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Isolation of eight novel *Caenorhabditis elegans* small RNAs

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Abstract

Eight novel small RNAs that were encoded in the regions corresponding to the introns of protein-coding genes were isolated from *Caenorhabditis elegans*. Seven of them showed a typical snoRNA secondary structure: one C/D snoRNA and six H/ACA snoRNAs. The remaining one RNA did not show any homology to other RNAs in a database. Four of the seven isolated snoRNAs could form base pairings with parts of rRNAs, suggesting that they are potential pseudouridilation sites and methylation sites. The results of our study suggest that there are more as-yet-unidentified small ncRNAs of which genes are located in the intron regions of protein-coding genes in *C. elegans*.

Keywords: H/ACA snoRNA; C/D snoRNA; Intron; Pseudouridine; rRNA methylation

1. Introduction

In the past decade, various small noncoding RNAs (ncRNAs), besides tRNAs, rRNAs and spliceosomal U snRNAs, have been isolated and their importance in the cell has been revealed (Eddy, 2001; Hüttenhofer et al., 2001; Gottesman, 2002; Marker et al., 2002; Storz, 2002; Yuan et al., 2003). The finding of two conserved box motifs of nucleolar RNAs, i.e., box C/D snoRNAs and box H/ACA snoRNAs, has shown that many small nucleolar RNAs function in rRNA processing and in nucleotide modification of rRNAs and spliceosomal U snRNAs (Bachellerie and Cavaille, 1998; Ofengand and Fournier, 1998; Hüttenhofer et al., 2002; Kiss, 2002). MRP RNA, of which the gene is related to a human disease, also exists in the nucleolus and functions in pre-rRNA processing (Ridanpää et al., 2001). This RNA also exists in mitochondoria and functions in the cleavage of RNA primers that are responsible for DNA replication. Recently, many tiny endogenous RNAs of about 20 nt in length, designated microRNAs (miRNAs), have been isolated from a wide range of organisms (Moss, 2002; Bartel, 2004). Some of them are thought to function in gene regulation of developmental timing or spatial patterning of cell fates by base pairing with target mRNA (Moss, 2000; Carrington and Ambros, 2003). Other examples of eukaryotic small ncRNA are 7SK RNA and BC1 RNA. The former functions in the regulation of RNA polymerase II activity (Nguyen et al., 2001) and the latter has been suggested to function in the translational regulation of specific mRNAs at synapses (Zalfa et al., 2003). These studies on small ncRNAs indicate their potentials and importance as well as their abundance in the biological process.

The presence of several *Caenorhabditis elegans* small ncRNAs, such as SRP RNA, SL1 and SL2 RNAs and Ro-associated Y RNA, besides miRNAs, tRNAs, rRNAs and spliceosomal U snRNAs, has been reported (Blumenthal and Steward, 1997; The *C. elegans* Sequencing Consortium, 1998; Labbe et al., 2000; Ambros et al., 2003; Grad et al., 2003; Lim et al., 2003). Twenty C/D snoRNA genes of *C. elegans* have so far been identified in *C. elegans* (Higa et al., 2002), but no H/ACA snoRNA has been isolated. RNase P RNA, which functions in pre-tRNA processing and is

Abbreviations: ncRNA, noncoding RNA; snoRNA, small nucleolar RNA.

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found in a variety of organisms from bacteria to mammals, has not been identified in *C. elegans*. It therefore appears that there are many as-yet-unidentified small ncRNAs in *C. elegans*.

In this study, we isolated 19 novel small stable RNA candidates by analysis of cDNA sequences prepared from small RNA fractions of *C. elegans*. Eight of the nineteen RNAs were encoded in the regions corresponding to the intron sequences of protein-coding genes. There were one C/D snoRNA, six H/ACA snoRNAs and one RNA, homologs of which have not been found in other known ncRNAs.

2. Materials and methods

2.1. Preparation of C. elegans total RNA

C. elegans (strain N2) was grown and maintained at 20 °C as described previously (Stirnagle, 1999). Collected *C. elegans* was washed with M9 buffer and stored at − 80 °C. RNAs were extracted from mixed-stage worms by TRIZOL Reagent (Invitrogen Life Technologies) according to the manufacturer's protocol with a minor change: ethanol instead of 2-propanol was used to precipitate RNAs. About 1 mg of total RNA was obtained from 1 g of *C. elegans*. *C. elegans* strain N2 and *E. coli* strain OP50 used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

2.2. Fractionation of RNA

The total RNA was fractionated by stepwise 2-propanol precipitation. One ml of RNA solution (0.5 mg/ml) was mixed with 100 μl 3M sodium acetate (pH 5.2) and 550 μl 2-propanol. The resulting precipitates containing large RNAs (>500 nt) and DNAs were collected by centrifugation at 12,000 rpm for 10 min at room temperature (33% 2-propanol precipitates). The supernatant was then mixed with 220 μl 2-propanol. The precipitates containing RNAs of about 50 to 500 nt in length (41% 2-propanol precipitates) were collected by cetrifugation, washed with 70% ethanol, dried, and dissolved in 300 μl of water. The contaminating DNAs were eliminated by DNase I digestion followed by phenol/chloroform treatment and ethanol precipitation.

2.3. cDNA synthesis and sequence analysis

The small RNAs were separated by 12% polyacrylamide/7 M urea denaturing gel electrophoresis. The gel was stained with ethidium bromide and irradiated with UV light to detect RNA bands. The RNAs from visible bands were extracted from the gel, and their cDNAs were synthesized by using a Takara cDNA synthesis kit (Takara Biochemicals, Tokyo) with 9 mer random primers. The cDNAs were cloned into the *Sma*I site of pUC19. Five cDNA clones from each RNA preparation were randomly chosen, and their sequences were determined by using an automatic DNA sequencer (DNA sequencer SQ-5500, Hitachi). The sequence reaction was performed with a Texas Red M13 forward primer and a Texas Red M13 reverse primer by using a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham LIFE SCIENCE).

2.4. Northern hybridization

Northern hybridization was performed as previously described (Sambrook et al., 2001) for each novel RNA candidate. The RNA from 41% 2-propanol precipitates (20 μg) was separated by 1.5% agarose gel electrophoresis and transferred to a Nylon membrane (Hybond-N^+, Amersham LIFE SCIENCE). Two oligonucleotide probes, having a part of the cDNA sequence and a complementary sequence to it, were used for the hybridization of each RNA. Hybridization was performed at 40 °C for 18 h with ^{32}P-labeled oligonucleotide probes. The membrane was then washed once in 5 × SSPE−0.1% SDS at 50 °C for 15 min and twice in 2 × SSPE−0.1% SDS at 50 °C for 15 min. The washed membrane was exposed to an Imaging Plate and analyzed by BAS3000 (FUJIFILM, Tokyo).

2.5. Primer extension

Oligonucleotides labeled with Texas Red by using a 5′ oligonucleotide Texas Red labeling kit (Amersham LIFE SCIENCE) according to the manufacturer's protocol were used as primers for the primer extension experiments. The primers had parts of each cDNA sequence, except for CeR-9. Since the cloned cDNA of CeR-9 was short, the primer was designed to have the 20-bp downstream sequence of the cDNA. The RNA from 41% 2-propanol precipitates (10 μg) was incubated at 65 °C for 10 min and immediately cooled on ice for 5 min. Reverse transcription was performed with RAV-2 reverse transcriptase (Takara Biochemicals) according to the manufacturer's protocol. Electrophoresis was performed on the 6% polyacrylamide/6.1 M urea denaturing gel at 1400 V for 8 h by using an automatic DNA sequencer (DNA sequencer SQ-5500, Hitachi). Sequencing of the *C. elegans* genomic DNA clones including each RNA coding region was performed with the same primers as those used for the primer extension reaction to determine the 5′end nucleotide sequences of the extension products. The genomic DNA clones were prepared by cloning PCR products of about 800 bp including the cDNA sequence, an upstream region of about 300 bp and a downstream region of about 300 bp of the cDNA sequence.

2.6. Search for target RNAs of CeR RNAs

Probable uridine targets of CeR-3, 4, 6, 7, 8 and 9 RNAs, which were H/ACA snoRNAs, were searched against *C. elegans* rRNAs, U snRNAs or SL RNAs. A bipartite antisense element of at least 9 nt against the CeR RNA

Table 1
Eight novel *C. elegans* small RNAs encoded in the introns of protein genes

RNA	cDNA length (bp)	Probable RNA size (nt)	Chromosome	Host gene	Homolog of host gene product	Host gene of *C. elegans* paralog[#]	Host gene of *C. briggsae* ortholog	Notes	Accession number
CeR-3	77	135	I	F53G12.10 intron 2	Ribosomal protein L30	C16A3.9 intron 2	CBG03905 (FPC0071) intron 2	H/ACA snoRNA	AB125752
CeR-4	83	132	III	R151.3 intron 1	Ribosomal protein L6	F28D1.7 intron 2	CBG16622 (FPC4044) intron 1	H/ACA snoRNA	AB125753
CeR-5	86	128	IV	Y38F2AR.12 intron 4	5-oxoprolinase	–	–(FPC4044) antisense		AB125754
CeR-6	96	128	III	B0412.4 intron 1	Ribosomal protein S29	B0412.4 intron 2	CBG15290 (FPC4010) intron 2	H/ACA snoRNA	AB125755
CeR-7	93	136	V	K07C5.4 intron 6	Nop56p	–	CBG09590 (FPC2220) intron 6	H/ACA snoRNA	AB125756
CeR-8	56	135	III	C16A3.9 intron 2	Ribosomal protein S13	F53G12.10 intron 2	CBG19687 (FPC4132) intron 2	H/ACA snoRNA	AB125757
CeR-9	33	137	V	W08G11.3 intron 4	*S. cerevisiae* Mlp2p	–	CBG22811 (–) intron 3	H/ACA snoRNA	AB125758
CeR-19	69	106	V	D1054.3 intron 2	*Oryza sativa* SGT1	–	CBG09701 (G45F20) intron 2	C/DsnoRNA	AB125759

[#] "–" indicates that no paralogous gene is found in the database.

internal loop, which was located at 13–16 nt upstream from box H or ACA, was searched for using the computer software program DNASIS ver. 3.5 (Hitachi Software Engineering). The bipartite duplex between the target RNA and CeR RNA was made up by two stems of at least 3 bp flanking the targeted uridine and the adjacent downstream nucleotide.

Probable methylation targets of CeR-19 RNA, which was a C/D snoRNA, were searched against *C. elegans* rRNAs, U snRNAs or SL RNAs by detecting the antisense elements of at least 9 nt against the upstream sequences of D and D′ boxes using the computer software program DNASIS ver. 3.5.

3. Results and discussion

3.1. Isolation of small ncRNAs

Total RNA prepared from mixed-stage *C. elegans* cells was fractionated according to size by stepwise addition of 2-propanol to precipitate RNAs. RNAs, mostly larger than 500-nt length, were precipitated by the first 2-propanol precipitation (final concentration of 2-propanol being 33%). RNAs of about 100 to 1500 nt in length were precipitated by the second 2-propanol precipitation (final concentration of 2-propanol being 41%). The 41% 2-propanol precipitates were fractionated by denaturing polyacrylamide gel electrophoresis and stained with ethidium bromide. Fifty-four RNA bands of about 100 to 1000 nt in length were detected in addition to those of 5.8S rRNA, 5S rRNA and tRNAs. The RNA extracted from each band was used to prepare cDNA clones. Five cDNA clones for each RNA band were randomly selected to determine their sequences. A computer homology search for cDNA sequences was performed using BLAST. The sequences of 106 cDNA clones were found in the *C. elegans* genomic DNA. Of the 106 cDNA clones, 85 had parts of nuclear or mitochondrion rRNA sequences. One cDNA clone included a part of the SRP RNA sequence, and another included a part of the U1 snRNA sequence. The other 19 cDNA sequences corresponded to *C. elegans* genome sequences that have not been predicted as known ncRNA genes, suggesting that these cDNAs were synthesized from novel RNAs, although the possibility that some are degradation products of mRNAs cannot be excluded.

The 19 novel RNA genes were located in various regions on the genome. One corresponded to the intergenic region, which had not been predicted to be protein or ncRNA coding region, and eight corresponded to the introns of protein-coding genes. Seven clones were parts of the exon sequences of protein genes. Two cDNA sequences

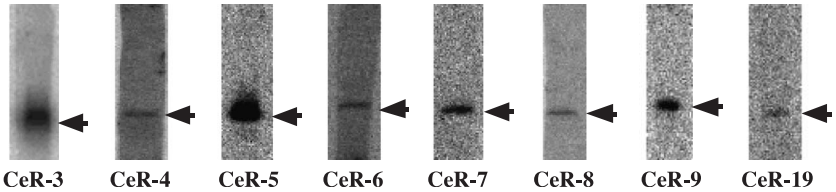

Fig. 1. Expression of CeR RNAs. Northern hybridization of the 41% 2-propanol precipitates of *C. elegans* RNA with oligonucleotide probes that have parts of complementary sequences of CeR-3, 4, 5, 6, 7, 8, 9 and 19. Arrows indicate signals.

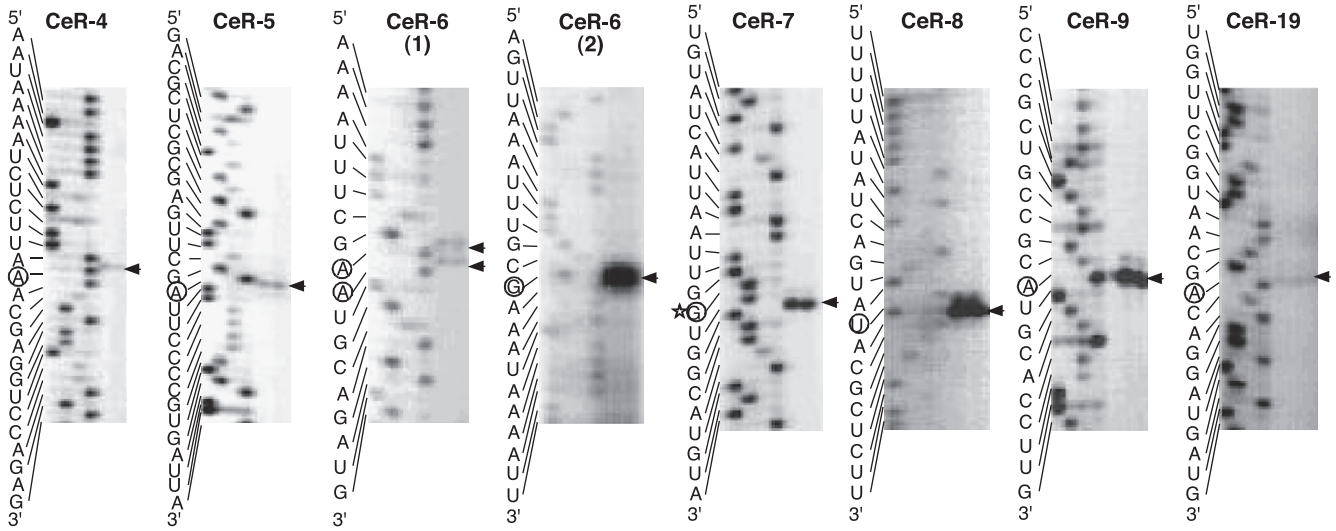

Fig. 2. Determination of the 5′end nucleotide sequences of CeR RNAs. Primer extension experiments were performed to determine the 5′end nucleotide sequences of CeR-4, 5, 6, 7, 8, 9 and 19. The sequences are shown at the left of each panel. The 5′end nucleotides are circled. Arrows indicate the primer extension products. The 5′end nucleotide sequence of CeR-7 is "G" as indicated by an asterisk, but the corresponding nucleotide of the K07C5.4 intron 6 in the database is "A" as shown by a closed triangle in Fig. 4 CeR-7.

contained both intron and exon sequences, and one contained both intergenic and exon sequences. Since many small ncRNAs, such as human snoRNAs, are encoded in introns of protein-coding genes, we further analyzed eight small ncRNA candidates, designated CeR-3, CeR-4, CeR-5, CeR-6, CeR-7, CeR-8, CeR-9 and CeR-19 RNA, the genes of which are located in the intron sequences of protein-coding genes. Table 1 shows the 8 CeR RNAs and their host genes.

3.2. Sequences of the eight CeR RNAs

To determine which strand of cDNAs encode the RNAs and to confirm their expressions in the cells, Northern hybridization was performed with two different probes for each RNA: a part of the cDNA sequence that corresponds to the intron sequence of the host protein-coding gene, and its antisense sequence. As expected, only probes having the antisense sequence of each intron showed a clear signal in each RNA (Fig. 1). The sequences of CeR-3 and CeR-8 cDNAs showed high similarity (43 nt out of 52 nt-cloned sequences [83 %] were identical), indicating that these RNAs are paralogous to each other. The sizes of all RNAs were similar, about 100 nt in length.

The 5′-end of each RNA was determined by primer extension experiments. As shown in Fig. 2, each RNA except for CeR-6 RNA exhibited a single extension product. For CeR-6 RNA, there were three possible 5′ end sequences: two weak signals at the successive nucleotide "A" positions (Fig. 2 CeR-6 (1)), and a strong signal at the nucleotide "G" located 56 bp downstream of the "A" positions (Fig. 2 CeR-6 (2)). Since cDNA of CeR-6 RNA includes 40-bp upstream sequences of nucleotide "G", the 5′ end of CeR-6 RNA may be nucleotide "A". Since nucleotide "G" is located at the 3′ end of the first hairpin region of the predicted secondary structure as discussed below (Fig. 4 CeR-6, shown by the open triangle), the reaction of the primer extension might stop at this position and give a strong signal.

The 3′-end of each RNA was estimated from the approximate size and the conserved nucleotide sequence in the introns encoding the RNA between *C. elegans* and *C. briggsae*.

Fig. 3. Comparison of the homologous intron sequences encoding CeR RNAs. The CeR RNA coding intron sequences were aligned with their paralogs and orthologs shown in Table 1. Closed triangles and open triangles indicate the 5′end sequences predicted by the primer extension experiment. Asterisks show the conserved nucleotides. The conserved region of each alignment is shown in a box. An underline shows the cloned cDNA sequence. Each box motif of H/ACA snoRNA and C/D snoRNA is shaded. CeF53G12.10I2, intron 2 of *C. elegans* F53G12.10 gene; CbFPC0071I2, intron 2 of *C. briggsae* CBG03905 gene; CeC16A3.9I2, intron 2 of *C. elegans* C16A3.9 gene; CbFPC4132I2, intron 2 of *C. briggsae* CBG19687 gene; CeR151.3I1, intron 1 of *C. elegans* R151.3 gene; CbFPC4044I1, intron 1 of *C. briggsae* CBG16622 gene; CeF28D1.7I2, intron 2 of *C. elegans* F28D1.7 gene; CbFPC0063I2, intron 2 of the F28D1.7 gene homolog identified in *C. briggsae* contig FPC0063; CeY38F2AR12I4, intron 4 of *C. elegans* Y38F2AR.12; CbFPC4044I5C, complementary sequence to intron 5 of the CeY38F2AR.12 gene homolog identified in *C. briggsae* contig FPC4044; CeB0412.4I1, intron 1 of *C. elegans* B0412.4 gene; CbFPC4010I2, intron 2 of *C. briggsae* CBG15290 gene; CeB0412.4I2, intron 2 of *C. elegans* B0412.4 gene; CeK07C5.4I6, intron 6 of *C. elegans* K07C5.4 gene; CbFPC2220I6, intron 6 of *C. briggsae* CBG09590 gene; CeW08G11.3I4, intron 4 of *C. elegans* W08G11.3 gene; CbCBG22811I3, intron 3 of *C. briggsae* CBG22811 gene; CeD1054.3I2, intron 2 of *C. elegans* D1054.3 gene; CbG45F20I2, intron 2 of the D1054.3 gene homolog identified in *C. briggsae* cosmid G45F20.


(CeR-3 and CeR-8)
CeF53G12.10I2
CbFPC0071I2
CeC16A3.9I2
CbFPC4132I2
(CeR-4)
CeR151.3I1
CbFPC4044I1
CeF28D1.7I2
CbFPC0063I2
(CeR-5)
CeY38F2AR12I4
CbFPC4044I5C
(CeR-6)
CeB0412.4I1
CbFPC4010I2
CeB0412.4I2
(CeR-7)
CeK07C5.4I6
CbFPC2220CI6
(CeR-9)
CeW08G11.3I4
CbCBG22811I3
(CeR-19)
CeD10543I2
CbG45F20I2

3.3. Sequence comparison with the C. briggsae homolog of each CeR RNA

Homologous host genes of the eight *C. elegans* CeR RNAs were found in the genome of *C. briggsae*, a closely related species of *C. elegans*. In the cases of CeR-3, 4, 7, 8 and 19, the RNA coding positions in the host genes were identical in the genomes of *C. elegans* and *C. briggsae*, while the situations were rather complicated for the other three cases. The corresponding sequence of CeR-5, of which the gene is located in the fourth intron of the Y38F2AR.12 gene, was found in the antisense strand of the position equivalent to the fifth intron of the homologous protein gene of *C. briggsae*. CeR-6 showed sequence similarity to both the first and the second introns of the B0412.4 gene in *C. elegans*. The identical sequence to CeR-6 RNA is present in the first intron of the B0412.4 gene of *C. elegans* and a very similar (77% identity) sequence is also present in the second intron of the same gene. On the other hand, only the second intron of the corresponding gene of *C. briggsae* showed sequence similarity to CeR-6. The CeR-9 sequence was found in the fourth intron of the *C. elegans* W08G11.3 gene, which is composed of eight exons and seven introns. The *C. briggsae* CBG22811 gene, which is an ortholog of the W08G11.3 gene, is composed of seven exons and six introns according to the data in WormBase (http://www.wormbase.org/). The homologous sequence of CeR-9 was found in the third intron of the CBG22811 gene.

The intron sequence in which each CeR RNA gene is located was aligned with its paralogous or orthologous gene sequences by using the computer program CLUSTALW (Fig. 3). The conserved region of each CeR RNA was about 100 to 140 nt in length (Fig. 3 and Table 1). The 5′ terminal sequence of each CeR RNA determined by the primer extension experiments was close to the 5′ end of the conserved region as shown by the closed triangles in Fig. 3.

3.4. Secondary structure predicted by the nucleotide sequence

In the conserved sequences of CeR-3, 4, 6, 7, 8 and 9 RNAs, characteristic H/ACA box motifs, box H (5′-ANANNA-3′) and box ACA (5′-ACA-3′), were found. The secondary structure of the conserved sequences of each RNA proposed by the computer program MFOLD also revealed a typical box H/ACA snoRNA secondary structure (Fig. 4). These RNAs are the first H/ACA box-type snoRNAs reported in *C. elegans*. CeR-3 and CeR-8 showed several compensatory changes in the stem regions to keep their secondary structures similar (Fig. 4, CeR-3 and CeR-8).

We also found typical C/D snoRNA structures in CeR-19 RNA: box C (5′-RUGAUGA-3′) and box D (5′-CUGA-3′) motifs, a stem structure composed of the upstream sequence of box C and the downstream sequence of box D, and box C′ (5′-RUGAUGA-3′) and box D′ (5′-CUGA-3′) motifs between box C and D motifs (Fig. 3, shaded region of each alignment).

CeR-5 RNA did not show any primary or secondary structural similarity to other RNAs reported so far in the databases, indicating that this is a novel eukaryotic RNA.

3.5. Target of CeR RNAs

Many snoRNAs function in the modification of rRNAs and U snRNAs by forming base pairs with target RNAs (Bachellerie and Cavaille, 1998; Ofengand and Fournier, 1998). Twelve potential base pairing interactions were detected between CeR RNAs and rRNAs (Fig. 5). These predicted 10 probable pseudouridylation sites and two methylation sites in rRNAs. Although only seven base pairings were detected between the internal loop of CeR-9 RNA and the neighbor of U2977 of 26S rRNA, we also included this interaction in the list. This is because the position corresponding to U2977 of *C. elegans* 26S rRNA in the 28S rRNA of several other organisms are modified to pseudouridine, indicating that this modification is evolutionarily conserved (Ofengand and Fournier, 1998). We cannot exclude the possibility that another unidentified *C. elegans* snoRNA guides the psuedouridilation of this position. The positions corresponding to U1058 of *C. elegans* 26S rRNA, the region of which makes base parings with CeR-7 RNA, are also modified in several other organisms (Ofengand and Fournier, 1998).

Target sequences of CeR-3, CeR-4 and CeR-8 RNAs could not be found in the sequence of *C. elegans* rRNAs, U snRNAs or SL RNAs. Recently, many snoRNAs with unknown targets have been found in other organisms (Hüttenhofer et al., 2002). Some snoRNAs may have functions other than modification of rRNAs and U snRNAs.

3.6. Location of CeR RNA genes

The locations of the eight CeR RNA genes are shown in Table 1. Four RNAs, CeR-3, 4, 6 and 8 RNAs, are encoded in the regions corresponding to the introns of the ribosomal protein genes, *rpl-30* (F53G12.10 gene), *rpl-6* (R151.3 gene), *rps-29* (B0412.4 gene) and *rps-13* (C16A3.9 gene), respectively. The CeR-4 RNA sequence is also found in the second intron of the F28D1.7 gene encoding the ribosomal protein S23 (*rps-23*). A sequence identical to that of CeR-6 RNA locates in the first intron of *rps-29*; the second intron of *rps-29* also has sequence homologous to CeR-6 RNA. The CeR-7 sequence is present in the gene encoding Nop56p, a protein component of box C/D snoRNP. CeR-5, 9 and 19 RNA genes are located in the introns of the Y38F2AR.12, W08G11.3 and D1054.3 genes, which are similar to the genes encoding *M. musculus* 5-oxoprolinase, *S. cerevisiae* Mlp2p and *O. sativa* SGT1, respectively. At present, the products of these three genes are not known to be related to the RNA-

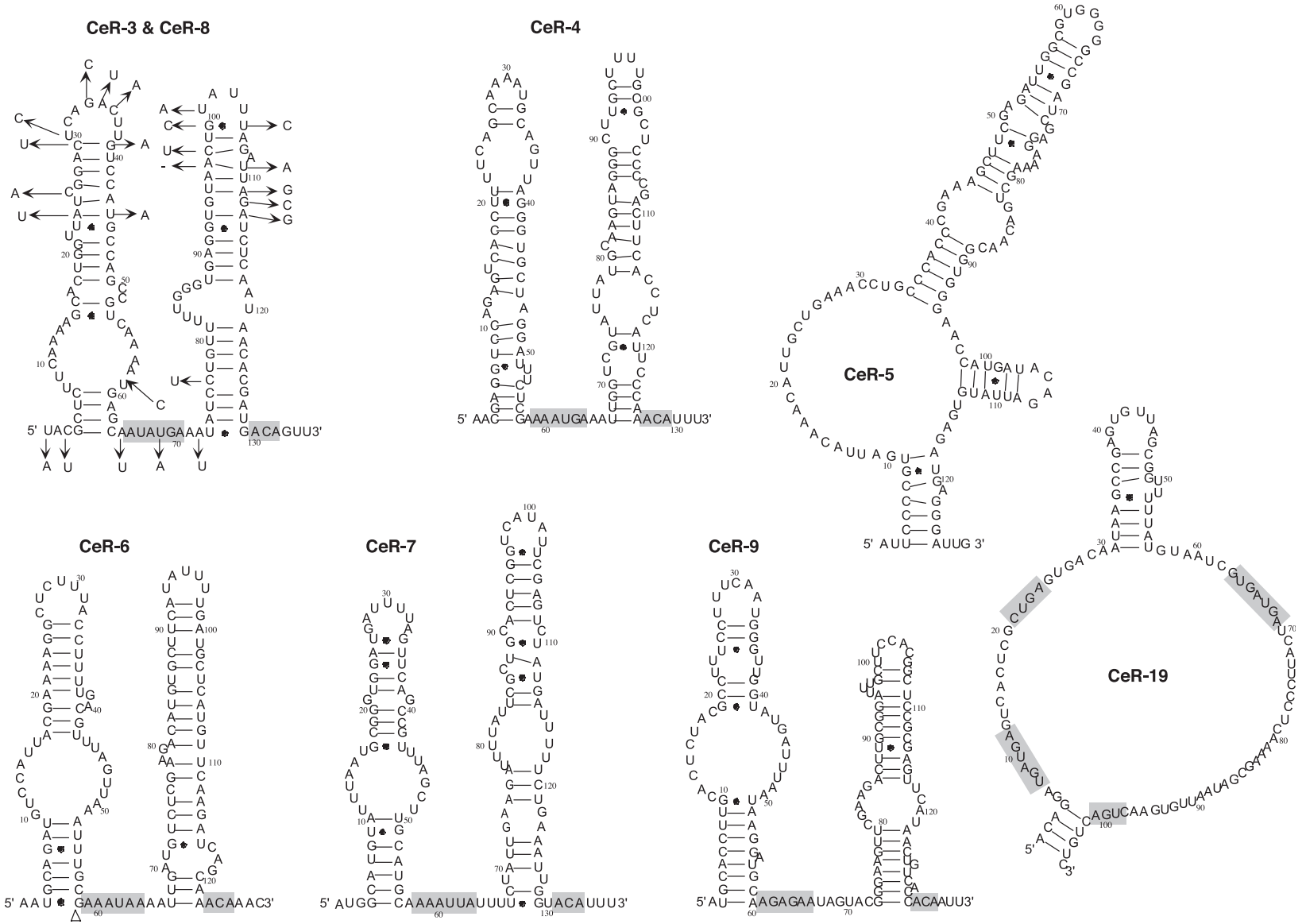

Fig. 4. Predicted secondary structures of eight CeR RNAs. Box motifs for CeR-3 and 8 and for CeR-4, 6, 7, 9 and 19 RNAs, which were predicted to be snoRNAs, are shaded. Since CeR-3 andCeR-8 RNAs are homologous, the secondary structure predicted from the CeR-8 RNA sequence is shown, and the different nucleotides of CeR-3 RNA are indicated by arrows. " – " shows a deletion. An open triangle in CeR-6 RNA reveals the corresponding nucleotide "G" of the signal in the primer extension experiment shown in Fig. 3 CeR-6 (2).

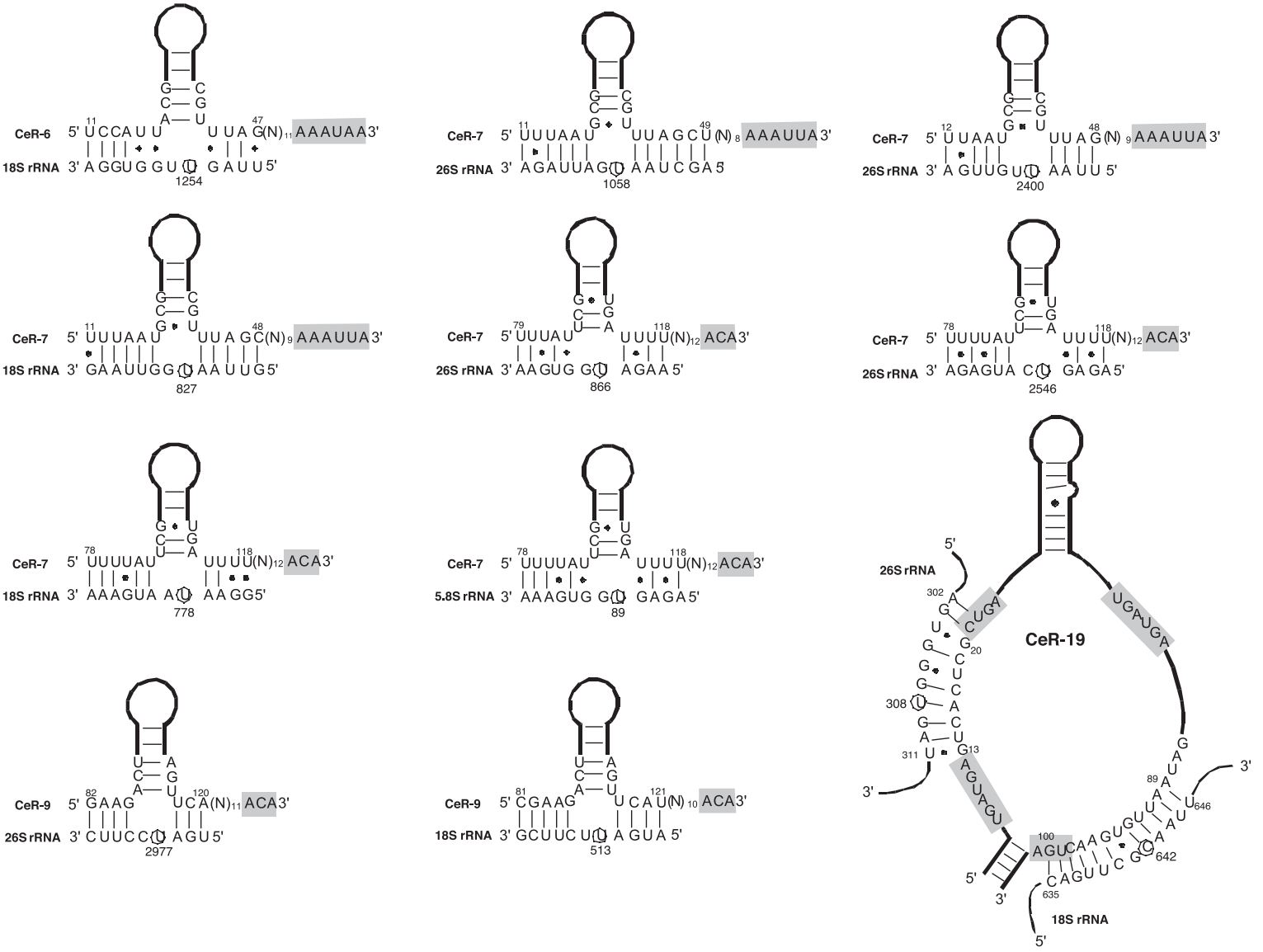

Fig. 5. Probable CeR RNA target nucleotides of rRNAs. Potential base pairings between CeR-6, CeR-7 or CeR-9 RNA and rRNAs. Circles indicate the U nucleotides that are probably modified to pseudouridines or nucleotides with 2′-*O*-methylation. Numbers show the position of each nucleotide in CeR RNAs or rRNAs.

mediated phenomena, in contrast to the host gene products of CeR-3, 4, 6, 7 and 8 RNAs, which are known to function in the translation—or translation-related machineries. Since the *C. briggsae* CeR-5 RNA gene is located in the intergenic region of the genome, it might have an independent promoter. It would be interesting to determine whether *C. elegans* CeR-5 RNA is also transcribed from its own promoter although its gene exists in the intron region of the protein-coding gene. Most *S. cerevisiae* snoRNA genes are monocistronic, while many vertebrate snoRNAs so far identified are encoded in the introns of protein- or non-protein-coding genes (Maxwell and Fournier, 1995; Hirose and Steitz, 2001). In *Drosophila*, most snoRNAs identified so far are encoded in the introns of protein-coding genes (Yuan et al., 2003). It is also known that many snoRNA genes are encoded in the introns of ribosomal protein genes (Sollner-Webb, 1993). In addition to CeR RNAs, there are 18 C/D snoRNA genes in *C. elegans* (Higa et al., 2002). Ten of these C/D snoRNA genes are located in the introns of the protein-coding genes, 5 are in the intergenic regions, and 3 correspond to parts of exons of the protein-coding genes The various coding strategies of snoRNAs in different organisms suggest that snoRNA genes are frequently amplified and moved among genomes during evolution. Our results suggest that there exist more ncRNAs in cells of *C. elegans*.

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References

Ambros, V., Lee, R.C., Lavanway, A., Williams, P.T., Jewell, D., 2003. MicroRNAs and other tiny endogenous RNAs in *C. elegans*. Curr. Biol. 13, 807–818.

Bachellerie, J.-P., Cavaille, J., 1998. Small nucleolar RNAs guide the ribose methylations of eukaryotic rRNAs. In: Henri Grosjean, R.B. (Ed.), Modification and Editing of RNA. ASM Press, Washington, DC, pp. 255–272.

Bartel, D.P., 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Blumenthal, T., Steward, K., 1997. RNA processing and gene structure. In: Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, J.R. (Eds.), *C. elegans*, vol. II. Cold Spring Harbor Laboratory Press, Plainview, New York, pp. 117–145.

Carrington, J.C., Ambros, V., 2003. Role of microRNAs in plant and animal development. Science 301, 336–338.

Eddy, S.R., 2001. Non-coding RNA genes and the modern RNA world. Nat. Rev., Genet. 2, 919–929.

Gottesman, S., 2002. Stealth regulation: biological circuits with small RNA switches. Genes Dev. 16, 2829–2842.

Grad, Y., Aach, J., Hayes, G.D., Reinhart, B.J., Church, G.M., Ruvkun, G., Kim, J., 2003. Computational and experimental identification of *C. elegans* microRNAs. Mol. Cell 11, 1253–1263.

Higa, S., Maeda, N., Kenmochi, N., Tanaka, T., 2002. Location of 2(ζ)-*O*-methyl nucleotides in 26S rRNA and methylation guide snoRNAs in *Caenorhabditis elegans*. Biochem. Biophys. Res. Commun. 297, 1344–1349.

Hirose, T., Steitz, J.A., 2001. Position within the host intron is critical for efficient processing of box C/D snoRNAs in mammalian cells. Proc. Natl. Acad. Sci. U. S. A. 98, 12914–12919.

Hüttenhofer, A., Kiefmann, M., Meier-Ewert, S., O'Brien, J., Lehrach, H., Bachellerie, J.P., Brosius, J., 2001. RNomics: an experimental approach that identifies 201 candidates for novel, small, non-messenger RNAs in mouse. EMBO J. 20, 2943–2953.

Hüttenhofer, A., Brosius, J., Bachellerie, J.P., 2002. RNomics: identification and function of small, non-messenger RNAs. Curr. Opin. Chem. Biol. 6, 835–843.

Kiss, T., 2002. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell 109, 145–148.

Labbe, J.C., Burgess, J., Rokeach, L.A., Hekimi, S., 2000. ROP-1, an RNA quality-control pathway component, affects *Caenorhabditis elegans* dauer formation. Proc. Natl. Acad. Sci. U. S. A. 97, 13233–13238.

Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B., Bartel, D.P., 2003. The microRNAs of *Caenorhabditis elegans*. Genes Dev. 17, 991–1008.

Marker, C., Zemann, A., Terhorst, T., Kiefmann, M., Kastenmayer, J.P., Green, P., Bachellerie, J.P., Brosius, J., Hüttenhofer, A., 2002. Experimental RNomics: identification of 140 candidates for small non-messenger RNAs in the plant *Arabidopsis thaliana*. Curr. Biol. 12, 2002–2013.

Maxwell, E.S., Fournier, M.J., 1995. The small nucleolar RNAs. Ann. Rev. Biochem. 64, 897–934.

Moss, E.G., 2000. Non-coding RNA's: lightning strikes twice. Curr. Biol. 10, R436–R439.

Moss, E.G., 2002. MicroRNAs: hidden in the genome. Curr. Biol. 12, R138–R140.

Nguyen, V.T., 2001. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. Nature 414, 322–325.

Ofengand, J., Fournier, M.J., 1998. The pseudouridine residues of rRNA: number, location, biosynthesis, and function. In: Henri Grosjean, R.B. (Ed.), Modification and Editing of RNA. ASM press, Washington, DC, pp. 229–253.

Ridanpää, M., van Eenennaam, H., Pelin, K., Chadwick, R., Johnson, C., Yuan, B., vanVenrooij, W., Pruijn, G., Salmela, R., Rockas, S., Mäkitie, O., Kaitila, I., de la Chapelle, A., 2001. Mutations in the RNA component of RNase MRP cause a pleiotropic human disease, cartilage-hair hypoplasia. Cell 104, 195–203.

Sambrook, J., Russell, D.W., Irwin, N., Janssen, K.A., 2001. Northern Hybridization. In: Argentine, J., Irwin, N., Janssen, K.A., McInery, N., Brown, D., Schaefer, S. (Eds.), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 7.42–7.45.

Sollner-Webb, B., 1993. Novel intron-encoded small nucleolar RNAs. Cell 75, 403–405.

Stirnagle, T., 1999. Maintenance of *C. elegans*. In: Hope, I.A. (Ed.), *C. elegans*. Oxford Univ. Press, Oxford, pp. 53–59.

Storz, G., 2002. An expanding universe of noncoding RNAs. Science 296, 1260–1263.

The *C. elegans* Sequencing Consortium, 1998. Genome sequence of the

nematode *C. elegans*: a platform for investigating biology. Science 282, 2012–2018.

Yuan, G., Klambt, C., Bachellerie, J.P., Brosius, J., Hüttenhofer, A., 2003. RNomics in *Drosophila melanogaster*: identification of 66 candidates for novel non-messenger RNAs. Nucleic Acids Res. 31, 2495–2507.

Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Penta, A.D., Reis, S., Oostra, B., Bagni, C., 2003. The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. Cell 112, 317–327.