Cell, Vol. 85, 115–124, April 5, 1996, Copyright ©1996 by Cell Press

RNase III Cleaves Eukaryotic Preribosomal RNA at a U3 snoRNP-Dependent Site

Sherif Abou Elela, Haller Igel, and Manuel Ares, Jr.
Center for the Molecular Biology of RNA
Biology Department
Sinsheimer Laboratories
University of California, Santa Cruz
Santa Cruz, California 95064

Summary

A yeast gene homologous to bacterial RNase III (*RNT1*) encodes a double-strand–specific endoribonuclease essential for ribosome synthesis. Two rRNA processing events are blocked in cells temperature sensitive for *RNT1*: cleavage at the snoRNA-dependent A0 site in the 5′ ETS and cleavage in the 3′ ETS. Recombinant RNT1 protein accurately cleaves a synthetic 5′ ETS RNA at the A0 site in vitro, in the absence of snoRNA or other factors. A synthetic 3′ ETS substrate is specifically cleaved at a site 21 nt downstream of the 3′ end of 28S rRNA. These observations show that a protein endonuclease collaborates with snoRNAs in eukaryotic rRNA processing and exclude a catalytic role for snoRNAs at certain pre-rRNA cleavage sites.

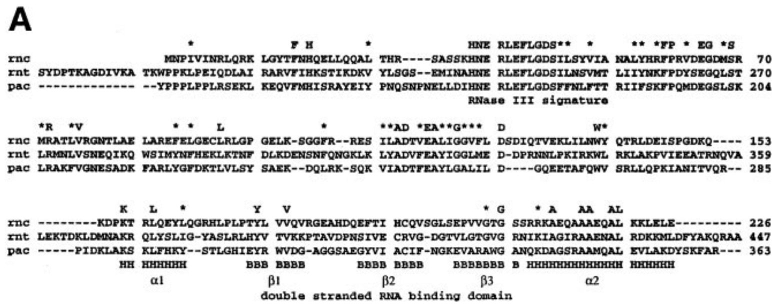
Introduction

In eukaryotes, ribosomal RNA is produced in the nucleolus as a large precursor transcript that is processed into mature 18S, 5.8S, and 28S rRNA by the removal of external transcribed spacers (5′ and 3′ ETSs) and internal transcribed spacers (ITS1 and ITS2). The events leading to mature rRNAs have been studied in vertebrates and lower organisms, revealing a complex processing machinery whose activity seems tightly coupled to ribosome assembly (for review see Eichler and Craig, 1994). Important components of this machinery include the small nucleolar ribonucleoprotein particles (snoRNPs), which consist of small nucleolar RNAs (snoRNAs) associated with proteins (Maxwell and Fournier, 1995). Although several nucleolar RNPs and proteins are essential for processing and ribosome assembly, little is known of their mechanisms of action or how pre-rRNA cleavage is linked to ribosome assembly.

U3, the most abundant and extensively studied snoRNA, is required for correct processing of the 5′ ETS in vertebrates, yeast, and archaebacteria (Kass et al., 1990; Hughes and Ares, 1991; Mougey et al., 1993; Potter et al., 1995). Cleavage at two U3-dependent sites (called A0 and A1) leads to 5′ ETS removal and 18S maturation in yeast (Hughes and Ares, 1991; Beltrame et al., 1994; Beltrame and Tollervey, 1995). Other snoRNAs required for efficient 5′ ETS removal include yeast U14 (Li et al., 1990), snR10 (Tollervey, 1987), snR30 (Morrissey and Tollervey, 1993), and vertebrate U22 (Tycowski et al., 1994). Thus far, vertebrate U8 is unique among snoRNAs for its role in processing 5.8S and 28S rRNAs (Peculis and Steitz, 1993). U3 base-pairs to the 5′ ETS (Beltrame and Tollervey, 1995), and U14 base-pairs with 18S sequences (Liang and Fournier, 1995), but neither of these interactions occurs adjacent to a cleavage site. The requirement for several snoRNAs in pre-18S rRNA processing has led to the hypothesis that a multi-snRNP processing complex akin to the spliceosome is responsible (for reviews see Maxwell and Fournier, 1995; Bachellerie et al., 1995). As more snoRNAs have been identified, a striking pattern of extensive complementarity with rRNA has emerged, leading to discussion of the hypothesis that snoRNAs assist correct folding of rRNA (Bachellerie et al., 1995; Steitz and Tycowski, 1995). How snoRNAs function in the processing pathway has yet to be determined.

The biochemical nature of the endonuclease activities required for eukaryotic rRNA processing has remained elusive. A crude in vitro system faithfully cleaves vertebrate 5′ ETS in a U3-dependent fashion (Kass et al., 1990; Mougey et al., 1993), but the enzymes responsible have not been isolated. Nucleolar endonucleases that cleave the 5′ ETS in single-stranded regions have been isolated from vertebrate cells (for review see Eichler and Craig, 1994), but their roles in the pathway of rRNA processing have been difficult to determine. A multifunctional ribonucleoprotein nuclease called MRP is responsible for a nonessential cleavage in the formation of the 5′ end of 5.8S rRNA in yeast (Schmitt and Clayton, 1993; Henry et al., 1994). Purified MRP cleaves synthetic mitochondrial RNA transcripts in vitro (Schmitt and Clayton, 1993), but cleavage of pre-rRNA by purified MRP has not yet been demonstrated.

In contrast, eubacterial pre-rRNA processing is carried out by a defined set of protein nucleases (for review see Apirion and Miczak, 1992). Key among these is RNase III, initially isolated by its ability to bind and cleave duplex RNA (Robertson et al., 1968). RNase III$^-$ mutants of Escherichia coli accumulate unprocessed pre-rRNA but are viable because an alternative pathway for the processing of 16S rRNA exists, and 23S rRNA retaining unprocessed extensions at its 5′ and 3′ ends can assemble into functional ribosomes (Gegenheimer et al., 1977). RNase III releases 16S and 23S rRNA from the primary transcript by cleaving within two extended RNA duplex structures formed by long-range interactions that pair the termini of each rRNA (Bram et al., 1980). RNase III has other functions in E. coli as well, including phage T7 mRNA processing, mRNA stability, and as an agent in antisense regulation (for review see Court, 1993). Eukaryotic homologs of RNase III have been identified by sequence comparisons (Xu et al., 1990; Iino et al., 1991; Rotondo et al., 1995; Kharrat et al., 1995); however, their role in pre-rRNA processing has not been addressed. We have examined the function of the yeast *RNT1* gene product, an RNase III-like enzyme. Our results demonstrate that RNase III is an essential endonucleolytic agent in yeast rRNA processing and indicate that the role of snoRNAs at some cleavage sites is limited to noncatalytic functions such as folding or presentation of pre-rRNA.



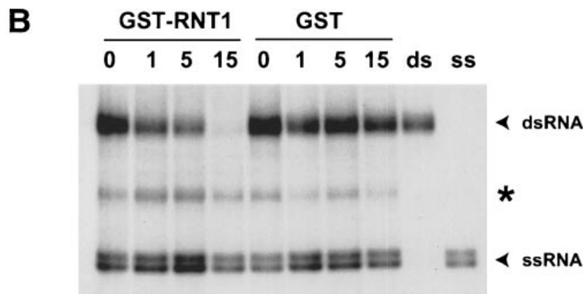


Figure 1. S. Cerevisiae *RNT1* Is Homologous to E. coli RNase III in Sequence and in Function

(A) Comparison of the predicted amino acid sequences of E. coli RNase III (top line, rnc), yeast *RNT1* (middle line, rnt), and S. pombe *pac1* (bottom line, pac). Identical amino acids are shown on top; similarity (L, I, V, M, F, Y, R, K, D, E, G, A) is indicated by asterisks. Only the C-terminal segments of the yeast proteins are shown and are aligned with the complete E. coli RNase III sequence. Additional identity and similarity exists between the two yeast proteins but is not indicated. Secondary structure elements of the double-stranded RNA-binding domain derived by nuclear magnetic resonance of E. coli RNase III (Kharrat et al., 1995) are indicated below the sequence.

(B) Cleavage of dsRNA in vitro by recombinant GST–RNT1. A double-stranded RNA (arrow, dsRNA, lane ds) and a single-stranded RNA (arrow, ssRNA, lane ss) were incubated with the GST–RNT1 fusion protein or with glutathione S–transferase (GST) as a control and fractionated on a nondenaturing polyacrylamide gel. Time of incubation in minutes is shown on top. Arrows indicate the position of migration of the double-stranded and single-stranded RNA substrates. The band indicated by the asterisk is a differently folded form of ssRNA.

Results

An Essential Yeast Gene Encodes a Double-Strand–Specific Ribonuclease Homologous to Bacterial RNase III

Sequencing of Saccharomyces cerevisiae DNA adjacent to the *CUS1* gene (Wells et al., 1996) revealed a homolog of E. coli RNase III (March et al., 1985) and Schizosaccharomyces pombe *pac1*, an RNase III homolog (Xu et al., 1990; Iino et al., 1991). We sequenced the S. cerevisiae *RNT1* gene (RNase III, GenBank: U27016), predicting a 473 amino acid protein. A 230 amino acid segment representing the C-terminal half of the *RNT1* protein (RNT1) shows 20% identity with full-length E. coli RNase III and 23% identity with the C-terminal half of *pac1* (Figure 1A). An identical 11 amino acid sequence (HNERLEFLGDS) is present in the three proteins. In addition, residues consistent with a double-stranded RNA-binding domain (Bass et al., 1994) similar to that found in E. coli RNase III (Kharrat et al., 1995) are observed at the C-termini of the proteins.

To examine the ability of RNT1 to cleave dsRNA, the *RNT1* coding region was fused to glutathione S–transferase (GST), expressed in an RNase III^{-} E. coli strain, and purified using glutathione agarose. When incubated with a mixture of labeled synthetic duplex RNA (dsRNA) and single-stranded RNA (ssRNA) substrates, the GST–RNT1 fusion protein preferentially cleaved the dsRNA without significantly cleaving ssRNA, whereas GST alone cleaved neither the dsRNA nor the ssRNA (Figure 1B).

To investigate the function of eukaryotic RNase III in vivo, a null allele of *RNT1* was constructed by disrupting the 11 amino acid signature sequence with a *HIS3* gene. A diploid strain carrying one wild-type and one disrupted copy of *RNT1* was sporulated and subjected to tetrad analysis. No more than two spores from each tetrad could form colonies, and the viable colonies never carried the *HIS3* gene that marks the disrupted *RNT1* locus, indicating that *RNT1* is an essential gene in S. cerevisiae (data not shown).

RNase III Is Required for Eukaryotic Pre-rRNA Processing In Vivo

To analyze the function of eukaryotic RNase III, we isolated a temperature-sensitive allele (*rnt1–1*) that supports growth at 26°C but not at temperatures higher than 30°C. We used the *rnt1–1* allele to monitor the effect of RNase III inactivation on the processing of rRNA. Wild-type or temperature-sensitive cells grown at 26°C were shifted to 37°C, and total RNA was extracted (Figure 2). When RNA samples derived from an equivalent dry mass of cells were separated on a denaturing gel and compared by ethidium bromide staining, we found that the rRNA content of *rnt1–1* cells decreased after the shift, indicating that RNT1 was necessary for the maintenance of mature rRNA. By 4 hr after the shift, mature rRNA species were greatly reduced, whereas the level of total tRNA remained constant (data not shown). Cell growth was arrested within 2 hr after the temperature shift (data not shown), suggesting that depletion of ribosomes was not the primary cause of growth arrest.

To determine which processing events are perturbed by the inactivation of RNase III, we identified transcripts that accumulate under restrictive conditions. Wild-type

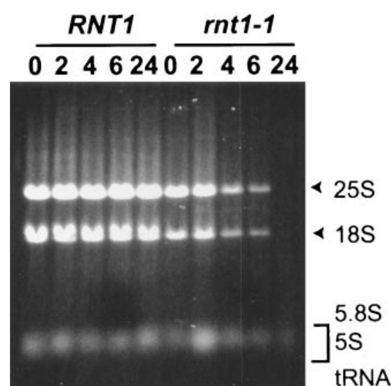


Figure 2. Ribosomal RNA Accumulation Is Blocked in Strains Carrying the ***rnt1–1*** Mutation

Ethidium–bromide-stained denaturing agarose gel of total RNA extracted from wild-type cells (*RNT1*) or from cells temperature-sensitive for RNase III (*rnt1–1*) after increasing incubation time (in hours) at 37°C. RNA from an equivalent dry weight of cells was loaded in each lane. Positions of the major RNA species are indicated by the arrows. The bracket indicates the position of migration of 5.8S, 5S, and tRNAs.

or *rnt1–1* cells were shifted to 37°C, and equal amounts of total RNA from each timepoint were separated, blotted to nylon membranes, and probed with oligonucleotides complementary to different parts of the pre-rRNA transcript (Figure 3). Probe A recognizes the 5′ ETS upstream of A0, a U3-dependent processing site that is cleaved early in the processing pathway. Significant and rapid accumulation of a large (35S) pre-rRNA and slower accumulation of a 23S transcript containing 18S rRNA sequences occurs in the *rnt1–1* strain after temperature shift (Figure 3A). Loss of 18S rRNA and accumulation of a 23S RNA is observed upon depletion of several snoRNAs (Tollervey, 1987; Hughes and Ares, 1991; Li et al., 1990; Morrissey and Tollervey, 1993; Beltrame et al., 1994) and indicates that cleavage at A0 and A1 is inhibited. An 18S probe confirms this and further shows that the 20S precursor of 18S rRNA does not accumulate (data not shown).

When a probe complementary to 5.8S rRNA is used (probe B), mature 5.8S rRNA and the normal 27S pre-rRNA intermediates containing the 5.8S, ITS2, and 25S rRNA sequences are detected in wild-type cells and in *rnt1–1* cells at permissive temperature (Figure 3B). In *rnt1–1* cells at restrictive temperature, the 35S rRNA precursor is detected, as well as unusual elongated forms of the 27S intermediates (designated 27S*). To determine whether 27S* is 3′ extended, we tested a probe complementary to a sequence downstream of the mature 3′ end of 25S rRNA (from position +96 to +112 relative to the 25S 3′ end; probe C). The 35S transcript and the 27S* forms of the 27S rRNA intermediates are detected, as well as transcripts longer than 25S rRNA (25S*). This suggests that RNase III cleaves a site near the 3′ end of 25S rRNA that removes the 3′ ETS. The existence of 27S* molecules in the mutant at restrictive temperature (Figures 3B and 3C) suggests that some processing in ITS1 continues in the absence of RNase III. Likewise, retention of the 3′ ETS in the 25S* transcripts (Figure 3C) would suggest that removal of ITS2 and 5.8S from the 27S* transcript continues. A transcript containing part of 25S and the 3′ extension is found in the mutant at restrictive temperature (transcript X); presumably, this is a stable degradation product of failed rRNA processing.

Inactivation of RNase III Blocks Cleavage at A0 and at a Site in the 3′ ETS

We monitored processing events in the 5′ and 3′ ETSs of pre-rRNA from *RNT1* and *rnt1–1* strains shifted to 37°C. Cleavage at A0 in the 5′ ETS was monitored using a primer complementary to the 3′ end of the 5′ ETS, just upstream of the mature 5′ end of 18S rRNA. This primer detects the 5′ end of the unprocessed 5′ ETS as well as the A0 cleavage site (Hughes and Ares, 1991; Beltrame et al., 1994). After the temperature shift, the signal corresponding to A0 cleavage decreased rapidly

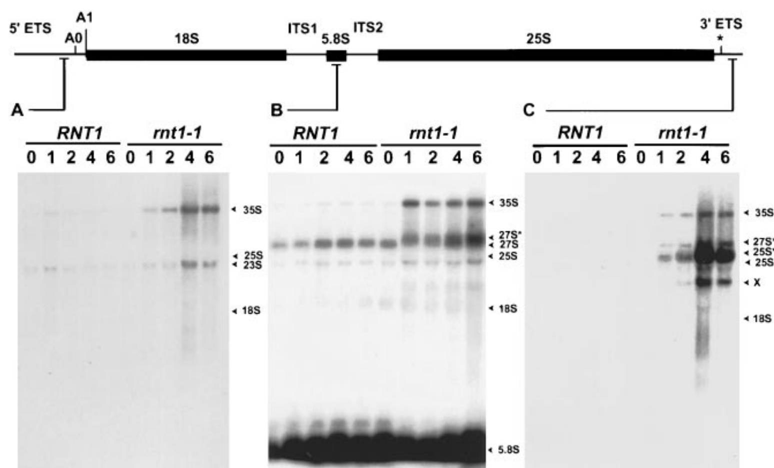


Figure 3. Transcripts Containing the ETSs Accumulate in the ***rnt1–1*** Mutant

RNA was isolated from wild-type (*RNT1*) or temperature-sensitive RNase III mutant (*rnt1–1*) cells after incubation at permissive temperature (0), or a shift to restrictive temperature for the indicated time (in hours), separated on denaturing gels, blotted, and probed with oligonucleotide probes complementary to different sequences in the pre-rRNA transcript (shown at top). ETS, external transcribed spacers; ITS, internal transcribed spacers; thick bars, mature rRNA sequences; asterisk, putative processing site in the 3′ ETS. (A) RNAs recognized by probe A. (B) RNAs recognized by probe B. (C) RNAs recognized by probe C. In A–C, migration of key RNA species is indicated at right. 35S, largest detectable pre-rRNA; 25S, 18S, 5.8S, mature rRNAs (identified by staining the blots with methylene blue); 27S, normal intermediate of 5.8S and 25S rRNA processing; 27S*, 3′ extended form of 27S; 25S*, 3′ extended form of 25S; X, unusual derivative of 25S* or 27S*.

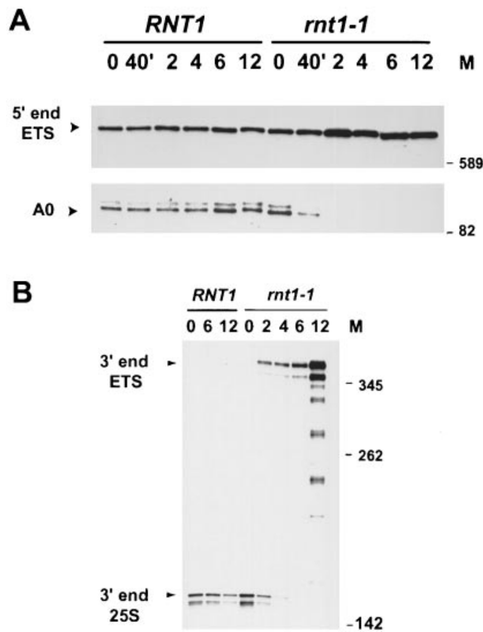


Figure 4. Cleavage at A0 in the 5′ ETS and at a Site in the 3′ ETS Is Inhibited in the ***rnt1–1*** Mutant at Restrictive Temperature

RNA was isolated from wild-type (*RNT1*) or temperature-sensitive RNase III mutant (*rnt1–1*) cells after incubation at permissive temperature (0), or a shift to restrictive temperature for the indicated time (in hours) and analyzed by primer extension (A) and RNase A and T1 protection (B).

(A) Cleavage at A0. RNA was annealed to an oligonucleotide complementary to the 3′ end of the 5′ ETS. Primer extension products corresponding to the 5′ end of the ETS and cleavage at A0 are indicated on the left. DNA markers are at right.

(B) 3′ extended rRNA. A uniformly labeled probe, complementary to positions −175 to +284 relative to the 25S rRNA 3′ end was annealed to RNA and digested with single-strand–specific ribonucleases. The positions of the 25S rRNA 3′ end and the ETS 3′ end are indicated on the left. The DNA size marker is indicated on the right.

and the signal corresponding to the 5′ end of the pre-rRNA primary transcript (Klemenz and Geiduschek, 1980, Bayev et al., 1980) increased in the *rnt1–1* mutant strain (Figure 4A). This indicates that cleavage at A0 is dependent on RNase III.

To investigate the role of RNase III at the 3′ end of 25S rRNA, we performed an RNase protection assay, using a probe that spans the 3′ end of 25S rRNA and includes sequences downstream (Figure 4B). RNA from wild-type cells or from *rnt1–1* cells grown at permissive temperature showed a single band corresponding to the mature 3′ end of the 25S rRNA, as well as slightly shorter bands derived from incompletely protected probe. After incubation of the *rnt1-1* strain under restrictive conditions, the signal corresponding to the mature 3′ end of the 25S rRNA decreases as a proportion of the total RNA, and two new signals appear that correspond to rRNA transcripts extending 195 and 210 nt past the mature 3′ end of 25S rRNA (Figure 4B). The appearance of these products is consistent with termination at the +210 site (van der Sande et al., 1989). We conclude that correct 25S rRNA 3′ end formation requires RNase III.

Primer extension experiments using oligonucleotides complementary to the 3′ end of ITS1 and to the 5′ end of 5.8S rRNA indicate that RNA ends consistent with cleavage at the internal processing sites A2, A3, B1 (L), and B1 (S) (Schmitt and Clayton, 1993; Henry et al., 1994) persist in the *rnt1–1* mutant at the restrictive temperature (data not shown). Primer extension using oligonucleotides complementary to the 3′ end of ITS2 do not reveal any processing sites blocked by the inactivation of RNase III (data not shown). Together with the appearance of the 23S, 27S*, and 25S* RNAs (see Figures 3A and 3C), these results suggest that, whereas 5′ and 3′ ETS processing requires RNase III, sites within the ITSs continue to be recognized by other elements of the processing machinery, for example MRP (Schmitt and Clayton, 1993; Henry et al., 1994). Although processing may continue at other sites, the overall rate may be too low to prevent detectable accumulation of 35S rRNA (see Figure 3) or to produce significant mature rRNA in the absence of RNase III (see Figure 2).

Yeast RNase III Cleaves a Model 5′ ETS Substrate at A0 in the Absence of Other Factors

To determine whether the 5′ ETS is cleaved by yeast RNase III, we synthesized a labeled transcript spanning the A0 site and incubated it with bacterially produced GST–RNT1 fusion protein. Structure models for the yeast 5′ ETS (Yeh and Lee, 1992; Venema et al., 1995) suggest that the A0 site is found near the base of an extended stem loop (Figure 5C), similar to sites cleaved by E. coli RNase III (Nicholson et al., 1988). The substrate includes sequences thought to comprise this structure, but lacks the U3-binding site (Beltrame and Tollervey, 1995). Incubation with GST–RNT1 generates two products consistent with processing at A0 (Figure 5A; P1 and P2). To identify sites of cleavage, we treated unlabeled substrate with the enzyme, annealed to a labeled oligonucleotide complementary to the 3′ end of the 5′ ETS (Beltrame et al., 1994), and extended with reverse transcriptase. To control for A0 processing, total yeast RNA was used as a template and a sequence ladder of the 5′ ETS was generated with the same primer, using plasmid DNA (Figure 5B). GST–RNT1 cleaved the synthetic 5′ ETS substrate at three positions identical to those observed at A0 in vivo, generating 5′ ends at −89, −90, and −91 relative to the 5′ end of 18S rRNA (Figures 5B and 5C). Based on this finding and on the observation that A0 cleavage is blocked in a temperature-sensitive RNase III mutant at restrictive temperature (see Figure 4A), we conclude that RNase III carries out endonucleolytic pre-rRNA processing at A0.

Yeast RNase III Cleaves a Model 3′ ETS Substrate 21 Nucleotides from the 3′ End of 25S rRNA

To test whether the 3′ ETS contains a yeast RNase III cleavage site, we incubated a labeled synthetic pre-rRNA spanning the 3′ end of 25S rRNA and including the 5′ half of the 3′ ETS with the GST–RNT1 fusion protein (Figure 6A). The substrate was cleaved, generating a fragment (P2) consistent with cleavage near the 3′ end of 25S rRNA. To map the cleavage site, we prepared

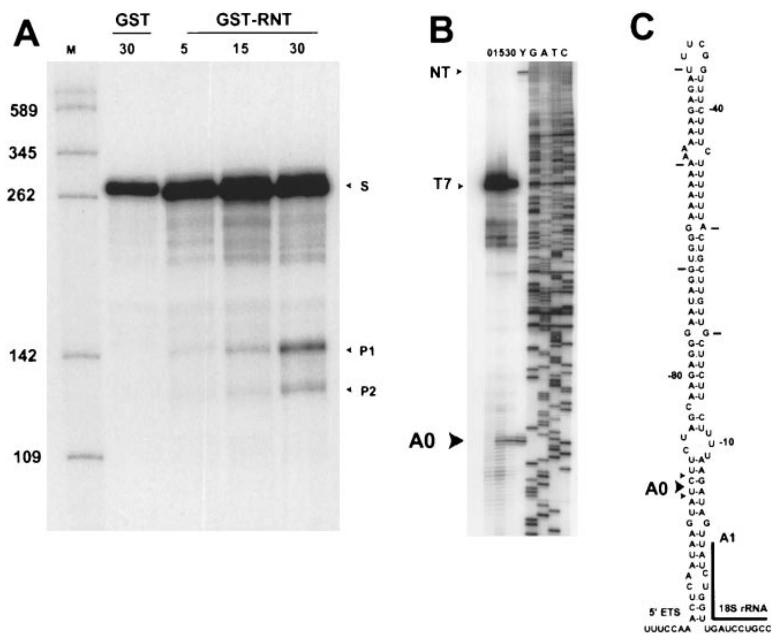


Figure 5. RNase III Cleaves a Model 5′ ETS at A0

(A) Cleavage of labeled RNA. A transcript spanning positions −180 to +28, relative to the mature 5′ end of 18S rRNA, was incubated with GST or a GST–RNT1 protein in vitro for the indicated time (in minutes). The input substrate (S) and products (P1 and P2) are indicated at right. DNA markers are at left.

(B) Mapping the site of cleavage. Primer complementary to the 3′ end of the 5′ ETS was extended on RNA extracted after incubation with GST-RNT1 protein. DNA sequence of the 5′ ETS using the same primer is shown on the right. A0 cleavage observed in vivo (arrow) is mapped using total wild-type yeast RNA (lane Y). The arrow labeled T7 indicates the 5′ end of the uncleaved substrate, and that labeled NT indicates the 5′ end of the unprocessed 5′ ETS from total yeast RNA (lane Y).

(C) Model for the pre-rRNA structure surrounding the 5′ ETS RNase III cleavage site at A0, indicated by the arrow.

unlabeled substrate containing additional plasmid sequence and treated it with GST–RNT1. An end-labeled primer complementary to the plasmid sequence was annealed and extended with reverse transcriptase, and the products were compared with a DNA sequencing ladder of the plasmid (Figure 6B). A major cleavage site was observed between two G residues at +21 and +22 relative to the 3′ end of mature 25S rRNA, as well as a minor site between +20 and +21 (Figures 6B and 6C). Because we had not mapped the 3′ ETS processing site(s) inhibited by RNAse III inactivation in vivo (see Figure 4), we were unable to judge the accuracy of the in vitro cleavage in the 3′ ETS.

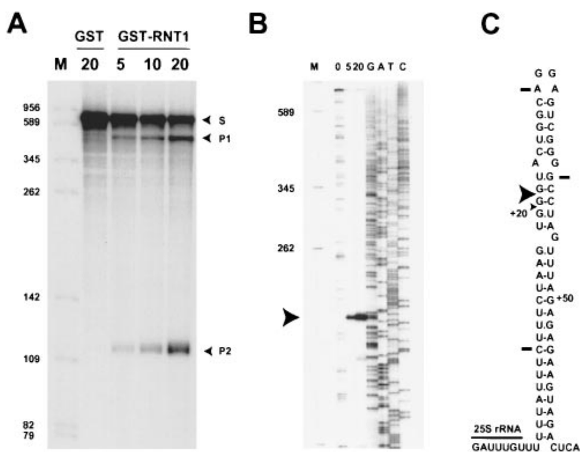


Figure 6. RNase III Cleaves a Model 3′ ETS In Vitro

(A) Cleavage of labeled RNA. A transcript spanning the mature 3′ end of the 25S rRNA was incubated with GST or a GST–RNT1 protein in vitro for the indicated time (in minutes). The input substrate (S) and products (P1 and P2) are indicated at right. DNA markers are at left.

(B) Mapping the site of cleavage in vitro. Primer complementary to a plasmid sequence present at the 3′ end of the substrate transcript was extended on RNA after incubation with GST–RNT1 protein. DNA sequence of the 3′ ETS using the same primer is shown on the right. The product corresponding to the cleaved RNA is indicated by the arrow.

(C) Model for the RNA structure surrounding the yeast 3′ ETS RNase III cleavage site, indicated by the arrow.

Discussion

We have identified an RNase III homolog in S. cerevisiae (Figure 1A) and shown that it is a double-strand–specific endoribonuclease (Figure 1B) essential for pre-rRNA processing (Figure 2). Inactivation of a temperature-sensitive form of yeast RNase III inhibits processing of the 5′ and 3′ ETSs and results in accumulation of the primary transcript of the rRNA genes (Figures 3 and 4). RNase III is required for A0 cleavage, a processing event known to be dependent on snoRNPs (Figures 3 and 4; Maxwell and Fournier, 1995). In the absence of other factors, a bacterially produced GST fusion of yeast RNase III cleaves a synthetic 5′ ETS substrate at A0 (Figure 5), and a synthetic 3′ ETS substrate 21 nt from the mature 3′ end of 25S rRNA (Figure 6). This defines two specific roles for RNase III in eukaryotic pre-rRNA processing, one of which also requires U3 snoRNA in vivo.

How Does RNase III Cooperate with snoRNAs?

Since RNase III catalyzes cleavage at a snoRNA-dependent processing site, it seems unlikely that snoRNAs form a catalytic RNA structure as postulated for the spliceosomal snRNAs (for reviews see Madhani and Guthrie, 1994; Ares and Weiser, 1995). Many snoRNPs are known or presumed to interact with pre-rRNA at sequences distant from the cleavage sites (Beltrame and Tollervey, 1995; Liang and Fournier, 1995; Bachellerie et al., 1995; Steitz and Tycowski, 1995). Also, multiple snoRNPs are required for processing at some sites (Maxwell and Fournier, 1995). Thus, pairing between the snoRNA and the substrate at the cleavage site cannot

be a general feature of snoRNA function. Recent work has shown that function of U3 in A0 processing requires U3 base pairing at a site in the 5′ ETS 125 nt upstream of the A0 processing site (Beltrame and Tollervey, 1995). The observation that U3 is dispensable for accurate cleavage at A0 in vitro (Figure 5) supports the hypothesis that the role of U3 in A0 cleavage in vivo is limited to a noncatalytic function. One possibility is that the U3 RNA–pre-rRNA interaction may prevent the pre-rRNA from following a nonproductive folding pathway that occludes the RNase III processing site. Alternatively, the interaction could be involved in assembling the pre-rRNA in a structured preribosomal complex that ensures presentation of the A0 site to RNase III. A third possibility is that RNase III could be bound directly to U3 snoRNP in vivo, and thus, U3 base pairing to the 5′ ETS (Beltrame and Tollervey, 1995) could serve to deliver RNase III to the processing site. In any case, the role of U3 snoRNA in A0 cleavage is likely to be structural rather than catalytic.

The relationship between the yeast A0 site and the vertebrate U3-dependent 5′ ETS cleavage site is not clear. In the mouse in vitro pre-rRNA processing system, cleavage at +650 occurs in a loop region of the 5′ ETS secondary structure model (Craig et al., 1991). The addition of RNA oligonucleotides complementary to the processing site or substrate mutations predicted to bury the cleavage site in a duplex region blocks processing, suggesting that the +650 site may be cleaved by a single-strand–specific nuclease (Craig et al., 1991). A purified nucleolar endonuclease with a single-stranded specificity is reported to cleave at +650 among other sites in the 5′ ETS (Shumard and Eichler, 1988). Thus, binding of evolutionarily conserved snoRNPs may expose different parts of the pre-rRNA for cleavage by nucleases with different structural requirements in different organisms, but with the same net effect on pre-rRNA processing.

We have been unable to detect A1 cleavage by RNase III in vitro. During processing in vivo, A0 precedes A1, and it is not known whether A1 processing strictly depends on cleavage at A0. Recently, mutations in the 5′ ETS near these processing sites have been tested in vivo (Venema et al., 1995). Mutations at the A1 site affect the accuracy and efficiency of A1 cleavage without effect on A0, despite disruption of the paired region containing the A0 cleavage site (Venema et al., 1995). E. coli RNase III cleaves internal loops adjacent to duplex regions (Nicholson et al. 1988), and if the yeast enzyme is similar, such mutant substrates might still be cleaved. Additional work will be needed to define the substrate requirements for A0 cleavage in vivo and in vitro, and to determine whether the RNase III dependence of A1 cleavage observed in vivo (Figure 3) is direct or indirect.

Role of RNase III in Removing the 3′ ETS

Studies using rRNA "minigenes" suggest that formation of the 3′ end of 25S rRNA in yeast takes place in three steps (Kempers-Veenstra et al., 1986; van der Sande et al., 1989; Yip and Holland, 1989). Transcription terminates about 210 nt from the mature 3′ end of 25S rRNA to generate a pre-rRNA containing the 3′ ETS. The 3′ ETS is then removed by two processing steps: endonucleolytic cleavage in the region spanning positions

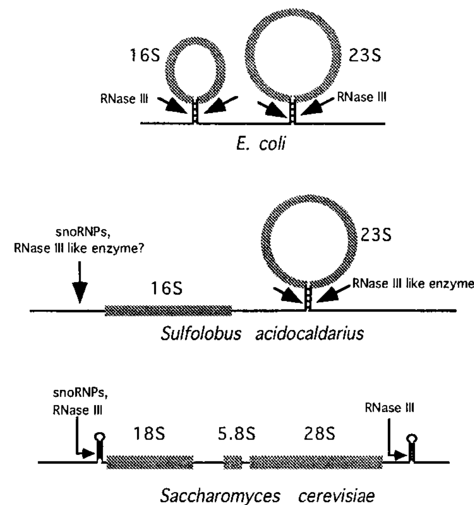


Figure 7. Pre-rRNA Processing in Representatives of Different Kingdoms

Processing sites are indicated by arrows. The positions of the mature rRNA sequences are indicated as stippled boxes or as circles.

+15 to +50, followed by exonucleolytic trimming to a metastable intermediate terminating at +7 (Veldman et al., 1980), and then finally to the mature 3′ end. Since RNase III is required for 3′ ETS removal in vivo (Figures 3C and 4B) and cleaves the 3′ ETS in vitro (Figure 6), we propose that endonucleolytic cleavage by RNase III within a stem loop in the 3′ ETS (Kempers-Veenstra et al., 1986; Melekhovets et al., 1994) leads to maturation of the 25S 3′ end. Because we did not identify the 3′ ETS processing sites that require RNAse III in vivo, we are not sure whether the cleavage product generated by the fusion protein in vitro is an accurate reflection of natural 3′ ETS processing. Cleavage at +21 is consistent with a 5′ terminus observed in cleaved minigene 3′ ETS transcripts in vivo (van der Sande et al., 1989). We find no indication in vivo for termination of transcription at the REB1-dependent polI termination sites reported from in vitro studies (Lang and Reeder, 1993).

In a variety of eukaryotes, the potential to form extended stem loop structures is present in the sequences following the mature 3′ end of the large rRNA (Kass et al., 1987; Gonzalez et al., 1990; Melekhovets et al., 1994), suggesting that RNase III-dependent removal of the 3′ ETS may be general. In frog oocytes, depletion of U8 snoRNA blocks 3′ ETS removal (Peculis and Steitz, 1993), showing that 3′ ETS processing is snoRNA dependent in vertebrates. Although neither a yeast U8 homolog nor a frog RNase III homolog has yet been identified, the 3′ ETS processing phenotypes of U8 and RNase III depletion are similar, and it seems possible that RNase III may cooperate with U8 snoRNA in removing the 3′ ETS.

Conservation and Divergence of RNase III Function

The roles of RNase III are different in the pre-rRNA processing pathways of eukaryotes and eubacteria (Figure 7). Eubacteria rely on two sets of long-range interactions

to pair the ends of 16S and 23S rRNA and use RNase III and other protein nucleases (Bram et al., 1980, Apirion and Miczak, 1992) seemingly without the aid of snoRNAs. Eukaryotes use a set of snoRNPs and, at least in yeast, RNase III, but do not pair the ends of the pre-rRNAs to generate RNase III substrates, relying instead on more locally restricted interactions (Figures 5 and 6). Sulfolobus acidocaldarius, an archaebacterium, uses a U3-like RNA in the processing of leader sequences at the 5′ end of pre-16S rRNA, a situation formally similar to the 5′ ETS of eukaryotes (Potter et al., 1995; Figure 7). As in eubacteria, archaebacterial 23S rRNA processing is thought to require pairing the ends of pre-23S rRNA. In this case, however, the duplex contains a conserved internal loop recognized by the tRNA intron endoribonuclease, an RNase III-like enzyme (Thompson and Daniels, 1988; Thompson et al., 1989). Thus, in the three different kingdoms, an overlapping set of components has been used to develop different strategies for pre-rRNA processing (Figure 7). The question of whether archaebacteria have RNase III and use it in conjunction with the U3-like RNA to carry out cleavage of the 5′ ETS remains open.

In bacteria, the stability of mRNAs is influenced by RNase III (Court, 1993). Though not absolutely essential in E. coli, RNase III contributes to growth (Court, 1993), and an RNase III homolog is conserved in even the smallest of bacterial genomes (Fraser et al., 1995). These observations and the presence of RNase III in yeast open the possibility that RNase III could be involved in other aspects of eukaryotic RNA metabolism, including mRNA turnover. The S. pombe homolog of RNase III, *pac1*, has been isolated as a suppressor of a meiosis mutation (Xu et al., 1990; Iino et al., 1991), as well as a suppressor of a defect in snRNA metabolism (Rotondo et al., 1995). The relationship between the recently discovered RNase III activity of *pac1* (D. Frendewey, personal communication) and the suppressor phenotypes is unclear in these cases, but the effects seem unlikely to be mediated through pre-rRNA processing. The S. cerevisiae *rnt1–1* mutant ceases growth within 2 hr after temperature shift (data not shown), a time when rRNA has not been substantially depleted (Figure 2), suggesting that yeast RNase III has an essential growth function in addition to rRNA processing. The involvement of RNase III in pre-rRNA processing in two kingdoms and its diverse roles in bacterial RNA metabolism suggest that there may be other roles for this ancient and conserved protein in eukaryotes.

Experimental Procedures

Plasmids, Strains, Media

Yeast was grown according to standard procedures (Rose et al., 1990). A diploid strain heterozygous for the *RNT1* gene disruption was constructed by standard techniques (Rothstein, 1991). An NcoI fragment carrying the *RNT1* gene was end-filled with Klenow and cloned in the SmaI site of pGEM-7Zf (Promega, Madison, WI), regenerating the NcoI sites. The plasmid was digested with EcoRI, which cleaves within the conserved RNase III signature sequence, and mixed with the BamHI fragment of the *HIS3* gene, and the ends were filled and ligated (Perbal, 1988). A plasmid containing the *rnt1::HIS3* insertion was cut with NcoI to release the insert and introduced into yeast strain SS330/SS328 (*MATa/MAT*α *ade2–101/ade2–101, his3–d200/his3–d200, ura3–52/ura3–52*). Southern blots of the His^+ transformants identified those heterozygous for the disruption. Tetrad dissection revealed the disruption to be lethal. Using the same plasmid, the *RNT1* gene was disrupted in haploid strain HI227 transformed with a *URA3* plasmid carrying a copy of *RNT1* (*MATa, leu2, trp1, ura3–52, lys 2*Δ, *his 3*–d200, *prb1–1122, pep4–3*, and *prc1–407*/ pRS316–*RNT1*). Successful gene disruption was confirmed by Southern blot analysis (Rose et al., 1990) and plasmid shuffling (Sikorski and Boeke, 1991).

Gene Isolation, Sequencing, and Sequence Alignment

Homology to bacterial RNase III was identified through an e-mail BLAST search (Altschul et al., 1990) at the National Library of Medicine (blast@ncbi.nlm.nih.gov). *RNT1* was sequenced (GenBank accession number U27016), using a series of subclones containing progressive unidirectional deletions produced with exonuclease III and S1 nuclease (Henikoff, 1984) as described in the Erase-a-Base System technical manual (Promega, Madison, WI).

Protein sequences (S. cerevisiae *RNT1*, GenBank accession number U27016; E. coli RNase III, GenBank accession number X02946; S. pombe *pac1,* GenBank accession number X54998) were aligned using CLUSTALW (Thompson et al., 1994) through the Baylor College of Medicine search launcher at http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi- align.html. Gap opening penalty was set at 18.0, and gap extension penalty was at 0.05. A gap was extended to increase alignment in the N-termini, and the double-stranded RNA-binding domain alignment was edited using the findings of Kharrat et al. (1995).

Isolation of the *rnt1–1* Mutation

The *RNT1* gene was subcloned as a BamHI–XhoI fragment in the *LEU2* plasmid pRS315 (Christianson et al., 1992). Mutations were introduced using hydroxylamine mutagenesis (Sikorski and Boeke, 1991). The plasmid (10 μg) was incubated in 500 μl of 1 M hydroxylamine at 75°C. Aliquots were collected at 20, 40, 60, and 90 min, and the reaction was stopped by adding 600 μl of 6 M NaI. DNA was removed from the reaction by binding to glass (Bio 101, Vista, CA). Each pool was amplified by transformation into E. coli XL1-Blue and introduced into yeast carrying a disrupted chromosomal *RNT1* gene and the *RNT1–URA3* plasmid. Transformants were replica plated to 5-fluoroorotic acid medium at different temperatures to remove the wild-type *RNT1* plasmid. Colonies that grew at 26°C but not at temperatures higher than 30°C were tested to determine whether the temperature-sensitive (ts) phenotype was due to a mutation in *RNT1*. One strain became temperature-resistant when retransformed with the *RNT1–URA3* plasmid. DNA was prepared from the ts strain, the mutagenized *LEU2* plasmid was recovered, and the insert was subcloned out. A new plasmid carrying the subcloned gene reproduced the ts phenotype after plasmid shuffling, confirming the location of the *rnt1–1* mutation.

To shift from permissive to restrictive temperature, wild-type or temperature-sensitive cells were grown to an OD_{600} of 0.6 in YEPD at 26°C, diluted with an equal volume of YEPD at 48°C, and incubated at 37°C.

Northern Blot Analysis

Total RNA was extracted from yeast cells and separated by electrophoresis, using 1.4% agarose gels containing 0.8% formaldehyde (Rose et al., 1990). The RNA was transferred to Zetabind nylon membrane (CUNO Incorporated, Meriden, CT) or Hybond-N (Amersham, Arlington Heights, IL) and stained with methylene blue to locate the 18 and 25S rRNAs (Good et al., 1994). Hybridization to end-labeled oligodeoxynucleotide probes (except probe C) was carried out at 30°C in 6× SSPE, 1% SDS, 2× Denhardt's, and 0.3 μg/ml denatured herring sperm DNA. The filters were washed in 6× SSPE at the same temperature and exposed to film. Probe C was hybridized at higher stringency by using 2.5× SSPE instead of 6× SSPE in the hybridization and wash steps. The probes used are probe A; 5′-CGGGTCTCTCTGCTGCC-3′, complementary to a 5′ ETS sequence upstream of A0; probe B; 5′-TTTCGCTGGGTTCTTCATC-3′, complementary to 5.8S rRNA; and probe C; 5′-CCCGGATCATAGAATTC-3′, complementary to sequences in the 3′ ETS.

Ribonuclease Protection

A probe complementary to the 3′ end of 25S rRNA and the 3′ ETS was derived by T7 transcription of a plasmid carrying the large HindIII fragment of the yeast rRNA gene and represented sequences from the HindIII site 284 nt downstream of the mature 3′ end of 25S, to the NheI site 175 nt upstream of the mature 3′ end of 25S. Total RNA (10 μg) was incubated at 42°C for 12 hr with 10^5 cpm of probe in 80% formamide hybridization buffer (Melton et al., 1984). The hybridization mix was digested with 40 μg/ml RNase A and 2 μg/ml RNase T1 for 1 hr at 30°C, extracted with phenol/chloroform, ethanol-precipitated, and loaded on 6% polyacrylamide gel.

Primer Extension

Primer extension reactions to map A0 in vivo were performed with 10 μg total yeast RNA and 0.2 ng of ^{32}P end-labeled oligonucleotide, essentially as described previously (Ares and Igel, 1990), except that annealing was for 60 min at 37°C following 2 min at 95°C. The oligonucleotide used in Figure 4A was J6: 5′ACTATCTTAAAACAAGCAACAAGCAG3′, complementary to the 5′ ETS. The oligonucleotide used in Figure 5B spanned the 5′ ETS/18S junction (Beltrame et al., 1994): 5′CCAAATAACTATCTAAAAG3′.

Expression of GST–RNT1 Fusion Protein and In Vitro Cleavage of dsRNA

A cloned copy of the *RNT1* gene was amplified using polymerase chain reaction to introduce NotI sites upstream and downstream of the coding region. The fragment was cut with NotI, ends were filled using Klenow (Perbal, 1988), and the fragment was cloned into SmaI site of pGEX-3X (Smith and Johnson, 1988) to generate an RNT1 fusion protein with GST at the amino terminus and 6 amino acids (AAGNSS) at the C-terminus. To avoid the possibility of contamination with RNase III from the bacterial host, the RNase III$^-$ strain of E. coli BL214 (Hfr, PO45, *thi-1*, *uraP119*, *rnc-105*, and *rel-1*) supplied by A. Nicholson (Nicholson et al., 1988) was used for all production of GST–RNT1. Protein was purified using glutathione agarose (Sigma, St. Louis, MO) as described by Smith and Johnson (1988), and was estimated to be about 90% pure by Coomassie staining after 10% SDS–polyacrylamide gel electrophoresis. Control GST protein was produced in parallel from pGEX-3X.

The duplex RNA substrate was produced by annealing two complementary strands of RNA derived by T7 transcription of polylinker sequence. Plasmid pRS316 cut with KpnI and pRS426 cut with SacI were transcribed, and the resulting 100 nt fragments were purified separately on 8% denaturing polyacrylamide gels. The two fragments were heated for 10 min at 65°C, mixed, and allowed to cool at room temperature for 1 hr. The 100 bp duplex RNA was then purified on a nondenaturing polyacrylamide gel. Single-stranded RNA control substrate was the 100 nt transcript derived from pRS316 cut with KpnI.

The 5′ ETS substrate was produced from a plasmid carrying a HindIII–NdeI fragment of rDNA blunted and cloned in the SmaI site of pRS426. The plasmid was cleaved with EcoRI and transcribed with T7 RNA polymerase to produce a 277 nt RNA containing 55 5′ polylinker nt, the last 180 nt of the 5′ ETS, the first 28 nt of 18S rRNA, and 14 nt of 3′ polylinker.

The 3′ ETS substrate was produced by subcloning a BamHI fragment from pRIB1 (originally derived from the EcoRI fragment spanning the 3′ end of 25S rRNA by the addition of BamHI linkers; Yip and Holland, 1989) into the BamHI site of pRS426. The plasmid was cleaved with NotI and transcribed with T7 RNA polymerase to produce a 698 nt RNA containing 82 5′ polylinker nt, the last 495 nt of 25S rRNA, the first 96 nt of the 3′ ETS, and 25 3′ polylinker nt. Cold substrate for primer extension mapping of the cleavage site was synthesized from the same plasmid cleaved with PvuII and has extra 3′ plasmid sequences, including the priming site for the reverse sequencing primer. The migration of P2 relative to DNA markers in Figure 6A is consistent with an RNA of chain length 100, owing to the greater mass of RNA. The migration of P1 is anomalous, for reasons we have not yet determined. The sequence of this cloned member of the rDNA repeat differs from the Saccharomyces Genome Database composite sequence for the rDNA repeat (http://genome-www.stanford.edu, file name: cseqXII_07) in having U rather than C at position +35 in the 3′ ETS.

To assay cleavage of dsRNA, we incubated 0.9 pmol of pure protein with 5.75 fmol of labeled 100 bp dsRNA at 30°C in a buffer containing 30 mM KCl, 10 mM $MgCl_2$, 30 mM Tris–HCl (pH 7.5), 5 mM spermidine, 0.1 mM DTT, 0.1 mM EDTA, and 0.4 mg/ml E. coli tRNA, in a total volume of 20 μl. Spermidine and tRNA are not essential for dsRNA-specific cleavage.

Cleavage of the labeled 5′ ETS substrate (3.3 pmol) was performed with 10 nmol of enzyme in 5 mM KCl, 10 mM $MgCl_2$, 30 mM Tris–HCl (pH 7.5), 5 mM spermidine, 0.1 mM DTT, 0.1 mM EDTA, and 0.4 mg/ml E. coli tRNA, in a total volume of 10 μl at 30°C for the indicated times. Unlabeled transcript was treated with the protein in the absence of tRNA as above. To map the cleavage site, we annealed 5 pmol of treated substrate to 10 pmol end-labeled 5′ ETS/18S primer (Beltrame et al., 1994) and extended with reverse transcriptase.

Cleavage of the labeled 3′ ETS substrate (5 fmol) was performed in 30 mM KCl, 10 mM $MgCl_2$, 30 mM Tris–HCl (pH 7.5), 5 mM spermidine, 0.1 mM DTT, 0.1 mM EDTA, and 0.4 mg/ml E. coli tRNA at 30°C, using 0.93 pmol of enzyme. To map the site of cleavage, we treated unlabeled substrate containing plasmid sequences, annealed it to end-labeled "reverse sequencing" primer (5′-TTTGTCGATACTGGTAC3′) and subjected to primer extension.

Acknowledgments

We thank Alan Nicholson for RNase III$^-$ E. coli, John Hughes and Liam Good for oligonucleotides, and Michael Holland for the pRIB1 plasmid. We thank David Frendewey for communicating unpublished observations. We are very grateful to David Engelke, David Frendewey, Skip Fournier, Rachel Green, Michelle Haynes, Ken Howe, Kate Lieberman, Rhonda Perriman, Barbara Sollner-Webb, Kazio Tycowski, Sandy Wells, Kevin Wilson, John Woolford, and Dong Yan for constructive comments on the manuscript.

This work was supported by grant GM40478 from the National Institute of General Medical Sciences. S. A. was supported by a grant to the RNA Center from the Lucille P. Markey Charitable Trust.

Received October 10, 1995; revised February 20, 1996.

References

Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Apirion, D., and Miczak, A. (1992). RNA processing in prokaryotic cells. Bioassays *15*, 113–120.

Ares, M. Jr., and Igel, A.H. (1990). Lethal and temperature-sensitive mutations and their suppressors identify an essential structural element in U2 small nuclear RNA. Genes Dev. *4*, 2132–2145.

Ares, M. Jr., and Weiser, B. (1995). Rearrangement of snRNA structure during assembly and function of the spliceosome. Prog. Nucl. Acids Res. Mol. Biol. *50*, 131–159.

Bachellerie, J.-P., Michot, B., Nicoloso, M., Balakin, A., Ni, J., and Fournier, M. (1995). Antisense snoRNAs: a family of nucleolar RNAs with long complementarities to rRNA. Trends Biochem. Sci. *20*, 261–264.

Bass, B.L., Hurst, S.R., and Singer, J.D. (1994). Binding properties of newly identified *Xenopus* proteins containing dsRNA-binding motifs. Curr. Biol. *4*, 301–314.

Bayev, A., Georgiev, O., Hadjiolov, A., Kermekchiev, M., Nikolaev, N., Skryabin, K., and Zakharyev, V. (1980). The structure of yeast ribosomal RNA genes. 2. The nucleotide sequence of the initiation site for ribosomal RNA transcription. Nucl. Acids Res. *8*, 4919–4926.

Beltrame, M., and Tollervey, D. (1995). Base pairing between U3 and the pre-ribosomal RNA is required for 18S rRNA synthesis. EMBO J. *14*, 4350–4356.

Beltrame, M., Henry, Y., and Tollervey, D. (1994). Mutational analysis of an essential binding site for the U3 snoRNA in the 5′ external transcribed spacer of yeast pre-rRNA. Nucl. Acids Res. *22*, 4057–4065.

Bram, R., Young, R., and Steitz, J. (1980). The ribonuclease III site flanking 23S sequences in the 30S ribosomal precursor RNA of *E. coli*. Cell *19*, 393–401.

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. Gene *110*, 119–122.

Court, D. (1993). RNase III: a double-strand RNA processing enzyme. In Control of mRNA Stability, G. Brawerman and J. Belasco, eds. (New York: Academic Press), pp. 70–116.

Craig, N., Kass, S., and Sollner-Webb, B. (1991). Sequence organization and RNA structural motifs directing the mouse primary rRNA-processing event. Mol. Cell. Biol. *11*, 458–467.

Eichler, D.C., and Craig, N. (1994). Processing of eukaryotic ribosomal RNA. Prog. Nucl. Acids Res. Mol. Biol. *49*, 197–239.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., et al. (1995). The minimal gene complement of *Mycoplasma genitalium*. Science *270*, 397–403.

Gegenheimer, P., Watson, N., and Apirion, D. (1977). Multiple pathways for primary processing of ribosomal RNA in *Escherichia coli*. J. Biol. Chem. *252*, 3064–3073.

Gonzalez, I.L., Chambers, C., Gorski, J.L., Stambolian, D., Schmickel, R.D., and Sylvester, J.E. (1990). Sequence and structure correlation of human ribosomal transcribed spacers. J. Mol. Biol. *212*, 27–35.

Good, L., Abou Elela, S., and Nazar, R.N. (1994). Tetrahymena ribozyme disrupts rRNA processing in yeast. J. Biol. Chem. *269*, 22169–22172.

Henry, Y., Wood, H., Morrissey, J.P., Petfalski, E., Kearsey, S., and Tollervey, D. (1994). The 5′ end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. EMBO J. *13*, 2452–2463.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene *28*, 351–359.

Hughes, J. M., and Ares, M., Jr. (1991). Depletion of U3 small nucleolar RNA inhibits cleavage in the 5′ external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. EMBO J. *10*, 4231–4239.

Iino, Y., Sugimoto, A., and Yamamoto, M. (1991). S. pombe *pac1*$^+$, whose overexpression inhibits sexual development, encodes a ribonuclease III-like RNase. EMBO J. *10*, 221–226.

Kass, S., Craig, N., and Sollner-Webb, B. (1987). Primary processing of mammalian rRNA involves two adjacent cleavages and is not species-specific. Mol. Cell. Biol. *7*, 2891–2898.

Kass, S., Tyc, K., Steitz, J.A., and Sollner-Webb, B. (1990). The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. Cell *60*, 897–908.

Kempers-Veenstra, A.E., Oliemans, J., Offenberg, H., Dekker, A.F., Planta, R.J., and Klootwijk, J. (1986). 3′-end formation of transcripts from the yeast rRNA operon. EMBO J. *10*, 2703–2710.

Kharrat, A., Macias, M.J., Gibson, T.J., Nilges, M., and Pastore, A. (1995). Structure of the dsRNA binding domain of *E. coli* RNase III. EMBO J. *14*, 3572–3584.

Klemenz, R., and Geiduschek, E.P. (1980). The 5′ terminus of the precursor ribosomal RNA of *Saccharomyces cerevisiae*. Nucl. Acids Res. *8*, 2679–2689.

Lang, W.H., and Reeder, R.H. (1993). The REB1 site is an essential component of a terminator for RNA polymerase I in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *13*, 649–658.

Li, H.D., Zagorski, J., and Fournier, M.J. (1990). Depletion of U14 small nuclear RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *10*, 1145–1152.

Liang, W.-Q., and Fournier, M. (1995). U14 base-pairs with 18S rRNA: a novel snoRNA interaction required for rRNA processing. Genes Dev. *9*, 2433–2443.

Madhani, H.D., and Guthrie, C. (1994). Dynamic RNA–RNA interactions in the spliceosome. Annu. Rev. Genet. *28*, 1–26.

March, P.E., Ahnn, J., and Inouye, M. (1985). The DNA sequence of the gene (*rnc*) encoding ribonuclease III of *Escherichia coli*. Nucl. Acids Res. *13*, 4677–4685.

Maxwell, E.S., and Fournier, M.J. (1995). The small nucleolar RNAs. Annu. Rev. Biochem. *64*, 897–934.

Melekhovets, Y.F., Good, L., Abou Elela, S., and Nazar, R.N. (1994). Intragenic processing in yeast rRNA is dependent on the 3′ external transcribed spacer. J. Mol. Biol. *239*, 170–180.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. *12*, 7035–7056.

Morrissey, J.P., and Tollervey, D. (1993). Yeast snR30 is a small nucleolar RNA required for 18S rRNA synthesis. Mol. Cell. Biol. *13*, 2469–2477.

Mougey, E.B., Pape, L.K., and Sollner-Webb, B. (1993). A U3 small nuclear ribonucleoprotein–requiring processing event in the 5′ external transcribed spacer of *Xenopus* precursor rRNA. Mol. Cell. Biol. *13*, 5990–5998.

Nicholson, A.W., Niebling, K.R., McOsker, P.L., and Robertson, H.D. (1988). Accurate *in vitro* cleavage by RNase III of phosphorothioate-substituted RNA processing signals in bacteriophage T7 early mRNA. Nucl. Acids Res. *16*, 1577–1591.

Peculis, B.A., and Steitz, J.A. (1993). Disruption of U8 nucleolar snRNA inhibits 5.8S and 28S rRNA processing in the Xenopus oocyte. Cell *73*, 1233–45.

Perbal, B. (1988). A Practical Guide to Molecular Cloning (New York: John Wiley & Sons).

Potter, S., Durovic, P., and Dennis, P.P. (1995). Ribosomal RNA precursor processing by a eukaryotic U3 small nucleolar RNA-like molecule in an archaeon. Science, *268*, 1056–1060.

Robertson, H.D., Webster, R.E., and Zinder, N.D. (1968). Purification and properties of ribonuclease III from *Escherichia coli*. J. Biol. Chem. *243*, 82–91.

Rose, M.D., Winston, F., and Hieter, P. (1990). Methods in Yeast Genetics: A Laboratory Course Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Meth. Enzymol. *194*, 281–301.

Rotondo, G., Gillespie, M., and Frendewey, D. (1995). Rescue of the fission yeast snRNA synthesis mutant snm1 by overexpression of the double-strand–specific Pac1 ribonuclease. Mol. Gen. Genet. *247*, 698–708.

Schmitt, M.E., and Clayton, D.A. (1993). Nuclear RNase MRP is required for correct processing of pre-5.8S rRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *13*, 7935–7941.

Shumard, C.M., and Eichler, D.C. (1988). Ribosomal RNA processing: limited cleavages of mouse preribosomal RNA by a nucleolar endoribonuclease include the early +650 processing site. J. Biol. Chem. *263*, 19346–19352.

Sikorski, R.S., and Boeke, J.D. (1991). In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. Meth. Enzymol. *194*, 302–318.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S–transferase. Gene *67*, 31–40.

Steitz, J., and Tycowski, K. (1995). Small RNA chaperones for ribosome biogenesis. Science *270*, 1626–1627.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of multiple sequence alignments through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucl. Acids Res. *22*, 4673–4680.

Thompson, L.D., and Daniels, C.J. (1988). A tRNA(Trp) intron endonuclease from *Halobacterium volcanii*: unique substrate recognition properties. J. Biol. Chem. *263*, 17951–17959.

Thompson, L.D., Brandon, L.D., Nieuwlandt, D.T., and Daniels, C.J. (1989). Transfer RNA intron processing in the halophilic archaebacteria. Can. J. Microbiol. *35*, 36–42.

Tollervey, D. (1987). A yeast small nuclear RNA is required for normal processing of preribosomal RNA. EMBO J. *6*, 4169–4175.

Tycowski, K.T., Shu, M.D., and Steitz, J.A. (1994). Requirement for intron-encoded U22 small nucleolar RNA in 18S ribosomal RNA maturation. Science *266*, 1558–1561.

van der Sande, C.A., Kulkens, T., Kramer, A.B., de Wijs, I.J., van Heerikhuizen, H., Klootwijk, J., and Planta, R.J. (1989). Termination of transcription by yeast RNA polymerase I. Nucl. Acids Res. *17*, 9127–9146.

Veldman, G.M., Klootwijk, J., de Jonge, P., Leer, R.J., and Planta, R.J. (1980). The transcription termination site of the ribosomal RNA operon in yeast. Nucl. Acids Res. *8*, 5179–5192.

Venema, J., Henry, Y., and Tollervey, D. (1995). Two distinct recognition signals define the site of endonucleolytic cleavage at the 5′-end of yeast 18S rRNA. EMBO J. *14*, 4883–4892.

Wells, S., Neville, M., Haynes, M., Wang, J., Igel, H., and Ares, M. Jr. (1996). *CUS1*, a suppressor of cold-sensitive U2 snRNA mutations, is a novel yeast splicing factor homologous to human SAP 145. Genes Dev. *10*, 220–232.

Xu, H.P., Riggs, M., Rodgers, L., and Wigler, M. (1990). A gene from S. pombe with homology to E. coli RNase III blocks conjugation and sporulation when overexpressed in wild-type cells. Nucl. Acids Res. *18*, 5304.

Yeh, L.-C., and Lee, J.C. (1992). Structure analysis of the 5′ external transcribed spacer of the precursor ribosomal RNA from Saccharomyces cerevisiae. J. Mol. Biol. *228*, 827–839.

Yip, M.T., and Holland, M.J. (1989). In vitro RNA processing generates mature 3′ termini of yeast 35 and 25S ribosomal RNAs. J. Biol. Chem. *264*, 4045–4051.