

Reporter mRNAs cleaved by Rnt1p are exported and degraded in the cytoplasm

Stacie Meaux¹, Mathieu Lavoie², Jules Gagnon², Sherif Abou Elela^{2,*} and Ambro van Hoof^{1,*}

¹Department of Microbiology and Molecular Genetics, University of Texas Health Science Center-Houston, Houston, TX 77030, USA and ²Département de Microbiologie et d'Infectiologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

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ABSTRACT

For most protein coding genes, termination of transcription by RNA polymerase II is preceded by an endonucleolytic cleavage of the nascent transcript. The 3' product of this cleavage is rapidly degraded via the 5' exoribonuclease Rat1p which is thought to destabilize the RNA polymerase II complex. It is not clear whether RNA cleavage is sufficient to trigger nuclear RNA degradation and transcription termination or whether the fate of the RNA depends on additional elements. For most mRNAs, this cleavage is mediated by the cleavage and polyadenylation machinery, but it can also be mediated by Rnt1p. We show that Rnt1p cleavage of an mRNA is not sufficient to trigger nuclear degradation or transcription termination. Insertion of an Rnt1p target site into a reporter mRNA did not block transcription downstream of the cleavage site, but instead produced two unstable cleavage products, neither of which were stabilized by inactivation of Rat1p. In contrast, the 3' and 5' cleavage products were stabilized by the deletion of the cytoplasmic 5' exoribonuclease (Xrn1p) or by inactivation of the cytoplasmic RNA exosome. These data indicate that transcription termination and nuclear degradation is not the default fate of cleaved RNAs and that specific promoter and/or sequence elements are required to determine the fate of the cleavage products.

INTRODUCTION

Processing of the nascent RNA polymerase II transcript by the cleavage and polyadenylation machinery generates

an uncapped 3' cleavage product. In the torpedo model for transcription termination, degradation of this nascent uncapped transcript by Rat1p triggers transcription termination (Figure 1A) (1,2). More recently, a variant of the torpedo model of transcription termination which involves the yeast endoribonuclease III Rnt1p, has been described (3,4). Cleavage sites for the endoribonuclease Rnt1p are found downstream of the canonical polyadenylation sites of many genes and Rnt1p cleavage is thought to act as a surveillance mechanism preventing transcription read-through from leaky or inefficient transcription termination (3,4). For example, cleavage of yeast *NPL3* transcripts is thought to occur co-transcriptionally, generating an uncapped nascent transcript. Degradation of this nascent transcript by Rat1p triggers transcription termination thus preventing the accumulation of aberrant dicistronic mRNAs. Rnt1p cleavage has also been reported within introns of pre-mRNAs and within mRNAs (5–7). It is presumed that cleavage within these transcripts targets them for rapid decay, but it remains possible that Rnt1p cleavage mediates transcription termination. Indeed, the exact decay pathway of such products remains largely unexplored.

Interestingly, Rnt1p-mediated cleavage does not always trigger transcription termination. For example, Rnt1p is also implicated in the processing of a variety of small stable RNAs, such as snRNAs and snoRNAs (8–11). Rnt1p-mediated cleavage of the 3' extended small RNA precursors is typically followed by further maturation by a 3' exoribonuclease complex named the nuclear exosome (Figure 1B) (12). On the other hand, Rnt1p-mediated cleavage at specific sites in the 5'-extended snoRNA precursors generates an uncapped 5' end that is accessible for further processing by the nuclear 5' to 3' exoribonuclease Rat1p (13). In some cases, multiple snoRNAs precursors are transcribed as a multimeric transcript (Figure 1C) (6,7)

*To whom correspondence should be addressed. Tel: +1 713 500 5234; Fax: +1 713 500 5499; Email: ambro.van.hoof@uth.tmc.edu
Correspondence may also be addressed to Sherif Abou-Elela. Tel: +1 819 564 5275; Fax: +1 819 564 5392; Email: sherif.abou.elela@usherbrooke.ca
Present address:

Stacie Meaux, Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA

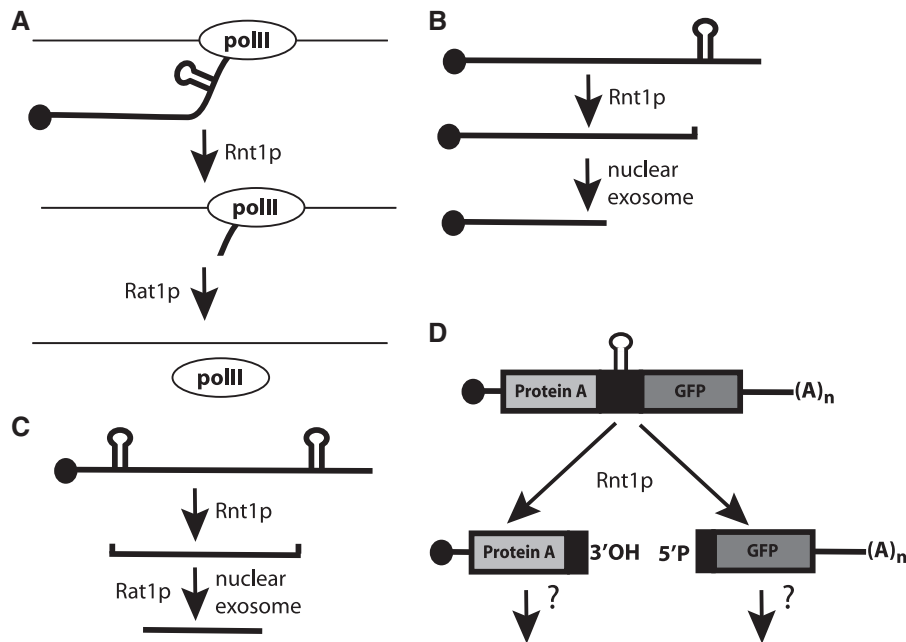


Figure 1. The fate of different Rnt1p cleaved RNA polymerase II transcripts. (A) Transcripts from some protein-encoding genes are cleaved by Rnt1p. Cleavage is followed by processing by Rat1p, the major nuclear 5' to 3' exoribonuclease, which causes RNA polymerase II to dissociate from the DNA. (B) Rnt1p cleaves precursors to some snoRNAs and snRNAs at a site downstream of the mature RNA. The cleavage product is further processed by the nuclear exosome. (C) Rnt1p cleaves precursors to other snoRNAs at a site upstream or downstream of the mature RNA, or at sites both upstream and downstream. Cleavage at upstream sites is followed by processing by Rat1p, but unlike in panel A this does not lead to transcription termination. (D) To determine which of these fates is the default fate of Rnt1p cleavage products, Rnt1p cleavage stem-loops were inserted between the coding regions of Protein A and GFP. Upon cleavage of the transcript by Rnt1p, two cleavage products will result. The 5' cleavage product will possess a 5' cap and a 3' hydroxyl in the place of a poly(A) tail. The 3' cleavage product will possess a poly(A) tail and a 5' monophosphate in the place of the 5' cap.

that is cleaved by Rnt1p resulting in monomeric intermediates which are further processed by Rat1p (14) and the nuclear exosome (15). Thus, it is clear that at least for a subset of substrates, Rat1p-mediated degradation of Rnt1p cleavage products does not lead to transcription termination. However, it is still not clear if Rnt1p cleavage normally triggers transcription termination and special features or RNA binding proteins prevent Rat1p-dependent termination in the case of snoRNAs. Another possibility is that Rnt1p cleavage only initiates transcription termination if other determinants are also present.

It is likely that the fate of RNA transcripts generated by endoribonucleolytic cleavage in the nucleus is dependent on the nature of the cleavage site and adjacent sequence. Insertion of a hammerhead ribozyme or a modified group I intron, which mediates internal cleavage of a reporter mRNA, generated cleavage products that are rapidly degraded by the cytoplasmic exoribonucleases (16). These are surprising observations since ribozyme-mediated cleavage is expected to occur rapidly while the RNA is still in the nucleus which is thought to have a tight surveillance mechanism that would degrade such cleaved RNAs. Instead, it seems that ribozyme-cleaved mRNAs are exported to the cytoplasm where they are rapidly targeted by the surveillance pathways. However, it is also possible that degradation of cleaved mRNAs in the cytoplasm arise from ribozyme cleavage after nuclear export.

It is also possible that nuclear RNA degradation is prevented by the structure or chemical properties of the ribozyme cleavage products that render them less susceptible to nuclear ribonucleases. For example, the 5' OH produced by hammerhead ribozyme cleavage should generate a poor Rat1p substrate (17).

In this study, we inserted different Rnt1p cleavage signals into an mRNA. Interestingly, cleaved mRNAs were detected for some Rnt1p signals, but not for others, and accumulation of cleaved reporter mRNAs reflects the inherent cleavage preference of the enzyme. This provides *in vivo* evidence that authentic Rnt1p signals are recognized with different efficiencies. In the reporter mRNA context, Rnt1p-mediated cleavage does not result in transcription termination or nuclear RNA degradation. Instead, the resulting 5' cleavage products are rapidly degraded by the cytoplasmic exosome, while the 3' cleavage products are rapidly degraded by the cytoplasmic Xrn1p. Thus, in comparable reporter mRNAs, the fate of Rnt1p cleavage products is very similar to the fate of ribozyme generated cleavage products. These results suggest that Rnt1p-mediated cleavage by itself is not sufficient to trigger transcription termination or nuclear degradation. Instead, we propose that transcription termination and nuclear RNA degradation require a combination of endonucleolytic cleavage signals and special primary or secondary structural motifs.

MATERIALS AND METHODS

Yeast strains

The *rnt1Δ* strain (yAV1084; *MATa*, *leu2-Δ0*, *lys2-Δ0*, *met15Δ0*, *ura3-Δ0*, *his3-Δ1*, *rnt1Δ::KANMX6*) was constructed by sporulating the *rnt1Δ::KANMX6* heterozygous diploid obtained from Open Biosystems. *ski7Δ*, *xrn1Δ* and *rrp6Δ* strains are isogenic to the wild-type BY4741 and were obtained from Open Biosystems. The temperature-sensitive *rat1-1* strain (yRP1781) was obtained from Dr Patricia J. Hilleren and Dr Roy Parker and has been described previously (18).

Plasmids

To analyze whether cleavage depended on the catalytic activity of Rnt1p. Plasmids containing a wild-type allele of *RNT1* [pRS315/GFP/RNT1 (19)], a catalytically-inactive allele of *RNT1* [pRS315/GFP/RNT1-D247R (19)], or an empty vector were introduced into an *rnt1Δ* strain.

Reporter mRNA constructs were generated by ligating complementary oligonucleotides into the BamHI site of pAV214 (16). The sites where Rnt1p cleaves are indicated with / marks in the sequences below (5,9,20–22). Note that the sequences of the expected cleavage products are identical except the 3' most 3–9 nt of the 5' product and the 5' most 6–8 nt of the 3' product. For example the expected 3' product of the MIG2 site starts with GgaaTtc, while the corresponding product of the U5 site starts with TAg aaCtc (differences in upper case).

pAV351 (ProteinA-MIG2-GFP) contains the sequence
5'GGATCCAAGAAAG/CTAGCGTACAGAAACA
GGAGTTTTTGACAGTAAGC/GGAATTCGG
ATCC3'.

pAV354 (ProteinA-U5-GFP) contains the sequence 5'GG
ATCCTTTTCTAC/A/AATTTGATTCATGAGTCCA
TGGAACAAATAT/A/TAGAACTCGGATCC3'.

pAV355 (ProteinA-5U-GFP) contains the sequence 5'GG
ATCCGAGTTCTATATATTTGTTCCATGGACTC
ATGAATCAAATTTGTAGAAAAGGATCC3'.

pAV356 (ProteinA-U5*-GFP) contains the sequence 5'G
GATCCTTTTCTACAAATTTGATTCATGACTCG
ATGGAACAAATATATAGAACTCGGATCC3'.

pAV398 (ProteinA-FIT2-GFP) contains the sequence 5'G
GATCCATCACTTGT/ATAATTTGTAATCGAGTT
CGGATAAGATGTATAC/GAATCTGGATCC3'.

pAV399 (ProteinA-snR47-GFP) contains the sequence
5'GGATCCAGA/A/AGGATATTGAACATGTTCA
GAAGAACGTGGTATCTTTT/TCCTTATGG
ATCC3'.

pAV400 (ProteinA-rRNA-GFP) contains the sequence
5'GGATCCTTCTTTT/TAAGTGGGTACTGGCAG
GAGCCGGGCCTAGTTTA/GAGAGAAGG
ATCC3'.

In vivo RNA analysis

Yeast strains were grown in SC-URA + 2% galactose to mid-log phase. The deletion strains were grown at 30°C, while the *rat1-1* strain was grown at room temperature

and incubated for 1 h at the restrictive temperature of 37°C. Wild-type control strains were analyzed under both conditions (e.g. in Figure 4A and C). For RNA decay analysis, the media was replaced with SC-URA + 4% glucose, total RNA was extracted, separated on 1.3% agarose gel and blotted by standard methods. Blots were probed with ³²P 5' end-labeled oligonucleotides specific for Protein A (5'TCTACTTTTCGGCGCCTGAG CATCATTTAGC3'), GFP (5'GCTGTTACAAACTCAA GAAGGACCATGTGG3'), *RPL41A* (5'GACATTACG ATACTCTTGAAAGAA3'), or the RNA subunit of the signal recognition particle (SRP) as a loading control (5'G TCTAGCCGCGAGGAAGG3'). Bands were detected and quantified using a STORM phosphorImager (GE Healthcare).

Protein analysis

Western blot analysis was performed according to standard techniques, using antibodies for Protein A (Sigma) and Pgk1p (Molecular Probes).

In vitro cleavage

Recombinant Rnt1p was produced in bacteria and FLPC-purified as previously described (23). Total RNA was isolated from *rnt1Δ* strains containing each plasmid grown to mid-log phase in SC-URA + 2% galactose. Fifteen micrograms of total RNA was then incubated in the absence or presence of 10 or 80 nM recombinant Rnt1p for 10 min at 30°C in 50 μl reaction buffer [30 mM Tris-HCl (pH 7.5), 5 mM spermidine, 0.1 mM DTT, 0.1 mM EDTA (pH 7.5), 10 mM MgCl₂] supplemented or not with 150 mM KCl. The reaction was stopped by phenol:chloroform extraction and analyzed as described in the 'In vivo RNA Analysis' section.

For cleavage of short stem-loops, RNA transcripts were generated by T7 RNA polymerase, gel purified and 5'-end-labeled using [γ -³²P]ATP as previously described (24). Cleavage reactions were performed by incubating 30 nM Rnt1p with 0.15 nM of 5'-end-labelled substrates for 10 min at 30°C in 20 μl reaction buffer, supplemented or not with 150 mM KCl, supplemented or not with 900 nM of unlabeled substrate. Cleavage products were separated by 20% denaturing PAGE and quantified as described (24).

RESULTS

Reporter design

Different Rnt1p signals were inserted into the middle of reporter genes expressed under the control of a galactose-inducible promoter (Figure 1D). The reporter gene (16) contains the coding region of the *Staphylococcus aureus* protein A ZZ domain followed by the coding region of the green fluorescent protein (GFP) gene and 3' flanking sequences of the *ADHI* gene that include a canonical transcription termination/polyadenylation site. Rnt1p normally cleaves a stem-loop structure capped with an AGNN tetraloop and the cleavage efficiency is dependent on the integrity and structure of the stem-loop motif.

Therefore, we tested the impact of a variety of stem-loops originating from the U5 snRNA (20), *snR47* snoRNA (9), mRNA [*MIG2*; (5) and *FIT2*; (22)], and the pre-rRNA 3' external transcribed spacer (3'ETS; 21). Except in the case of *FIT2*, each of these signals has been shown to trigger Rnt1p-mediated cleavage in their natural context. The sequences used include the tetraloop and 22–23 nt from the natural substrates on each side of the tetraloop. The insertion of these sequences into the reporter construct preserves the coding frame and does not add any premature stop codons. Mfold (25) was used to confirm that the intended stem-loop is predicted to be the most energetically stable structure (data not shown).

Rnt1p cleaves reporter mRNAs *in vivo*

To evaluate the cleavage efficiency of the reporter transcripts by Rnt1p, each reporter plasmid was transformed into a wild-type yeast strain and into the isogenic strain carrying a deletion of the *RNT1* gene. After galactose-induced expression of the reporter mRNA, total RNA was extracted from the transformed strains and analyzed by northern blotting. ProteinA-GFP reporters lacking an Rnt1p cleavage signal or containing a hammerhead ribozyme were included to serve as controls. As shown in Figure 2A, hybridization with a probe specific for the 5' half of the reporter (Protein A) revealed two distinct mRNA species. The slower migrating species corresponds

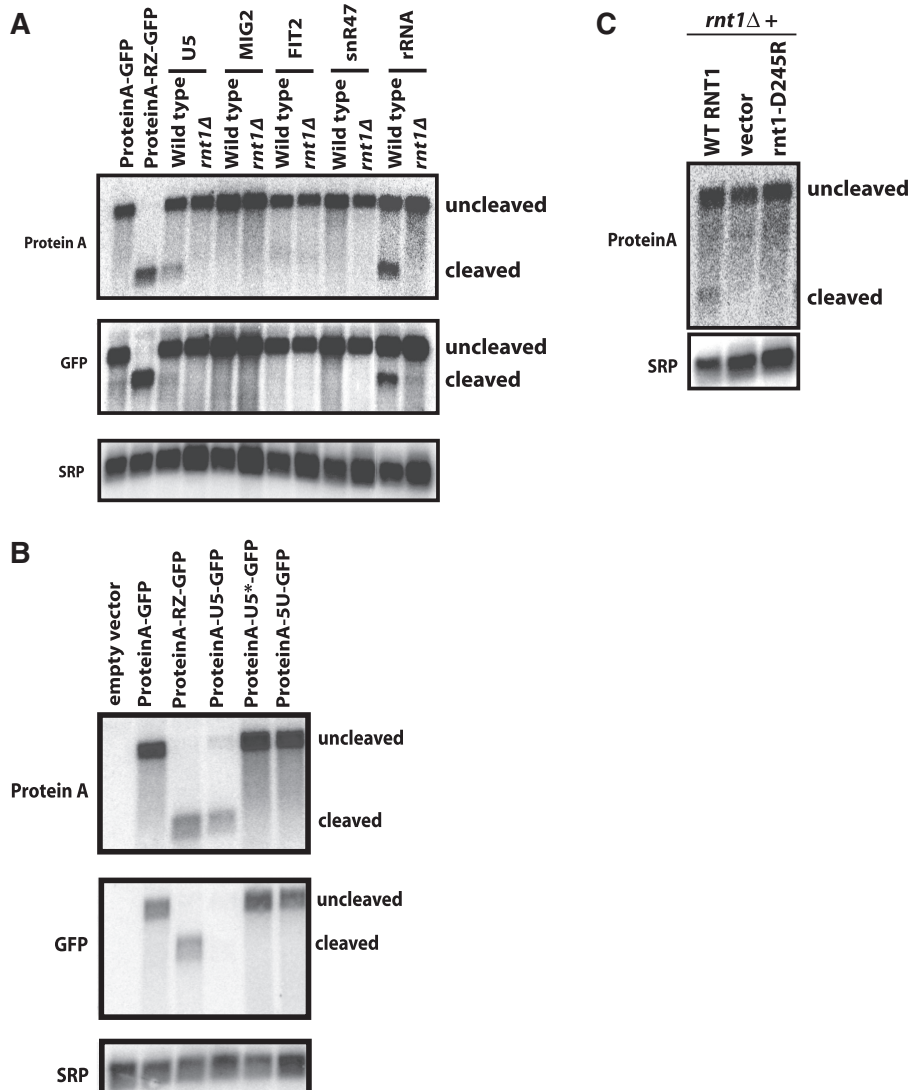


Figure 2. Rnt1p cleaves reporter mRNAs *in vivo*. (A) Northern blot analysis of total RNA extracted from wild-type or isogenic *rnt1Δ* yeast strains transformed with the indicated plasmids. (B) Northern blot analysis of total RNA extracted from wild-type yeast transformed with the Protein A-U5-GFP plasmid and two variants that should not be recognized by Rnt1p: Protein A-U5*-GFP contains point mutations of the U5 sequence that disrupt Rnt1p recognition and Protein A-5U-GFP contains the U5-derived sequence in the inverted orientation. (C) Northern blot analysis of RNA isolated from an *rnt1Δ* strain transformed with the Protein A-U5-GFP reporter construct and either an empty vector, a plasmid expressing wild-type Rnt1p, or a plasmid expressing a catalytically inactive Rnt1p (D245R).

to the expected size of the uncleaved reporter transcript, indicating that all reporters are effectively transcribed. More importantly, a faster migrating fragment corresponding to the expected size of the cleaved 5' portion of the reporter is observed in the wild-type strain transformed with the constructs containing the ribozyme control, U5 or rRNA cleavage signal. Cleaved reporter mRNAs with U5- or rRNA-derived sequences were not detected in an isogenic *rnt1Δ* strain, suggesting that the smaller species were indeed cleavage products produced by Rnt1p. Similarly, 3' cleavage products (GFP) were detectable for these same constructs in the wild-type strain, but not in an *rnt1Δ* strain (Figure 2A). Interestingly, both 5' and 3' cleavage products can be observed at the same time for the cleaved construct, thus suggesting that Rnt1p cleavage was not sufficient to prevent further transcription of the downstream GFP fragment. As expected, deleting *RNT1* had no effect on the RNAs produced from the ribozyme control (data not shown). We also detected low levels of smaller RNAs from the construct with the *FIT2*-derived Rnt1p site, but these RNAs could also be detected in an *rnt1Δ* strain, suggesting that they do not result from Rnt1p-mediated cleavage. For the constructs with Rnt1p sites derived from *snR47* and *MIG2*, we failed to detect any putative cleavage products. Thus it appears that some, but perhaps not all, of our reporter mRNAs can be efficiently cleaved by Rnt1p *in vivo*.

To further show that the ProtA-U5-GFP construct was an Rnt1p substrate, we altered the stem-loop sequence. In one control, the AGNN loop sequence and the top base pair of the stem were changed (GAGTCC changed to GACTCG; Figure 2B; construct ProteinA-U5*-GFP), and in a second control we inserted the U5-derived sequence backwards (Figure 2B; construct ProteinA-5U-GFP). Neither of these controls yielded any cleavage product, consistent with the known specificity of Rnt1p. Finally, the D245R point mutation in the catalytic site of Rnt1p (19) also disrupted the accumulation of the cleavage product (Figure 2C). Overall, we conclude that the reporter mRNAs with U5 snRNA and rRNA derived sequences are cleaved by Rnt1p *in vivo* and that this cleavage is dependent upon the AGNN stem-loop.

Accumulation of cleaved reporter mRNAs *in vivo* reflects substrate preference of Rnt1p

The fact that cleavage could not be observed for three of our reporter transcripts was somewhat surprising given that these cleavage signals are all derived from known natural Rnt1p substrates. Since the potential products generated after Rnt1p cleavage would be very similar from one construct to another, we believe that failure to detect cleavage products of mRNAs with *MIG2*, *FIT2* and *snR47* stem-loops is not caused by the fast decay of these specific transcripts *in vivo*, although we could not completely rule out this possibility. Another possible explanation for the lack of accumulation of cleaved reporter mRNAs is that Rnt1p may have an intrinsic preference for the U5 and rRNA stem-loops. To test this possibility we isolated total RNA from the *rnt1Δ* strain containing each of the five constructs. This total RNA was then

incubated with recombinant Rnt1p purified from *Escherichia coli* and submitted to northern blot analysis (probing for GFP). This analysis showed that all five AGNN stem-loop mRNAs are specifically cleaved under non-physiological conditions that favor RNA-protein interactions (high concentrations of Rnt1p and low salt buffer; Figure 3A). This result indicates that all transcripts can form the expected secondary structure *in vitro*. Importantly, we could not detect specific cleavage products of the control lacking an AGNN stem-loop, although the uncleaved mRNA seems unspecifically degraded in the presence of Rnt1p under these conditions. Reducing the enzyme concentration and increasing KCl to physiological concentration (150 mM) suppresses the unspecific degradation of the control lacking the stem-loop

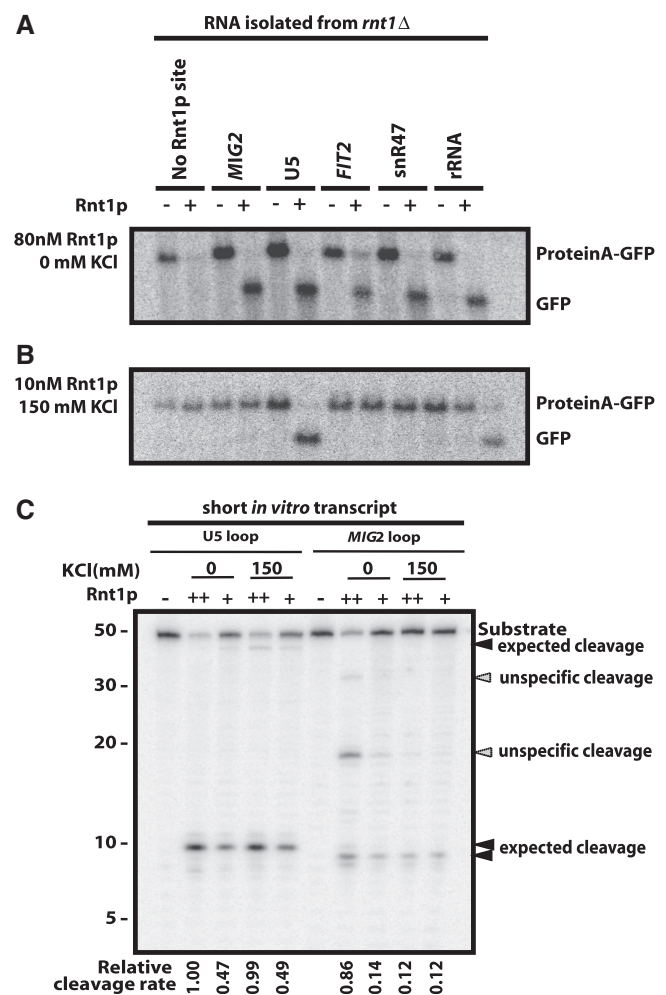


Figure 3. Differences in the accumulation of cleaved reporter mRNAs *in vivo* correlate with differences in recognition by Rnt1p. (A and B) Total RNA was isolated from an *rnt1Δ* strain containing each of the indicated plasmids and incubated for 10 min with either high concentration (80 nM) of Rnt1p and no KCl added (A) or low concentration (10 nM) of Rnt1p and 150 mM KCl (B). Each sample was then submitted to northern blot analysis and probed for GFP. (C) Short RNAs containing Rnt1p cleavage sites from U5 or MIG2 were generated by T7 RNA polymerase and 5'-end-labeled. Substrates were incubated with or without purified Rnt1p, either in the presence or absence of potassium chloride as indicated.

and reveals that the mRNAs with Rnt1p sites from *U5* snRNA and rRNA were preferentially cleaved (Figure 3B). These results signify that although all reporter transcripts have the potential to be cleaved by Rnt1p, only the *U5* and rRNA reporters are cleaved under physiological conditions. Thus, the differences in cleaved reporter mRNA accumulation *in vivo* correlate with differences in interactions of Rnt1p with these AGNN stem-loops.

It was not clear at this point whether Rnt1p's preferential cleavage of some substrates is a secondary effect of the insertion of the different cleavage signals in an mRNA, out of their normal context, or if Rnt1p has inherent preferences for certain AGNN stem-loops. To test if the stem-loops themselves show a difference in their ability to be cleaved by Rnt1p, we compared the cleavage of small, *in vitro*-transcribed *U5*-derived or *MIG2*-derived recognition sequences. We chose these two substrates because our *in vivo* and *in vitro* studies suggested that the *U5* stem-loop is a good substrate for cleavage while the *MIG2* stem-loop is cleaved less efficiently. Following *in vitro* transcription and 5' end labeling, each substrate was incubated with or without purified Rnt1p, either in the absence or presence of 150 mM KCl. Interestingly, the cleavage of these short stem-loop substrates mirrored those seen with the corresponding reporter mRNAs. Indeed, both substrates were cleaved in low salt concentrations when Rnt1p is in excess (enzyme:RNA ratio of 200:1). Increasing the salt concentration did not affect the cleavage efficiency of the *U5* stem-loop, but strongly inhibited cleavage of the *MIG2*-derived substrate. Consistently, the cleavage of the *MIG2* substrate was less efficient than that observed with *U5* under more physiological conditions (150 mM KCl and enzyme:RNA ratio of 1:30). We conclude that different sites for Rnt1p exhibit different cleavage efficiencies and that the newly constructed *in vivo* reporters faithfully reflect the substrates inherent cleavage efficiency and thus constitute a good tool for testing Rnt1p cleavage *in vivo*.

The 5' product of an Rnt1p cleaved reporter mRNA is degraded by cytoplasmic RNases

It was previously shown that mRNAs cleaved by a hammerhead ribozyme are degraded by cytoplasmic enzymes (16,26), suggesting that the unadenylated 5' cleavage product is exported from the nucleus. However, we could not exclude the possibility that the cleavage by the ribozyme occurred in the cytoplasm after export of the full-length capped and polyadenylated mRNA from the nucleus. Given the nuclear localization of Rnt1p (19,27,28), our newly designed ProteinA-U5-GFP construct now allows us to evaluate the fate of mRNAs that are cleaved in the nucleus.

In order to identify the cellular location where the cleavage products are degraded, we tested the impact of impairing nuclear or cytoplasmic exoribonucleases on the stability of Rnt1p cleavage products. If an Rnt1p cleavage product is degraded in the nucleus, it should be stabilized by the inactivation of the nuclear 5' exoribonuclease Rat1p and/or the nuclear exosome. On the other hand, if the cleaved RNA is degraded in the cytoplasm it is

expected to be more stable upon inactivation of the cytoplasmic 5' exoribonuclease Xrn1p and/or the cytoplasmic exosome. As shown in Figure 4, the Rnt1p 5'-end cleavage product was equally unstable in wild-type cells, cells carrying a temperature-sensitive mutation in *RAT1* and grown at the restrictive temperature, or cells lacking the nuclear exosome component Rrp6p. In contrast, in strains lacking the cytoplasmic exosome cofactor Ski7p or the cytoplasmic exonuclease Xrn1p, the half-life of the 5' cleavage product is increased 4- and 2-fold, respectively (Figure 4), suggesting that the 5' cleavage product is exported to the cytoplasm. If Rnt1p 5' cleavage product is exported to the cytoplasm, it should be available to the translational machinery. Western blot analysis using antibodies against Protein A indicated that the unadenylated cleavage product is translated in wild-type cells, and the amount of the protein produced is increased in *ski7Δ* cells, where the 5' cleavage product is more stable (Figure 4D). Thus, both mRNA stability measurements and western blot analysis indicates that the Rnt1p 5' cleavage product is exported to the cytoplasm.

We have previously shown that introducing a ribozyme motif within the reporter constructs generates an unadenylated cleavage product that is exported and translated (16,29). To determine the polyadenylation state of Rnt1p cleavage products we used oligo(dT)-dependent RNase H digestion. As expected, the vast majority of the 5' product of Rnt1p cleavage was not polyadenylated (Figure 4E). We conclude that the Rnt1p 5' cleavage product is predominantly degraded by cytoplasmic exoribonucleases and suggest that Rnt1p-cleaved mRNAs are not retained in the nucleus for an extended period of time, but instead are readily exported from the nucleus.

In addition to the cleavage products, we also detected a significant amount of uncleaved ProteinA-U5-GFP mRNA. This uncleaved mRNA pool is stabilized in an *xrn1Δ* strain, but not in strains with mutations in *RRP6*, *RAT1* or *SKI7*. This Xrn1p-mediated decay suggests that the uncleaved mRNA represents a pool of mRNA that escapes Rnt1p cleavage and is exported from the nucleus as a typical mRNA. Consistent with this interpretation is that mutation of the Rnt1p-recognition site does not significantly stabilize the uncleaved mRNA (data not shown) and that the uncleaved mRNA is translated into a protein A-GFP fusion protein (Figure 4D). Together these observations indicate that the uncleaved pool of ProteinA-U5-GFP mRNA that is detected by northern blot is not a precursor to the cleaved pool, but instead is degraded like a normal mRNA that lacks an Rnt1p cleavage site.

The 3' product of an Rnt1p cleaved reporter mRNA is degraded by Xrn1p

When analyzing the fate of the 3' GFP cleavage product, we discovered that, in a wild-type background, this product was present at extremely low levels (Figure 5A) and extremely unstable (Figure 5B). In the *xrn1Δ* strain, steady state levels were increased ~18-fold compared to wild-type (Figure 5A), and its half-life was greatly increased (half-life of 22 min; Figure 5D). In contrast, the stability was not affected by inactivation of the nuclear 5'-3'

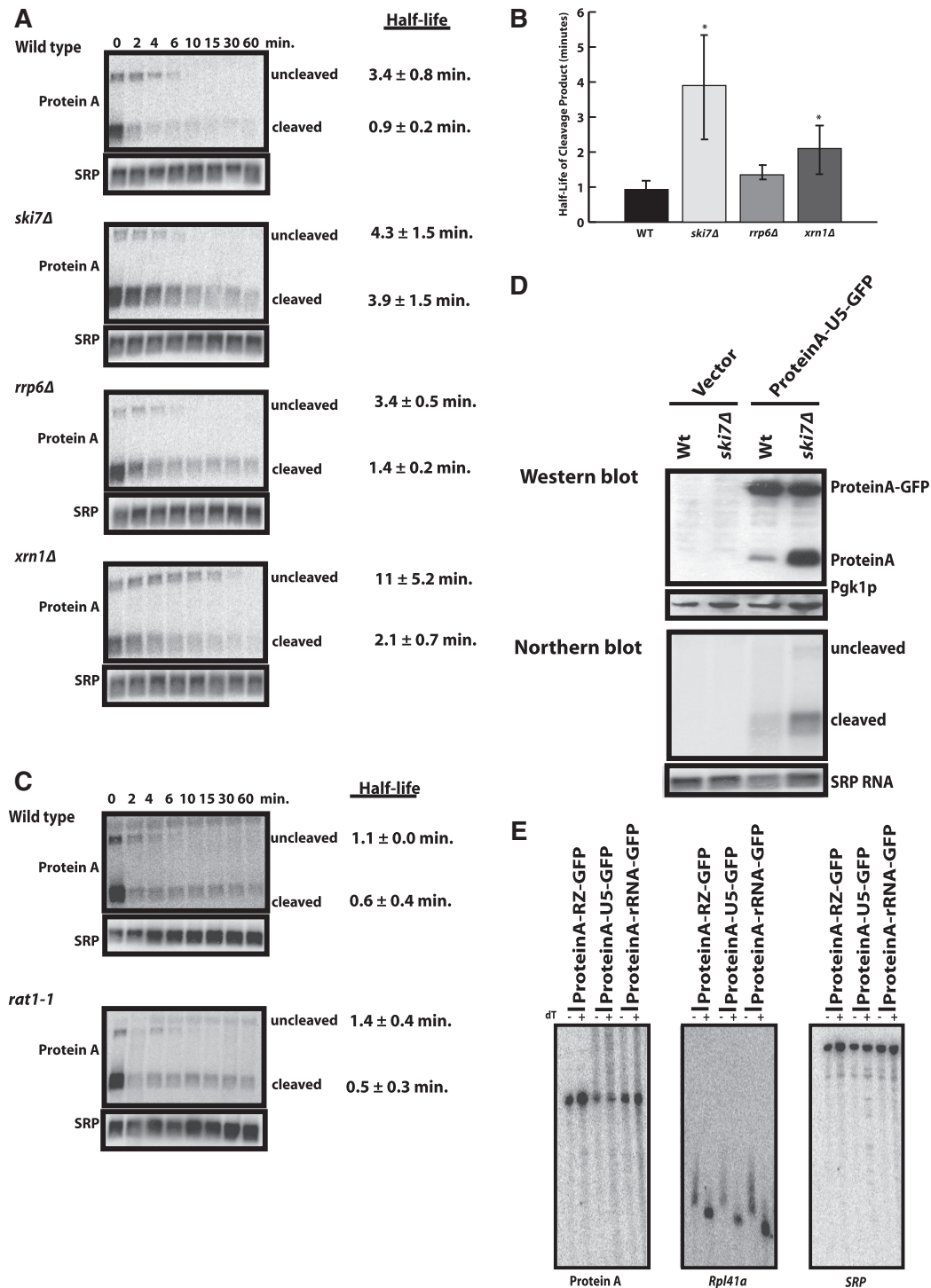


Figure 4. The unadenylated 5' cleavage product is degraded by cytoplasmic RNases. (A) Isogenic wild-type, *ski7Δ*, *rrp6Δ* or *xrn1Δ* strains containing the Protein A-U5-GFP reporter were grown to mid-log phase at 30°C. Transcription of the reporter gene was terminated by addition of glucose and total RNA was isolated at the indicated times. RNA was submitted to northern blot analysis and probed for Protein A or SRP RNA. Half-lives for uncleaved and cleaved RNAs with standard deviations are indicated. Each experiment was performed four times. (B) Bar graph of the half-lives of the Protein A cleavage product in mutants tested in panel A, with standard deviations. Asterisk indicates that the average half-life is significantly different from the half-life in wild-type ($P < 0.05$). (C) Wild-type yeast or a yeast strain with a temperature-sensitive allele of *RAT1*, both expressing the Protein A-U5-GFP reporter, were grown to mid-log phase in media containing galactose at 23°C and then incubated at 37°C for 1 h to inactivate the Rat1-1p. Media was then replaced with media containing glucose to terminate transcription of the reporter and aliquots were taken at the times indicated. Decay rates for uncleaved and cleaved RNAs are indicated. (D) The 5' cleavage product is translated. Isogenic wild-type and *ski7Δ* strains were transformed with empty vector or the Protein A-U5-GFP plasmid as indicated. Total protein and RNA was isolated from a culture grown to mid-log phase and submitted to western and northern blot analysis, respectively. Western blots were probed for Protein A and Pgk1p, to control for loading, while northern blots were probed for Protein A and the RNA subunit of the signal recognition particle (SRP). (E) The 5' cleavage product is unadenylated. Shown is a polyacrylamide northern blot of total RNA extracted from wild-type yeast transformed with the indicated plasmids.

(continued)

exoribonuclease Rat1p (Figure 5C), or by inactivation of the nuclear or cytoplasmic exosome (data not shown). These results suggest that Xrn1p is mainly responsible for the decay of the 3' cleavage product, and therefore that it is not retained in the nucleus for an extended period of time, but instead readily exported from the nucleus.

DISCUSSION

Many examples in the literature appear to indicate that Rnt1p-mediated cleavage during non-coding RNA processing is more efficient than cleavage of stem-loops in mRNAs. However, it is difficult to directly compare Rnt1p cleavage efficiency between substrates in their natural context (between snRNA U5 and *MIG2* mRNA for example) because many variables can affect substrate recognition, enzyme catalysis, and fate of the cleavage products. In addition, Rnt1p cleavage of some substrates may be influenced by features outside the stem-loop itself, such as interacting proteins or specific RNA folding (11,30). Introducing different Rnt1p cleavage sites derived from varying natural substrates into a common mRNA context provides a useful tool to study Rnt1p-mediated cleavage and the fate of mRNA cleavage products that are generated in the nucleus.

Interestingly, not all stem-loops resulted in the accumulation of cleaved reporter mRNAs. We have shown that this is at least in part because the inserted U5 stem-loop is recognized much more efficiently than the *MIG2* signal. *In vitro* experiments confirmed that this preferential cleavage is caused by specific features within the AGNN stem-loops themselves. One of many possible explanations is the presence of a GC-rich region near the cleavage site of the *MIG2* stem. Such regions can negatively affect Rnt1p cleavage of short substrates *in vitro* (24), but it was not known if this effect would also apply *in vivo*. More importantly, an independent study published during the writing of this manuscript has reported similar conclusions (31). Basically, it was shown that Rnt1p activity *in vivo* was highly variable when synthetic hairpins were inserted into the 3' untranslated portion of a transcript and that *in vivo* activity could be correlated with *in vitro* cleavage rate. Babiskin and Smolke (31) showed that most of their synthetic hairpins selected for efficient cleavage in one reporter context maintained their cleavage efficiency in a second reporter context. Our results suggest that *MIG2*, *FIT2* and *snR47* stem-loops are not well cleaved outside of their natural context. At least for snR47, cleavage of the endogenous substrate is efficient, with little if any pre-snR47 detectable in cells containing a functional Rnt1p (9). Thus, the *MIG2*, *FIT2* and *snR47* stem-loops may not fold properly in the context of a reporter, or cleavage of

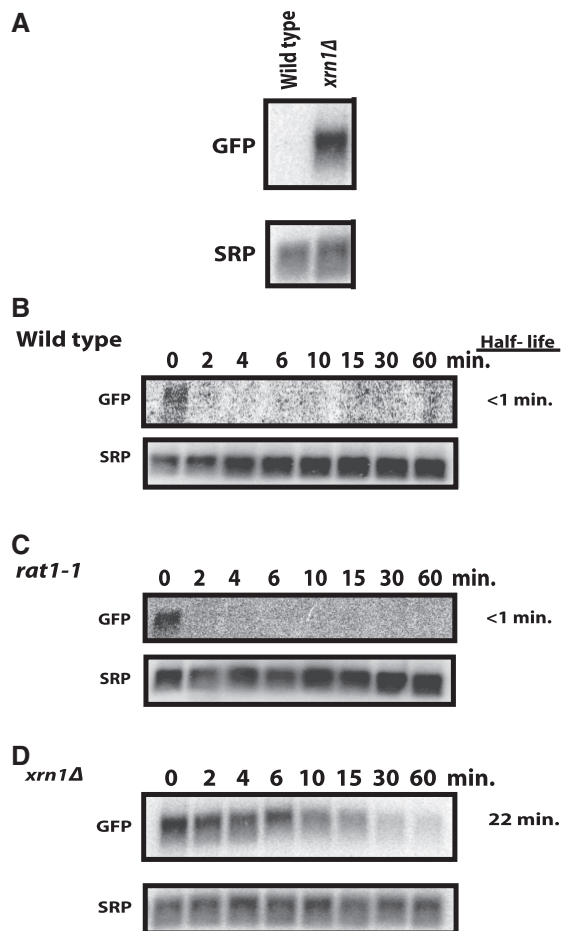


Figure 5. The uncapped 3' cleavage product is degraded by the cytoplasmic Xrn1p. (A) Isogenic wild-type and *xrn1Δ* strains containing the Protein A-U5-GFP reporter were grown to mid-log phase in media containing galactose and submitted to northern blot analysis. Blots were probed for GFP and the RNA subunit of the signal recognition particle (SRP) as indicated. (B–D) The degradation rate of cleaved GFP mRNA was determined in wild-type, *xrn1Δ* and *rat1-1* strains as described in Figure 4.

these natural stem-loops (as opposed to selected synthetic hairpins) requires additional features not present in reporter constructs. Finally, although unlikely, we have not been able to rule out that these reporter mRNAs are in fact cleaved, but that the cleavage products are very rapidly degraded by one or more RNases.

In addition to constituting a convenient tool to evaluate the elements that control Rnt1p activity, our reporter system allows us to study the impact of cleavage on gene expression. For example, it was shown that Rnt1p could mediate transcription termination of transcripts (such as *NPL3*) in the absence of a poly-A signal downstream of the ORF, thus preventing the accumulation of a

Figure 4. Continued

The RNA was treated with RNase H in the presence or absence of oligo(dT) as indicated. The shift in mobility for the *RPL11A* mRNA indicates that it contains a poly(A) tail that is removed by RNase H treatment, while the lack of a shift in mobility of the Protein A mRNA and the RNA subunit of the signal recognition particle (SRP) indicates that these RNAs do not have a poly(A) tail. For each panel, the indicated strains were grown to mid-log phase at 23°C prior to total RNA isolation. The blots were hybridized with probes specific for Protein A, *RPL11A* and the RNA subunit of the SRP as a loading control as indicated.

polycistronic transcript. Similarly, one might expect that cleavage of the reporter transcript would terminate transcription and prevent expression of the downstream sequence (GFP). Clearly our results indicate that this is not the case. Although we cannot tell if cleavage occurred co- or post-transcriptionally, the fact that both 5' and 3' cleavage products can be observed at the same time (Figure 2A) indicates that an Rnt1p target site is not sufficient for transcription termination. Moreover, if Rnt1p cleavage triggered Rat1p-dependent transcription termination, one would expect to observe in a *rat1-1* mutant strain higher expression levels of the 3' cleavage product, without an effect on the stability of the 3' cleavage product. In contrast to this expectation, there was no large increase in the levels of the 3' cleavage product in a *rat1-1* strain (Figure 5). We conclude that cleavage of our reporter constructs did not lead to significant levels of transcription termination.

This conclusion raises the question of what features of Rnt1p cleavage trigger or prevent transcription termination. Our data indicate that different Rnt1p sites are cleaved with different efficiencies. One possible hypothesis could be that fast Rnt1p cleavage is required for co-transcriptional cleavage and thus for triggering transcription termination. Interestingly, the U5 snRNA and rRNA sites that were efficiently cleaved in our reporter mRNAs, are naturally present 3' of the mature RNA and cleavage at the endogenous rRNA site facilitates transcription termination (32,33). In contrast, slow Rnt1p cleavage would rather result in post-transcriptional cleavage. Consistently, the natural position for Rnt1p site in snR47 is 5' of the mature snoRNA, where it facilitates processing, but not transcription termination. If this site triggered transcription termination by the torpedo model it would prevent snoRNA production. Importantly, our results suggest that the required differences in timing of cleavage are in part determined by Rnt1p site itself (Figure 3C). Some Rnt1 target sites may be cleaved rapidly, allowing for the cleavage to trigger transcription termination at the 3' end of genes. Conversely, Rnt1p target sites upstream of mature snoRNAs may need to be cleaved slower, allowing for continued transcription and processing. *In vivo* Rnt1p-mediated cleavage could be further reduced by alternative RNA or RNP structures, or increased by recruiting Rnt1p to the site. RNA polymerase II pausing after transcribing an Rnt1p site could also result in increased co-transcriptional RNA cleavage, and increased transcription termination. Thus, endogenous genes that undergo Rnt1p-mediated transcription termination may contain additional sequences outside the stem-loop that either cause very efficient cleavage by Rnt1p, or transcriptional pausing downstream of the Rnt1p site.

The downstream events that follow Rnt1p cleavage on non-coding RNA substrates, such as rRNA, snoRNAs and snRNAs are well characterized (12,15). However, the fate of cleaved mRNAs was still obscure. In this study, we have observed that the 3' product of Rnt1p cleavage was very unstable in wild-type cells, but significantly stabilized upon deletion of the *XRN1* gene, which encodes the major cytoplasmic 5' exoribonuclease. In

contrast, inactivation of Rat1p had no detectable effect on the stability of this fragment. Since Rnt1p has been localized to the nucleus by several independent methods (19,27,28), we conclude that our reporter mRNAs are cleaved in the nucleus, and that the 3' fragment is degraded following export to the cytoplasm. This fate is the same as what we have previously shown for hammerhead ribozyme cleavage products, and thus likely presents a general degradation pathway for cleaved mRNAs. This implies that endogenous Rnt1p cleavage products contain specific features to specify their further processing. Interestingly, the endogenous *RPS22B* and *RPL18A* mRNAs, which both encode ribosomal proteins, both contain an Rnt1p site within an intron. The cleaved *RPS22B* pre-mRNA accumulates in a *rat1-1* strain, while the cleaved *RPL18A* pre-mRNA accumulates in an *xrn1Δ* strain. Although both accumulate to even higher levels in the double mutant, this shows that these apparently very similar Rnt1p products have different downstream fates (7).

The 5' fragment resulting from Rnt1p cleavage of our reporter mRNAs was stabilized by mutations inactivating the cytoplasmic exosome, and to a lesser extent by deleting *XRN1*. This 5' fragment also is translated. We conclude that this 5' fragment is exported to the cytoplasm, where it is degraded. This fate resembles what we have previously described for the 5' cleavage product of a hammerhead ribozyme, and thus likely is the default pathway for cleaved mRNAs. Since endogenous snRNAs and snoRNAs are further processed by the nuclear exosome, this suggests that they too contain specific features to direct such further processing and that Rnt1p cleavage by itself is not sufficient to dictate the fate of the cleavage products.

Both the poly(A) tail and the cap structure have been suggested to facilitate mRNA export from the nucleus. Our findings confirm that neither is absolutely required for this process. Previously, we and others have shown that ribozyme-cleaved mRNAs also enter the cytoplasm (16,34,35), but an important limitation to these studies is that it is hard to prove that ribozyme cleavage occurred before nuclear export. The strategy of using Rnt1p should be widely applicable to generate unadenylated mRNAs specifically in the nucleus. Furthermore, Rnt1p cleavage products contain a 3' OH and a 5' monophosphate (36–38), while hammerhead ribozymes generate a 2'3' cyclic phosphate and a 5' OH (39,40). Thus, Rnt1p products are chemically the same as the cleavage products of most nuclear and cytoplasmic endo- and exoribonucleases. The demonstration of the utility of Rnt1p to cleave mRNAs in the nucleus complements other tools, such as the use of an *rlg1-100* strain to specifically generate cleaved *HAC1* mRNA in the cytoplasm (41), and the use of allosteric ribozymes to temporally regulate reporter mRNA cleavage (42). Finally, our reporter construct should provide a very convenient tool in order to identify the regulatory features, such as promoter sequences or regions flanking endogenous cleavage signals, which trigger Rnt1p-mediated transcription termination and dictate the fate of the cleavage products.

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