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RNA polyadenylation in Archaea: not observed in *Haloferax* while the exosome polynucleotidylates RNA in *Sulfolobus*

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The addition of poly(A) tails to RNA is a phenomenon common to all organisms examined so far. No homologues of the known polyadenylating enzymes are found in Archaea and little is known concerning the mechanisms of messenger RNA degradation in these organisms. Hyperthermophiles of the genus *Sulfolobus* contain a protein complex with high similarity to the exosome, which is known to degrade RNA in eukaryotes. Halophilic Archaea, however, do not encode homologues of these eukaryotic exosome components. In this work, we analysed RNA polyadenylation and degradation in the archaea *Sulfolobus solfataricus* and *Haloferax volcanii*. No RNA polyadenylation was detected in the halophilic archaeon *H. volcanii*. However, RNA polynucleotidylation occurred in hyperthermophiles of the genus *Sulfolobus* and was mediated by the archaea exosome complex. Together, our results identify the first organism without RNA polyadenylation and show a polyadenylation activity of the archaea exosome.

Keywords: RNA polyadenylation; exosome; Archaea
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INTRODUCTION

RNA polyadenylation is a general phenomenon common to all organisms examined. Eukaryotic messenger RNA is polyadenylated post-transcriptionally by the poly(A) polymerase, a process important for its function and longevity. In bacteria and most organelles (excluding mammalian and trypanosome mitochondria), RNA molecules are not stably polyadenylated at the mature 3′ end (Dreyfus & Regnier, 2002; Bollenbach *et al*, 2004; Gagliardi *et al*, 2004). Instead, polyadenylation is part of the RNA degradation process. Recently, the involvement of RNA polyadenylation in the degradation of yeast nuclear-encoded RNAs and human mitochondrial RNAs was discovered (Lacava *et al*, 2005; Slomovic *et al*, 2005; Vanacova *et al*, 2005).

The main polyadenylation enzyme in *Escherichia coli* is poly(A) polymerase I (Mohanty & Kushner, 2000). However, in spinach chloroplasts and in certain bacteria, the post-transcriptional addition of heteropolymeric poly(A)-rich tails is carried out by polynucleotide phosphorylase (PNPase; Mohanty & Kushner, 2000; Bollenbach *et al*, 2004). Following the addition of poly(A) or poly(A)-rich tails, the polyadenylated RNA molecules are degraded rapidly by the hydrolytic exoribonucleases of the RNase II/R family and by the phosphate-dependent exoribonuclease, PNPase.

PNPase is a highly conserved enzyme and *pnp* genes are found in all genomes examined, excluding those of Archaea, yeast, Mycoplasma and apparently those of trypanosomes as well (Zuo & Deutscher, 2001; Anantharaman *et al*, 2002; Yehudai-Resheff *et al*, 2003). It is a trimeric enzyme, in which each subunit comprises two RNase PH domains, an S1 domain and a K homology (KH) domain (Symmons *et al*, 2002). The exosome is another RNA-degrading complex that is present in all eukaryotes, and is composed of several exoribonucleases and RNA-binding proteins (Allmang *et al*, 1999; van Hoof & Parker, 1999). It resembles the PNPase with regard to the number of RNase PH, S1 and KH domains, suggesting similar structural properties (Symmons *et al*, 2002; Raijmakers *et al*, 2004). Many archaeal genomes encode homologues of the core subunits of the eukaryotic exosome; this led to the suggestion of an exosome-like complex for RNA degradation in Archaea (Koonin *et al*, 2001). The existence of an exosome-like complex was experimentally shown in *Sulfolobus solfataricus* (Evguenieva-Hackenberg *et al*, 2003; Lorentzen *et al*, 2005). It contains four previously predicted exosomal subunits, which are the orthologues of the yeast proteins Rrp4, Rrp41, Rrp42 and Csl4.

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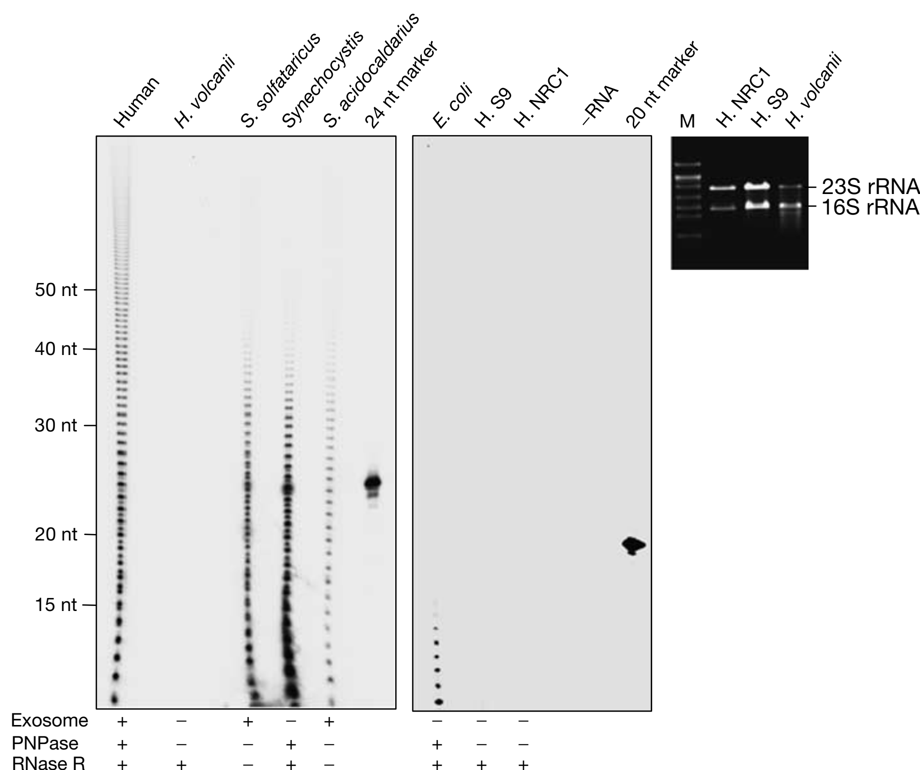

Fig 1 | Detection of poly(A) tails in Archaea. RNA was purified from human, *Synechocystis*, *Escherichia coli* and Archaea cells. The RNA was labelled with [^{32}P]pCp at the 3′ end and digested to completion with RNase T1 and RNase A. The remaining poly(A) tails were fractionated on denaturing polyacrylamide gel electrophoresis alongside a ^{32}P-labelled 24- or 20-nt oligonucleotide size marker and detected by autoradiography. The presence of the archaeal exosome, polynucleotide phosphorylase (PNPase) and/or RNase R homologue in these organisms is indicated at the bottom. *H. volcanii*, *Haloferax volcanii*; *S. solfataricus*, *Sulfolobus solfataricus*; *S. acidocaldarius*, *Sulfolobus acidocaldarius*; H. S9 and H. NRC1, *Haloferax salinarum* S9 and NRC1, respectively; −RNA, a reaction carried out with no addition of RNA. An ethidium bromide staining of an agarose gel, in which the halophile RNAs were fractionated, is shown on the right. M-RNA ladder, high range (Fermentas Inc.).

Halophilic Archaea, however, lack the corresponding genes for these exosome proteins and other known poly(A) polymerases (Koonin *et al*, 2001; Zuo & Deutscher, 2001). This suggests that RNA polyadenylation does not take place in halophilic archaea, or if it does, that an as yet unknown enzyme catalyses this process. As *Haloferax volcanii* is available for molecular, genetic and biochemical studies, it was chosen as a representative halophilic archaeon in our studies.

We show here that *H. volcanii* is the first organism described in which no RNA polyadenylation takes place. In contrast, RNA polyadenylation is found in *S. solfataricus* and is catalysed by the exosome.

RESULTS

No RNA polyadenylation in halophilic Archaea

To test for RNA polyadenylation in Archaea, purified RNA from three halophilic and two hyperthermophilic Archaea was labelled with ^{32}P at the 3′ end. It was then digested with the endoribonucleases RNase A and RNase T1, which cleave following the nucleotides G, U and C. Therefore, only poly(A) tails located at the 3′ end are detectable in this assay. Poly(A) tails were detected in RNA prepared from *S. solfataricus* and *Sulfolobus acidocaldarius* as well as in RNA from human cells, cyanobacteria or *E. coli* (Fig 1). However, no poly(A) tails were observed in *H. volcanii* and *H. salinarum* S9 and NRC1. The absence of poly(A) tails in *H. volcanii* was also confirmed by the inability to detect any poly(A) tails by the oligo(dT)-primed reverse transcription PCR (RT–PCR) technique (Fig 2). Furthermore, a protein extract from *H. volcanii* cells, which was active in RNA degradation, was completely devoid of any RNA polyadenylation activity (not shown). However, the possibility of post-transcriptional addition of 1–4 adenosines or other nucleotides in *H. volcanii* was not excluded by these methods. Therefore, the nucleotide sequences at the 3′ end of transcripts derived from two genes were determined using the circled RT–PCR method. Circularized *H. volcanii* RNAs were submitted to RT–PCR with the use of specific primers that enabled one to examine the joining site of the 16S ribosomal RNA molecules and the *rnr* transcripts. The sequence of 85 related clones showed no clone with the post-transcriptional addition of nucleotides that are not encoded by the genomic DNA. As a control, applying this technique to an *S. solfataricus* transcript showed the addition of such nucleotides in 6 out of 19 clones (Fig 2B; supplementary Fig 1 online). We concluded that RNA is polyadenylated in *Sulfolobus* but not in *H. volcanii*. Thus, *H. volcanii* is the first organism to be described in which no RNA polyadenylation takes place.

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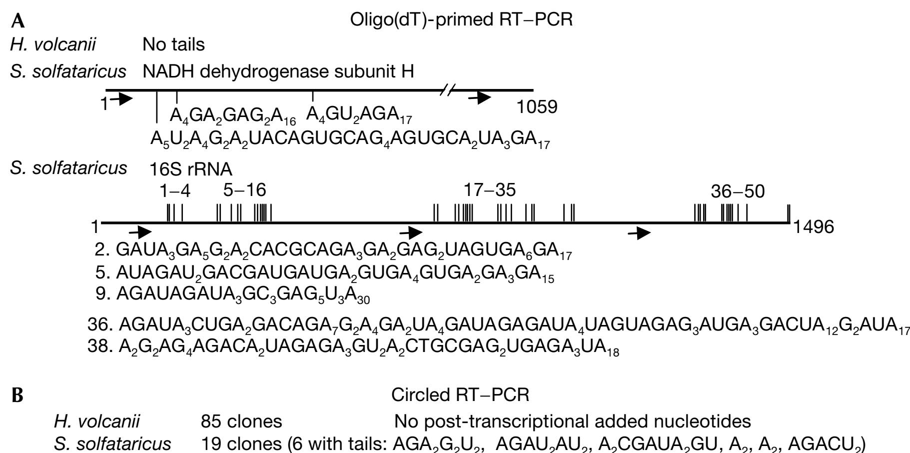

Fig 2 | Analysis of the added tails by oligo(dT)-primed or circled reverse transcription–PCR (RT–PCR). RNA prepared from *Haloferax volcanii* or *Sulfolobus solfataricus* was analysed for polyadenylated tails using the oligo(dT)-primed RT–PCR (**A**) and circled RT–PCR (**B**) methods. The gene-specific oligonucleotides used for the PCR amplification are indicated by arrows. The relative locations of the tails are indicated by vertical lines, and several sequences are presented. The sequences of the PCR primers and of the entire poly(A)-rich tails are shown in supplementary Table S1 online.

Heterogeneous poly(A)-rich tails in *S. solfataricus*

Tails produced by PNPase were shown to be heterogeneous, consisting of the other three nucleotides in addition to adenosines. In contrast, poly(A) polymerase synthesizes homopolymeric poly(A) tails (Mohanty & Kushner, 2000; Rott *et al*, 2003). As *S. solfataricus* expresses neither PNPase nor an apparent poly(A) polymerase, we examined whether its RNAs contain poly(A) tails that are heterogeneous or homogenous in sequence. Oligo(dT)-primed RT–PCR analysis of the tails showed heterogeneous sequences containing all four nucleotides, similar to those generated by PNPase in bacteria and organelles (Fig 2; supplementary Table S1 and Fig S1 online). The detection of poly(A) tracks of 30, 23, 21 and 20 nt, which are longer than the oligo(dT)$_{17}$ used to prime the RT reaction, verified that poly(A) tracks of these lengths, as shown in Fig 1, were indeed present in *S. solfataricus* (Fig 2; supplementary Table 1 online).

The exosome is the polynucleotidylation enzyme

The detection of poly(A)-rich tails in *S. solfataricus* and *S. acidocaldarius* raised the question as to which enzyme catalyses polynucleotidylation in these Archaea. The poly(A) polymerases of eukaryotes and bacteria belong to different classes of the nucleotidyltransferase superfamily, together with the CCA-adding enzymes (Yue *et al*, 1996). *H. salinarum,* NRC1, *S. solfataricus* and most other Archaea with sequenced genomes contain only a single gene for a CCA-adding enzyme (Anantharaman *et al*, 2002). For *Archaeoglobus fulgidus* and *Sulfolobus shibatae,* a close relative of *S. solfataricus*, the specific CCA-adding function of these enzymes was observed (Yue *et al*, 1996; Xiong & Steitz, 2004), which suggests that they are not poly(A) polymerases. The other polyadenylating enzyme in bacteria and organelles is PNPase. As described above, although lacking PNPase, an exosome-like complex, which structurally and functionally resembles PNPase, is present in *S. solfataricus*, and its subunits are encoded in other archaeal genomes (Evguenieva-Hackenberg *et al*, 2003; Lorentzen *et al*, 2005). The similarity of the archaeal exosome and PNPase, together with the detection of heterogeneous tails, suggested the archaeal exosome as a good candidate for a polynucleotidylation enzyme in *Sulfolobus*.

To test our hypothesis, we studied RNA polyadenylation by the *S. solfataricus* exosome. We purified recombinant *S. solfataricus* Rrp41, Rrp42 and Csl4 proteins from *E. coli* and reconstituted a 240 kDa complex *in vitro*. The exosome complex showed both polyadenylation and RNA degradation activities (Fig 3), and similarly to PNPase, the mode of action was dependent on the presence of ADP and phosphate (Yehudai-Resheff *et al*, 2003). As expected from proteins of a thermophilic organism living at 80 °C, the activities were higher at 60 and 80 °C than at 37 °C. Indeed, when assayed at 80 °C, a polyadenylation signal lower than that at 60 °C was obtained because of the combined action of polyadenylation and degradation activities. None of the subunits alone showed polyadenylation or degradation activity, excluding the possibility that the observed reactions are due to traces of contaminating *E. coli* proteins (Fig 3B).

Our results show that a complex of three different exosomal subunits from *S. solfataricus* can both polyadenylate and degrade RNA. A cell-free extract of *S. solfataricus* also showed strong polyadenylation and degradation activities that were dependent on the presence of ADP and phosphate, respectively (Fig 4). To show that this activity is exosome specific, two rounds of co-immunoprecipitation with Rrp41-specific antibodies were carried out to reduce the amount of exosome complex in the extract to about 6%, as determined by immunoblot analysis (Fig 4B). The exosome depletion resulted in a significant decrease of polyadenylation and RNA degradation activity of the cell-free extract (Fig 4). Quantification using phosphorimager of the amount of polyadenylated RNA, assayed at 60 °C for 15 min, disclosed a reduction of about 70%. In addition, the immunoprecipitated archaeal exosome showed ADP- and phosphate-dependent RNA polyadenylation and degradation activities, respectively (Fig 4). This experiment confirms that the exosome-like complex has an important role in polynucleotidylation and degradation of RNA in *S. solfataricus.*

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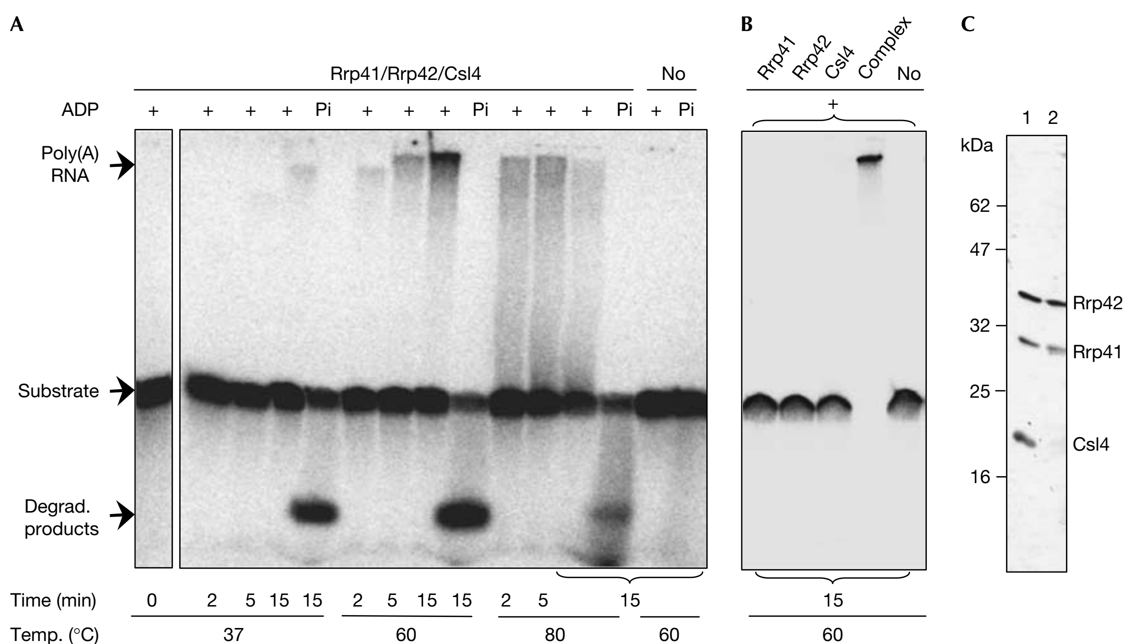

Fig 3 | Reconstituted *Sulfolobus solfataricus* exosome can polyadenylate and degrade RNA. (**A**) Reconstituted Rrp41/Rrp42/Csl4 complex was incubated with ^{32}P-labelled poly(A) of 30 nt with the addition of 20 mM ADP, as indicated. In the lanes labelled Pi, the ADP was replaced by 10 mM K_2HPO_4. The incubation temperatures are indicated at the bottom. At the time points indicated, RNA was isolated and analysed by denaturing polyacrylamide gel electrophoresis and autoradiography. No, RNA with no protein added. (**B**) Polyadenylation activity was observed only with the reconstituted complex (complex) but not when each protein alone was assayed. The polyadenylation products are >500 nt long and the degradation products are about 10 nt long, as determined from a sequencing gel analysis. (**C**) Silver-stained protein gel showing the Rrp41/Rrp42/Csl4 and the Rrp41/Rrp42 complexes (about 50 ng of each protein; lanes 1 and 2, respectively).

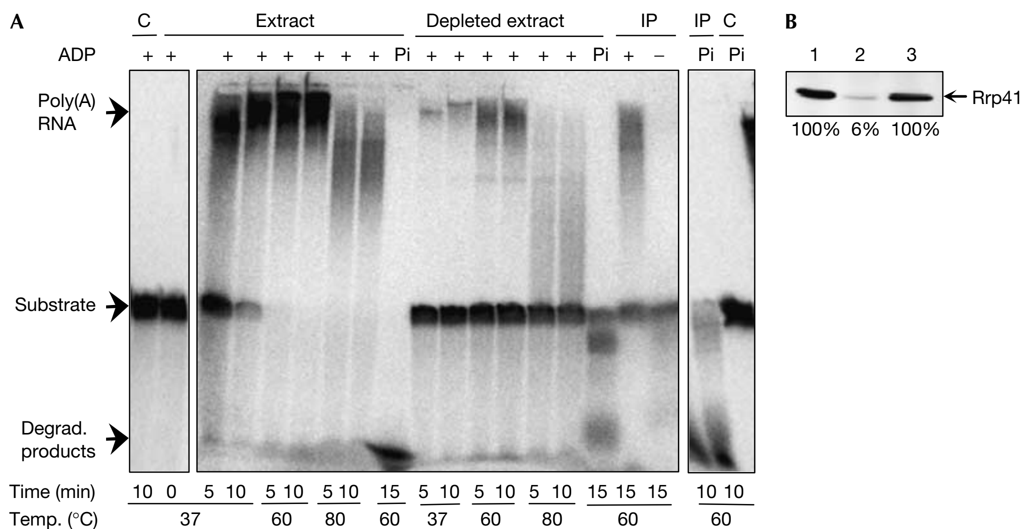

Fig 4 | Depletion of the exosome from *Sulfolobus solfataricus* cell-free extract resulted in a concomitant reduction of RNA polyadenylation and degradation activities. (**A**) *S. solfataricus* cell-free extract, an extract depleted by immunoprecipitation of the archaeal exosome and the immunoprecipitated archaeal exosome (IP) were analysed for RNA polyadenylation and degradation activities. Phosphoimages of polyacrylamide gel electrophoresis gels show resolved RNA substrate (5′-end-labelled 30-nt poly(A)), polyadenylated and degradation products. The presence of 20 mM ADP or 10 mM K_2HPO_4 (Pi) is indicated above the panels. No ADP or Pi was added to the reaction mixture in the lane labelled (−). The incubation time and incubation temperature are indicated at the bottom. C, control without proteins. (**B**) Monitoring the amount of the exosome. Immunoblot decorated with Rrp41 antibodies of the cell-free extract (lane 1), the depleted extract (lane 2) and the depleted extract using the pre-immune serum as a control (lane 3).

DISCUSSION

So far, every organism tested has shown post-transcriptional polyadenylation of RNA (Dreyfus & Regnier, 2002; Edmonds, 2002). Owing to the lack of homologues to known polyadenylating enzymes, we proposed that the halophilic Archaea may function without polyadenylation. These organisms contain no PNPase or exosome, no bacterial-type poly(A) polymerase and only a single nucleotidyltransferase gene encoding a CCA-adding enzyme. Indeed, no RNA polyadenylation could be detected in *H. volcanii* by 3′-end labelling and ribonuclease digestion, by oligo(dT)-primed RT–PCR, by analysing polyadenylation activity in a cell-free protein extract or by the circled-RNA RT–PCR technique. This is the first organism described that metabolizes RNA without any polyadenylation.

In the absence of an exosome or PNPase, the exoribonuclease RNase R homologue is the obvious candidate for the exonucleolytic RNA degradation activity. Indeed, we found that this enzyme is expressed and is required for viability in *H. volcanii* and therefore has an important role in the polyadenylation-independent degradation pathway (data not shown). Our results show unexpectedly that poly(A)-dependent RNA degradation is used by thermophiles but not by halophiles. RNA structures at high temperature are less stable; however RNA structures are expected to be stabilized at high-salt concentration. The explanation for this phenomenon could be partially related to the differences in the activities of the RNase R homologue and the archaeal exosome in degrading structural RNA molecules. In addition, nothing is known so far about RNA conformations under *in vivo* conditions in extremophiles. It is conceivable that in these organisms, other factors, which are yet to be identified, are involved in the stabilization or destabilization of RNA structures.

Our work showed very different results with regard to polyadenylation in two representative members of the Archaea. No RNA polyadenylation was detected in the halophilic Archaea, which do not contain an exosome complex, whereas heterogeneous tails of mainly fragmented RNA molecules were observed in hyperthermophiles that belong to the genus *Sulfolobus*, which contains the exosome-like complex. Indeed, we showed the RNA polynucleotidylation activity of this complex and that depletion of the exosome from the cell-free extract resulted in a significant decrease in the polynucleotidylation activity.

The initial step in the RNA degradation process in many bacteria and organelles is thought to be endonucleolytic cleavage (Kushner, 2002; Even *et al*, 2005). Following this cleavage, the proximal cleavage product is either degraded by exoribonucleases or subjected to polyadenylation/polynucleotidylation by poly(A) polymerase or PNPase and then degraded (Coburn & Mackie, 1999; Dreyfus & Regnier, 2002; Bollenbach *et al*, 2004). Our results indicate that in Archaea, the basic principles of this mechanism were preserved during the course of evolution, despite the fact that the existence of the exosome resembles the eukaryotic RNA degradation system. Indeed, many Archaea contain genes related to RNase E and RNase J1/J2 (Anantharaman *et al*, 2002; Even *et al*, 2005), which are believed to generally mediate endonucleolytic cleavage, initiating the degradation process. Further steps in RNA degradation may be poly(A) dependent and carried out by the exosome-like complex in *Sulfolobus*, or poly(A) independent and carried out by RNase R in halophilic Archaea.

Recently, an RNA surveillance mechanism in the yeast nucleus involving the eukaryotic exosome and RNA polyadenylation was described (Lacava *et al*, 2005; Vanacova *et al*, 2005). In addition, RNA polyadenylation was recently described to be involved in the degradation of human mitochondrial RNA (Slomovic *et al*, 2005). This disclosed that polyadenylation as a signal for RNA degradation was conserved through evolution in bacteria, archaea and in the organelles and nucleus of eukaryotes. However, although we show here that the archaeal exosome, similar to the bacteria and organelle PNPase, is carrying out the polynucleotidylation reaction, in the yeast nucleus this reaction is not carried out by the exosome. Instead, a poly(A) polymerase (Trf4p) that is associated with the exosome polyadenylates the RNA, which is then targeted for degradation by the exosome. Further analysis is required to determine whether the eukaryotic exosome, like the archaeal one, has polyadenylation activity or not. The structural resemblance of the exosome and the PNPase complex, and the fact that the archaeal exosome shows polynucleotidylation activity similar to that of PNPase, strongly suggests that PNPase and the exosome evolved from an RNA-degrading/polynucleotidylation complex that was already present in the last universal common ancestor of the three domains of life. Why halophilic Archaea lost polyadenylation later in evolution remains elusive.

METHODS

Organism. *H. volcanii* and *H. salinarum* S9 and NRC1 cells were grown at 42 °C in a medium containing 3.4 M NaCl (Dyall-Smith, 2004). *S. acidocaldarius* and *S. solfataricus* (strain P2) were grown as described (Evguenieva-Hackenberg *et al*, 2003). RNA was isolated using the ‘hot phenol’ method (Rott *et al*, 2003). A soluble protein extract of *S. solfataricus* was prepared by sonicating freshly grown logarithmic cells (1.7 g) resuspended in 6 ml of TMN buffer (Allmang *et al*, 1999). Cell debris was removed by centrifugation at 10,000*g* and the extract stored in aliquots at −80 °C.

Determination of poly(A) tails. First, 20 μg of Archaea, *E. coli* and *Synechocystis* (Rott *et al*, 2003) and 3 μg of human RNA (CCRF-CEM cancer cell line) were 3′-end-labelled with [^{32}P]pCp and T4 RNA ligase for 24 h at 4 °C. Then, the RNA was digested with 25 μg of RNase A and 300 U of RNase T1 for 1 h at 37 °C (Lisitsky *et al*, 1996). Poly(A) tails were resolved in polyacrylamide sequencing gels containing 7 M urea and detected by autoradiography. Analyses of the poly(A) tails by oligo(dT)-primed RT–PCR and circled RT–PCR were carried out as described (Lisitsky *et al*, 1996; Perrin *et al*, 2004).

***In vitro* RNA degradation activity assay.** Degradation assays using the *S. solfataricus* cell-free extract (100 ng protein) or reconstituted exosome (10 ng of each) were carried out with a 5′-end-labelled 30-meric poly(A) substrate. The assay buffer contained 20 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MnCl_2$, 10 mM K_2HPO_4, 0.1 mM EDTA, 2 mM dithiothreitol, 12% glycerol, 375 mM trehalose and 2 U RNasin. For polyadenylation assays, the K_2HPO_4 was replaced by 20 mM ADP. The reaction products were resolved in a 10% denaturing polyacrylamide gel and were analysed by autoradiography.

Reconstitution of the *Sulfolobus solfataricus* exosome. The genes encoding the *S. solfataricus* proteins Csl4, Rrp41 and Rrp42 (Evguenieva-Hackenberg *et al*, 2003) were cloned between the *Xho*I and *Nde*I restriction sites of the pET-116 expression vector. Proteins were produced in *E. coli* BL21(DE3) and purified by

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Ni-NTA affinity chromatography. The His_6 tag was cleaved off followed by further purification using size-exclusion chromatography on a Superdex 200 column. An exosomal subcomplex consisting of Rrp41 and Rrp42 was reconstituted by mixing Rrp41 with an excess of Rrp42 (1.5 molar ratio) in a buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 10% glycerol and 1 mM dithiothreitol for 30 min at 25 °C. Stable Rrp41–Rrp42 protein complexes were purified on a Superdex 200 size-exclusion chromatography column. A larger exosomal complex was reconstituted by mixing the purified Rrp41–Rrp42 complex at a 1:2 molar ratio with Csl4 (assuming the Rrp41–Rrp42 complex to form a hetero-hexamer) using the same protocol, and was purified on a Superdex 200 column.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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