ACADEMIC PRESS

Biochemical and Biophysical Research Communications 297 (2002) 1344–1349

BBRC

www.academicpress.com

Location of 2′-*O*-methyl nucleotides in 26S rRNA and methylation guide snoRNAs in *Caenorhabditis elegans*

Sayomi Higa, Noriko Maeda, Naoya Kenmochi,[1] and Tatsuo Tanaka*

Department of Biochemistry, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

Received 20 August 2002

Abstract

Many nucleotides in rRNAs are modified. We devised a method to locate 2′-*O*-methyl nucleotide residues using a conventional DNA sequencer. We found 38 2′-*O*-methyl nucleotides in the 26S rRNA of *Caenorhabditis elegans* using this method. Fourteen of the 38 residues are conserved in both human and yeast rRNAs and 14 residues are conserved in either human or yeast rRNA. The remaining 10 nucleotides are uniquely methylated in *C. elegans* 26S rRNA. We searched the *C. elegans* genomic sequence for small nucleolar RNAs (snoRNAs), which guide the methylation of ribose residues, and predicted 18 snoRNA sequences that are expected to guide the methylation of some of these nucleotide residues.

Keywords: rRNA; snoRNA; 2′-*O*-methylation; Modification; *Caenorhabditis elegans*

Many nucleotides in mature rRNAs undergo covalent, posttranscriptional modifications, in all organisms. These modifications occur on precursor RNAs during ribosome synthesis and are essentially of three types: base methylation, methylation of the 2′-hydroxyl group of ribose residues, and base rotation conversion of uridine residues to pseudouridine [1]. Methylation of the 2′-hydroxyl group of ribose is the most prominent of these modifications. The number of reported methylated residues is 54 in the yeast *Saccharomyces cerevisiae* [2] and about 105–107 in mammals including humans and mice [1,3]. However, the number of methylated residues is still uncertain, even in most intensively studied human rRNA [4]. Although virtually all of the modified nucleotides appear to be confined to the most conserved and functionally important regions of rRNA, the function of these modifications remains largely enigmatic. A well-characterized model organism is required for functional studies. The nematode *Caenorhabditis elegans* is one such organism; however, modification of *C. elegans* rRNA has not been reported. The rRNA sequences of more and more species have become available through rDNA sequencing. Yet information about the modified nucleotides cannot be obtained simply sequencing the rDNA. Recently, some convenient methods for locating 2′-*O*-methyl nucleotides were devised [5]. We further improved one of these methods to incorporate use of an automatic DNA sequencer. With this method, we located 38 2′-*O*-methyl nucleotides on *C. elegans* 26S rRNA.

The selection of ribose residues for 2′-*O*-methylation is directed by C/D box small nucleolar RNAs (snoRNAs) [6]. snoRNAs contain common features, including one or two 10–21 nucleotide antisense elements that complement mature rRNA and conserved sequence motifs called boxes C, C′, D, and D′ [7]. Substrate modification occurs in the complementary sequence, five nucleotides upstream of the box D or D′ [8,9]. Since snoRNAs have been detected in yeast, plants, and vertebrates, they appear to be ubiquitous in eukaryotes. Recently, small RNAs were identified as Archaean homologues of eukaryotic snoRNAs [10,11]. Although genomic sequencing has almost been completed and the expressed sequences of *C. elegans* are being studied extensively, only two snoRNAs have been registered in the DDBJ/EMBL/GenBank database with Accession Nos. Z75111 and Z75112. We searched for snoRNAs that might be important for the methylation of the sites

* Corresponding author. Fax: +81-98-895-1404.
E-mail address: ttanaka@med.u-ryukyu.ac.jp (T. Tanaka).
[1] Present address: Central Research Laboratories, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan.

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0006-291X(02)02377-X

described here and found 18 candidate sequences in the *C. elegans* genome.

Materials and methods

Culture and RNA preparation. Wild-type *C. elegans* (N2, Bristol) was cultured at 20 °C in liquid following the description of Koelle and Herman (http://www.dartmouth.edu/artsci/bio/ambros/protocols/warm-protocols.html). Four days after inoculation, worms were harvested by centrifugation at 3000 rpm for 3 min, suspended in 0.1 M NaCl, recentrifuged at 2000 rpm for 3 min, resuspended in 0.1 M NaCl, left on ice for 5 min before the addition of an equal amount of 60% sucrose, and centrifuged at 3500 rpm for 5 min. The fluffy top layer was collected and washed again with 0.1 M NaCl. The total worm RNA was prepared using TRI reagent (Sigma) following manufacturer's protocol.

Mapping 2′-O-methyl nucleotides. The method described by Maden [5] for mapping 2′-*O*-methyl groups in RNA was modified to incorporate the use of an automatic DNA sequencer instead of a radioisotope and radioautography. A 11 μl solution containing 10 μg total worm RNA and 1 pmol 5′-Texas red-labeled primer was treated at 70 °C for 5 min and then placed on ice for 5 min. An appropriate amount of dNTP and 5 μl of 5× concentrated reverse transcriptase reaction buffer [250 mM Tris–HCl (pH 8.3), 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine, and 50 mM DTT], 20 U RNase inhibitor, and 30 U AMV reverse transcriptase (Promega) were added to the solution, adjusted with distilled water to a final volume of 25 μl, and incubated for 1 h at 42 °C. Primer extension product was precipitated in 89% ethanol, washed with 80% ethanol, dried in vacuo, and dissolved in 10 μl distilled water, before addition of 3 μl loading dye mixture. The primer extension products and position markers were resolved on a sequencing gel on a Hitachi SQ-5500 sequencer. The position marker was a sequence ladder produced from the same primer and PCR-amplified rRNA gene as the template.

Oligonucleotide primers. A total of 40 oligonucleotide primers were made for approximately every 100 nucleotides along the *C. elegans* 26S rRNA. The primers were 19–25 nucleotides long and designed to have T_m's of around 58 °C. Nucleotide synthesis and 5′-end labeling with Texas red were completed by Hokkaido System Science (Sapporo, Japan) or Hitachi Instruments Service (Tokyo, Japan). Some cases, we labeled synthetic oligonucleotides using the 5′ oligonucleotide Texas red labeling kit (Amarsham) according to manufacturer's instructions.

Prediction of snoRNA sequences. We used two methods to screen the methylation guide snoRNA: a snoscan [2] computer program with default parameters and a largely manual method as follows. We searched the *C. elegans* genomic sequence using BLAST [12] under the assumption that snoRNA contains a sequence complementary to 13 rRNA nucleotides with a methyl nucleotide at the fifth position, followed by box D (CUGA). Of the resulting sequences, we selected sequences of about 100 nucleotides containing box C (RUGAUGA) and short inverted sequences at the 5′- and 3′-ends to ensure we included the stem structure. The candidates obtained from both methods were critically reexamined to verify that they complemented the methylation sites, contained complete boxes C, C′, D, and D′, and contained matching 5′- and 3′-ends. Thus, only sequences with high probability of being methylation guide snoRNAs were selected.

Results and discussion

Mapping of 2′-O-methyl nucleotides

Locating 2′-*O*-methyl nucleotides on an RNA molecule had been laborious until some clever methods were recently devised [3]. One of the most convenient methods is based on the fact that 2′-*O*-methyl nucleotides impede reverse transcriptase at low dNTP concentrations but not at elevated dNTP concentrations [5]. We modified the method to use an automatic DNA sequencer instead of a radioisotope and radioautography. Replacement of radiolabeled primers with Texas red-labeled ones enables the switch to the conventional automatic DNA sequencer. Fig. 1 shows a typical printout of the analyzer. The bands of the modified nucleotides, which diminish as the dNTP concentration increases, are clearly recognized, as indicated by arrows in Fig. 1. Automatic DNA sequencers are fairly easy to use and provide rapid results that are highly reliable, since the band image intensity and contrast are freely adjustable.

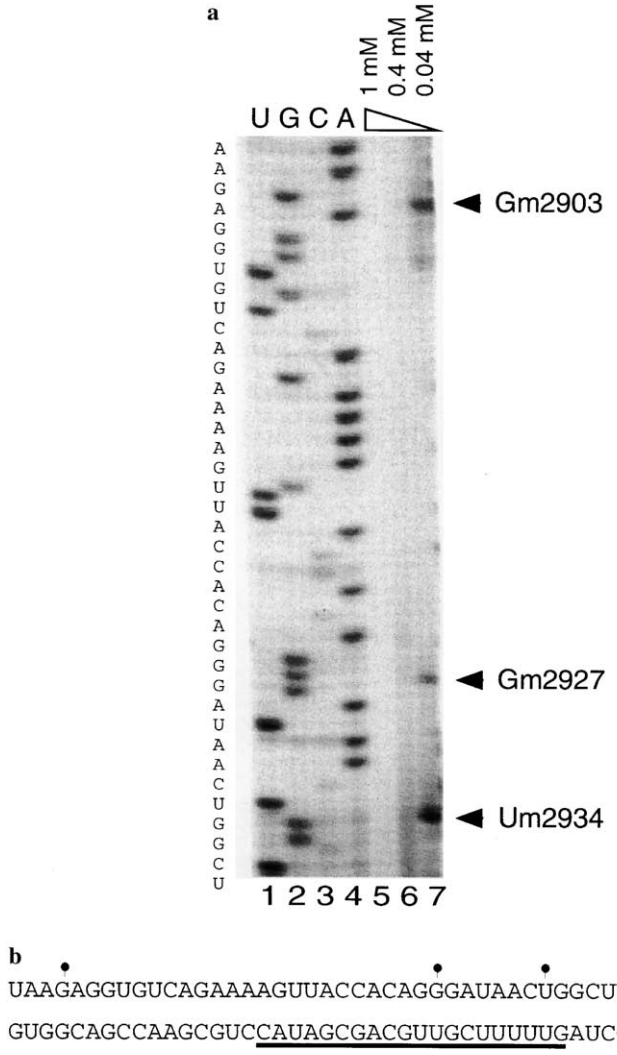

Fig. 1. Printout of an automatic sequencer run. (a) Lanes 1–4: sequence ladder produced using the same primer as lanes 5–7 and cDNA as the template. Lanes 5–7: reverse transcriptase products at the dNTP concentrations of 1, 0.4, and 0.04 mM, respectively. The area corresponds to residues 2901–2938 of 26S rRNA and the sequence appears on the left-hand side. The arrowheads show bands of Gm2903, Gm2927, and Um2934. (b) The 26S rRNA sequence from residues 2900 to 2979. Filled circles indicate 2′-*O*-methyl nucleotides. The underlined sequences are positions of the primer.

Also advantageous, the procedure was automated in that the 2′-*O*-methyl nucleotides were located by the programmed sequencer.

Detection of 2′-O-methylated nucleotides

Using the 40 primers, we searched from the 5′-end to the vicinity of the 3′-end of 26S rRNA in *C. elegans* and found 38 2′-*O*-methyl nucleotides (Table 1). Comparing this number with the reported 64 and 37 2′-*O*-methyl residues in human 28S rRNA and yeast 25S rRNA [3], we suspect that some 2′-*O*-methyl nucleotides are still missing. This is highly probable: locating 2′-*O*-methyl nucleotides is so difficult that the total number of 2′-*O*-methyl nucleotides in most intensively studied human rRNA is still uncertain [4]. The structure of rRNA is highly conserved, so that a specific nucleotide in *C. elegans* can be correlated to that in another species such as human and yeast. As shown in Table 1, 14 of the 38 2′-*O*-methyl nucleotides are conserved in both human 28S rRNA and *S. cerevisiae* 25S rRNA and 14 are conserved in either human rRNA or yeast rRNA. The remaining 10 are unique to *C. elegans*, suggesting that the location of 2′-*O*-methyl nucleotides is less conserved than the nucleotide sequence. Although 2′-*O*-methyl nucleotides are widely dispersed in 26S rRNA, two clusters of methyl nucleotides were remarkable at the positions of 2310–2530 and 2840–3090. These correspond to the last one-third of domain IV and domain V, as shown in Fig. 2. The modified nucleotides in *Escherichia coli* rRNAs have been reported to occur within or close to the functional center of the ribosome [13]. The most important function of the 50S ribosomal subunit is to catalyze peptide bond formation. The location of the peptidyl transferase center appears to be in domain V of 23S rRNA. The structure of the ribosome was recently determined with atomic resolution and three-dimensional positions of the nucleotide residues of rRNA were clearly shown [14]. The fine structure of the peptidyl transferase center and its reaction mechanism were proposed [15]. Applying the model to eukaryotes, many 2′-*O*-methyl nucleotides are in the near vicinity of the peptidyl transferase center, but may not directly affect the reaction. Nazar et al. [16] suggested that methylation of the single nucleotide U14 in 5.8S rRNA shifts the rRNA to a more open structure and destabilizes the 5.8S rRNA–28S rRNA interaction. On the other hand, in vivo evidence suggests that 2′-*O*-methylation is important for RNA structural stability [6,17]. RNA methylations may elicit different effects on in vitro RNA from those on in vivo RNA–protein complex and the location of the methylation may determine the different influences. At any rate, 2′-*O*-methylation may fine-tune the structure of rRNA and control flexibility and stability important for its function and interaction with proteins. However, no clear functional role has been assigned to any of the modified nucleotides. Sirum-Connolly et al. [18] demonstrated that mutants lacking the PET56 gene, which is required for the formation of 2′-*O*-methylguanosine within the peptidyl transferase center in mitochondrial 21S rRNA, are deficient in production of functional large ribosomal subunits. This observation suggests that at least one posttranscriptional modification in 21S rRNA is required for the assembly of large subunits [18]. Tollervey et al. [19] generated temperature-sensitive NOP1 gene mutants (NOP1 encodes the snoRNA-associated protein fibrillarin) and showed that inhibition of methylation in 25S rRNA or its precursor mildly inhibited rRNA processing. Disruption of the U24 snoRNA gene prevented methylation of three nucleotides but did not affect the growth rate or accumulation of precursor and mature

Table 1
2′-*O*-Methyl nucleotides in *C. elegans* 26S rRNA[a]

C. elegans	*H. sapiens*	*S. cerevisiae*
Am395	Am391	
Gm668		
Am678	Am1306	Am647
Gm860	Gm1501	Gm803
Am931		Am874
Gm963	Gm1604	Gm906
Am1185	Am1849	Am1131
Cm1502	Cm2328	Cm1435
Am1514	Am2340	Am1447
Um1544		
Um1955		
Um1981	Um2814	Um1886
Am2317	Am3687	
Gm2343	Gm3713	
Am2359	Am3729	Am2254
Am2384	Am3754	Am2279
Gm2391		Gm2286
Um2417	Um3787	
Am2429	Am3799	
Am2448		
Cm2486	Cm3856	
Gm2498		
Um2524	Um3894	Um2419
Um2762	Um4187	
Um2841	Um4266	Um2726
Gm2903		Gm2788
Gm2927		Gm2812
Um2934		
Cm2991	Cm4416	
Am2999		
Gm3029	Gm4454	
Um3033	Um4458	Um2918
Am3058	Am4483	Am2943
Cm3060		Cm2945
Cm3071	Cm4496	Cm2956
Um3087		
Um3109		
Am3159		

[a] The first column is a list of detected 2′-*O*-methyl nucleotides. The second and third columns show the corresponding nucleotides in humans and yeast, respectively [1,2].

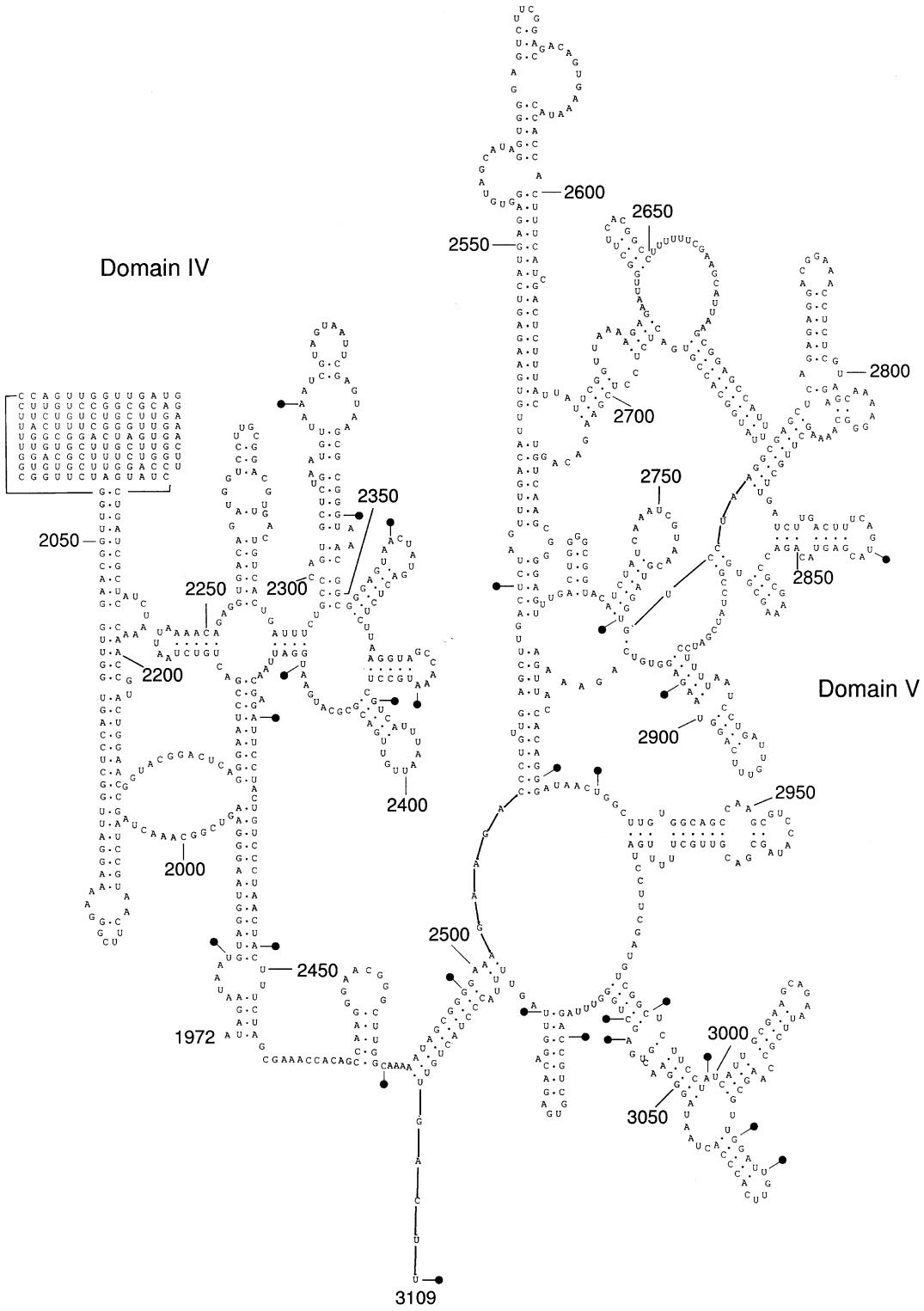

Fig. 2. Locations of 2′-*O*-methyl nucleotides in domains IV and V of 26S rRNA. The secondary structure of the region was drawn by a RnaViz [21] computer program. Nucleotides methylated at the 2′-*O* positions are indicated by filled circles. Nucleotide numbers are indicated for every 50 residues.

rRNAs [8]. Thus, further work is required to elucidate the role 2′-*O*-methyl nucleotides play in ribosomal function and formation. *C. elegans* is certainly one of the most suitable organisms for such studies.

Prediction of snoRNA sequences in the C. elegans genome

rRNA 2′-*O*-methylation in eubacteria is mediated simply by specific methyltransferases, whereas the

Fig. 3. Predicted snoRNAs. Predicted snoRNAs are shown along with the complementary rRNA sequence including the 2′-*O*-methyl nucleotides (shown by filled circles). Boxes C, C′, D, and D′ are shown by capital letters. Short inverted repeat sequences are shown by slanting letters. U18 and U15 are the DDBJ/EMBL/GenBank database names, with Accession Nos. Z75111 and Z75112.

reaction in eukaryotes involves several proteins and is guided by snoRNAs [20]. Searching for snoRNAs that guide methylation of nucleotides located in this experiment was fascinating. Finding novel snoRNAs on genomic DNA sequences is as complicated as locating new coding sequences. Before this work, only two snoRNA sequences were reported on the DDBJ/EMBL/GenBank database under Accession Nos. Z75111 and Z75112. We scanned the *C. elegans* genomic sequence with the snoscan [2] computer program and manually, as described in the Materials and methods. Consequently, we found 18 putative snoRNA genes (Fig. 3). Although we selected only highly probable sequences, 18 snoRNA genes for 38 methylation sites is an unsatisfactory prediction. A more sensitive and reliable algorithm for searching snoRNAs is needed. Furthermore, the predicted snoRNA methylation guide genes reported here should be confirmed experimentally to ensure that they are expressed as snoRNAs.

Acknowledgment

We are grateful to the *Caenorhabditis* Genetics Center for providing the Bristol N2 strains used in this study.

References

[1] B.E.H. Maden, The numerous modified nucleotides in eukaryotic ribosomal RNA, Prog. Nucleic Acids Res. Mol. Biol. 39 (1990) 241–303.
[2] T.M. Lowe, S.R. Eddy, A computational screen for methylation-guide snoRNAs in yeast, Science 283 (1999) 1168–1171.
[3] B.E.H. Maden, M.E. Corbett, P.A. Heeney, K. Pugh, P.M. Ajuh, Classical and novel approaches to the detection and localization of the numerous modified nucleotides in eukaryotic ribosomal RNA, Biochimie 77 (1995) 22–29.
[4] A. Rebane, H. Roomere, A. Metspalu, Locations of several novel 2′-*O*-methylated nucleotides in human 28S rRNA, BMC Mol. Biol. 3 (2002) 1.
[5] B.E.H. Maden, Mapping 2′-*O*-methyl groups in ribosomal RNA, Methods 25 (2001) 374–382.
[6] P.P. Dennis, A. Omer, T. Lowe, A guided tour: small RNA function in Archaea, Mol. Microbiol. 40 (2001) 509–519.
[7] A.G. Balakin, L. Smith, M.J. Fournier, The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions, Cell 86 (1996) 823–834.
[8] Z. Kiss-Laszlo, Y. Henry, J.-P. Bachellerie, M. Caizergues-Ferrer, T. Kiss, Site-specific ribose methylation of preribosomal RNA: a novel function for small nuclear RNAs, Cell 85 (1996) 1077–1088.
[9] M. Nicoloso, L.H. Qu, B. Michot, J.-P. Bachellerie, Intron-encoded, antisense small nucleolar RNAs: the characterization of nine novel species points to their direct role as guide for the 2′-*O*-ribose methylation of rRNAs, J. Mol. Biol. 260 (1996) 178–195.
[10] A.D. Omer, T.M. Lowe, A.G. Russell, H. Ebhardt, S.R. Eddy, P.P. Dennis, Homologs of small nucleolar RNAs in Archaea, Science 288 (2000) 517–522.
[11] C. Gaspin, J. Cavaille, G. Erauso, J.-P. Bachellerie, Archaeal homologs of eukaryotic methylation guide small nucleolar RNAs: lessons from the *Pyrococcus* genomes, J. Mol. Biol. 297 (2000) 895–906; J. Mol. Biol. 300 (2000) 1017–1018, erratum.
[12] T.L. Madden, R.L. Tatusov, J. Zhang, Applications of network BLAST server, Methods Enzymol. 266 (1996) 131–141.
[13] R. Brimacombe, P. Mitchell, M. Osswald, K. Stade, D. Bochkariov, Clustering of modified nucleotides at the functional center of bacterial ribosomal RNA, FASEB J. 7 (1993) 161–167.
[14] N. Ban, P. Nissen, J. Hansen, P.B. Moore, T.A. Steitz, The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution, Science 289 (2000) 905–920.
[15] P. Nissen, J. Hansen, N. Ban, P.B. Moore, T.A. Steitz, The structural basis of ribosome activity in peptide bond synthesis, Science 289 (2000) 920–930.
[16] R.N. Nazar, A.C. Lo, A.G. Wildeman, Effect of 2′-*O*-methylation on the structure of mammalian 5.8S rRNAs and the 5.8S–28S rRNA junction, Nucleic Acids Res. 11 (1983) 5989–6001.
[17] K.R. Noon, E. Bruenger, J.A. McCloskey, Posttranscriptional modifications in 16S and 23S rRNAs of the archaeal hyperthermophile *Sulfolobus solfataricus*, J. Bacteriol. 180 (1998) 2883–2888.
[18] K. Sirum-Connolly, T.L. Mason, Functional requirement of a site-specific ribose methylation in ribosomal RNA, Science 262 (1993) 1886–1889.
[19] D. Tollervey, H. Lehtonen, R. Jansen, H. Kern, E.C. Hurt, Temperature-sensitive mutations demonstrate roles for yeast fibrillarinin pre-rRNA processing, pre-rRNA methylation, and ribosome assembly, Cell 72 (1993) 443–457.
[20] T. Kiss, Small nucleolar RNA-guided post-transcriptional modification of cellular RNAs, EMBO J. 20 (2001) 3617–3622.
[21] P. De Rijk, R. De Wachter, RnaViz, a program for the visualization of RNA secondary structure, Nucleic Acids Res. 25 (1997) 4679–4684.