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RESEARCH PAPER

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Distinct activities of several RNase J proteins in methanogenic archaea

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RNA degradation plays an important role in the control of gene expression in all domains of life, including Archaea. While analyzing RNA degradation in different archaea, we faced an interesting situation. The members of a group of methanogenic archaea, including *Methanocaldococcus jannaschii*, contain neither the archaeal exosome nor RNase II/R homologs. However, looking for potential ribonucleases revealed proteins related to the recently discovered ribonuclease RNase J. RNase J is unique among known ribonucleases because it may combine endo- and 5'→3' exo-ribonucleolytic activities in a single polypeptide. Here, we report the characterization of the ribonuclease activities of three RNase J homologs encoded in the genome of the methanogenic archaeon *Methanocaldococcus jannaschii*. The analysis of the recombinant archaeal proteins purified from *E. coli* revealed an optimal activity at 60°C. Whereas mjRNase J1 and -J3 displayed exclusively 5'→3' exonucleolytic activity, mjRNase J2 is an endonuclease with no apparent exonuclease activity. The exonucleolytic activity of both mjRNase J1 and -J3 is enhanced in molecules harboring monophosphate at the 5' end. mjRNase J3, and to some extent mjRNase J2, degrade ssDNA. Together, these results reveal that in archaea lacking the exosome and RNase II/R, RNA and perhaps also DNA are possibly degraded by the coordinated activities of several RNase J proteins. Unlike bacteria, in archaea RNase J proteins provide separately the exo- and endonucleolytic activities that are probably essential for RNA degradation.

Introduction

Similar to eukaryotes, prokaryotes and organelles, RNA processing and degradation in archaea plays an important role in the control of gene expression and is essential for life.[1] RNA-degrading enzymes are conserved in archaea, bacteria and organelles, as well as in the cytoplasm and nucleus of eukaryotes. For example, 3'→5' exonucleolytic digestion is carried out by the hydrolytic enzymes of the RNase II/R family that are present in almost all organisms.[2-4] Another conserved enzyme responsible for this activity is polynucleotide phosphorylase (PNPase), present in most bacteria and organelles.[2,5] Furthermore, the archaeal exosome, found in several groups of archaea, is structurally and functionally similar to PNPase, though it is composed of several proteins, each of which is related to the corresponding domain of the PNPase.[6-8] Another conserved ribonuclease is RNase J. This enzyme was discovered in the bacterium *Bacillus subtilis* in a search for an endonuclease with similar functions to *E. coli* RNase E.[9] RNase J from *B. subtilis* has both 5'→3' exonucleolytic and endonucleolytic activities.[10-13] Homologs of the *B. subtilis* protein are present in the genomes of most bacteria, archaea and plants, in which it is predicted to be localized to the chloroplast.[9,14,15]

RNase J belongs to the β-CASP subfamily of the zinc-dependent metallo-β-lactamase (MBL) superfamily. Enzymes of this group include important proteins acting on nucleic acid substrates and are widely distributed throughout the bacterial, archaeal and eukaryal domains.[14,16] Structurally, RNase J is active as a dimer in which each monomer contains three globular domains: β-lactamase core, β-CASP core and C-terminal domain (**Fig. 1**).[12] Although members of the MBL are found in all three domains of life and are located in almost every genome sequenced to date, their highest genomic representation is found in archaea.[9,17] Functionally, MBL are metalloenzymes, usually requiring one or two zinc ions for their activity; they use one water/hydroxyl molecule as a ligand and act on a wide variety of substrates.[14,18] Among the proteins of the MBL family that function as hydrolytic nucleases and have been implicated in the metabolism of nucleic acids are tRNase Z, RNase J and CPSF-73.[16] The importance of the β-CASP domain lies in the fact that it participates along with motifs II and III of the MBL superfamily in Zn^{+2} binding, which often results in nuclease activity (**Fig. 1**).

Structurally, MBL has a common four-layered αββα conformation and shares five conserved motifs: Asp (motif I), His-X-His-X-Asp-His (motif II), His (motif III), Asp (motif IV), and His (motif V) (**Fig. 1**).[14,17-19] The domain immediately C-terminal to the metallo-β-lactamase domain is the β-CASP domain. Its name is derived from the metallo-β lactamase-associated CPSF

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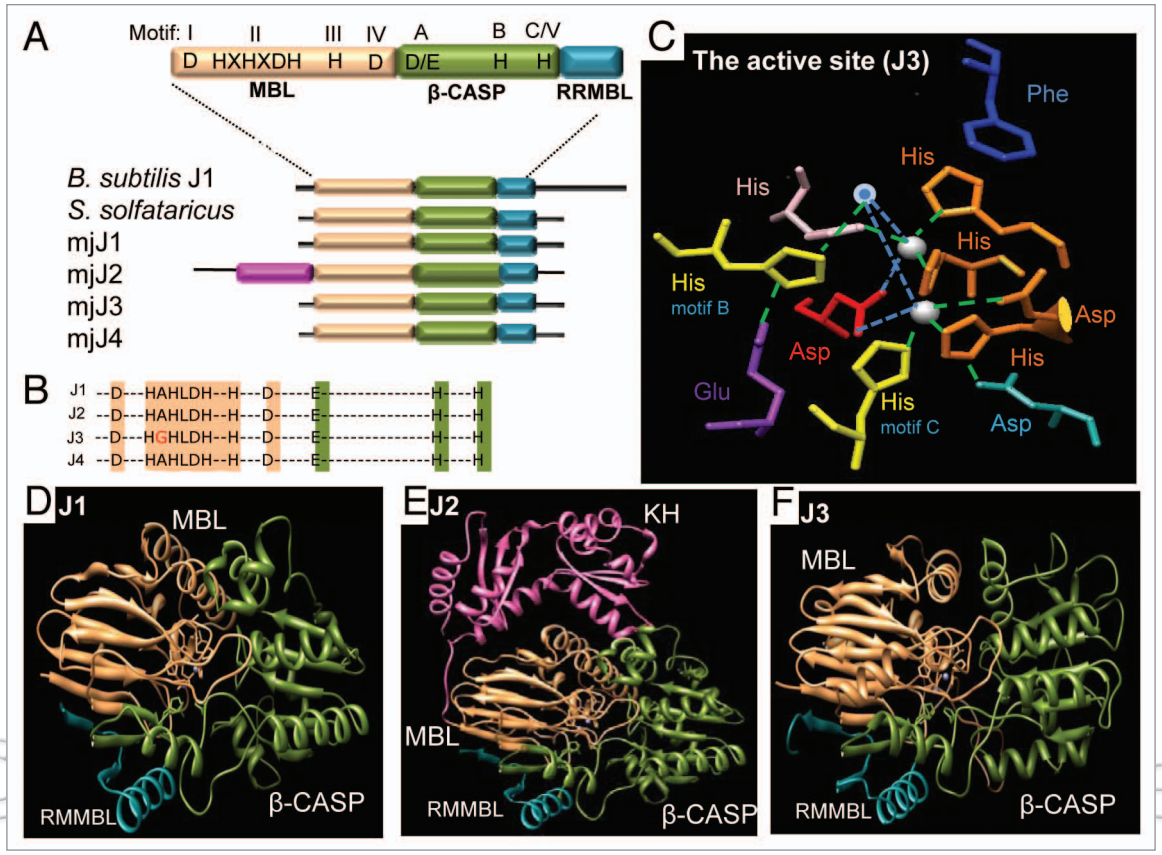


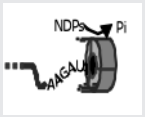
Figure 1. mjRNase J homologs motif alignment and structural modeling. (A) A schematic representation of the motifs found in RNase J of *B. subtilis*, *S. solfataricus* and the four RNase J homologues found in the *M. jannaschii* genome; MBL in colored sandy brown, β-CASP in green, RMMBL in blue and KH domain in pink. (B) Depiction of mjRNase J homologs multiple sequence alignment showing the amino acids of the MBL and β-CASP domains, using Clustal W and JalView. (C) Homology modeling of the active site of mjRNase J3. The models of the active site of the four *M. jannaschii* mjRNase J proteins were similar to the one presented. The amino acids of motif I-V of the MBL domain and the (A–C) motifs of the β-CASP domain are shown, as well as the two Zn ions (silver spheres). Hydrogen bonds are dissipated with dashed lines. (D–F) Models were created using *T. thermophilus* PDB code 3bk2 as a template for mjRNase J3 and *P. horikoshii* PDB code 3af5 as a template for mjRNase J1 and -J2. mjRNaseJ1, -J2 and -J3 models scored 33%, 59% and 38% identity to their template models. The different domains are colored using the same colors as in (A). Amino acids participating in functional active site are shown in sticks and colored in corresponding to the motifs they belong to. The Zn ions are marked as silver spheres.

Artemis SNM1/PSO2 proteins that act on nucleic acids. CPSF-73, the cleavage and polyadenylation specificity factor, is involved in pre-mRNA 3' processing.[20,21] The rest of the family is involved in DNA repair.[16] In-depth sequence analysis showed that members of the β-CASP family contain three specific motifs denoted motifs A, B and C, where motif A has an acidic residue (Asp or Glu), motif B has a His residue and motif C has a His residue in the case of RNA-acting proteins or a hydrophobic amino acid in DNA-acting proteins (**Fig. 1**).[18]

In our search for an organism in which RNA polyadenylation does not occur, the group of halophilic archaea and several members of the group of methanogenic archaea were found.[8] Further analysis of the mechanism of RNA polyadenylation and degradation in different archaea revealed a functional division into three groups (**Table 1**). The first group includes the halophiles belonging to the Euryarchaeota Halobacterium, those living at high salt concentrations. These archaea do not have the archaeal exosome and do not exhibit polyadenylation to assist in RNA degradation.[8] Exonucleolytic degradation in this organism is carried out by the 3'→5' hydrolase, RNase R.[22] In addition, the genomes of members of this group contain yet uncharacterized RNase J homologs. The second group is characterized by the presence of the archaeal exosome and includes the hyperthermophiles (Crenarchaeota Sulfolobus) and some of the methanogens (Euryarchaeota Methanosarcisa) (**Table 1**). In this group, polyadenylation (polymerizing heteropolymeric tails) and 3'→5' exonucleolytic degradation are carried out by this complex, similar to PNPase in bacteria and chloroplasts.[8] In addition, several RNase J homologs are present. The third group includes anoxygenic hyperthermophilic methanogenic archaea (Euryarchaeota Methanococcus) that lack the exosome or RNase II/R. Consequently, these are the only organisms that potentially do not harbor a 3'→5' exonucleolytic RNA degradation activity, and the question what enzyme degrades RNA in these organisms remained a mystery until the discovery of RNase J.[22]

Archaeal RNase J from three different hyperthermophilic strains has been characterized. Analyzing two RNase J enzymes from the Euryarchaeota *Pyrococcus abyssi* and

Table 1. The RNA degradation enzymes and poly(A)-rich tails in different archaea

		RNase J (# of homologs)	Archaeal exosome	RNase R/II	RNA tails
					
Halophiles		+(3)	-	+	-
Hyperthermophiles		+(5)	+	-	+
Methanogens	group A	+(2)	+	-	+
	group B	+(4)	-	-	-

According to the mechanism of RNA degradation and, in particular, the enzymes performing the exonucleolytic activity, the archaea domain can be functionally divided into three groups. The first one consists of the halophiles (referred as Euryarchaeota Halobacterium in the classical taxonomy of archaea), in which no polyadenylation occurs and the exonucleolytic degradation is performed by an RNase II/R enzyme and possibly also by RNase J. The second group consists of the hyperthermophiles (Crenarchaeota Sulfolobus) and some of the methanogens (Euryarchaeota Methanosarcina). These organisms harbor the archaeal exosome that degrades and polyadenylates RNA. In addition, like all other archaea, these organisms contain RNase J. The third group, consisting of the remaining methanogens (Euryarchaeota Methanococcus), contains only RNase J-type enzymes. The numbers in parentheses in the RNase J column display the number of RNase J homologs in each group of archaea; *H. volcanii* is used for halophiles, *S. solfataricus* for hyperthermophiles, *M. mazei* for the group A of methanogens and *M. jannaschii* for the group B of hyperthermophilic methanogens.

Thermococcus kodakaraensis and the Crenarchaeon *Sulfolobus solfataricus* revealed a 5'→3' exonucleolytic activity that depends on the phosphorylation state of the 5' end, with no endonucleolytic activity.[23,24] These proteins are also able to digest single-stranded DNA. In the case of *S. solfataricus,* binding of translation initiation factor a/eIF2(γ) to the 5'-end of mRNA counteracts the 5'→3' exoribonucleolytic activity of Sso-RNase J in vitro.[24,25] Members of this group also contain the exosome that exonucleolytically degrades RNA in the 3'→5' direction, as well as other RNase J homologs, of which one or more could provide the endonuclease activity required for RNA degradation (**Table 1**). Indeed, single nucleotide analysis using high throughput sequencing of the *S. solfataricus* transcriptome, revealed enormous amounts of endonucleolytic digestion products, indicating the present of an efficient endoribonuclease.[26] Two RNase J homologs, those of the archaea strains *Methanosarcina mazei* and *Pyrococcus horikoshii*, have been crystallized and their structures explored, revealing similarities to the bacterial enzyme and the eukaryotic factor CPSF-73.[17,19] Several of the archaeal RNase J proteins contain also two KH domains, known to function in RNA binding in many proteins.

In this study, we searched for RNase J homologs within Euryarchaeon *Methanocaldococcus jannaschii*, a representative methanogenic archaea lacking the exosome and RNase R. We asked whether the several RNase J homologs present in the genome of this organism are responsible for both the exo- and endonucleolytic activities needed for RNA degradation. We report the characterization of three out of four of its homologs, termed mjRNase J1, mjRNase J2 and mjRNase J3. mjRNase J1 and mjRNase J3 are 5'→3' exonucleases displaying high activity on 5' mono- than triphosphorylated transcripts and lacking any endonucleolytic activity. However, mjRNase J2, which is mostly homologous to human cleavage and polyadenylation factor CPSF-73, displays only endonucleolytic activity that is insensitive to the number of phosphates at the 5' end. Additionally, we found that mjRNase J2 and -J3 are able to degrade ssDNA. Together, these results imply that the endo- and exonucleolytic activities serving RNA degradation in methanogenic archaea are carried out by several RNase J enzymes. Unlike the bacteria *B. subtilis*, in which RNase J1 displays both endo- and exonucleolytic activities, in *M. jannaschii*, one enzyme is exclusively an endonuclease, and the other two display only exonucleolytic activity.

Results

Several RNase J homologs are present in the genome of a methanogen. To detect RNase J homologs in the methanogenic archaea, we searched the genome of *M. jannaschii* using the NCBI genome PSI Blast tool while using the RNase J1 from *B. subtilis* as a query. Four potential homologs of RNase J were found and named mjRNase J1, mjRNase J2, mjRNase J3 and mjJ4, (**Fig. 1**). Multiple sequence alignment of these RNase J homologs disclosed the conserved motifs of the metallo-β-lactamase and the β-CASP families.[16] The sequence alignment displays the conserved motifs I–IV, comprising the metallo-β-lactamase superfamily, and the A-C motifs, which constitute the β-CASP family (**Figs. 1 and S1 and S2**). Motif II (HXHXDH), which is well conserved among all MBL archaeal homologs, has an alanine residue in the second position, with the exception of mjRNase J3, which contains, similar to RNase J1 of *B. subtilis*, a glycine at this specific position (**Figs. 1B and S2**). mjRNase J2 contains the addition of two copies of a KH domain, which is characteristic of many RNA-binding proteins as well as some archaeal RNase J homologs.[17,27]

As described above, the RNase J homologs are the only conserved ribonucleases identified so far in methanogenic archaea not containing the exosome. Therefore, we decided to examine these proteins for exonucleolytic and/or endonucleolytic activities. Because we faced difficulties in the expression and purification of the mjJ4 protein, it was not further characterized. In

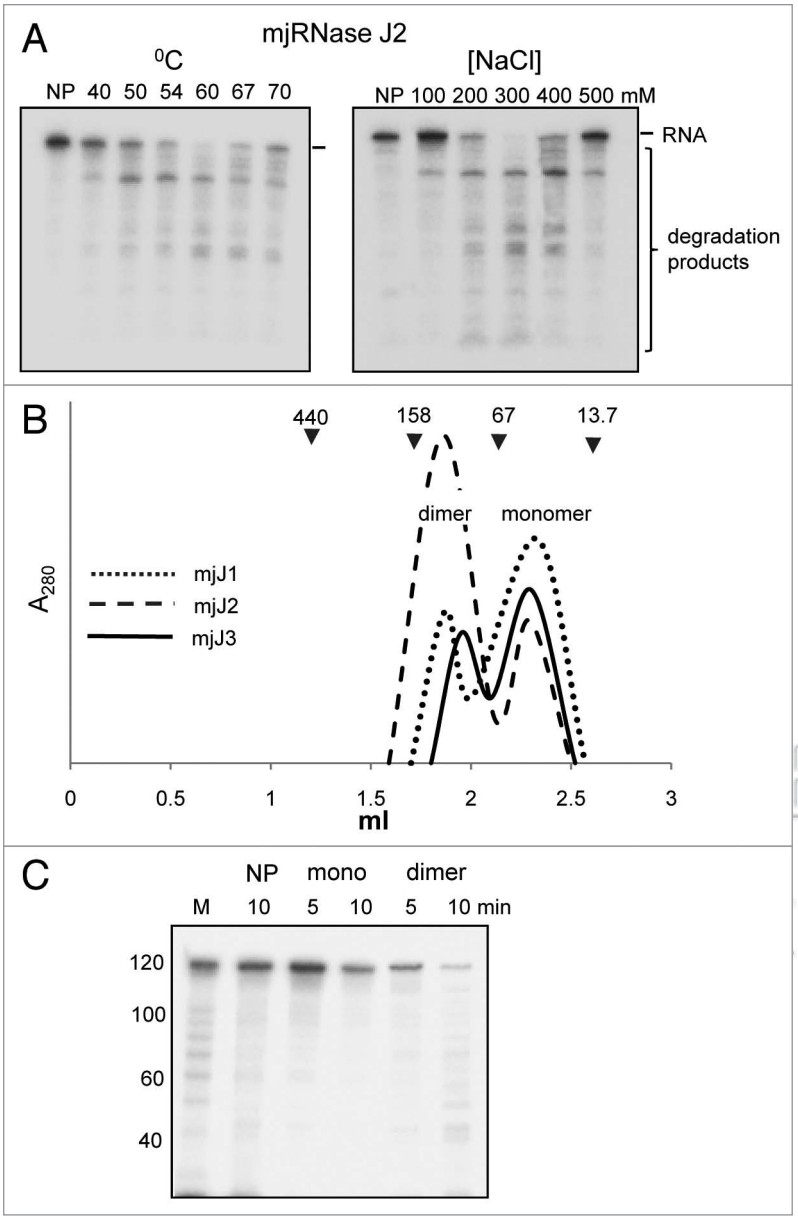


Figure 2. mjRNase J proteins display optimal activity at 60°C and 300 mM $NaCl_2$ and form homodimers at 60°C. (A) A uniformly labeled RNA corresponding to the 3' end of the spinach chloroplast *petD* transcript (2 fmol, 120 nt long) and containing monophosphate at the 5' end, was incubated for 1 h with mjRNase J2 at the indicated temperatures (left) and salt concentrations (right). Following incubation, the RNA was purified and analyzed by 10% urea-PAGE and autoradiography. NP: RNA incubated with no protein. (B) Purified proteins were incubated for 10 min at 60°C and then fractionated (at 4°C) with an analytical Superdex 200 column to detect monomers and oligomeric complexes. No dimers were formed without heating the proteins. The migration pattern of size markers and the elution peaks of the monomer and dimer forms are shown: mjRNase J1 in dotted line, J2 in dash line and J3 in solid line. (C) mjRNase J2 is active in its dimeric form. The dimeric form of the enzyme was purified using preparative Superdex 200 following 10 min incubation at 60°C for the dimeric form and without heating for the monomeric one. RNA degradation activity was then analyzed as described in (A) for short incubation time of up to 10 min since the formation of the dimeric form occurs during the incubation.

addition, the prediction of the structural similarity of this protein to the bacterial RNase J was relatively low (21% based on PDBsum). The genes of the other three homologs were cloned to an *E. coli* expression vector, produced in bacteria and purified (Materials and Methods and **Fig. S3**). These proteins were named mjRNase J1, mjRNase J2 and mjRNase J3. mjRNase J1 and -J3 migrated in SDS-PAGE according to their calculated molecular weights of 52.5 kDa and 50 kDa, respectively. mjRNase J2 migrated at about 85 kDa; while its calculated molecular weight is 72.5 kDa (**Fig. S3**). Because the temperature of the living environment of *M. jannaschii* can rise up to 120°C, the initial purification step was "cooking" the soluble proteins at 70°C. This step ensured the inactivation of any residual activity of contaminant ribonucleases from the host bacteria (**Fig. 2D**). Since *M. jannaschii* are found in very high-temperature and high-salinity environments, we first determined the optimal temperature and salt concentration for ribonuclease activity. Each of the three proteins was incubated with [^{32}P]-RNA at different temperatures and salt concentrations. As shown in **Figure 2** for mjRNase J2, ribonucleolytic activity was optimal at 60°C and 300 mM NaCl. This activity was significantly inhibited at temperatures below 40°C and salt concentrations below 100 mM (**Fig. 2A**). Similar results were obtained for mjRNase J1 and mjRNase J3 (not shown). In addition, similar to what have been reported for bacterial RNase J[10-12,23,28] and eukaryote CPSF-73,[21] where amounts of 0.5–6.0 μg enzyme were used for the RNA degradation assays, relatively high concentration of the archaeal enzymes was used in order to obtain reasonable observed activity.

Next, we analyzed the complex formation of these proteins using analytical size-exclusion column. The three proteins eluted mostly as monomers. However, upon heating to 60°C, the formation of the dimeric form was observed, as also reported for the bacteria and archaea proteins (**Fig. 2B**).[12,17] The significant amount of monomeric form as shown in **Figure 2B** could be obtained since following heating to 60°C, the separation on size exclusion column was performed at low temperature, causing the dissociation of the dimeric state. Since activity was very low at room temperature, the dimeric conformation at 60°C may represent the ribonuclease active conformation. In order to explore this hypothesis, the monomeric and dimeric forms were purified using a preparative size exclusion column and immediately tested for ribonuclease activity at 60°C. Since the dimeric state is rapidly formed at 60°C the assay was restricted to short time points. The results disclosed significant lower activity of the monomeric state as compared to the dimeric one (**Fig. 2C**). When longer incubation was applied, the difference was less significant

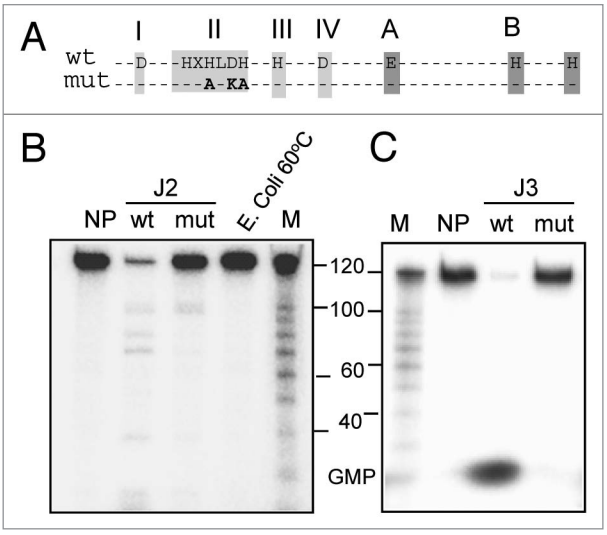


Figure 3. Mutating amino acids at motif II inhibits ribonucleolytic activity. (A) The motif II amino acid sequence of mjRNase J2 and J3 and the two histidines and aspartate that were mutated. (B) Wildtype and mutated mjRNase J2 were incubated with 5' [^{32}P] RNA for 1 h at 60°C. In addition, an *E. coli* protein extract heated to 60°C for 1 h was analyzed to observe the lack of any residual *E. coli* ribonuclease activity (lane labeled *E. coli* 60°C). Following incubation the RNA was analyzes by urea-PAGE and autoradiography. NP-incubation with no protein. M-RNA length markers. (C) Wild type and mutated mjRNase J3 were analyzed as described in (B) for mjRNase J2.

(not shown). Together, these results suggest that the archaeal enzyme is active in the dimeric stage which is formed upon heating to high temperature.

Point mutations of the conserved amino acids in motif II inhibited ribonuclease activity. In order to further characterize the enzyme we targeted the Zn ions required for the activity with chelators. EDTA treatment at 60°C was unable to extract the Zn ion and inhibit activity. However, treatment with imidazole inhibited the activity of mjRNase J1 and J3 (not shown). In order to further explore the active site, two of the conserved histidines and the aspartate of MBL motif II that bind the Zn ion (**Fig. 1**) were site directed modified. Ribonucleolytic activity of the mutated protein was severely inhibited indicating the importance of these amino acids in the formation of the active site, as was also described for other RNase J members and the human CPSF-73 (**Fig. 3**).[11,12,20,25] To further verify the lack of any activity of residual ribonucleases of the bacteria expression host, an *E. coli* protein extract was heated at 60°C as performed with the archaeal proteins. The heated extract was devoted of any ribonucleolytic activity, indicating that the observed activities were only performed by the archaeal enzymes (**Fig. 3B**).

mjRNase J1 and -J3 prefer a 5' monophosphorylated RNA for degradation. Structural insight into the dual activity of *Thermus thermophilus* RNase J revealed that the bacterial RNase J has a 5'-monophosphorylated nucleotide binding pocket and that its activity is enhanced when the substrate is a 5'-monophosphorylated RNA, as compared to a triphosphorylated one.[12] The archaeal enzymes exhibit a similar preference for monophosphate at the 5' end.[23,24] To examine the effect of 5' mono- or triphosphorylated RNAs on mjRNase J activity, the proteins were incubated at 60°C with 120 nt transcripts harboring either mono- or triphosphates at the 5' end (**Fig. 4**). Both mjRNase J1 and -J3 displayed significantly higher degradation activity on the 5' end of mono- compared to triphosphorylated transcripts. Since the 5' end of primary transcripts in archaea is characterized by triphosphates, the preference of these two enzymes for monophosphorylated RNA could provide clues regarding the protective role of the triphosphorylated 5' end of the primary transcript, as with many transcripts in bacteria.[4,9,24] Unlike the results of mjRNase J1 and -J3, mjRNase J2 showed no preference and degraded both 5' mono- and triphosphorylated substrates at similar rates

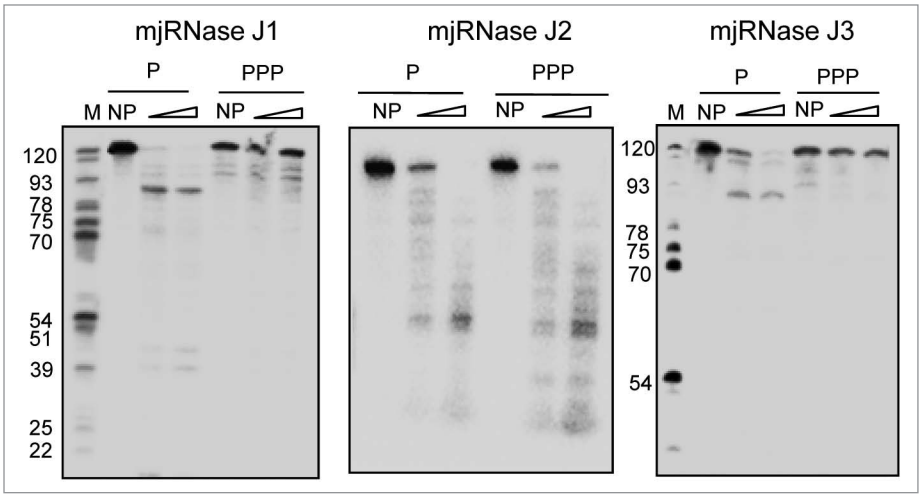


Figure 4. mjRNase J1 and mjRNase J3 preferentially degrade RNAs with 5' monophosphate. A uniformly labeled RNA (2 fmol) containing either mono- (P) or tri-phosphates (PPP) at the 5' end was incubated with mjRNase J1, mjRNase J2 or mjRNase J3 at 60°C for 30 and 60 min. Following incubation, the RNA was purified by phenol extraction and ethanol precipitation and analyzed by 20% urea-PAGE and autoradiography. The length of each size marker, in nucleotides, is shown to the left. NP: RNA incubated with no protein.

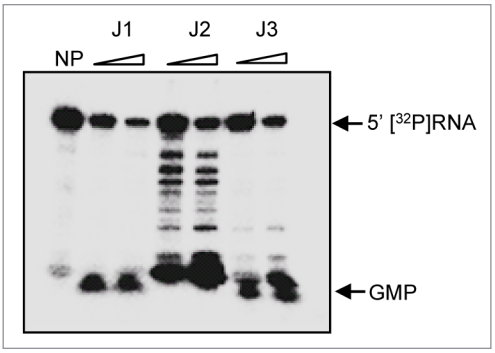


Figure 5. mjRNase J1 and mjRNase J3 are 5' to 3' exonucleases, but mjRNase J2 is an endonuclease. The three nucleases of *M. jannaschii* were incubated with 5' monophosphorylated and 5'-labeled (5'-P, 5'-[^{32}P]) RNA. Labeled RNA (3 fmol) was incubated for 30 or 60 min with 1.2 pmol of mjRNase J1, 0.8 pmol of mjRNase J2 or 1 pmol of mjRNase J3. Following incubation at 60°C, the reaction mixture was mixed with formamide, heated to 85°C and analyzed by 10% urea-PAGE and autoradiography. NP: RNA incubated with no protein.

(**Fig. 4** middle part). The sensitivity of their activities to the nature of the substrate's 5' end suggests that mjRNase J1 and -J3 are 5'→3' exonucleases, an activity expected of RNase J homologs (see below).

mjRNase J1 and -J3 are 5'→3' exonucleases, but mjRNase J2 is an endonuclease. To explore the nature of the ribonuclease activities of the three proteins, whether exo- or endoribonucleases, several assays were applied. In the first assay, the digestion of an RNA substrate [^{32}P]-labeled at the 5' end was analyzed. Under these conditions, when the degradation products of the reactions with mjRNase J1 and -J3 were examined in urea-PAGE, the amount of full-length RNA decreased during the incubation, and the accumulation of GMP was observed (**Fig. 5**). These results strongly suggest a 5'→3' exonucleolytic degradation. In contrast, in the case of mjRNase J2, the accumulation of numerous intermediate degradation products but not accumulation of GMP was observed, suggesting an endonuclease or 3'→5' exonuclease activity (**Fig. 5**).

In order to determine whether mjRNase J2 is an endonuclease and to further examine the exonuclease activities of mjRNase J1 and -J3, the products of the degradation assays were analyzed by thin-layer chromatography (TLC). The three proteins were independently incubated with RNA molecules uniformly labeled with [^{32}P]-UTP and containing 5'-monophosphate (5'-P) or 5'-triphosphate (5'-PPP) at 60°C for 4 h. The same analysis was also performed with the same RNA molecule containing 5' end-labeled 5'-monophosphate (5'-[^{32}P]). For comparison, the *E. coli* PNPase was also assayed. This phosphorolytic enzyme is a 3'→5' exonuclease, producing nucleotide diphosphates as degradation products.[5] The RNase J proteins are predicted to hydrolytically degrade the RNA-producing nucleotide monophosphates. In agreement with the results presented in **Figures 4 and 5**, UMP was observed for mjRNase J1 and -J3, and the activity was enhanced when the 5' end of the RNA was monophosphorylated, as compared to the reaction with RNA having three phosphates at that position (**Fig. 6**). In the case of mjRNase J2, no product could be detected on the TLC plate, implying that under these conditions, this protein displays exclusively endonucleolytic activity. To further verify the 5'→3' directionality of the degradation activity of mjRNase J1 and -J3, a 5' monophosphorylated substrate in which only the 5' guanosine was [^{32}P]-labeled, was used. Similarly, in this case, GMP product accumulated in the reaction products

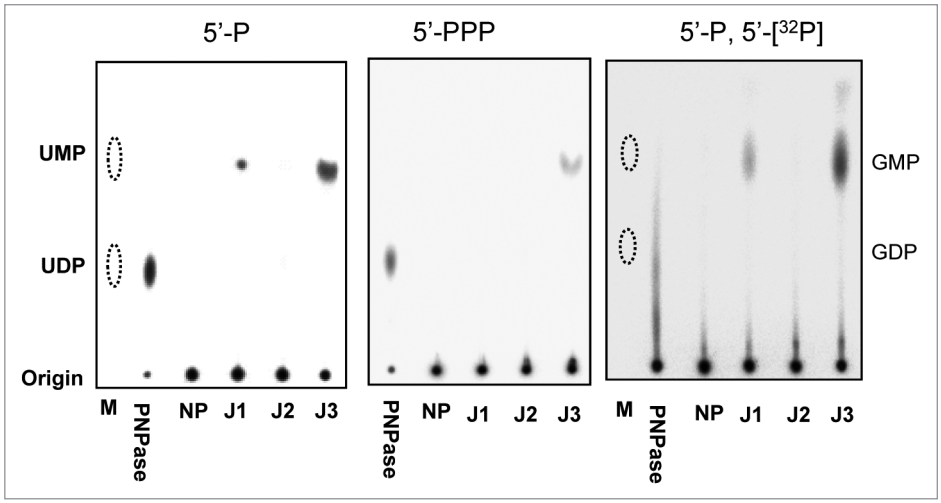


Figure 6. 5'-dependent 5'→3' exonuclease activities of mjRNase J1 and mjRNase J3. The three nucleases of *M. jannaschii* were incubated with uniformly [^{32}P]UTP labeled 5'-monophosphorylated (5'-P) or 5'-triphosphosphorylated (5'-PPP) RNAs (left and middle parts). In addition, 5'-monophosphorylated and 5'-labeled (5'-P, 5'-[^{32}P]) RNA was assayed (right part). *E. coli* PNPase was also analyzed to compare exonuclease activities. Following incubation for 4 h at 60°C (37°C for PNPase), the nucleotides were separated by TLC and observed by autoradiography. The migration patterns of the nucleotide markers analyzed on the same plate and visualized by fluorescence quenching are indicated with dotted circles (lane M). Note that in the experiments described in (A and B), the RNA was labeled uniformly with [^{32}P]UTP, whereas in the experiment described in (C), the RNA was 5'-labeled with [^{32}P]GTP. For the experiments shown in (A and B), 4 fmol of uniformly labeled RNA was incubated with 1.2 pmol of mjRNase J1, 0.8 pmol of mjRNase J2, 1 pmol of mjRNase J3 and 1.2 pmol of *E. coli* PNPase. For the experiment described in (C), 3 fmol of 5'-labeled RNA was used.

of mjRNase J1 and -J3 but not mjRNase J2 (**Fig. 6** right part). Together, these results demonstrate that mjRNase J1 and -J3 are 5'→3' exonucleases and that mjRNase J2 is an endonuclease with no detectable exonuclease activity. We were still left with the possibility that similar to the RNase J1 of *B. subtilis*, mjRNase J1 and -J3 have, in addition to the exonucleolytic activity, a low endonucleolytic activity. To examine this possibility, we assayed the digestion of circular RNA.

To determine if mjRNase J1 and -J3 have endonucleolytic in addition to exonucleolytic activity, even if it is very low, a circular [^{32}P]-labeled 34 nt substrate was incubated with each of the proteins individually. Degradation of such a substrate requires endonucleolytic activity. Indeed, as observed in **Figure** 7 for the *E. coli* PNPase that served as an internal control, the circular RNA remained intact. Though the three proteins degraded the linear transcript, only mjRNase J2 was able to degrade the circular molecule (**Fig.** 7). Therefore, mjRNase J1 and -J3 display only exonuclease activity. Together, these results imply that unlike the bacterial *B. subtilis* RNase J1, which shows both endo- and 5'→3' exonucleolytic activities in one protein, the three mjRNase J proteins display a clear division of work. Whereas mjRNase J1 and -J3 show exclusively 5'→3' exo- without any detectable endonucleolytic activity, mjRNase J2 displays only endo- without any exonucleolytic activity. mjRNase J2 is the first RNase J to be characterized exclusively as an endonuclease with no exonuclease activity.

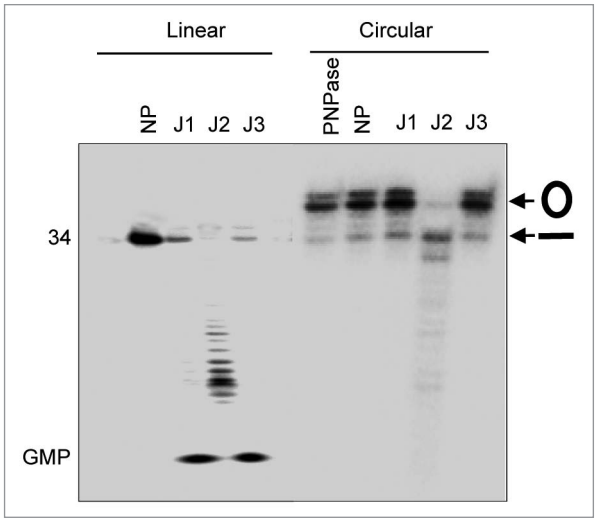


Figure 7. mjRNase J2, but not -J1 or -J3, displays endonuclease activity. A 34 nt fragment corresponding to the 3' end of the spinach chloroplast *petD* transcript was [^{32}P]-labeled at the 5' end and then circularized with T4 ligase. Both linear and circular substrates (2 fmol) were incubated with 0.5 pmol of *E. coli* PNPase, mjRNase J1 (3 pmol), mjRNase J2 (4 pmol) and mjRNase J3 (2 pmol) at 60°C (37°C for PNPase) for 120 min. Following incubation, the RNA was analyzed by 20% urea-PAGE and autoradiography. *E. coli* PNPase, a phosphorylase and 3'→5' exoribonuclease that exclusively degrades linear RNA, was used as an internal control. NP: RNA incubated with no protein. A schematic presentation of the circular and linear molecules is indicated to the right.

Not just ribonucleases; mjRNase J2 and -J3 degrade single-stranded DNA. To further analyze the enzymatic activity of mjRNase J1, -J2 and -J3, the proteins were incubated at 60°C with 5' [^{32}P]-labeled ssDNA. The products were then analyzed by fractionation, either by urea-PAGE or TLC, allowing for the characterization of the activity of the proteins on ssDNA. The results, presented in **Figure 8**, show no activity for mjRNase J1. However, low activity could be detected for mjRNase J2, and mjRNase J3 displayed remarkable exonucleolytic activity in which the ssDNA was rapidly disappeared and a mono-nucleotide product accumulated (**Fig. 8**). Based on the results obtained with RNA as a substrate, it could be assumed that the mjRNase J2 low degradation activity resulted from an endonucleolytic cleavage of the ssDNA. Accordingly, no accumulation of mono-nucleotide was observed on the TLC plate (**Fig. 8C**). However, it should be noted that such low activity could result from a very low amount of product that could be under the limit of detection of this system. Since the DNA was [^{32}P]-labeled at the 5' end and only mono-nucleotide was observed following the digestion, we concluded that mjRNase J3 is a highly active ssDNA 5'→3' exonuclease, whereas mjRNase J2 displays low activity on such a substrate, much as does an endonuclease.

In order to analyze the relative activity of mjRNase J3 on RNA compared to ssDNA, a competition assay was performed. In this experiment, increasing amounts of non-radioactive ssDNA were added to the reaction mixture to compete the degradation of [^{32}P] RNA of the same sequence. As shown in **Figure 8B**, the addition of ssDNA at an equal molar concentration already inhibited the degradation of the RNA to some extent, showing about equal activity of mjRNase J3 on these molecules. On the other hand, dsDNA was not able to compete the activity on ssDNA (**Fig. 8B**). Degradation of ssDNA has previously been shown for the RNase J of the archaea *Pyrococcus abyssi* and *Thermococcus kodakaraensis*.[23]

Taken together, our results reveal a division of labor between the several RNase J members in the enzymatic activities of RNA degradation in the archaeon *M. jannaschii*. In the absence of any other known exo- and endoribonuclease in this organism, RNase J proteins provide both activities in different proteins.

Discussion

While analyzing RNA degradation and polyadenylation in different organisms and organelles, a group of hyperthermophilic and methanogenic archaea that did not contain any of the known 3'→5' exonucleases was identified (**Table 1**). Moreover, because no organism lacking RNase II, RNase R, PNPase and the exosome had been found and at that time RNase J was yet to be discovered, the question what enzyme is responsible for RNA degradation in this group of archaea could not be answered.[22,29] However, the discovery of RNase J in *B. subtilis* as both a 5'→3' exonuclease and an endonuclease and the observation of several homologs in archaeal genomes raised the possibility that in this group of hyperthermophilic methanogens, RNA degradation is carried out entirely by RNase J enzymes. To explore this possibility, we analyzed in this work the ribonuclease properties of three out of four RNase J homologs in *Methanocaldococcus jannaschii*. Indeed, the results show that these proteins can provide both

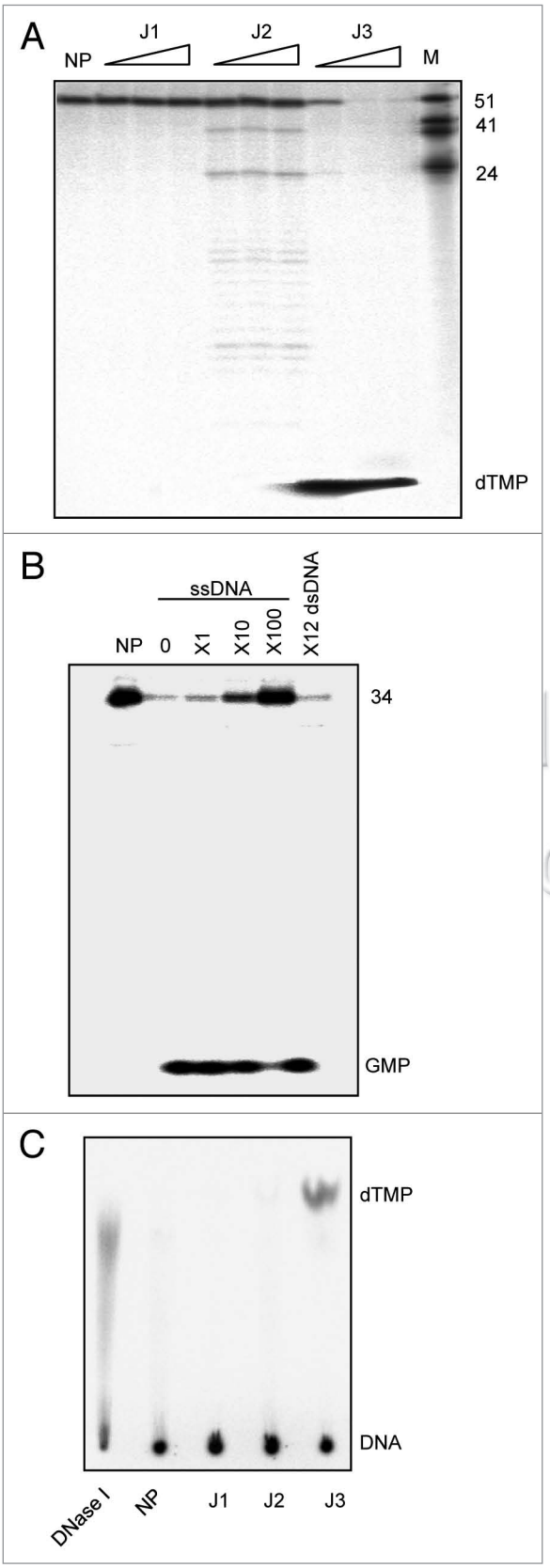


Figure 8. mjRNase J2 and mjRNase J3, but not mjRNase J1, degrade ssDNA. 5' [^{32}P]-labeled ssDNA (51 nt, 3 fmol) was incubated with mjRNase J1 (3 pmol), mjRNase J2 (4 pmol), and mjRNase J3 (2 pmol) for 1, 3 and 6 h at 60°C. *E. coli* DNase I was used as an internal control. The degradation products were then analyzed by denaturing 20% PAGE (A) or TLC (C) and autoradiography. (B) Competition of ssDNA for the ribonucleolytic activity of mjRNase J3. mjRNase J3 (1 pmol) was incubated with 5' [^{32}P] labeled 34 nt RNA (2.75 fmols). Non radioactive single stranded DNA (ssDNA) of the same sequence was added to the reaction at the molar excess as shown at the top of the figure. Double stranded DNA of 1,350 nt (dsDNA) was analyzed as a competitor as well. The nonradioactive competitor DNA was added to the reaction mixture before the [^{32}P]RNA. Incubation was for 1 h at 60°C. NP: no protein added to the reaction mixture. M: ssDNA size markers of known lengths, as shown to the right. dTMP: deoxythymidine monophosphate.

endonucleolytic and 5'-dependent 5'→3' exonucleolytic activities in the archaeal cell. Therefore, these two activities could potentially provide the RNA degradation and perhaps also the RNA processing requirements of the cell. This is the only organism described that potentially lacks a 3'→5' exonuclease. Accordingly, and because it lacks the archaeal exosome that is responsible for the polyadenylation of RNA in that organism, no posttranscriptionaly added tails have been identified.[22]

The first RNase J to be characterized, that of the bacterium *B. subtilis*, has both endo- and exonucleolytic activities that are located in the same active site.[10-12] However, the two archaeal RNase J enzymes characterized so far display exclusively exonuclease activity, as was also found here for the two homologs, mjRNase J1 and mjRNase J3.[23,24] Surprisingly, one of the enzymes, mjRNase J2, displayed exclusively endonuclease activity. The experimental conditions used included several substrates and various physiological parameters. Therefore, this is the first described RNase J homolog lacking any exonucleolytic activity. Accordingly, the cleavage activity was insensitive to the number of phosphates at the 5' end. Homology modeling analysis of the different proteins using the X-ray diffraction-solved structure of the *T. thermophilus* RNase J and the two archaeal homologs did not reveal any differences in the vicinity of the catalytic site that might be responsible for the exclusively endonuclease activity of mjRNase J2, as compared to the exonucleolytic activities of mjRNase J1 and mjRNase J3 (**Fig. 1**).[12,17,19] Outside of the catalytic site, a significant difference among the three proteins is the addition of the two KH domains in mjRNase J2, well known in other RNA-binding proteins. Whether the addition of these two KH domains is responsible for the exclusive endonucleolytic activity of mjRNase J2 remains to be determined by further investigation. It is also interesting to note that the archaeal RNase J proteins lack the C-terminal domain that characterizes the bacterial enzymes and is essential for activity of the *T. thermophilus* RNase J.[12]

While we were conducting this research, the structures of three archaeal RNase J homologs, based on protein crystallographic analysis, were reported.[17,19,27] These structures also showed similarities to the eukaryotic CPSF-73, which performs the endonucleolytic cleavage in the polyadenylation of mRNAs.[21] Additionally, the endonucleolytic activity of mjRNase J2 resembles the results obtained with human CPSF-73, which is a sequence-independent endonuclease.[21] Consequently, based on sequence, structure,

molecular weight, and its endonuclease behavior, the methanogenic mjRNase J2 is similar to the eukaryotic cleavage and polyadenylation β-lactamase protein CPSF-73. This may suggest an interesting evolutionary relationship between these two proteins. Interestingly, and unlike mjRNase J2, CPSF-73 also displays exonucleolytic activity in the processing of histone transcripts.[20]

In archaea, the 5' end of primary transcripts typically contains triphosphates.[24] The 5' end could be a monophosphorylated if it is formed by processing of the primary transcript.[1] Therefore, similar to bacterial primary transcripts with triphosphorylated 5' termini, which are more protected against exonucleolytic degradation, a similar situation could account for mjRNase J1 and -J3. This also resembles the situation in eukaryotic mRNAs that are protected at the 5' end by the cap nucleotide. In *M. jannaschii*, a 5' monophosphorylated transcript is established following cleavage of the primary transcript by mjRNase J2 or another endonuclease, such as RNase Z (see below). This cleavage product, containing a 5' monophosphate, is therefore much more susceptible to exonucleolytic degradation by mjRNase J1 and -J3. In this respect, the degradation system in *M. jannaschii* may resemble that of *E. coli*, in which the initial step in RNA degradation is the removal of two phosphates from the 5' end, and that of eukaryotes, in which the decapping of mRNA promotes its degradation by the 5'→3' exonuclease Xrn1.[24,30]

Archaeal mRNAs are often characterized by very short or leaderless 5' untranslated regions.[25,26] Though most of the transcripts contain triphosphates at the 5' end that protect them from RNase J digestion, binding of translation initiation factor a/eIF2(γ) to this region protects the transcript from 5'→3' degradation by RNase J as well.[25] Hence, the involvement of RNA-binding proteins and factors such as a/eIF2(γ) in the control of the degradation of different transcripts by the RNase J members, as well as the coordination between translation and degradation, is hypothesized to take place.

An interesting observation concerning the activities of mjRNase J2 and -J3 was their ability to degrade ssDNA. Similar ssDNA degradation activity has been reported for the archaeal RNase J of *Pyrococcus abyssi* and *Thermococcus kodakaraensis*.[23] The β-CASP family includes three DNA repair proteins: Artemis, Snm1 and Pso2. Based on sequence analysis, motif C of the β-CASP family is characterized by a histidine residue for RNA-degrading enzymes and a valine residue for DNA-degrading proteins. There is a histidine residue at motif C of the β-CASP domain for both mjRNase J2 and -J3. The DNA repair proteins Artemis and Snm1/Pso2 are associated with DNA interstrand cross-links, where the DNA is covalently adducted in between its strands.[31,32] The human paralog of yeast Snm1/Pso2 is Snm1A, has also two homologs, Snm1B (Apollo) and Snm1C (Artemis). Whereas all human Snm1 proteins exhibit 5'→3' exonucleolytic degradation on ssDNA, Snm1A and Artemis also degrade dsDNA.[31,33] In addition, Artemis cleaves ssDNA and RNA (with the association of DNA-PK_{CS}) as an endonuclease.[34] Here, we described an endo- or 5'→3' exonucleolytic activity of two mjRNase J proteins on ssDNA but not dsDNA. Exploring a possible function of mjRNase J2 and -J3 in the metabolism of DNA in archaea awaits further studies.

In this work, we report a member of Euryarchaota Methanococcus hyperthermophilic archaea in which RNA degradation is possibly performed exclusively by RNase J proteins. These proteins provide both endo- and 5'→3' exonucleolytic activities, in the absence of the known enzymes responsible for the 3'→5' degradation activities. An interesting evolutionary question is whether this group of archaea reflects the initial situation of ancient organisms, in which the RNA degradation mechanism was the 5'→3' activity exhibited by one or more members of the RNase J family, which would provide both the endo- and exonucleolytic activities. Under this scenario, the 3'→5' degradation mechanisms, involving the polyadenylation and the phosphorolytic and hydrolytic activities, evolved later in bacteria and other groups of archaea like the Euryarchaeota Halobacterium (halophiles) and Crenarchaeota Sulfolobus (hyperthermophiles), which today contain the archaeal exosome.[5] An alternative scenario would imply the elimination of the 3'→5' degradation mechanism from this group of Euryarchaeota Methanococcus archaea during evolution. The presence of a group of archaea in which RNA degradation is possibly exclusively performed by RNase J homologs, together with the observation that homologs of RNase J are widely present in almost all genomes sequenced, suggest that this enzyme is an ancient and key player in the evolution of RNA degradation processes.

Materials and Methods

Identification of *M. jannaschii* RNase J homologs and multiple sequence alignment. The *B. subtilis* RNase J1 sequence, obtained from the NCBI sequence library, was PSI-Blasted against the sequence of the *Methanocaldococcus jannaschii* genome. Following three iterations and obtaining reliable homology scores, four homologs were found; RNase J1 (NP_247130), RNase J2 (NP_248231.1), RNase J3 (NP_247856.1) and Mj4 (NP_247010). The selected protein sequences were virtually folded using HHpred—a server for protein remote homolog detection. The results were further used as an input to MODELLER, a server that builds 3D models by comparing to a known protein structure. *P. horikoshii* protein (PDB 3af5) was used as a template for mjRNase J1 and J2. *T. thermophilus* protein (PDB 3bk2) was used as a template for mjRNase J3. The reason for using two different models is that the best scored protein in the data bank was used for each protein. mjRNaseJ1, J2 and J3 models scored 33%, 59% and 38% identity to their template models, respectively. Multiple sequence alignment was done using Clustal W and was analyzed using Jalview.

Cloning, expression and purification of the mjRNase J homologs. *M. jannaschii* RNase J homologs were PCR-amplified using genomic DNA and the appropriate oligonucleotide primers (**Table S1**) and cloned into the BamHI and SalI restriction sites of the bacterial expression vector pQE-30 (Qiagen Inc.). In this way, six sequential His residues were added at the protein N-terminus. The point mutations were introduced using the site-directed mutagenesis kit (Stratagene Inc.,) and the oligonucleotide primers indicated in **Table S1**.

E. coli M15 pREP4 cells were transformed with the mjRNase J3 expression plasmid and grown at 30°C in LB medium to an OD_{600} of 0.6. Protein expression was induced with 0.1 mM IPTG for 16 h at 18°C. For expression of mjRNase J2 and mjRNase J1, the bacteria were grown at 30°C in LB containing 0.5 M sorbitol and 50 mM HEPES to an OD_{600} of 0.6, cooled down to 18°C and then incubated 1 h with the addition of 300 mM benzyl alcohol. Expression was induced with 0.1 mM IPTG for 16 h at 18°C. To purify proteins, bacteria were disrupted with a microfluidizer, cleared by centrifugation and heated at 70°C for 30 min (mjRNase J1 and mjRNase J3) or 1 h (mjRNase J2). Following centrifugation, the soluble fraction was applied to a Ni column, eluted with 250 mM imidazole, dialyzed with buffer A (50 mM Na_3PO_4 pH 7.0, 300 mM $NaCl_2$, 10% glycerol, 4 mM $MgCl_2$ and 1 mM DTT) and applied to a heparin column following elution with buffer A containing 1.5 M NaCl. The final purification step employed a MonoQ column in which mjRNase J3 bound to the column, whereas mjRNase J2 and mjRNase J1 were obtained in the flow-through. The purified proteins were fast-frozen and stored at -80°C.

Preparation of the RNA substrates for the degradation analysis. The DNA construct used for the transcription of 120 nt RNA corresponding to the 3' end of the spinach chloroplast *pet*D gene has been described before in reference 35. In vitro transcription with T7 RNA polymerase and radioactive labeling were performed as previously described in reference 36. RNA molecules containing 5' monophosphate were obtained by the addition of 150 mM GMP to the transcription reaction mixture.[37] 5' end labeling was done by first removing a phosphate from the 5' end with calf intestinal alkaline phosphatase and subsequently adding the [^{32}P] with T4 polynucleotide kinase and γ-[^{32}P]ATP.[37] Circular RNA substrate was obtained by annealing an RNA molecule harboring a 5' monophosphate and 5' [^{32}P]-end-labeled produced as described above and ligating with T4 ligase at 16°C for 16 h. The ligation product was then digested with *E. coli* PNPase to remove any remaining linear transcripts.

RNA degradation assays. In vitro RNA degradation assays were performed using the recombinant proteins and either [^{32}P] UTP-uniformly labeled or 5' [^{32}P]-end-labeled RNA substrates as described above. In a common reaction, a protein (30 μM) was incubated at 60°C with 0.02 μM RNA in buffer A for the times indicated in the Figure legends. Following incubation, the RNA was either purified by phenol extraction and EtOH precipitation or directly analyzed by denaturing PAGE or TLC chromatography.[36]

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Note

Supplemental material can be found at:
www.landesbioscience.com/journals/rnabiology/article/16604

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