17. D. A. Baylor and A. L. Hodgkin, *J. Physiol. (London)* **234**, 163 (1973).
18. J. L. Schnapf, B. J. Nunn, M. Meister, D. A. Baylor, *ibid.* **427**, 681 (1990).
19. T. Yagi and P. R. MacLeish, *J. Neurophysiol.* **71**, 656 (1994).
20. D. A. Baylor, B. J. Nunn, J. L. Schnapf, *J. Physiol. (London)* **390**, 145 (1987).
21. For rods, the sensitivity at 660 nm compared to that at 500 nm was taken as 2.6×10^{-4}. Relative sensitivity values were calculated from rod suction electrode recordings (5) after correction for axial illumination by applying Beer's law and assuming a peak axial optical density of 0.35 (5). For cones, the sensitivity at 660 nm relative to that at 500 nm was estimated to be 0.13 (red cone) and 7.4×10^{-3} (green cone). Values from suction recordings (*20*) were corrected assuming a peak axial density of 0.17.
22. The after-hyperpolarization could be selectively eliminated by background lights expected to desensitize rods but not cones.
23. R. Nelson, *J. Comp. Neurol.* **172**, 109 (1977).
24. J. D. Conner and D. I. A. MacLeod, *Science* **195**, 698 (1977); J. D. Conner, *J. Physiol. (London)* **332**, 139 (1982); R. Knight and S. L. Buck, *Vision Res.* **33**, 2203 (1993).
25. R. G. Smith, M. A. Freed, P. Sterling, *J. Neurosci.* **6**, 3505 (1986).
26. D. A. Baylor and R. Fettiplace, *Cold Spring Harbor Symp. Quant. Biol.* **40**, 529 (1976); J. L. Schnapf and D. R. Copenhagen, *Nature* **296**, 862 (1983); D. R. Copenhagen, J. F. Ashmore, J. L. Schnapf, *Vision Res.* **4**, 363 (1983).
27. We thank D. A. Baylor, S. L. Buck, D. R. Copenhagen, J. C. Horton, J. H. LaVail, and A. Stockman for comments on the manuscript. This work was funded by a grant from the Plum Foundation and grants RO1–EY07642 and F32–EY06399 from the National Eye Institute. Additional support was obtained from That Man May See and Research to Prevent Blindness.

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Ribosomal RNA Precursor Processing by a Eukaryotic U3 Small Nucleolar RNA–Like Molecule in an Archaeon

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An RNA-containing endonuclease that catalyzes the excision and maturation of the 16*S* ribosomal RNA (rRNA) from the rRNA primary transcript (pre-rRNA) in the hyperthermophilic archaeon *Sulfolobus acidocaldarius* has been characterized. The ribonucleoprotein was inactivated by micrococcal nuclease treatment and inactivation was reversed by reconstitution with bulk RNA. A 159-nucleotide RNA with sequence and structural similarity to U3 small nucleolar RNAs of eukaryotes copurified with the endonuclease activity. Oligonucleotide-targeted ribonuclease H inactivation of the U3-like RNA component also abolished processing activity. A motif within the U3 homolog is complementary to the region around the three cleavage sites in the pre-RNA substrate. Thus, U3-mediated processing of pre-rRNA is not specific to eukaryotes; its origin predates the divergence of archaea and eukaryotes.

At least two endonuclease activities participate in the processing of the primary transcript from the single copy 16S-23S rRNA operon in *S. acidocaldarius* (*1*). The first of these endonucleases is the well-characterized, helix-processing enzyme unique to all archaea (*2*, *3*). This endonuclease excises introns from intron-containing tRNA and rRNA gene transcripts and, in most species, excises pre–16S and 23S rRNAs from the primary rRNA transcript (*1*–*4*). The enzyme has a rigorously defined substrate specificity; it acts on two three-base bulges, located on opposite strands within an extended helix, that are separated by 4 base pairs (bp). With respect to rRNA processing, the enzyme is analogous (but not homologous) to the helix-specific ribonuclease (RNase) III of bacteria (*5*). In *S. acidocaldarius*, this activity excises pre–23S but not pre–16S RNA from the primary rRNA transcript (*1*).

Although the 16S RNA sequence is surrounded by a long inverted repeat in the primary transcript, it is unlikely that the putative helix is used or required for excision or maturation events in the 5′ external transcribed spacer (ETS). Instead, the 5′ ETS is cleaved at three positions by the second endonuclease activity, which we now show resembles the U3-containing ribonucleoprotein (RNP) particles responsible for processing of pre-rRNA in the nucleolus of eukaryotic cells (*6*). The first two cleavages occur ~99 and 31 nucleotides (nt) upstream of the 16S RNA sequence, and the third occurs at the 5′ ETS–16S RNA junction in order to produce the 5′ end of mature 16S RNA. We have shown previously that a cell-free extract of *S. acidocaldarius* will cleave a synthetic RNA substrate that contains the 5′ ETS and the first 72 nt of 16S RNA sequence at or close to the expected three positions (*1*). This observation indicates that 5′ ETS processing does not require formation of the 16S RNA processing helix, and that maturation at the 5′ end of 16S RNA requires no more than 72 nt of 16S RNA sequence and does not require concomitant small ribosomal subunit assembly. The endonuclease activity was sensitive to RNase A digestion, indicating that it contained an RNA component essential for activity.

Primer extension analysis was used to position precisely the 5′ ends of intermediates and products resulting from in vitro cleavage of an SP6 RNA polymerase–transcribed substrate RNA by an activity purified from cell-free extract (*7*) (Fig. 1). The primer was complementary to positions 57 to 35 of 16S rRNA. The observed products indicated that major cleavages occur at positions −99 (site 1) and −31 (site 2) of the 5′ ETS and at position +1 (site 4) at the 5′ ETS–16S RNA junction. These intermediates and the mature 16S RNA product were virtually identical to those observed in vivo (*1*, *8*). Additional minor extension products with 5′ ends near sites 1, 2, and 4 were observed; these could represent alternative positions for endonucleolytic processing or, in some instances, nuclease trimming at the 5′ end of the intermediates or products.

We also directly visualized the accumulation of product fragments that resulted from the cleavage of ^{32}P-labeled substrate RNA. Three of the four products accumulated in a time-dependent manner (Fig. 2, A and B). The fourth product was too short to visualize on the electrophoresis gel.

RNase A sensitivity of processing activity in crude cell extracts (*1*) was supported by incubating partially purified processing activity with micrococcal nuclease at 37°C before increasing the temperature to 75°C for 15 min and then adding the substrate RNA (*9*). No specific processing was evident when the partially purified activity was treated with the nuclease (Fig. 2C, lanes 2 and 3). The inactivated processing activity was then reactivated by reconstitution with bulk *S. acidocaldarius* RNA (3 to 15 μg) for 15 min at 75°C, after which substrate RNA was added and incubation was continued for a further 15 min at 75°C (Fig. 2C, lanes 4 to 8). Reactivation was partial with the use of 6 μg of RNA and essentially complete with >6 μg of RNA. Bulk RNA from several other archaeal or bacterial species was ineffective in heterologous reconstitution (*7*). The reconstituted activity was itself sensitive to redigestion by micrococcal nuclease. The reconstituted mixture was transferred back to 37°C and a second dose of micrococcal nuclease (100 ng or 1 μg) was added, before returning the sample again to 75°C and adding substrate RNA; no processing was evident (Fig. 2C, lanes 9 to 12).

The presence of an RNA component in the purified processing activity was detected

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by reverse transcription and polymerase chain reaction (PCR) analysis (*10*). This procedure yielded a product band of 148 bp. The product was cloned, sequenced, and used to probe genomic DNA. A 1.2-kb Hind III–Eco RV genomic fragment hybridized to the probe and, by nucleotide sequencing, was shown to encode the RNA that had been amplified by PCR. To ensure that the PCR product was an authentic component of the processing activity, we performed Northern (RNA) hybridization with material obtained by phenol extraction of the processing activity at various steps in a procedure that resulted in a 200-fold purification. Both the genomic probe and the PCR product probe hybridized to an RNA of ~150 nt that copurified with the processing activity (Fig. 3). The nucleotide sequences of the PCR and genomic clones were determined and the position of the authentic 5′ end of the RNA was localized by primer extension analysis. The PCR product was found to lack 11 nt from the 5′ end of the in vivo RNA from which it was derived.

The 159-nt RNA exhibits sequence and potential structural similarity to U3, the most abundant and well-characterized small nucleolar RNA (snoRNA) of eukaryotes (*6*) (Fig. 4A). In *Saccharomyces cerevisiae*, depletion of U3 inhibits multiple events required for 18*S* RNA maturation and the accumulation of small ribosomal subunits (*11*). A U3-containing complex appears to bind 230 nt upstream of the ETS–18*S* RNA junction and initiates three cleavage events: The first occurs 89 nt upstream of the ETS–18*S* RNA junction, the second at the ETS–18*S* RNA junction to generate

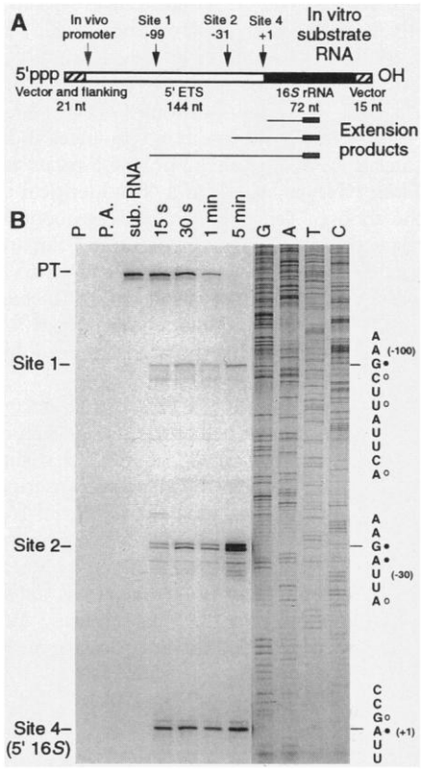

Fig. 1. Localization of in vitro cleavage sites within the 5′ ETS. (**A**) Schematic representation of the synthetic in vitro–transcribed substrate RNA, indicating the three major sites of cleavage at positions −99, −31, and +1. The extension products generated with an oligonucleotide primer complementary to positions 57 to 35 of 16S rRNA as well as in vitro processing intermediates are also shown. (**B**) Nonradioactive substrate RNA was partially processed for 15 s to 5 min (*7*). The resulting intermediates were subjected to primer extension analysis and the extension products were detected by electrophoresis on a denaturing polyacrylamide gel and autoradiography. Lanes: P, control in which the primer, in the absence of the processing activity or substrate RNA, was subjected to extension analysis; P. A., control in which the processing activity, in the absence of added substrate RNA, was subjected to extension analysis; and sub. RNA, control in which the in vitro–transcribed substrate RNA, in the absence of processing activity, was subjected to extension analysis. The next four lanes represent extension analysis on RNA intermediates that accumulate after exposure to processing activity for 15 s, 30 s, 1 min, or 5 min, respectively. The band at the top of the gel (labeled PT) is the extension product derived from full-length substrate RNA. All primer extensions were performed in the presence of 2.5 μg of bulk yeast RNA as carrier. The G, A, T, and C lanes are a dideoxy sequence ladder generated by the 5′-phosphorylated oligonucleotide primer and plasmid pPD1105 as template. The DNA(+) strand sequence is indicated on the right with the major (●) and minor (○) extension stops indicated.

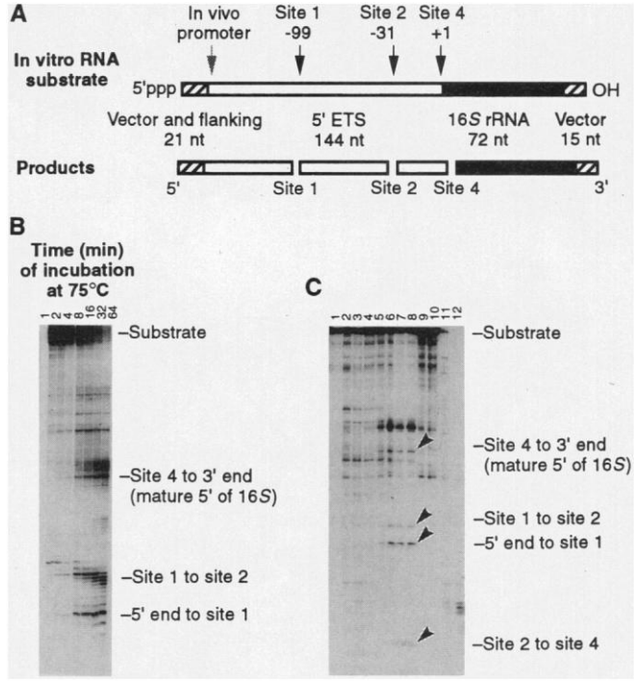

Fig. 2. Detection of partially purified processing activity and its sensitivity to micrococcal nuclease digestion. (**A**) Schematic representation of the in vitro–transcribed substrate RNA and the four-limit cleavage products. (**B**) Substrate RNA labeled with [α-^{32}P]CTP was incubated for 0, 1, 2, 4, 8, 16, 32, or 64 min with purified processing activity at 75°C (*1*, *7*). Products were separated on a denaturing 8% polyacrylamide gel and detected by autoradiography. (**C**) Processing activity was inactivated by treatment with micrococcal nuclease (100 ng per assay) in the presence of Ca^{2+} at 37°C for 15 min and subsequently reconstituted at 75°C by addition of bulk RNA. After reconstitution for 15 min at 75°C, ^{32}P-labeled substrate was added and incubation continued for a further 15 min. Products were separated on a denaturing 8% polyacrylamide gel and detected by autoradiography. Lanes: 1, substrate RNA; 2 and 3, activity treated with micrococcal nuclease and assayed without reconstitution; 4 to 8, activity treated with micrococcal nuclease, reconstituted with 3, 6, 9, 12, or 15 μg of bulk RNA, and assayed; 9 and 10, same as lanes 4 and 7, but retreated after reconstitution with 100 ng of micrococcal nuclease for 15 min at 37°C before returning again to 75°C for the addition of substrate; 11 and 12, same as lane 7 but retreated after reconstitution with 1 μg of micrococcal nuclease for 15 or 30 min, respectively, at 37°C.

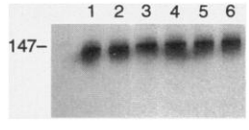

Fig. 3. Copurification of a small RNA with 5′ ETS–processing activity. The activity responsible for cleavage of the 5′ ETS–containing substrate RNA was purified from crude cell lysates. At each step of purification, RNA was prepared by phenol extraction and subjected to Northern analysis with the genomic clone that encodes the U3-like RNA as a probe. The lanes represent various steps in a procedure that results in a 200-fold purification: 1, crude extract; 2, 35% saturated ammonium sulfate fraction; 3 and 4, fractions 10 and 11 from Superose 6 (Pharmacia) chromatography; 5, MonoQ ion exchange chromatography (Pharmacia); 6, Hi-trap heparin chromatography (Pharmacia). Details of the purification procedure and composition of the processing activity will be described in a subsequent publication (*24*). The RNA extractions at the different stages of purification were normalized to a constant amount of a major 30-kD protein that copurifies with the processing activity. On an immunoblot, this protein cross-reacted with human antibodies to fibrillarin. The anti-fibrillarin antibodies coprecipitate the same protein and RNA components that copurify with the processing activity (*21*). The position of a 147-nt RNA size standard is indicated on the left.

the mature 5′ end of 18S RNA, and the third within the first internal transcribed spacer (ITS1) close to the junction with 5.8S RNA (*11*, *12*). Similar U3-dependent cleavages have been observed in the *Xenopus* and mammalian pre-rRNA processing pathways (*13*, *14*).

The U3 snoRNAs from eukaryotes exhibit a common secondary structure, contain five separate sequence elements (boxes A, C′, B, C, and D) that are highly conserved, and, together with the protein fibrillarin, constitute two of the components of a larger RNP complex (*6*). Box A is the most highly conserved region of U3. In yeast and mammals, nucleotides within or close to box A are implicated in the binding of U3 to the ETS region of pre-rRNA (*12*, *15*). Structural analysis of human and *Xenopus* U3 indicates that the box B sequence is single-stranded and therefore available for a potential interaction with substrate RNA (*16*, *17*). However, no clearly conserved complementarity between box B and sequences at or near U3-dependent cleavage sites has been identified. Box C is thought to participate in the association of the RNA with fibrillarin (*15*).

Although shorter than most eukaryotic U3 RNAs, the *S. acidocaldarius* U3-like RNA retains the conserved box A, C′, B, C, and D sequences at the appropriate positions and can be readily folded into a U3-like secondary structure (Fig. 4A). Alignment of the five box sequences indicates that, over these 62 nt, the *S. acidocaldarius* RNA is 56, 50, and 48% identical to the mouse, *Xenopus*, and yeast sequences, respectively (Fig. 5). In the same comparison, the yeast sequence is 71 and 63% identical to those of mouse and *Xenopus*, respectively. We conclude that the RNA we have identified is the archaeal homolog of eukaryotic U3 snoRNA.

Processing of the 5′ ETS substrate occurs in vitro in the absence of the second half of the long inverted repeat sequence that surrounds 16S rRNA in the primary transcript. This result indicates that the potential 16S RNA processing helix that is a universal feature in virtually all bacterial and archaeal rRNA operons may not be required for 5′ ETS processing in *S. acidocaldarius*. We therefore reexamined the *S. acidocaldarius* 5′

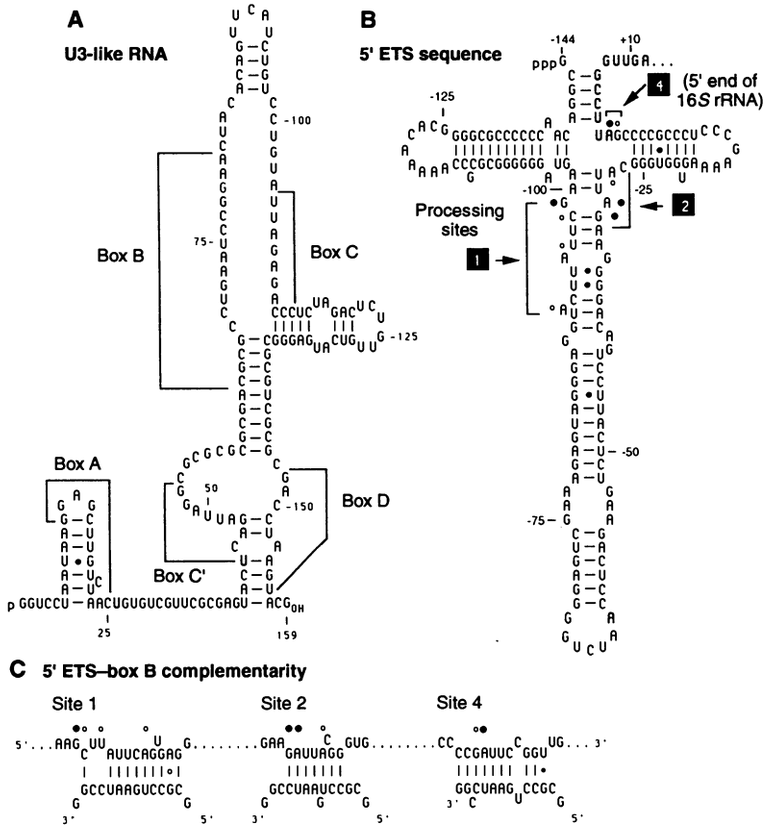

Fig. 4. Sequence and secondary structure of and cleavage site complementarity between U3-like RNA and 5′ ETS RNA. (**A**) The RNA component of the processing activity is 159 nt in length and is folded into a eukaryotic U3–like secondary structure (*6*, *23*). The conserved sequence motifs are box A (positions 12 to 25), box C′ (44 to 53), box B (65 to 81), box C (105 to 114), and box D (148 to 156). (**B**) The 5′ ETS is folded into the predicted cloverleaf secondary structure with the use of the program RNA fold (*25*). Processing occurs at sites 1, 2, and 4 near positions −99, −31, and +1, respectively. (**C**) The core of box B in the U3-like RNA exhibits complementarity to the sequences surrounding processing sites 1, 2, and 4 in the 5′ ETS.

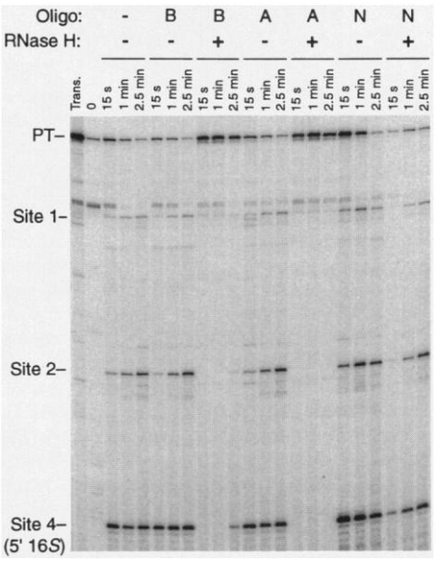

Fig. 6. Oligonucleotide-targeted RNase H inactivation of the processing activity. The substrate, processing intermediates, and products of the processing reaction were detected with the primer extension assay described in Fig. 1. Extension products were separated on a denaturing 8% polyacrylamide gel and analyzed by autoradiography. Portions of the processing reaction mixture were removed for analysis after 15 s, 1 min, and 2.5 min. The first two lanes correspond to the in vitro transcript (Trans.) and the zero time control (0) in the normal processing reaction. The oligonucleotides (Oligo) used for targeting RNase H digestion were box B oSP7 (B), box A oSP2 (A), and the nonspecific oSP5 (N).

Fig. 5. Alignment of the conserved U3 RNA box A, C′, B, C, and D motifs. The five conserved motifs from three eukaryotic U3 RNAs (mouse, Mus; *Xenopus*, Xen; *S. cerevisiae*, Sce) are aligned with the corresponding motifs in the U3-like RNA from *S. acidocaldarius* (Sac). The *S. acidocaldarius* box D sequence is extended by 2 nt over that shown in Fig. 4 in order to conform to the length of the eukaryotic box D sequences. A consensus sequence (Con) has been derived; uppercase nucleotides are conserved in all three eukaryotic RNAs; lowercase nucleotides are conserved in two of the three RNAs. Bold dots (●) indicate identities between the U3-like RNA of *S. acidocaldarius* and the consensus sequence.

	Box A	Box C′	Box B	Box C	Box D
Mus:	GGAUCAUUUCUAUA..GAGGACGAGG..UGAGCGUGAAGCCGGCU..UUGAUGAUCG..AGUCUGAGUGG				
Xen:	GGAUCAUUUCUAUA..GAGGAAGAGG..UGAGCGUGAAGUGAGCU..UUGAUGAACG..AGGCUGAGUGG				
Sce:	GGAUCAUUUCUAUA..GUUGAUGAGG..AGAGUGAGAAACCGAAA..AUGAUCUUGA..AGUCUGACAAG				
Con	GGAUCAUUUCUAUA..GagGA-GAGG..uGAGcGuGAAgccggcu..uUGAUgaucg..AGuCTGAgugG				
	●●● ● ● ●●●●	●● ●●●	● ●● ●●●● ●●●●	●● ●● ●	●● ●●●
Sac:	GGAGCUUGUCUAAC..UCAGAUUAGG..CGCGCCUGAAUCCGGAA..UUAGAGACCC..GACCUAAGUAC				

ETS for an alternative secondary structure. The 144-nt 5′ ETS together with the first 6 nt of 16S rRNA can be folded into a cloverleaf structure (Fig. 4B), in which sites 1, 2, and 4 are in close proximity. The major cleavages within sites 1 and 2 occur 5′ to the G(−99) and A(−31) residues that form a noncanonical base pair near the base of the downward helix. Maturation of 16S rRNA at position +1 occurs in a short unpaired region between the rightward and upward helices. Examination of the U3-like RNA sequence indicates that the core of box B exhibits substantial complementarity to the nucleotide sequence around processing sites 1, 2, and 4 in the substrate RNA (Fig. 4C). It is not known whether this complementarity is important for site recognition or whether it plays a role in the cleavage reaction. In eukaryotes, the potential for complementarity between U3 box B and cleavage site sequences is either absent or much less significant.

In yeast, processing at the 5′ ETS–18S RNA junction requires several snoRNAs, including U3 and U14, as well as fibrillarin and other proteins (6, *18*). To confirm the importance of the *S. acidocaldarius* U3-like RNA in 5′ ETS processing, we subjected the U3-like RNA to oligonucleotide-targeted RNase H digestion (*19*). The two targeting oligonucleotides were complementary to the U3-like RNA between positions 85 to 69 and 32 to 12 and overlap, respectively, the highly conserved box B and box A regions. The presence of either the box B or box A oligonucleotides or a nonspecific oligonucleotide had no effect on the normal processing reaction (Fig. 6). Similarly, digestion with RNase H in the presence of the nonspecific oligonucleotide had essentially no effect on processing activity. In contrast, digestion with RNase H in the presence of the box B oligonucleotide markedly reduced the rate of consumption of the substrate and accumulation of intermediates and product, and digestion in the presence of the box A oligonucleotide appeared to inactivate processing activity completely.

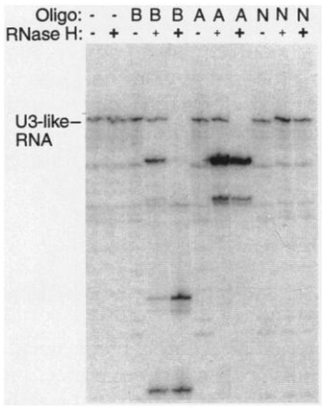

Fig. 7. Oligonucleotide-targeted RNase H ablation of the U3-like RNA component of the processing activity. Processing activity was incubated in the absence or presence of specific or nonspecific oligonucleotides and then digested with RNase H (*20*). The RNA was recovered from the control and treated samples, and subjected to Northern analysis with the U3-like cDNA fragment from plasmid pPD1156 as probe. The specific oligonucleotides were box B oSP7 (B) and box A oSP2 (A) and the nonspecific oligonucleotide was oSP5 (N).

To show that the RNase H–targeted digestion specifically ablates the U3-like RNA component of the processing activity, we performed Northern analysis (Fig. 7). Processing activity was incubated with the specific box B or box A oligonucleotides, or the nonspecific oligonucleotide, and either digested or not digested with RNase H (*20*). The RNA recovered from the respective samples was subjected to Northern hybridization with the U3-like complementary DNA (cDNA) as probe. In the control sample and in the samples with an oligonucleotide but without RNase H digestion, the U3-like RNA was unaltered. Similarly, RNase H digestion in the presence of the nonspecific oligonucleotide had no effect on the mobility of the RNA. In contrast, RNase H treatment in the presence of the box B or box A oligonucleotides resulted in virtually complete ablation of the U3-like RNA component. These results, together with the processing results in Fig. 6, indicate that the integrity of the box A sequence is essential for in vitro processing of the 5′ ETS substrate. The residual processing activity present after RNase H treatment in the presence of the box B–specific oligonucleotide may indicate either that ablation was incomplete or that residual U3-like RNA fragments produced as a result of cleavage within box B retain some activity. For example, if box B is used to locate the cleavage sites within the substrate RNA, removal of the box B sequence might be expected to reduce the rate of the reaction.

Although we were able to reconstitute processing activity after RNase inactivation by the readdition of bulk unfractionated RNA from *S. acidocaldarius*, attempts to reconstitute activity with the use of *S. acidocaldarius* U3-like RNA transcribed in vitro from an SP6 promoter have so far been unsuccessful. The in vitro–transcribed RNA lacked several nucleotides at the 5′ end; these may be essential for function or correct folding. Furthermore, the processing complex contains several additional RNAs that have been identified by pCp end-labeling. A cDNA copy of one of these RNAs has been cloned and sequenced; it contains box C and D motifs but has not been further characterized (*21*).

Our results raise several questions. First, what is the evolutionary origin and phylogenetic distribution of RNP particles containing a U3-like RNA? And second, what was the primordial function of these particles? All eukaryotes possess U3-containing snoRNPs and these are essential for processing and maturation of small-subunit rRNA (6, *11*, *13*, *14*). We have shown that a processing activity containing an essential U3-like RNA is also present in the crenarchaeota (or eocytic) branch of the archaea. If the presence of a fibrillarin-like protein is indicative of this type of processing, then it is likely that the same activity is also present in the euryarchaeota (or methanogen-halophile) branch of archaea in which fibrillarin-like genes have been identified (*22*). We have recently observed that human antibodies to fibrillarin cross-react with a major *S. acidocaldarius* protein that copurifies with the *S. acidocaldarius* processing activity (*21*). We suggest that this activity may be universally present in the archaea and that its primary (and perhaps primordial) function may be to mediate the 5′ end maturation of small-subunit rRNA. As far as we are aware, to date, fibrillarin or U3 RNA-like molecules have not been detected in eubacteria.

In summary, our results demonstrate that the use of RNP particles for the processing and maturation of rRNAs is an established feature that predates the divergence of archaea and eukaryotes. Furthermore, the in vitro processing assay described here and the ability to reconstitute processing activity after nuclease inactivation suggest that it may soon be possible to address directly the role of U3 RNA in the endonucleolytic cleavages of RNA and the more complex process of ribosome subunit assembly.

REFERENCES AND NOTES

1. P. Durovic and P. P. Dennis, *Mol. Microbiol.* **13**, 229 (1994).
2. L. D. Thompson, L. D. Brandon, D. T. Nieuwlandt, C. J. Daniels, *Can. J. Microbiol.* **35**, 36 (1989).
3. R. A. Garrett, J. Dahlberg, N. Larson, J. Kjems, A. Mankin, *Trends Biochem. Sci.* **16**, 22 (1991); P. P. Dennis, in *General and Applied Aspects of Halophilic Microorganisms*, F. Rodriguez-Valera, Ed. (Plenum, New York, 1991), pp. 251–258.
4. J. Chant and P. P. Dennis, *EMBO J.* **5**, 1091 (1986).
5. D. Court, in *Control of mRNA Stability*, G. Brawerman and J. Belasco, Eds. (Academic Press, New York, 1993).
6. S. J. Baserga and J. A. Steitz, in *The RNA World*, R. F. Gesteland and J. F. Atkins, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), pp. 359–382; B. Sollner-Webb, K. Tyc, J. A. Steitz, in *Ribosomal RNA: Structure, Evolution, Gene Expression and Functions in Protein Synthesis*, R. A. Zimmermann and A. E. Dalberg, Eds. (CRC Press, Boca Raton, FL, in press); M. J. Fournier and E. S. Maxwell, *Trends Biochem. Sci.* **18**, 131 (1993); T. Hartshorne and N. Agabian, *Nucleic Acids Res.* **22**, 3354 (1994); K. T. Tycowski, M. Shu, J. A. Steitz, *Genes Dev.* **7**, 1176 (1993).
7. Substrate RNA used in the in vitro processing reaction was transcribed with SP6 RNA polymerase from plasmid pPD1105 that had been linearized with Eco RI. The 257-nt transcript contains the entire 144-nt

5′ ETS and the first 72 nt of the 16S RNA sequence sandwiched between 21 nt of vector and 5 nt of host flanking sequence at the 5′ end and 15 nt of vector sequence at the 3′ end. When required, the RNA was uniformly labeled with [α-^{32}P]CTP (cytidine triphosphate) or 3′ end-labeled with pCp with the use of RNA ligase. Processing was performed at 75°C as described (*1*) with purified activity instead of crude cell extract. For partial processing, ~1 pmol of nonradioactive substrate RNA was incubated with processing activity for 15 s to 5 min. Reactions were terminated by phenol extraction and the RNA was recovered after ethanol precipitation. Sites of in vitro cleavage were mapped by primer extension with a 5′ end-labeled oligonucleotide complementary to positions 57 to 35 of the 16S rRNA sequence. Primer (0.5 pmol) was annealed to partially processed RNA in the presence of 2.5 μg of yeast carrier RNA by incubation for 2 min at 92°C, 10 min at 47°C, and 20 min at 20°C. An enzyme mix containing deoxynucleoside triphosphates and Superscript II reverse transcriptase (100 units per reaction) (BRL) was added, and extension was performed at 47°C for 45 min. The extension products were recovered after phenol extraction and ethanol precipitation and were displayed on a denaturing polyacrylamide gel. Bulk in vivo RNA from *S. acidocaldarius* used for S1 nuclease protection and for reconstitution was isolated from growing cell cultures by boiling in SDS lysis buffer, phenol extraction, ethanol precipitation, and centrifugation through 5.8 M CsCl [P. P. Dennis and J. Chow, in *Archaea—A Laboratory Manual*, F. Robb *et al.*, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, in press)]. Bulk RNA alone showed no processing activity. Bulk RNAs isolated from *Escherichia coli*, *Bacillus subtilis*, or *Haloferax volcanii* were inactive in heterologous reconstitution.

8. The product with a 5′ end at position +1 and the intermediate with a 5′ end at −31 are identical to those observed in vivo (or in vitro) by S1 nuclease protection. The intermediate with a 5′ end at position −99 is 3 or 4 nt longer than the in vivo (or in vitro) intermediate detected by S1 nuclease protection; the difference presumably represents limited S1 digestion at the end of the protected DNA probe. For unknown technical reasons, we have been unable to generate high-quality primer extension products with bulk in vivo RNA as template.
9. The limit processing assay was performed for 1 to 64 min at 75°C with ^{32}P-labeled substrate as described (*1*) but with purified activity instead of crude cell extract. The product RNA fragment that extends from the 5′ end of the transcript to site 1 migrates anomalously fast in the electrophoresis gel, probably because of an extended hairpin containing nine consecutive G • C base pairs (Fig. 4B). The identity of this fragment was verified by increasing or decreasing the length of the flanking sequence at the 5′ end of the substrate RNA and observing the expected mobility shift of this fragment. The identity of the fragment from site 4 to the 3′ end was confirmed by RNA ligase–mediated 3′ end-labeling of the substrate RNA with pCp. Bands that are not labeled in Fig. 2 are either uncharacterized processing intermediates or are the result of thermal or contaminating nuclease–mediated degradation of the ^{32}P-labeled substrate RNA.
10. A complementary DNA (cDNA) clone of the U3-like RNA was obtained after reverse transcription and PCR amplification of RNA extracted from the purified processing activity. Two complementary oligonucleotides (JC1, 5′-CGACGGATAGAAGAATTCTGTTCGTTGGAG; and its complement, JC2) were used for first-strand synthesis. Briefly, JC1 was blocked at the 3′ end with terminal transferase and dideoxy adenosine triphosphate and phosphorylated at the 5′ end with polynucleotide kinase and adenosine triphosphate (ATP). The modified oligonucleotide was then ligated to the RNA extracted from the processing activity with RNA ligase. The complementary oligonucleotide JC2 was used to prime first-strand synthesis with avian myeloblastosis virus reverse transcriptase, and the product was tailed with terminal transferase and ATP. The tailed product was amplified by PCR with Race 37 (CGAGCTGCGTCGACAGGCT$_{17}$) and JC2 as primers. The product was cloned directly into the pCR2 vector to yield plasmid pPD1156. The cDNA insert was used to identify the chromosomal gene on a 1.2-kb Eco RV–Hind III fragment. The genomic fragment was cloned into plasmid pSP73 (Promega) to give plasmid pPD1158. The nucleotide sequence of both the cDNA and the chromosomal gene were determined (*23*).
11. J. M. X. Hughes and M. Ares, *EMBO J.* **10**, 4231 (1991); J. M. X. Hughes, D. A. M. Konings, G. Cesarini, *ibid.* **6**, 2145 (1987).
12. M. Beltrame and D. Tollervey, *ibid.* **11**, 1531 (1992).
13. S. Kass, K. Tyc, J. A. Steitz, B. Sollner-Webb, *Cell* **60**, 897 (1990).
14. R. Savino and S. A. Gerbi, *EMBO J.* **9**, 2299 (1990).
15. S. J. Baserga, W. Yang, J. A. Steitz, *ibid.* **10**, 2645 (1991).
16. K. A. Parker and J. A. Steitz, *Mol. Cell. Biol.* **7**, 2899 (1987).
17. C. Jeppesen, B. Stebbins-Boaz, S. A. Gerbi, *Nucleic Acids Res.* **16**, 2127 (1988).
18. D. Tollervey, H. Lehtonen, M. Carmo-Fonseca, E. C. Hurt, *EMBO J.* **10**, 573 (1991).
19. Approximately 150 pmol of the specific oligonucleotides oSP7 (5′-GTAGTTCCGCATTCAGG) or oSP2 (5′-CGACACAGTTAGACAAGCTCC), complementary to the box B and box A regions of *S. acidocaldarius* U3-like RNA (positions 85 to 69 and 32 to 12, respectively), or nonspecific oligonucleotide oSP5 (5′-ATAAGCTTAATAATGCCGCCGTAGCT) was mixed with 5 μl of processing activity (obtained after precipitation with 35% ammonium sulfate; purified ~10-fold), heated to 85°C, and cooled slowly to 37°C. Buffer [10 μl of 60 mM tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1.5 mM dithiothreitol, bovine serum albumin (45 μg/ml), 4% (v/v) glycerol] was added together with 2 units of *E. coli* RNase H (Pharmacia). Incubation was continued for 10 min at 37°C and 20 min at 30°C (*13*). The treated processing activity was then heated to 75°C for 15 min, substrate was added, and incubation was continued. Portions of the mixture were removed at specified time intervals and analyzed by the primer extension assay described in Fig. 1. For the control reactions, the extract alone or the extract plus specific or nonspecific oligonucleotides was treated in a parallel manner except that no RNase H was added. In other quantitative experiments, we have estimated by densitometric analysis that the rate of consumption of substrate after RNase H digestion in the presence of the oSP7 box B oligonucleotide (Fig. 6) is ~5% of that in the control reactions.
20. Processing activity was incubated with specific oligonucleotides oSP7 or oSP2, or nonspecific oligonucleotide oSP5, and digested with RNase H as described (*19*). The RNA obtained after phenol extraction and ethanol precipitation was subjected to electrophoresis on a denaturing 6% polyacrylamide gel, electroblotted onto a Hybond N+ membrane (Amersham), and fixed by brief exposure to ultraviolet light. The membrane was probed under standard conditions (*13*) at 42°C for 16 hours with the ^{32}P-labeled product generated from the U3-like cDNA fragment from plasmid pPD1156 (*10*) with random oligonucleotide primers.
21. S. Potter and P. P. Dennis, unpublished results.
22. K. A. Amiri, *J. Bacteriol.* **176**, 2124 (1994).
23. The GenBank accession numbers for the U3-like sequence and the 5′ ETS sequences are U13990 and U05018, respectively.
24. S. Potter and P. P. Dennis, in preparation.
25. M. Zucker and M. Steigler, *Nucleic Acids Res.* **9**, 133 (1981).
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Revival and Identification of Bacterial Spores in 25- to 40-Million-Year-Old Dominican Amber

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A bacterial spore was revived, cultured, and identified from the abdominal contents of extinct bees preserved for 25 to 40 million years in buried Dominican amber. Rigorous surface decontamination of the amber and aseptic procedures were used during the recovery of the bacterium. Several lines of evidence indicated that the isolated bacterium was of ancient origin and not an extant contaminant. The characteristic enzymatic, biochemical, and 16S ribosomal DNA profiles indicated that the ancient bacterium is most closely related to extant *Bacillus sphaericus*.

Microorganisms have been isolated from various types of ancient materials, including salt crystals, deep earth cores, and fossilized animals and plants (*1*, *2*). All such claims of ancient origin have faced the criticism of being a result of modern environmental contamination based on the sense that viable, ancient isolates are unlikely. This skepticism stems primarily from the extrapolation of survival curves of modern bacteria that suggest that accumulated damage and macromolecular decay would preclude viability (*3*).

Morphological and biochemical data about ancient bacteria are scarce, precluding detailed studies of bacterial metabolism, origins, and evolution. Sequence data derived from the 16S ribosomal RNA (rRNA) have been used to construct a phylogenetic tree for modern prokaryotes (*4*). Such data from both ancient bacterial DNA and *Bacillus* spp. from amber samples of known age can be used to analyze bacterial phylogeny and the rate of nucleotide substitution for various genes in this taxon.

Bacillus is an ancient and ubiquitous bacterial genus characteristically capable of

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