

Distinct Pathways for snoRNA and mRNA Termination

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Summary

Transcription termination at mRNA genes is linked to polyadenylation. Cleavage at the poly(A) site generates an entry point for the Rat1/Xrn2 exonuclease, which degrades the downstream transcript to promote termination. Small nucleolar RNAs (snoRNAs) are also transcribed by RNA polymerase II but are not polyadenylated. Chromatin immunoprecipitation experiments show that polyadenylation factors and Rat1 localize to snoRNA genes, but mutations that disrupt poly(A) site cleavage or Rat1 activity do not lead to termination defects at these genes. Conversely, mutations of Nrd1, Sen1, and Ssu72 affect termination at snoRNAs but not at several mRNA genes. The exosome complex was required for 3' trimming, but not termination, of snoRNAs. Both the mRNA and snoRNA pathways require Pcf11 but show differential effects of individual mutant alleles. These results suggest that in yeast the transcribing RNA polymerase II can choose between two distinct termination mechanisms but keeps both options available during elongation.

Introduction

Dynamic interactions of various factors with RNA polymerase II (Pol II) promote initiation, elongation, or termination of transcription. Accurate termination is important because stopping transcription too late or too early can disrupt proper gene expression. Termination by Pol II is tightly coupled to mRNA 3' end processing, and multiple proteins, RNA/DNA sequences, and chromatin configurations may be involved (Proudfoot et al., 2002; Proudfoot, 2004; Buratowski, 2005).

Two major models have been proposed for transcription termination of protein coding genes. The “anti-terminator” or “allosteric” model proposes that transcription through the poly(A) signal changes the properties of the elongating Pol II complex, perhaps by dissociation of positive elongation factors or recruitment of ter-

mination factors (Logan et al., 1987). Indeed, this model is supported by evidence for exchange of factors associated with polymerase at the 3' end of genes (Kim et al., 2004a) and genes where termination apparently occurs without cleavage (Osheim et al., 1999; Tran et al., 2001; Sadowski et al., 2003). The “torpedo” model postulates that cleavage of the nascent RNA transcript at the poly(A) site transmits a signal to Pol II, leading to the destabilization of the elongation complex (Connelly and Manley, 1988). Recently, the 5' to 3' exoribonuclease Rat1/Xrn2 was shown to degrade the RNA transcript downstream of a poly(A) cleavage or cotranscriptional cleavage (CoTC) site, and this degradation somehow promotes transcription termination (Kim et al., 2004b; West et al., 2004). These findings strongly support the torpedo model. Since both models have experimental support, it seems likely that termination can occur by more than one mechanism. One pathway or another may predominate in particular gene contexts.

In addition to mRNAs, Pol II transcribes noncoding RNAs such as the small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). snoRNAs are required for maturation of pre-rRNAs (cleavage and base modification), and they are divided into two classes (C/D and H/ACA) based on conserved secondary structures (Kiss, 2002). Each class of snoRNAs is associated with a specific set of proteins to form stable snoRNPs. Recent studies indicate that snoRNP assembly occurs cotranscriptionally (Morlando et al., 2002; Ballarino et al., 2005; Yang et al., 2005). So far, 66 snoRNAs have been identified in *Saccharomyces cerevisiae*, and 42 of them are independently transcribed (Samarsky and Fournier, 1999).

The 3' ends of snoRNAs are not polyadenylated by the mRNA poly(A) polymerase (Pap1), but the basis for this difference with mRNAs is still unclear. One proposal is that snoRNA 3' end formation is a variation of the mechanism used for mRNAs. Cleavage factor IA, which is required for mRNA 3' end formation, could promote cleavage of snoRNA transcripts without subsequent polyadenylation of the 3' ends (Fatica et al., 2000; Morlando et al., 2002). Additionally, 3' extended forms of snoRNAs accumulate in strains with mutations in the exosome, a complex of 3' to 5' exonucleases, indicating that longer premature snoRNA transcripts are processed by 3' trimming (Allmang et al., 1999; van Hoof et al., 2000). Several proteins have been implicated in snoRNA 3' end formation. Mutations in either of two RNA-binding proteins (Nrd1 and Nab3) or a helicase (Sen1) cause defective termination at multiple snoRNA genes (Steinmetz et al., 2001; Ursic et al., 2004). Subunits of the APT complex (Pti1, Ref2, Ssu72, and Swd2), which associate with the mRNA cleavage/polyadenylation factor Pta1, are also implicated in snoRNA termination (Dheur et al., 2003; Ganem et al., 2003; Steinmetz and Brow, 2003; Cheng et al., 2004; Dichtl et al., 2004). Recently, a role for the Paf1 complex in snoRNA 3' end formation was reported (Sheldon et al., 2005). However, the mechanism or mechanisms by which these proteins promote snoRNA termination are not understood.

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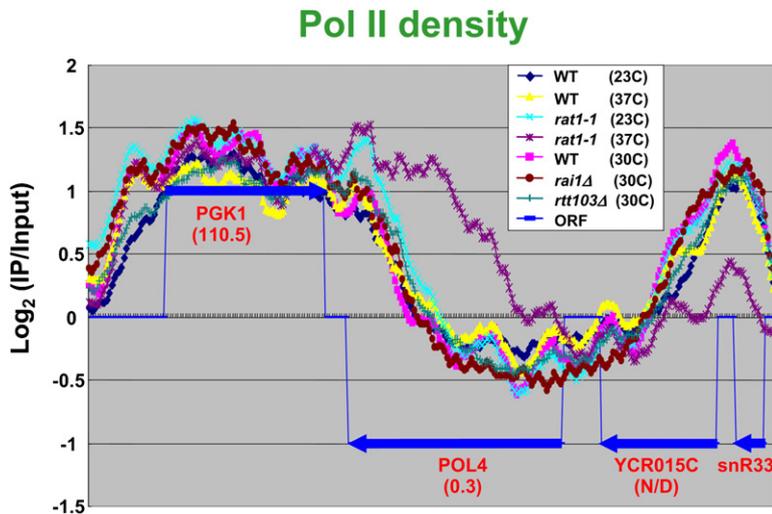


Figure 1. Transcriptional Termination of *SNR33* by Pol II Is Not Dependent on Rat1
Chromatin immunoprecipitated with anti-Rpb3 was amplified and analyzed using tiled microarrays of chromosome III as described (Kim et al., 2004b). The *PGK1-SNR33* region is shown. The y axis indicates the log₂ value of the immunoprecipitation (IP) to input ratio, and the x axis represents position along the chromosome. Dark blue arrows denote open reading frames (ORFs). Note that *PGK1* transcription is from left to right, while *SNR33* is from right to left. The numbers in parentheses show the transcription rates (mRNA/hr), with N/D being below the detection limit (Holstege et al., 1998).

Here, we compare factor requirements for mRNA and snoRNA 3' end formation. Chromatin immunoprecipitation (ChIP) experiments show that Rat1 and the known mRNA cleavage factors are present at snoRNA genes, as are Sen1, Nrd1, and Nab3. However, we find that termination at *SNR13* or *SNR33* is normal in strains defective for Rat1 activity or mRNA 3' end cleavage. Conversely, mutations in the Nrd1/Nab3/Sen1 complex lead to defects in snoRNA 3' end formation but do not affect 3' ends at several mRNAs tested. These results suggest that snoRNA termination can occur by a mechanism distinct from the mRNA torpedo model. The predominant snoRNA pathway may involve cleavage-independent termination and subsequent 3' trimming by the exosome/Nrd1 complex. However, the ChIP results suggest that elongating Pol II interacts with both machineries and that the choice between pathways is made while transcribing.

Results

Rat1-Independent Transcription Termination of snoRNA Genes

We previously showed that the exonuclease Rat1/Xrn2 promotes transcription termination by yeast Pol II in vivo (Kim et al., 2004b). In this study, crosslinked chromatin was immunoprecipitated for the Pol II subunit Rpb3 and used to probe a microarray covering *Saccharomyces cerevisiae* chromosome III. At most mRNA genes with a clear Rpb3 signal, crosslinking of Pol II extended further at the 3' end in the mutant as compared to wild-type cells. For example, the highly transcribed *PGK1* gene (transcription rate 110.5 mRNA/hr; see Holstege et al., 1998) shows 3' extended Pol II crosslinking into the *POL4* locus (0.3 mRNA/hr, which is below the level of detection by ChIP for Rpb3) in *rat1-1* cells at the nonpermissive temperature (Figure 1).

Even though it is robustly transcribed by Pol II, transcriptional termination of the nearby H/ACA class snoRNA gene *SNR33* was not affected by the *rat1-1* mutation (Figure 1). Indeed, in the *rat1-1* mutant no termination defects were observed at any of the other snoRNAs on chromosome III (*SNR43*, *SNR65*, and *SNR189*; data not shown). However, Pol II density on

all of these snoRNA genes was slightly reduced in the mutant. Since Rat1 also participates in the 5' processing or degradation of snoRNA precursors (Lee et al., 2003), we speculate there may be a regulatory mechanism to reduce snoRNA transcription when snoRNA precursors accumulate.

To investigate the mechanism used during transcription termination of snoRNAs, we monitored two snoRNA genes: the C/D class *SNR13* and the H/ACA type *SNR33* (Figure 2A). The recruitment of several known RNA processing factors to these snoRNA genes was monitored using ChIP assays with TAP-tagged factors. Because of the small size of these genes, it was not possible to easily resolve 5' and 3' crosslinking. Proteins involved in cleavage and polyadenylation of mRNAs, including subunits of CFIA (Rna14, Rna15, and Pcf11), CFIB (Hrp1), and CPF (Cft2), were strongly recruited to *SNR33* (Figure 2B) and *SNR13* (data not shown). Similar results were seen with subunits of the APT complex, a set of proteins associated with CPF (Nedea et al., 2003). Crosslinking of Ssu72, a C-terminal domain (CTD) phosphatase important for mRNA and snoRNA 3' end formation, to snoRNA genes was previously reported (Nedea et al., 2003).

Surprisingly, despite the fact that snoRNA termination is unaffected in the *rat1-1* mutant strain, Rat1 is clearly recruited to the *SNR33* gene (Figure 2B). A similar recruitment pattern was observed on *SNR13* (data not shown). To confirm Rat1-independent termination of snoRNAs as seen on the microarray, conventional ChIP assays with anti-Rpb3 antibody were performed using *rat1-1* cells. As a control to show that we can detect snoRNA termination defects in the ChIP assay, temperature-sensitive strains mutated in *SSU72* were used to generate readthrough transcription (Ganem et al., 2003). When shifted to 37°C for 30 min, *rat1-1* cells showed a 3' extended Pol II signal at the *ADH1* gene, but *ssu72-2* has no effect (Figure 3A). Conversely, a 3' extension of Pol II crosslinking at *SNR33* was seen in *ssu72-2* and *ssu72-3* cells but not in the *rat1-1* mutant (Figure 3B). As seen in the ChIP/microarray experiment, Pol II crosslinking to snoRNAs was slightly reduced in the *rat1-1* strain. In agreement with the ChIP results, *SNR13* and *SNR33* readthrough transcripts were detected by northern

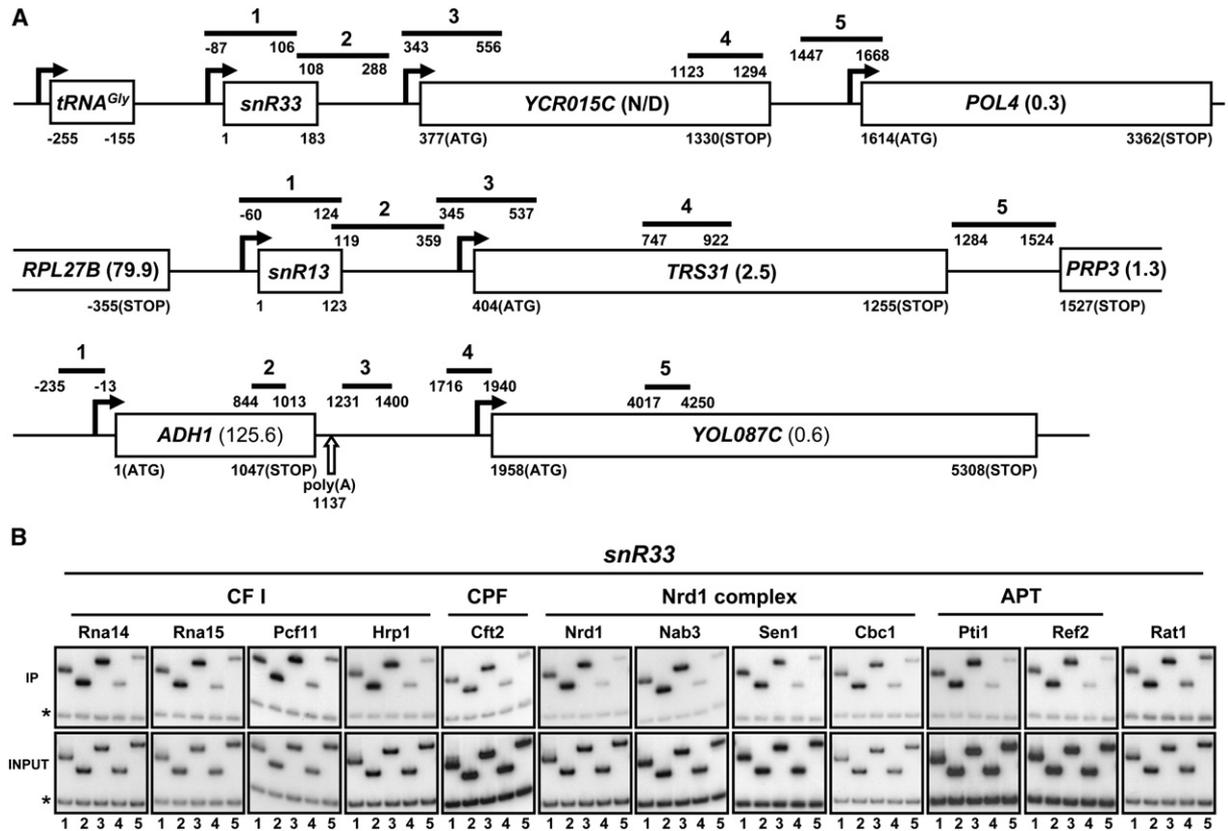


Figure 2. Crosslinking of RNA 3' Processing Factors to *SNR33*

(A) Schematic diagrams of the *SNR33*, *SNR13*, and *ADH1* gene-flanking regions. The coding region (*ADH1*) or mature transcript (*SNR33* and *SNR13*) is represented by an open box. Black arrows indicate promoters and direction of transcription. Open arrow in *ADH1* indicates the position of the major polyadenylation site. Bars above genes show the positions of PCR products used in the ChIP assays. Numbers in parentheses show the transcription rates (mRNA/hr).

(B) ChIP analysis was carried out on the *SNR33* gene in strains carrying the indicated TAP-tagged proteins. Precipitated chromatin was PCR amplified with primers shown in (A). The top band is the *SNR33*-specific band, while the common lower band (marked by asterisk) is an internal background control from a nontranscribed region on chromosome V. The input panel shows control used to normalize the PCR amplification efficiency of each primer pair. Similar results were seen for *SNR13* (data not shown).

blotting in the *ssu72* mutants but not in the *rat1-1* mutant (Figure 3C). Therefore, termination of transcription at these snoRNA genes is not dependent upon Rat1, and this finding suggests that snoRNA termination may occur by a mechanism different from that used at mRNA genes.

Cleavage-Independent Termination of snoRNA Genes

Rat1-independent termination at snoRNA genes raises the question of whether there is cleavage of nascent RNA transcripts at the 3' end of these RNAs. For mRNA genes, endonucleolytic cleavage of the transcript by the polyadenylation machinery generates an uncapped 5' phosphorylated RNA end that is thought to provide an entry site for Rat1-dependent degradation. These events contribute to termination by a mechanism that is still unclear. To see whether a similar mechanism applies to snoRNAs, termination was assayed in mutant strains where mRNA cleavage is inhibited.

Pcf11 is a component of cleavage factor IA (Rna14, Rna15, Pcf11, and Clp1). Pcf11 binds the CTD of the Pol II largest subunit and is required for pre-mRNA 3' end processing (Amrani et al., 1997; Licatalosi et al., 2002). Keller and colleagues characterized a useful col-

lection of conditional alleles of *PCF11* (Sadowski et al., 2003) as follows: *pcf11-2* leads to defective CFIA activity but has normal CTD binding; *pcf11-13* lacks CTD-binding ability but still supports mRNA cleavage in vitro; *pcf11-9* is defective for both CFIA activity and CTD-binding ability. At mRNA genes like *ADH1* and *PMA1*, the *pcf11-2* and *pcf11-9* mutants, but not *pcf11-13*, have termination defects (Figure 4A and see Figure S1 in the Supplemental Data available with this article online; note that the effect of *pcf11-2* is much stronger on *PMA1* than *ADH1*). This argues that at mRNAs, cleavage of the nascent RNA transcripts is necessary for termination. In contrast, transcription readthrough at snoRNAs was seen in *pcf11-9* and *pcf11-13* but not *pcf11-2* cells, indicating that CTD interaction but not cleavage was important at these genes. Rpb3 crosslinking levels to snoRNA genes and *PMA1* were greatly reduced in the *pcf11-9* strain at the nonpermissive temperature. The basis for this is unclear, but it may be an indirect effect because the cells are dying. In *rna14-1* strains, which also have mRNA cleavage defects, snoRNA termination was normal (data not shown).

Cleavage/polyadenylation factor Brr5/Ysh1 (the yeast homolog of CPSF-73) is thought to be the 3'

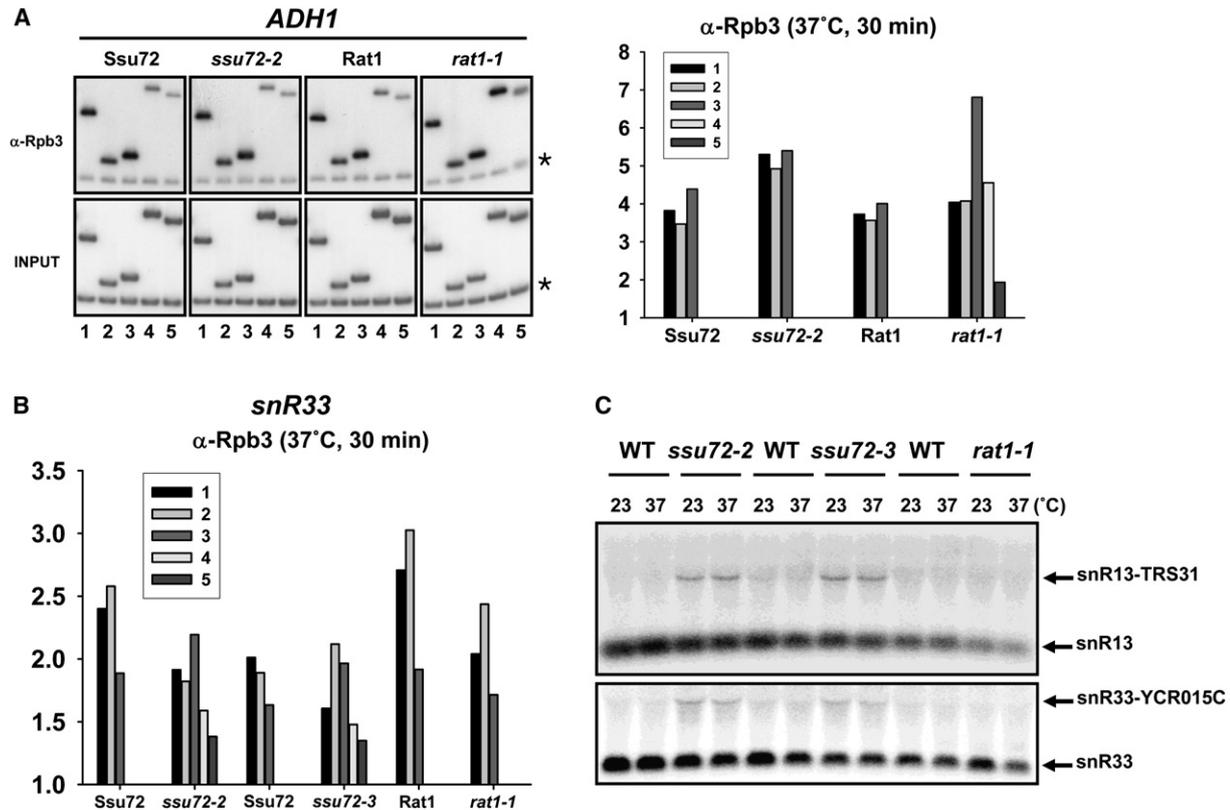


Figure 3. Transcription Termination of *SNR13* and *SNR33* Is Not Dependent upon *Rat1*

(A) ChIP analysis with anti-Rpb3 antibody on *ADH1* in isogenic wild-type, *ssu72-2*, and *rat1-1* strains. Cells were shifted to nonpermissive temperature (37°C) for 30 min. The left panel shows PCR products, and numbers below each lane refer to PCR primers as in Figure 2A. The right panel shows quantification of results. The y axis is the ratio of *ADH1* signal relative to the internal negative control after normalizing to the input controls. A value of 1.0 therefore indicates no signal above background. Crosslinking of Rpb3 (i.e., Pol II) extended beyond position 4 in the *rat1-1* strain, confirming its role in mRNA termination. In contrast, the *ssu72-2* mutation has no effect on termination of *ADH1*.

(B) ChIP was performed on *SNR33*. Two *ssu72* mutants, but not *rat1-1*, show termination defects. Similar results (data not shown) were seen with *SNR13*.

(C) Northern blot analysis of total RNAs isolated at 23°C or 37°C (30 min). Mature form and readthrough transcripts of *SNR13* or *SNR33* are indicated by arrows.

endonuclease responsible for cleavage at the poly(A) site (Ryan et al., 2004). More recently, CPSF-73 has also been implicated in the endonucleolytic 3' end processing of the nonpolyadenylated metazoan histone pre-mRNAs (Dominski et al., 2005; Kolev and Steitz, 2005). To investigate whether Brr5 has a role in termination, we depleted Brr5 in vivo using a temperature-inducible degron strain (Zhelkovsky et al., 2006). Upon Brr5 depletion, termination was defective at all mRNA genes tested (Figure 5 and Figure S2). In contrast, termination at *SNR13* and *SNR33* was normal (Figures 5B and 5C), further indicating that cleavage by the polyadenylation machinery is not required for snoRNA termination.

We considered whether another protein might function as an endonuclease to generate an exonuclease entry site. Rnt1 is the yeast RNase III and is involved in processing and maturation of multiple RNAs, including ribosomal and snoRNAs. Endonucleolytic cleavage by Rnt1 generates entry points for Rat1 and the related Xrn1 nuclease to carry out subsequent 5' trimming of unprocessed snoRNA precursors (Lee et al., 2003). Although Rnt1 cleaves nascent snoRNA transcripts (Lee et al., 2003), its deletion does not lead to any defects

in termination of snoRNA or mRNA genes (Figure S3). We also tested for a role of the nuclear exosome in termination, since exosome and Rnt1 participate in RNA 3' end trimming and degradation of snoRNAs (Allmang et al., 1999). Deletion of nuclear exosome subunit Rrp6 did not affect termination in either mRNAs or snoRNAs (Figure S3).

Requirement of Distinct Protein Factors for snoRNA Termination

The Nrd1 complex includes Nrd1, Nab3, and Sen1 (Steinmetz et al., 2001; Dheur et al., 2003; Ganem et al., 2003; Ursic et al., 2004; Vasiljeva and Buratowski, 2006). This complex is required for termination at snoRNA genes, perhaps in combination with other factors (Steinmetz et al., 2001; Dheur et al., 2003; Ganem et al., 2003; Ursic et al., 2004). However, the Nrd1 complex is also detected by ChIP at mRNA genes (Nedea et al., 2003), suggesting it may function at both classes of genes. Accordingly, we tested whether mRNA termination is affected in *nrd1-51*, *nrd1-102*, and *sen1-1* conditional mutants. Termination at the *ADH1* and *PMA1* genes was not affected at the nonpermissive temperature (Figure 6A and Figure S4),

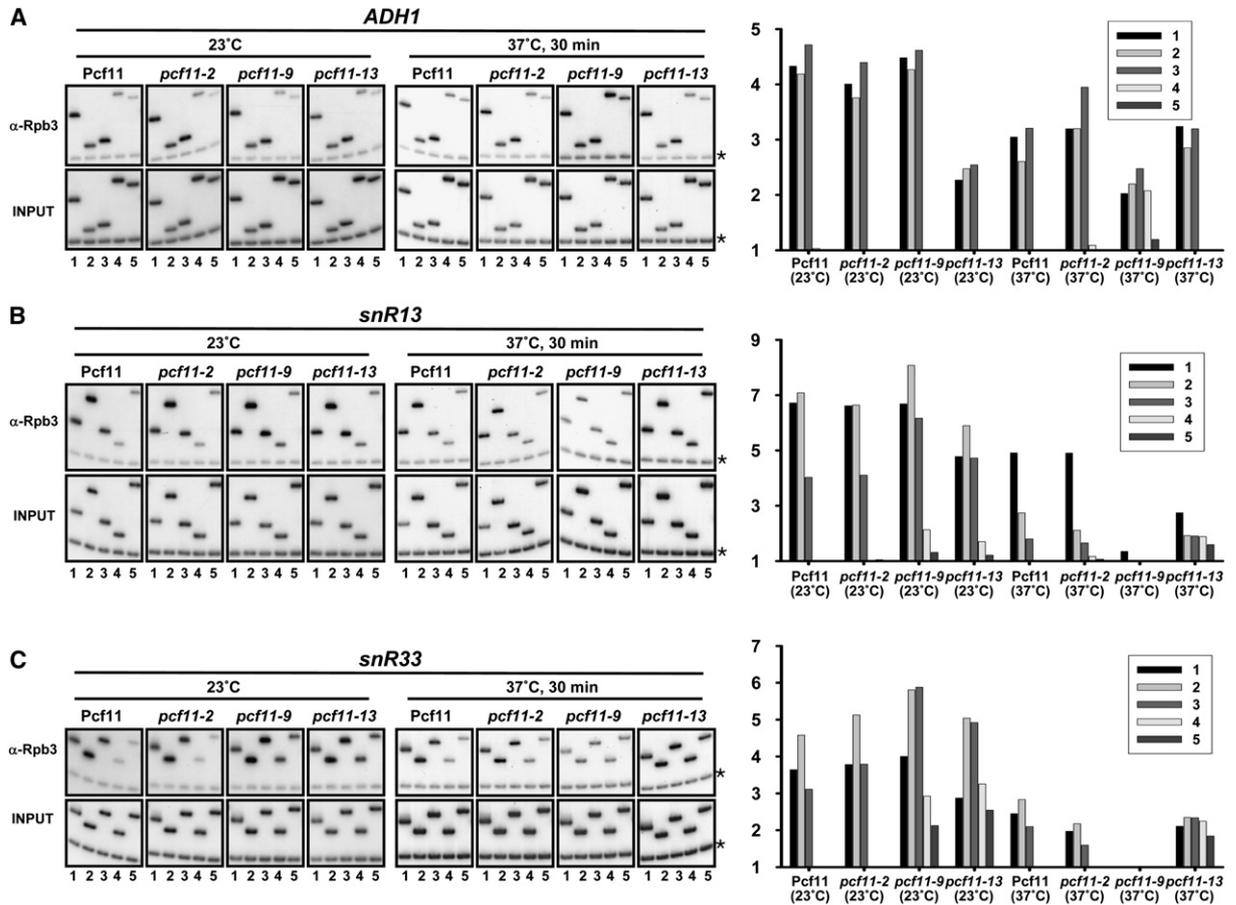


Figure 4. Allele-Specific Effects of PCF11 Mutations on mRNA and snoRNA Termination

Wild-type, *pcf11-2*, *pcf11-9*, and *pcf11-13* cells were shifted to nonpermissive temperature (37°C) for 30 min, and α-Rpb3 ChIP assays were carried out. Quantification data are shown in the right panels. (A) *ADH1* gene. (B) *SNR13* gene. (C) *SNR33* gene. See also Figure S1 for the *PMA1* gene.

suggesting that although these factors may be present at mRNA genes they are not necessary for normal termination. In contrast, *SNR13* showed a strong termination defect in *nrd1* and *sen1* mutants (Figure 6B). Somewhat surprisingly, *sen1-1* and two *ssu72* mutants exhibited transcriptional readthrough at *SNR33*, but termination defects at *SNR33* were not observed in *nrd1-51* or *nrd1-102* mutants (Figures 6C, 3B, and 3C). Considering that Nrd1 is a sequence-specific RNA-binding protein, this variation in Nrd1 dependency within snoRNA genes is probably due to different sequence contexts at the 3' end. Indeed, while *SNR13* scores very highly for predicted Nrd1 sites, *SNR33* does not (Carroll et al., 2004). The cap-binding complex associates with the Nrd1 complex (Vasiljeva and Buratowski, 2006) and crosslinks to both mRNA and snoRNA genes (Figure 2 and data not shown). However, deletion of the Cbc1 subunit gene did not result in termination defects at either class of genes (Figure S3).

Detection of Precursor and Readthrough snoRNA Transcripts

To confirm the ChIP results and to analyze how defective termination affects RNA synthesis, northern blot assays were used to monitor *ADH1*, *SNR13*, and *SNR33*

transcripts (Figure 7). Transcripts were separated by agarose (Figure 7A) or acrylamide (Figures 7B and 7C) gel electrophoresis. While a single major transcript was observed for each gene in wild-type cells, two types of new snoRNA transcript species were observed in mutant strains. One class was 200–300 nucleotides (nt) longer than the processed transcript. Based on the Pol II ChIP data (Figures 1 and 3–6), this RNA is the size expected for the full-length transcription product before 3' trimming by exosome (pre-snoRNA). Indeed, this transcript is seen in an exosome mutant (*rrp6Δ*) where termination is normal but processing is partially defective (Figure 7A, lanes 12–15; Figure 7B, lanes 3 and 4; Figure 7C, lanes 3 and 4). Interestingly, a small amount of pre-snoRNA is also seen in *nrd1* mutants at nonpermissive temperature (Figure 7A, lanes 7 and 9; Figure 7C, lanes 17 and 19). We confirmed that these RNAs were not due to 5' extension by RT-PCR (data not shown). Since *nrd1* mutants have no termination defect on this gene (Figure 6C), we believe the appearance of pre-snoRNA reflects the other function of Nrd1 in recruiting and activating exosome at snoRNA 3' ends (Vasiljeva and Buratowski, 2006).

The second new class of transcript seen in mutant strains represents transcriptional readthrough of the

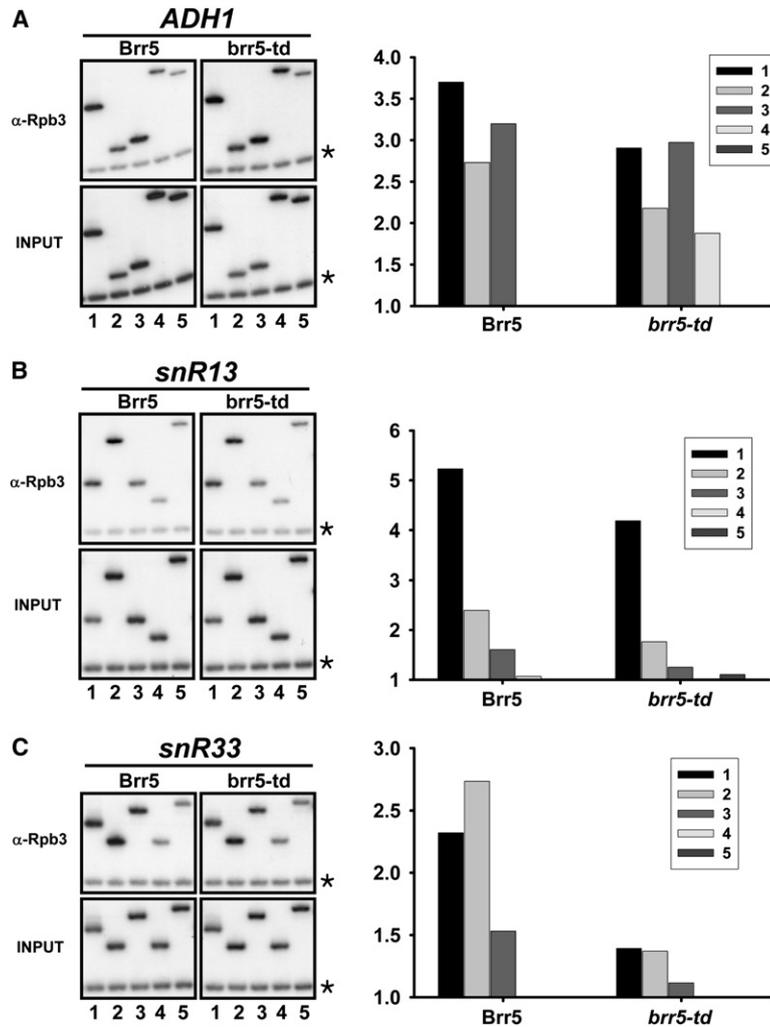


Figure 5. Termination of snoRNAs Does Not Require the mRNA Cleavage Factor Brr5

Wild-type and degron-tagged Brr5 (*brr5-td*) cells were incubated at 37°C for 100 min to deplete Brr5 in vivo, and analyzed by α -Rpb3 ChIP assays. Quantification data are shown in the right panels. (A) *ADH1* gene. (B) *SNR13* gene. (C) *SNR33* gene. See also Figure S2 for analysis of the *PMA1* gene and immunoblotting to show Brr5 depletion.

normal snoRNA terminator followed by termination at the poly(A) site of the adjacent mRNA gene (Steinmetz et al., 2001; Ganem et al., 2003). The appearance of this transcript correlated perfectly with transcriptional readthrough as defined by downstream Pol II crosslinking. Readthrough transcripts were seen for both *SNR13* and *SNR33* in a *sen1* mutant, as well as at *SNR13* in *nrd1* mutants. Importantly, readthrough of the snoRNA terminators was not seen with *rna14* or *rna15* mutants (Figure 7A, lanes 4–6; Figure 7B, lane 5; Figure 7C, lane 5 and data not shown), suggesting that these subunits of polyadenylation factor CFIA are not essential for snoRNA termination. These mutant strains did show a reduction in levels of the *ADH1* transcript, the expected result because mRNA transcripts that are not properly polyadenylated get degraded (Torchet et al., 2002; Milligan et al., 2005).

The results with CFIA subunit Pcf11 were more complicated. The *pcf11-13* allele exhibits readthrough transcription on *SNR13* and *SNR33*, but not *ADH1*, in agreement with the Pol II ChIP. The more severe *pcf11-9* allele causes readthrough of *ADH1* and *SNR33*. However, no snoRNA readthrough transcripts can be seen on the northern blots in *pcf11-2* cells. In agreement with the ChIP experiments, the RNA analysis indicates that

snoRNA termination requires the CTD interaction function of Pcf11, but not the other function or functions of CFIA that are important for mRNA cleavage.

Discussion

Although Pol II transcribes both mRNAs and sn/snoRNAs, only mRNAs are polyadenylated. The polyadenylation machinery is necessary for proper termination at mRNA genes, in part because mRNA cleavage provides an entry point for the Rat1/Xrn2 exonuclease, which somehow contributes to proper termination (Kim et al., 2004b; West et al., 2004). The termination mechanism of snoRNAs is still unclear. In *Saccharomyces cerevisiae*, the Nrd1, Nab3, and Sen1 proteins are known to be important (Steinmetz et al., 2001; Ganem et al., 2003; Ursic et al., 2004), but it has been suggested that the polyadenylation machinery and exosome may also be involved. Here, we show that the exosome is necessary for proper 3' trimming, but not termination, of snoRNAs. Furthermore, transcription termination of snoRNA does not require Rat1 exonuclease or the presumed mRNA endonuclease Brr5/Ysh1, indicating that snoRNA termination does not occur by the torpedo mechanism used by mRNAs. Conversely, mutations in

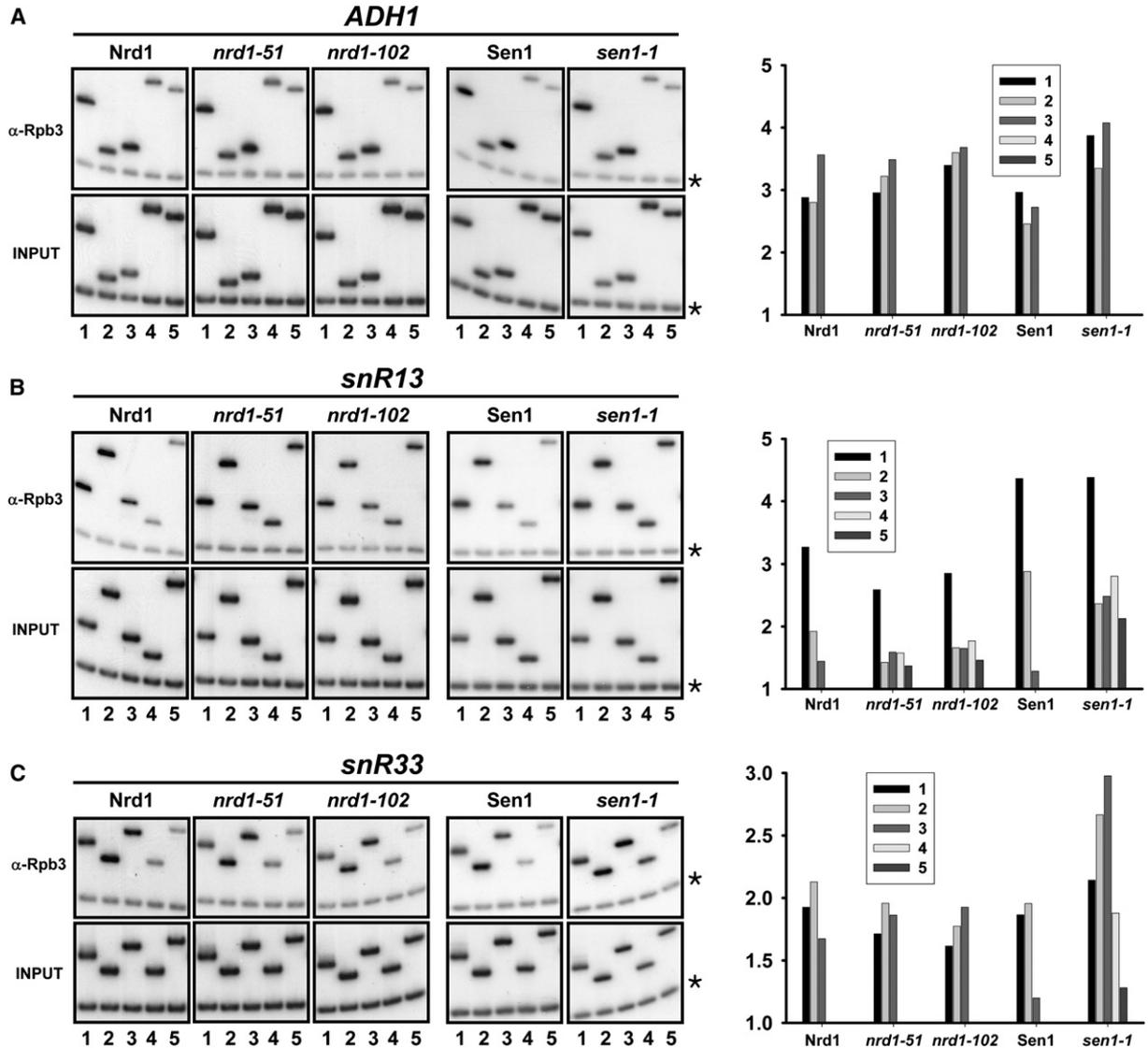


Figure 6. Effects of *NRD1* and *SEN1* Mutants on Transcription Termination
Isogenic wild-type, *nrd1-51*, *nrd1-102*, and *sen1-1* cells were shifted to nonpermissive temperature (37°C) for 30 min, and α -Rpb3 ChIP was carried out. Quantifications of data are shown in the right panels. (A) *ADH1* gene. (B) *SNR13* gene. (C) *SNR33* gene.

NRD1 or *SEN1* do not cause termination defects at several mRNAs. Therefore, there must be at least two termination pathways for Pol II in *Saccharomyces cerevisiae* (Figure 8). Both pathways appear to require Pcf11, although perhaps not the same functions of this protein. Surprisingly, ChIP experiments show that poly(A) factors and Rat1 are recruited to snoRNAs, suggesting that elongating Pol II maintains both pathways as possible options until relatively late in the transcription cycle.

Pcf11 Is Used by Both Pathways

It is likely that the termination pathways have some overlap, because both use Pcf11, a subunit of the polyadenylation factor CFIA. However, different mutant alleles of Pcf11 have distinct effects (Figures 4 and 7 and Figure S1), consistent with the finding that Pcf11 CTD binding and polyadenylation functions are separable (Sadowski et al., 2003). Pcf11-2 causes defects in mRNA

cleavage and termination yet does not affect snoRNA termination. Pcf11-13, which does not affect mRNA cleavage activity but is defective for CTD binding (Sadowski et al., 2003), causes a strong termination defect on the snoRNAs tested, but not at mRNAs. Pcf11-9 is defective for mRNA cleavage and CTD binding (Sadowski et al., 2003), and we find that this allele affects termination at both classes of genes. Consistent with a general role, it has recently been shown that Pcf11 can directly trigger in vitro termination of Pol II in a CTD-dependent manner (Zhang et al., 2005).

Pcf11 is a subunit of the CFIA polyadenylation factor, so it might be expected that other subunits of this complex would also be involved in snoRNA termination. However, in our ChIP and northern assays, the *rna14* or *rna15* mutants were defective for termination of mRNAs but not snoRNAs (Kim et al., 2004a, data not shown). This finding appears to conflict with a previous

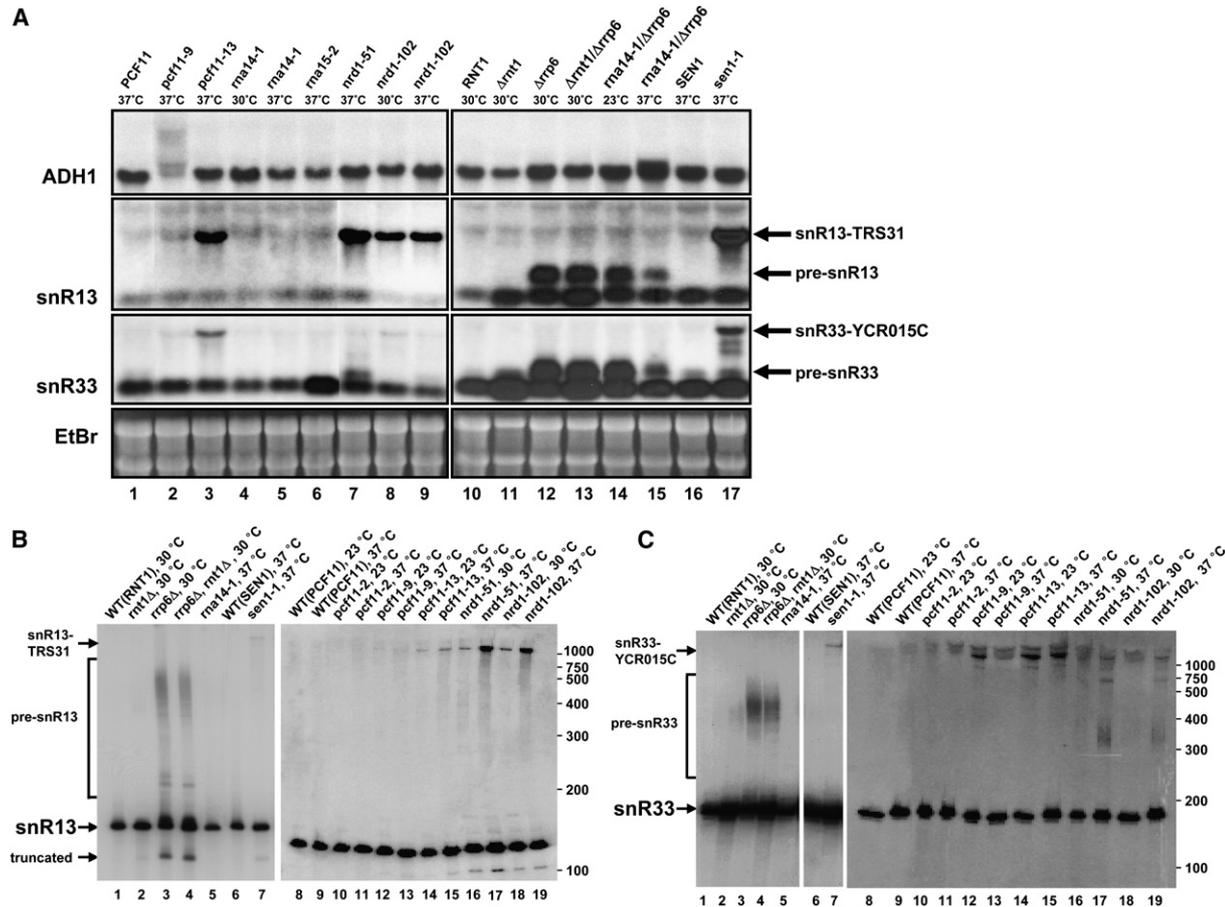


Figure 7. Northern Blot Analyses of *ADH1*, *SNR13*, and *SNR33* Genes

Total RNA was isolated from the indicated mutant cells grown at indicated temperatures. (A) RNA was resolved in 1.2% agarose gels. Full-length pre-snoRNA and readthrough transcripts are indicated by arrows. (B and C) RNA was resolved on 6% polyacrylamide gels and probed for *SNR13* and *SNR33* transcripts, respectively. The truncated snR13 species has previously been described and is thought to be a decapped, 5' truncated breakdown product of the mature snR13 species (Rasmussen and Culbertson, 1998).

report that 3' end formation of reporter transcripts containing snoRNA 3' sequences was disrupted in *rna14-1* and *rna15-2* mutants (Morlando et al., 2002). They also detected endogenous snR13-TRS31 transcripts by primer extension in *rna15-2* cells shifted to the nonpermissive temperature. A possible explanation for the discrepancy is that the previous study used much longer temperature shifts than we did (several hours versus 30 min). It may be that after prolonged time at the nonpermissive temperature the Rna14 and Rna15 mutants have an indirect effect on Pcf11 protein levels or activity. Therefore, there is agreement that the CFIA complex is important for snoRNA termination, but it remains to be determined whether individual subunits other than Pcf11 have a direct role. Other studies also suggest that Ssu72 and the APT complex, a set of proteins peripherally associated with polyadenylation factor CPF, contribute to snoRNA 3' end formation (Dheur et al., 2003; Nedea et al., 2003). Therefore, there may be an early stage in which the two pathways overlap.

The snoRNA Termination Pathway

Instead of polyadenylation, the 3' ends of sn/snoRNAs undergo nucleolytic trimming of a longer precursor tran-

script by RNase III (Rnt1) and the exosome (Allmang et al., 1999; van Hoof et al., 2000). Unlike mRNAs, 3' processing of snoRNAs may be separate from termination. We saw normal snoRNA termination in strains lacking Rnt1 or the nuclear exosome subunit Rrp6 (Figure S3). In cells lacking Rrp6, snoRNA transcripts with 3' extensions of a few hundred nucleotides were detected (Figure 7), a result also recently observed in genome-wide studies (Davis and Ares, 2006; Houalla et al., 2006). ChIP experiments to monitor Pol II at *SNR13* and *SNR33* showed that these extended transcripts are the size expected for full-length transcripts before exosome processing. These pre-snoRNA transcripts show size heterogeneity of about 100 nt (Figure 7B), probably due to two factors. First, like mRNA genes, snoRNA genes may have a window of termination sites rather than a single discrete site. Second, it is likely that these preprocessed transcripts have short poly(A) tails (van Hoof et al., 2000; Morlando et al., 2002). The alternative poly(A) polymerases Trf4 and Trf5 modify RNAs that are targeted to the exosome for 3' processing and/or degradation (Lacava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005; Eggecioglu et al., 2006). Houalla et al. (2006) found that deletion of *RRP6* led to an increase of transcripts

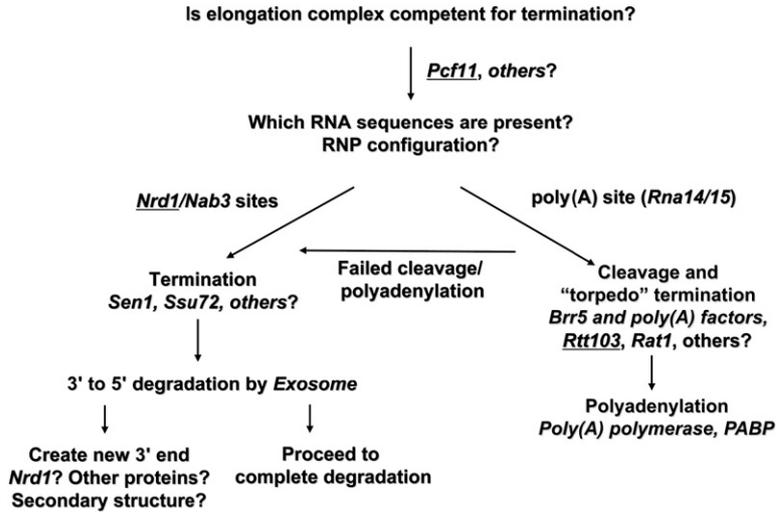


Figure 8. A Possible Decision Pathway for Termination and 3' Processing by Pol II
See Discussion for details. Proteins are italicized, CTD-binding proteins are underlined.

spanning the 5' end of *TRS31* upstream into *SNR13*, leading to the suggestion that exosome could be involved in *SNR13* termination. However, we suspect that the primer extension experiment in that report may have detected the stabilized pre-snr13 transcripts we observe in the absence of Rrp6. Our results suggest that exosome is not a termination factor for snoRNAs.

The ChIP analysis shows that snoRNA termination is normal in *Rat1* or *Brr5* mutants that exhibit strong mRNA termination defects, suggesting that snoRNAs do not terminate by the *Rat1* torpedo mechanism. Instead, the strongest candidate for a snoRNA-specific terminator complex is that containing *Nrd1*, *Nab3*, and *Sen1* (Conrad et al., 2000; Steinmetz et al., 2001). *Sen1* has helicase activity (Kim et al., 1999), and it is speculated that it may actively displace Pol II. *Nrd1* has multiple functions including sequence-specific RNA binding and CTD binding, and mutations in either function can affect snoRNA termination (Conrad et al., 2000; Steinmetz et al., 2001). Surprisingly, in our experiments *nrd1-51* and *nrd1-102* mutants had strong termination defects at *SNR13* but not *SNR33* (Figure 6). In a computer analysis prediction of *Nrd1*- and *Nab3*-binding sites, *SNR13* was ranked much higher than *SNR33* and shown to be more dependent upon *Nrd1*/*Nab3* for termination (Carroll et al., 2004). In *nrd1* mutants, RNA analysis revealed preprocessed *SNR33* transcripts that were similar to those seen in exosome mutants (Figure 7). This is presumably because *Nrd1* also interacts with the nuclear exosome, recruiting it to substrates and increasing its exonuclease activity (Vasiljeva and Buratowski, 2006). We believe that the sequence-specific binding activities of *Nrd1* and *Nab3* may recruit both *Sen1* and exosome to snoRNA substrates. Depending on the particular sequences and rate-limiting steps at a snoRNA gene, different *Nrd1* mutants could manifest defects in either *Sen1*-dependent termination or exosome-dependent 3' trimming.

The Two Termination Pathways Serve as "Back-Up" to Each Other

Several observations argue that *Nrd1* and *Sen1* do not normally function at mRNA terminators. In our experiments, *SEN1* or *NRD1* mutants caused Pol II to read

through snoRNA termination sequences but not several mRNA terminators tested. This agrees with a genome-wide analysis of Pol II crosslinking in which less than 1% of mRNA-encoding genes showed readthrough in a *sen1* mutant (Steinmetz et al., 2006 [this issue of *Molecular Cell*]). Interestingly, these genes were generally quite short (less than 500 bp), as are most snoRNAs. Similarly, microarray studies showed that very few mRNA transcripts are affected in *nrd1-5* mutant cells (Houalla et al., 2006). Many of the exceptions (notably including the *NRD1* mRNA itself) have *Nrd1*- and *Nab3*-binding sites and are downregulated by the *Nrd1*/exosome complex (Arigo et al., 2006a; Houalla et al., 2006). These exceptions may, at least in part, use the snoRNA termination pathway.

It was recently discovered that the yeast genome produces a large number of cryptic noncoding transcripts (CUTs) that are degraded by the exosome/TRAMP complex (Wyers et al., 2005; Davis and Ares, 2006). It appears that degradation of many of these cryptic transcripts is also dependent upon *Nrd1* (Arigo et al., 2006b; Thiebaut et al., 2006). *Nrd1* may be involved in termination as well as exosome recruitment at these loci. Therefore, the snoRNA termination/processing pathway and the termination/degradation pathway for cryptic transcripts are likely to be closely related. The only feature required to distinguish these two pathways is a signal to block exosome from completely degrading the transcript, such as secondary structure or a bound protein. We have found that *Nrd1* binding itself can block the progression of exosome (Vasiljeva and Buratowski, 2006).

When termination fails at *SNR13* or *SNR33*, transcription continues on to the poly(A) site of the adjacent mRNA gene. When the cleavage/poly(A) termination pathway fails, it is likely that the polymerase continues until it can be terminated using the snoRNA-type pathway. This would explain why Pol II eventually terminates even when *Rat1* is inactivated (Kim et al., 2004b; West et al., 2004). The minimal *Nrd1*- and *Nab3*-binding sites (GUAA/G and UCUU, respectively) occur frequently in the yeast genome (Carroll et al., 2004). This mechanism may also recruit exosome, which is known to be important for the degradation of nonpolyadenylated transcripts.

Choosing between Pathways

How does Pol II choose which of the two termination pathways to use? In mammalian cells, snRNA identity appears to be specified by promoter sequences (de Vegvar et al., 1986; Hernandez and Weiner, 1986). Instead of TFIID, these promoters recruit an alternative TBP complex known as SNAPc (Henry et al., 1995). These promoter-bound factors may in turn recruit a specific snRNA elongation/termination complex such as the recently described Integrator complex (Baillat et al., 2005). *Saccharomyces cerevisiae* does not have either SNAPc or Integrator; rather, snRNA and snoRNA promoters are thought to use the same Pol II initiation complex as mRNA promoters.

Another possible signal for specifying RNA identity could be cotranscriptional assembly of either mRNPs or snoRNPs, as influenced by sequences within the transcript. Mutations of snoRNP components affect snoRNA 3' end formation and termination (Morlando et al., 2004; Ballarino et al., 2005). Similarly, the hnRNP-like proteins Hrp1 and Npl3 have been shown to affect mRNA 3' end formation (Kessler et al., 1997; Bucheli and Buratowski, 2005). Cotranscriptionally assembled RNA/protein complexes may interact with the downstream 3' end-processing machineries and guide Pol II into a specific termination pathway.

Undoubtedly, another essential step in the decision process is recognition of RNA sequences at the 3' end of genes by the specific 3' processing complexes. Although yeast polyadenylation sequences appear to be rather degenerate, some consensus sequences have been derived (Dichtl and Keller, 2001; Gross and Moore, 2001). For snoRNA genes, there may be multiple and/or strong Nrd1- and Nab3-binding sites (Figure 8).

Surprisingly, both classes of 3' RNA processing factors appear to be recruited to Pol II-transcribed genes in yeast regardless of whether they encode mRNA or snoRNA. Here, we show that the poly(A) factors and Rat1 can be crosslinked to snoRNA genes. Conversely, Nrd1 and Sen1 are seen at both the 5' and 3' ends of mRNAs (Nedea et al., 2003; Steinmetz et al., 2006). Several proteins associated with the polyadenylation factors (Pcf11, Ssu72, Ref2, and Pti1) have been implicated in both pathways, suggesting their activities could be part of the decision-making process (Dheur et al., 2003; Nedea et al., 2003; Steinmetz and Brow, 2003; Cheng et al., 2004; Dichtl et al., 2004). By CHIP, we cannot determine whether both snoRNA and mRNA 3' processing complexes are simultaneously bound to a single Pol II or whether the factors are dynamically exchanging. In either case, the elongating yeast Pol II apparently keeps the two different termination options available until the last stage of transcription in preparation for the appropriate RNA sequences to emerge.

Experimental Procedures

Yeast Strains

Strains used in this study are listed in Table S1.

Chromatin Immunoprecipitations

CHIP procedures and quantification were performed as described (Kim et al., 2004a). For temperature-shift experiments, cells were incubated at 23°C until OD₅₉₅ reached 1.0. Equal amount of media prewarmed to 51°C was then added, and cells were further incubated at

37°C for 30 min. For depletion of degenon-tagged Brr5 in vivo, incubation time was extended to 100 min at 37°C. Primers used in PCR are listed in Table S2. Variation between duplicate PCR reactions is less than 5% (as measured by the internal control PCR product), and variation between experiments was typically less than 20%.

Microarray Experiments

Amplification of DNAs and hybridization were carried out as previously described (Kim et al., 2004b).

Northern Blot Analysis

Total RNA was isolated using hot phenol extraction from cells grown at 23°C, 30°C, or after temperature shift to 37°C for 30 min. About 80 µg of total RNA was loaded onto 1.2% agarose (MOPS-formaldehyde) gel, or about 15 µg of total RNA was loaded onto denaturing acrylamide gels (6%). RNA was transferred onto nylon membranes, and prehybridization was carried out for 2 hr at 42°C in a solution containing 50% formamide, 5× Denhardt's solution, 5× SSPE, 0.1% SDS, and 200 µg/ml single-stranded DNA. Hybridization was performed in the same conditions with a radiolabeled probe for ~16 hr, and the membranes were washed in 2× SSC/0.1% SDS at RT or 42°C. After washes, the membranes were exposed to X-ray film or analyzed by phosphorimager analyzer (BAS1500, Fuji). DNA fragments were amplified from genomic loci with the following primers (see Figure 2) and used as templates for random oligo labeling: ADH1 (#2up and #3low), snR13 (#1up and #2low), and snR33 (#1up and #2low).

Supplemental Data

Supplemental Data include four figures and two tables and can be found with this article online at <http://www.molecule.org/cgi/content/full/24/5/723/DC1/>.

Acknowledgments

We thank W. Keller, E. Steinmetz, D. Brow, J. Corden, G. Chanfreau, D. Ursic, and M. Culbertson for strains and plasmids; D. Brow, J. Corden, and D. Libri for communicating unpublished results; and J. Corden and D. Brow for helpful discussions. This research was supported by grants GM56663 to S.B. and GM68887 to C.M. from the US National Institutes of Health. L.V. is a Fellow of the Leukemia and Lymphoma Society. M.K. is supported by the Charles A. King Trust Postdoctoral Fellowship (Charles A. King Trust, Bank of America, Co-Trustee [Boston, Massachusetts]).

Received: July 11, 2006

Revised: October 17, 2006

Accepted: November 13, 2006

Published: December 7, 2006

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Accession Numbers

The chromatin immunoprecipitation/microarray datasets in Figure 1, which also appeared in Kim et al. (2004b), have been deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/projects/geo/>) under accession number GSE6301.