rna14-1*	Amrani et al. (1997)
YSB213	MATa *ade1 ade2 lys2 gal1 ura3-52*	Briggs and Butler (1996)
YSB214	MATa *ade1 ade2 lys2 gal1 ura3-52 rrp6::KAN*	Briggs and Butler (1996)
YSB215	MATa *ade1 ade2 lys2 gal1 ura3-52 pap-1-1*	Briggs and Butler (1996)
FY41	MATa *leu2-Δ1 trp1-Δ63 ura3-52*	Zhao et al. (1999)
FY1284	MATa *ade8 his4-917δ ura3-52 pta1-2*	Zhao et al. (1999)
YT2	MATa *ade2 leu2 his3 trp1 ura3 yth1::TRP1*Ycplac33-YTH1 (*CEN4 URA3 YTH1*)	Tacahashi et al. (2003)
YT3	MATa *ade2 leu2 his3 trp1 ura3 yth1::TRP1*Ycplac33-YTH3 (*CEN4 URA3 yth1ΔN1*)	Tacahashi et al. (2003)
CW04	MATa *ura3-1 ade2-1 his3-11,15 leu2-3,112 trp1-1*	de la Cruz et al. (1998)
MS157-1A	MATa *ura3-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 dob1-1*	de la Cruz et al. (1998)
YRP1222	MATa *ade1-100 his4-519 leu2-3,112 ura3-52 rrp4-1*	Anderson and Parker (1998)
YSB501	MATα *ura3 leu2 his3 trp1 nab2::HIS3* pAC717 (*NAB2 CEN URA3*)	Marfatia et al. (2003)
YSB502	MATα *ura3 leu2 his3 trp1 nab2::HIS3* pAC1152 (*nab2-1 CEN URA3*)	This study
YSB503	MATα *ura3 leu2 his3 trp1 nab2::HIS3 trf4::KANMX* pAC717 (*NAB2 CEN URA3*)	Marfatia et al. (2003)
YSB504	MATα *ura3 leu2 his3 trp1 nab2::HIS3 trf4::KANMX* pAC1152 (*nab2-1 CEN URA3*)	This study
YSB1002	MATα *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0*	Winzeler et al. (1999)
YSB1005	MATα *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 rrp6*::KANMX	Winzeler et al. (1999)
YSB1017	MATα *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 trf4*::KANMX	Winzeler et al. (1999)
YSB1020	MATα *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 trf5*::KANMX	Winzeler et al. (1999)
YSB1060	MATα *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 air1*::KANMX	Winzeler et al. (1999)
YSB1061	MATα *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 air2*::KANMX	Winzeler et al. (1999)
YSB3001	MATa *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 URA3*::CMV-tTA	Mnaimneh et al. (2004)
YSB3002	MATa *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 URA3*::CMV-tTA KANMX-tetO7-*RRP43*	Mnaimneh et al. (2004)
YSB3003	MATa *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0 URA3*::CMV-tTA KANMX-tetO7-*RRP46*	Mnaimneh et al. (2004)
YSB3017	MATa *his3-Δ0 leu2-Δ0 lys2-Δ0 ura3-Δ0URA3*::CMV-tTA KANMX-tetO7-*MTR4*	Mnaimneh et al. (2004)

TABLE 2. Oligonucleotides

Name	Sequence
OSB5	GGAATTCCGGGTGGTTTCGTCCACTACGGTG
OSB151	GTCTAGCCGCGAGGAAGG
OSB157	GGCCAGCAATTTCAAGTTA
OSB184	CATACGCGCACAAAAGCAGAG
OSB217	GCTCTAGAGCTCCTCCGCAAACCAGTTTTACG
OSB218	CCATCGATGGCCCAGTCTGTCCTTAACTC
OSB272	TCTAGTGGATCCCCCCGCTGAATTC
OSB273	CCCGGGGGGATCCACTAGTTCTAG
OSB290	GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT
OSB313	GGGATGGAGTTGCAGGAGC
OSB316	TCCTCCGCAAACCAGTTTTACGCACCAAGAACAAGATACGGAAATGAACTGA
OSB317	ATGATTACGCCAAGCTATTTAGGTGACACTATAGATCCTCCGCAAACCAGTTTTACG
OSB349	TTTTTTTTTTTTTTT
OSB491	ATTTCAAAAAAAAAAAAAAAAAAAAAAAAAAAGTGTACGCATGTAACATTATACTG
OSB493	GCAATAAATATAAAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCACATGCGTACATTGTAATATGAC
OSB494	CAGTATAATGTTACATGCGTACACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATTTGAAATATAAATAACG
OSB497	GCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCACGGATTAGAAGCCGCC
OSB498	CTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCCCAAGAAAAATATG
OSB544	ATGATTACTCCAAGCTATTTAGGTGACACTATAGCCCCTTTTCCTTTGTCGATATC
OSB545	AAGGGCGATGCAATAAAAGTGGAAG
OSB574	ACGTCACGGCGGATGGAATTCTTGTATTAG
OSB575	TCTCTTGCCTTTCCACGATATTTGATCACG
OSB769	GCAAGCGTATCTCTCGAAACAC
OSB772	CTAGCCAGCTCGTCTACGACAG

20 units of Ribolock (Fermentas) for 1 h at 37°C. Four micrograms of the decapped RNA was treated with 20 units of RNA ligase (New England Biolabs) in 400 μL of the manufacturer's buffer containing 20 units of Ribolock for 12 h at 15°C. The products were precipitated with ethanol, collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 12 μL of H$_2$O. cDNA synthesis was carried out on 6 μL of the ligated RNA with 200 units of M-MLV reverse transcriptase (Invitrogen) in 20 μL of the manufacturer's first-strand synthesis buffer containing 20 units of Ribolock, 0.5 mM dNTP, and 25 pmol of OSB769 for 50 min at 37°C, followed by incubation for 15 min at 70°C. PCR was carried out on 1 μL of the cDNA using OSB316 and OSB772. The products were purified using a Qiaquick PCR Purification Kit (QIAGEN) and cloned directly into pGEM-T-easy (Promega). DNA sequence analysis was carried out on individual clones.

Messenger RNA 3′-end processing of mRNA in vitro

The protocol for 3′-end processing of synthetic radiolabeled pre-mRNA substrates was performed as described in Butler et al. (1990). Oligonucleotides OSB317, which encodes the SP6 promoter, and OSB218 (3′ *NAB2* primer) were used in PCR with plasmid DNA templates pGFPNAB2 and pNAB2-U$_{26}$ to generate DNA templates for in vitro mRNA transcription. Templates for transcription to produce *CYC1* RNAs were produced in two steps. First, for production of the *CYC1*-A$_{26}$ substrate, separate PCR reactions were performed using oligonucleotides OSB491 and OSB498 or OSB493 and OSB497 using plasmid pRP497 (Muhlrad and Parker 1999) as a template. These products were mixed together and used as a template for PCR with OSB497 and OSB498.

Likewise, for production of the *CYC1*-U$_{26}$ substrate, separate PCR reactions were performed with oligonucleotides OSB493 and OSB498 or OSB494 and OSB497 using plasmid pRP497 as a template. These products were mixed together and used as a template for PCR with OSB497 and OSB498. The *CYC1*-normal substrate was made by PCR using OSB497 and OSB498 and pRP497 as a template. The resulting PCR products were cloned into HindIII-digested YCplac22 by gap repair. Isolated plasmid clones were recovered, and the DNA sequence of the *CYC1* region was determined. Next, the *CYC1* portions of the *CYC1*-normal, *CYC1*-A$_{26}$, and *CYC1*-U$_{26}$ substrates were PCR-amplified with OSB544, which encodes the SP6 RNA polymerase promoter, and OSB545. The products were used in SP6 RNA polymerase transcription reactions in vitro to produce ^{32}P-labeled RNA transcripts (Butler et al. 1990).

RNA analysis

Total RNA was isolated from yeast strains grown to an A$_{600}$ of 0.8–1.2 as described (Patel and Butler 1992), and Northern Blot analysis was carried out as described (Briggs et al. 1998). Oligonucleotides used for detection of RNA in Northern blot analyses are described in Table 2. The procedure for generating [γ-^{32}P]ATP-labeled DNA oligonucleotide probes was described in Briggs et al. (1998). 7S rRNA, *SCR1* RNA, and *ACT1* mRNA were detected by ^{32}P-N-terminal-labeled DNA oligonucleotides OSB157, OSB151, and OSB184, respectively. 5′ [α-^{32}P]CTP-labeled DNA random primed hexamer probes were generated using the Random Primers DNA Labeling System (Invitrogen). *GFP-NAB2* fusion transcripts were detected with ^{32}P-labeled random-primed labeled probes to GFP generated from a PCR template

generated with primers OSB272 and OSB273 using pGFP-N-FUS as a template. *NAB2* mRNA was detected with ^{32}P-labeled random-primed probes generated from a BamHI, EcoRI *NAB2*-containing fragment of pGAD424-NAB2 (Roth et al. 2005). *TRF4* mRNA was detected with ^{32}P-labeled random-primed labeled probes generated from a PCR template with primers OSB574 and OSB575 using YSB1001 genomic DNA as a template.

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