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A novel experimental approach for systematic identification of box H/ACA snoRNAs from eukaryotes

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ABSTRACT

Box H/ACA snoRNAs represent an abundant group of small non-coding RNAs mainly involved in the pseudouridylation of rRNAs and/or snRNAs in eukaryotes and Archaea. In this study, we describe a novel experimental method for systematic identification of box H/ACA snoRNAs from eukaryotes. In the specialized cDNA libraries constructed by this method with total cellular RNAs from human blood cells, the high efficiency of cloning for diverse box H/ACA snoRNAs was achieved and seven novel species of this snoRNA family were identified from human for the first time. Furthermore, the novel method has been successfully applied for the identification of the box H/ACA snoRNAs from *Drosophila* and the fission yeast, demonstrating a powerful ability for systematic analysis of box H/ACA snoRNAs in a broad spectrum of eukaryotes.

INTRODUCTION

The small nucleolar RNAs (snoRNAs) represent an abundant family of small non-coding RNAs in both eukaryotes and Archaea (1–3). Except for RNase MRP, the snoRNAs are divided into two classes, box C/D snoRNAs and box H/ACA snoRNAs based on the conserved motifs and structural features (4). Box C/D snoRNAs share two conserved motifs, the 5′ end box C (RUGAUGA) and the 3′ end box D (CUGA), whereas the box H/ACA snoRNAs exhibit a common hairpin–hinge–hairpin-tail secondary structure with the H (ANANNA) motif in the single-stranded hinge region and an ACA triplet located 3 nt upstream of the 3′ termini (5). Several snoRNAs, such as U3, snR30, U8, U17 and RNase MRP, are required for specific cleavage of pre-rRNAs (6,7). However, the majority of known snoRNAs play important roles in the post-transcriptional modification of rRNAs and snRNAs. Box C/D snoRNAs serve as guides for site-specific 2′-O-ribose methylation while box H/ACA snoRNAs direct the conversion of uridine to pseudouridine at specific residue of rRNAs or snRNAs (8,9). In addition to rRNA and snRNA targets, snoRNAs or their homologs are involved in the methylation of other cellular RNAs such as tRNA in Archaea (10). Remarkably, snoRNAs may act on mRNA and play a role in the regulation of RNA editing (11). With the increasing number of snoRNAs, especially orphan snoRNAs, identified from various organisms, the high diversity, both in genomic organization and function, of snoRNAs are demonstrated and are far more complex than has been anticipated (12–16).

Now there are two major methods for large-scale search of snoRNAs, i.e. computational and experimental approaches. The box C/D snoRNAs possess conserved motifs such as box C and D, and 10–21 nt complementarity to rRNAs or snRNAs, which enable the successful development of computational identification of C/D snoRNA guides from database on a genome-wide scale (17). However, the box H/ACA snoRNAs are so far identified mainly by experimental approaches owning to the less conserved motifs and shorter functional elements. The general strategy of experimental methods for the snoRNA identification is to construct various cDNA libraries encoding small RNA molecules. For instance, the experimental RNomics approach by size-fractioning total RNAs or nuclear RNAs was widely used and promoted a great advance for the genomic survey of various small RNAs in several model organisms (18–20). A more specific experimental approach for isolation of box H/ACA snoRNAs was performed by using co-immunoprecipitation that takes advantage of specifically association between box H/ACA snoRNAs and the nucleolar protein Gar1p (21,22). Nevertheless, the methods are either complicated and time-consuming or not specific for the box H/ACA snoRNA family.

In an attempt to develop a simple and specific method for the identification of box H/ACA snoRNAs, here we introduce a new strategy which was mainly based on the application of an anchored primer for the conserved triple nucleotides at the

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3′ termini of box H/ACA snoRNAs. This approach is easy to perform for identifying the target sequences from total cellular RNAs and has been successfully applied for systematic analysis of box H/ACA snoRNAs in different eukaryotes.

MATERIALS AND METHODS

Construction of cDNA libraries

The human blood cells were obtained from blood donor (NO. 10655956-01). Total cellular RNAs were isolated according to the guanidine thiocyanate–phenol–chloroform procedure described by Chomoczynski *et al*. (23). Of the total RNAs 30 μg were tailed with ATP by using poly(A) polymerase (Takara) and subsequently reverse-transcribed into the first-stranded cDNAs using [γ-^{32}P]dATP labeled anchor primer dT_{16}-TGT and AMV reverse transcriptase (Promega). The reaction mixture was size-fractioned on a denaturing 8% polyacrylamide gel (8 M urea and 1 × TBE buffer). cDNAs with sizes ranging from 120 to 185 nt and from 185 to 270 nt were excised and eluted from the gel in NES buffer (0.5 M NH_4Ac, 1 mM EDTA and 0.1% SDS). The selected cDNA was tailed with dGTP at the 3′ end by using terminal deoxynucleotidyl transferase (Takara). G-tailed cDNAs were then amplified by PCR with a forward primer dT_{23} carrying a HindIII restriction site and a reverse primer $polyC_{16}$ carrying a BamHI restriction site. Then amplified fragments sized 190–240 and 220–400 nt were excised and eluted from the gel in NES buffer, respectively. After being digested with HindIII and BamHI, the purified PCR products were inserted into the corresponding restriction sites of plasmid pUC18 and transformed into *Escherichia coli* DH5α as described previously (24).

DNA sequence analysis

The cDNA libraries were screened by PCR with the P47 and P48 universal primer pair. Only the recombinant plasmids carrying fragments in the range 50–500 bp were selected to sequence. Sequencing was performed with an automatic DNA sequencer (Applied Biosystems, 377) using the Big Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystens).

Genomic locations of the sequences from the cDNA library were analysed using the BLAST program from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). The secondary structures of the box H/ACA snoRNAs were analysed by an mfold program [(25), http://www.bioinfo.rpi.edu/applications/mfold/old/rna/].

Northern blot analysis

The probe was labeled with 5′ end [γ-^{32}P]dATP. An aliquot of 20 μg total RNA was separated by 8% polyacrylamide gel containing 8 M urea and electrotransferred onto nylon membrane (Hybond-N^+; Amersham), followed by UV light irradiation for 2 min. Prehybridization, hybridization and detection were carried out according to the recommended procedures of Roche Molecular Biochemicals. The membrane was prehybridized and hybridized in high-SDS concentration hybridization buffer at 42°C. The Nylon membrane was washed in 2 × SSPE solution (including 0.1% SDS) twice for 10 min at room temperature. Filters were exposed to a phosphor screen and analysed by the Typhoon 8600 variable mode imager.

Oligonucleotides

Oligonucleotides were synthesized and purified by Sangon Co. (Shanghai, China). The primers for reverse transcription: dT_{16}-TGT, 5′-TTTTTTTTTTTTTTTTNNNTGT-3′ (N points to any one of ATGC); dT_{23}, 5′-CCCCAAAAGCTTTTTTTT-TTTTTTTTTTTTTTT-3′. The following oligonucleotides were used in the construction and screening of the human cDNA libraries: dT_{23} and $polyC_{16}$, 5′-GGAATTCGGATCCC-CCCCCCCCCCCCC-3′; P47, 5′-CGCCAGGGTTTTCCCA-GTCACGAC-3′; P48, 5′-AGCGGATAACAATTTCACACA-GGA-3′. The following oligonucleotides were used for northern blot analyses of novel snoRNAs: P107, 5′-CCTGTTGG-GTGTGTTGTAAA-3′; P108, 5′-CCGTGT GGGAGCCAT-TCTCTGAT-3′; P109, 5′-TATGTTGGAGAAATATAC-3′.

RESULTS

Strategy for construction of a cDNA library enriched in box H/ACA snoRNAs with ACA-anchored primer

Box H/ACA snoRNAs only have characteristic secondary structure and short common motif sequences, which seems impossible to be oligonucleotide anchored effectively. But note that a conserved ACA motif exactly positions three nucleotides upstream of the 3′ termini of this snoRNA type. If extracted RNAs are tagged at the 3′ end and then submitted to reverse transcription using a special primer matching both the tag and the adjacent terminal nucleotides of the box H/ACA snoRNAs, the cDNA fraction for the target RNAs could be trapped and enriched in a cDNA library.

In this study, an original strategy to generate a specific cDNA library enriched in box H/ACA snoRNA was developed. First of all, total cellular RNAs were A-tailed and then reverse-transcribed into cDNAs employing primer dT_{16}-TGT, which ensures the preferential amplification of H/ACA snoRNAs whereas precludes the reverse transcription of other RNAs or fragments with poly(A) tails. The PCR products were size-fractioned from the gel according to the length of box H/ACA snoRNAs and then used to construct cDNA library. Thus the library enriched in ACA-tailed sequences could be obtained (Figure 1).

High efficiency of the approach for identifying diverse box H/ACA snoRNAs from human blood cells

By using the ACA-anchored primer, a special cDNA library (library I) encoding 140–160 nt RNAs was constructed with total cellular RNAs from human blood cells. A total of 115 clones from the library were randomly sequenced and ~64% of sequences were found to be specific for box H/ACA snoRNAs (Figure 2). Although some snoRNA sequences were overrepresented, 34 species of the snoRNAs were revealed, including 16 snoRNAs identified only once in this analyses (Supplementary Table 1). One third of cDNA sequences of the analysis were derived from the known snRNA, rRNA and mRNA fragments, respectively. Whereas, in the control library constructed with the same sized cDNAs and the oligo(dT) primer, an improved strategy derived from experimental

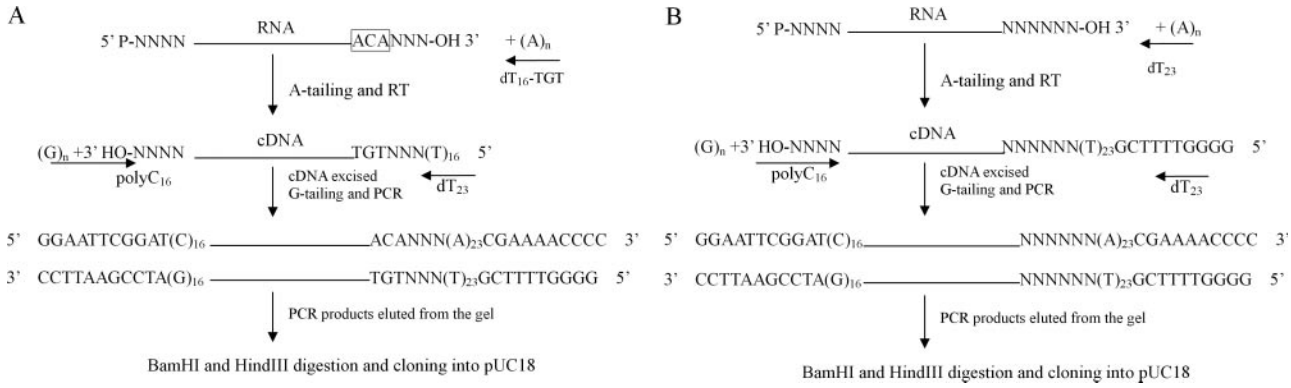

Figure 1. Strategy for construction of a specialized cDNA library enriched in box H/ACA snoRNAs. Total cellular RNAs were isolated and tailed with poly(A). The first cDNA strand was synthesized with anchor primer (**A**) or ordinary primer (**B**). The cDNA sized in a specific range was excised and eluted from the gel. Poly(G) tail was added to the 3′ end of the selected cDNA and then converted into double-stranded DNAs by PCR with two primers polyC16 and oligo(dT). The amplified DNA was digested, purified and cloned into a plasmid vector.

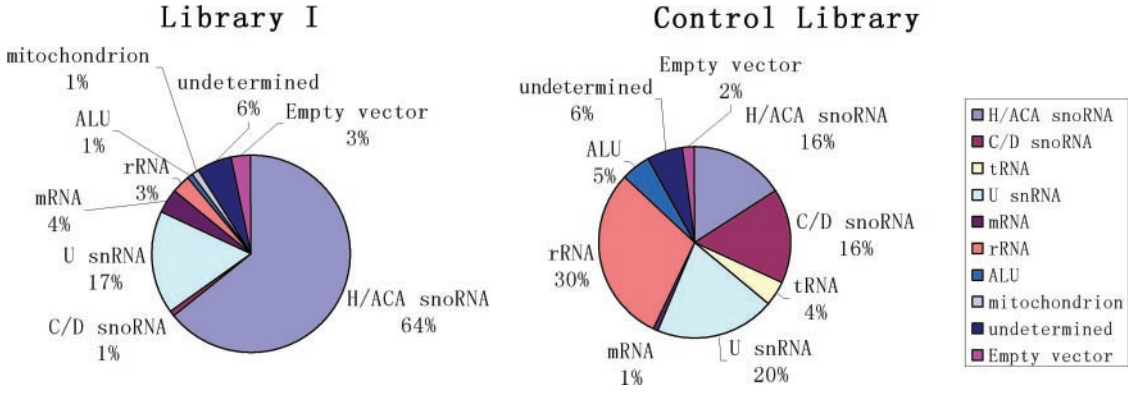

Figure 2. Sequence analysis of all chosen cDNA clones from human blood cell libraries. Library I, derived from cDNAs sized 120–185 nt with ACA-anchor primer; Control Library, cDNA derived from cDNAs sized 120–185 nt with ordinary primer. Clones representing different RNA categories are shown as percentage of total clones.

RNomics (18,26), a high percentage of contamination of abundant cellular RNA fragments was observed and only 16% of the sequences could be assigned to box H/ACA snoRNAs (Figure 2).

Considering that some box H/ACA snoRNAs such as U17 are much longer than usual, we also constructed another ACA-anchored library (library II) with cDNA fractions sized 185–300 nt. In this library, ~19% of cDNA sequences could be assigned to box H/ACA snoRNAs and more clones were ascribed to the rRNA or mRNA fragments. However, our experiments demonstrated that <6% of the cDNA sequences were box H/ACA snoRNAs in the control library constructed with non-anchored primer.

From the analyses of 240 clones in the two anchored libraries, we identified 96 box H/ACA snoRNA sequences that represent 46 species of this RNA family. The majority of cDNA sequences were full-length box H/ACA snoRNAs, showing a high quality of the cloning approach. Notably, all box H/ACA snoRNAs presented in the control library were also determined in the anchored library except ACA25 that has a different AUA motif, other than ACA, at the 3′ end. The amount of the identified snoRNAs from the analyses represented more than half of the total known 85 box H/ACA snoRNAs in human cells. Evidently, a high efficiency of cloning for box H/ACA snoRNAs was achieved through the anchor primer with size-fraction strategy.

Identification of seven novel human box H/ACA snoRNAs

Seven new species among the 47 box H/ACA snoRNAs were experimentally identified from the human cells. Although the sequences were divergent to some extent, the four snoRNAs were recognized as functional homologs in mammals and so named after their counterpart as U19-2, ACA3-2, HBI-61 and HBI-80, respectively (Table 1). However, three novel H/ACA snoRNAs, named as U107, U108 and U109, were identified for the first time in mammals and then were further detected positively by northern blot (Figure 3). Remarkably, the three snoRNAs belong to different categories according to their structure and function. U109 is a typical guide RNA which was predicted to direct a known pseudouridylation of the sixth residue of U1 snRNA (Figure 4). The identification of U109 implies a complete list of box H/ACA snoRNA for the human U1 snRNA because there are only two pseudouridylated nucleotides in this small spliceosomal RNA. The adjacent Ψ5 is guided by ACA47 snoRNA which was identified in a large-scale of analysis previously (21). U108 was predicted to guide

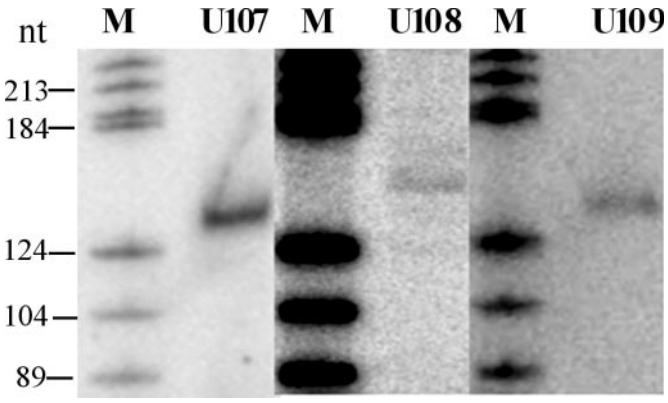

Figure 3. Positive detection of the three new box H/ACA snoRNAs by northern blot. Lane M, molecular weight markers (pBR322 digested with HaeIII and 5′ end labeled with [γ-^{32}P]ATP).

a pseudouridylation of 28S rRNA at U372, but the nucleotide has not been reported as a known pseudouridylated site in the human rRNAs. A possible role of chaperon function of U108 in 28S rRNA folding was suggested. In contrast to the two former, U107 does not possess any sequence complementarity to rRNAs and therefore represents an orphan snoRNA whose function remains to be elucidated.

All of the novel snoRNAs are intron-encoded by their host genes. The snoRNA gene variants are usually encoded by the same host genes, such as ACA3 and ACA3-2 which are located in the second and fourth intron of RPL27A, respectively. The three new isoforms of U107 were also found in the 10th intron of *Mage*D gene family, respectively. Intriguingly, U19-2 is intron-encoded by a protein-coding gene ATP6V0E while its isoform U19 was previously found in the intron of a non-protein-coding gene (27), demonstrating a different regulation for the expression of the snoRNAs in human.

Table 1. Seven novel human box H/ACA snoRNAs identified from the cDNA libraries

Clone name	SnoRNA name	Iso	Length (nt)	Exp	Modification	Antisense element	Homolog	Chr	Location	Accession no.
Hbc-1	U107	3	131	N blot	n.d.	n.d.	–	X	Intron 10 of MAGED2	AM055729
Hbc-2	U108	2	147	N blot	n.d.	7 + 3 nt (5′)	–	10	Intron 11 of CWF19L1	AM055730
Hbc-3	U109	2	134	N blot	U1-Ψ6	5 + 5 nt (3′)	–	5	Intron 1 of MGC23909	AM055742
Hbc-4	U19-2	3	204	cDNA	28S-Ψ3731	5 + 5 nt (5′)	U19	5	Intron 3 of ATP6V0E	AM055743
					28S-Ψ3733	6 + 4 nt (3′)				
Hbc-5	ACA3-2	2	131	cDNA	28S-ψ3889	7 + 3 nt (5′)	ACA3	11	Intron 4 of RPL27A	AM055744
Hbc-6	HBI-61	3	178	cDNA	n.d.	n.d.	MBI-61	3	Intron 3 of EIF2A	AM055745
Hbc-7	HBI-80	1	102	cDNA	18S-ψ1625	7 + 4 nt (3′)	MBI-80	7	Intron 3 of TBRG4	AM055746

Iso, numbers of isoforms; Len, length of the snoRNA gene; Exp, expression situation, cDNA, N blot, snoRNA was identified from a cDNA sequence or northern blotting analysis; Chr, chromosomal location of the snoRNA gene.

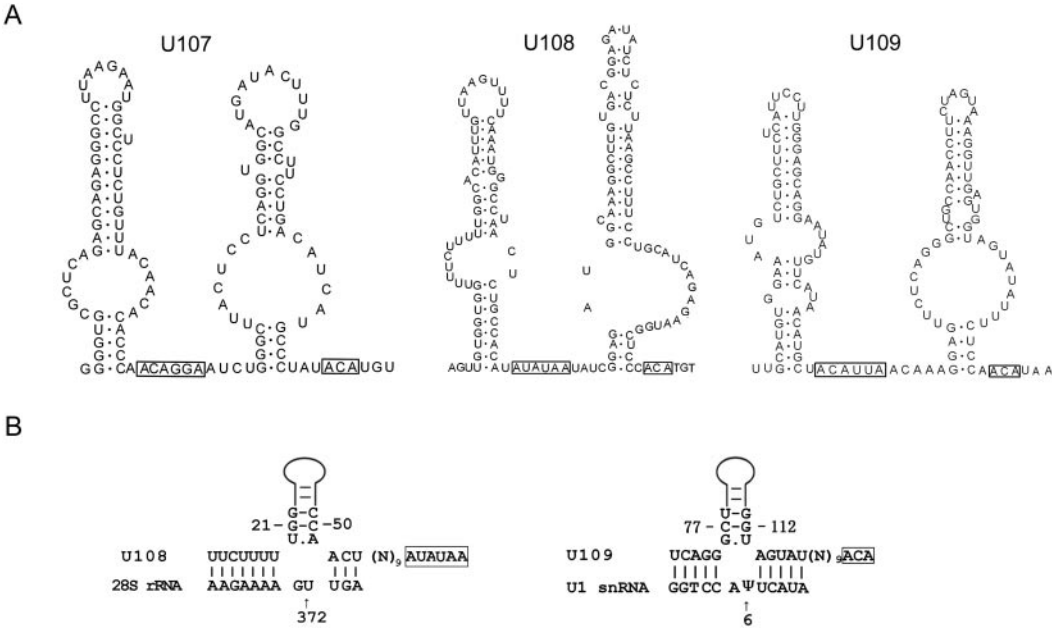

Figure 4. The structure and function of novel box H/ACA snoRNAs. (**A**) Proposed secondary structures for U107, U108, U109, the H and ACA motifs are boxed. (**B**) Predicted base-pairing of U108 with 28S rRNA; bipartite base-pairing interaction of the U109 (top strand) to their predicted target site U1-Ψ6 (bottom strand). The hairpin structural element in the guide RNA that interrupts the base-pairing interaction is shown. Arrows indicated predicted modified sites.

An approach for identification of box H/ACA snoRNAs from different eukaryotes

The approach was applied to analyse box H/ACA snoRNAs in another model eukaryotes such as *Drosophila melanogaster* and *Schizosaccharomyces pombe*. A primer-anchored library was constructed with total cellular RNAs from the adults of the fruit fly and the cDNAs used for cloning ranged in size from 120 to 180 nt. As expected, a high efficiency for trapping ACA-tailed RNAs was observed. The library contained 33% of the clones for ACA-tailed sequences and 17 different box H/ACA snoRNAs were identified from a random analysis of 100 clones (Supplementary Table 2). Similarly, in the yeast library, 39% of the clones belong to the ACA-tailed sequences and 17 different box H/ACA snoRNAs were characterized from a random sequencing of 163 clones (Supplementary Table 3). Although a limited screening of the libraries by DNA sequencing, the amount of the snoRNAs identified from the analyses represented an important percentage of the known box H/ACA snoRNAs in these organisms according to our update collection (14,28).

Obviously, the strategy does work well in distant eukaryotes owing to the phylogenetic conservation of the ACA motif in this snoRNA family. The method appears suitable for systematic screening of box H/ACA snoRNAs from a broad spectrum of eukaryotes even if the genome data of these organisms are still not available.

DISCUSSION

In this study, we developed a novel experimental approach for box H/ACA snoRNA identification by using the ACA-anchored primer with size-fractioning technique. The approach can produce a specific cDNA library enriched in ACA-tailed sequences, the contamination from abundant cellular RNAs, especially small RNAs such as 5.8S and 5S rRNAs that have a similar size to the snoRNAs, was efficiently precluded at the first step of reverse transcription. As compared with the previously reported methods (18,21), the novel approach is obviously simpler, but with higher efficiency for the detection of a specific family of snoRNA. For example, the cDNA libraries constructed by the standard approach of experimental RNomics contained only 3% clones for snmRNA candidates in mouse and 6.3% in the fruit fly before selection (18,20). While our libraries contained 64, 33 and 39% of the clones for the box H/ACA snoRNAs in human, *Drosophila* and the fission yeast, respectively. Furthermore, since the approach can easily be used in different organisms, it provides us with a powerful tool for systematic analysis of box H/ACA snoRNAs in eukaryotes and even Archaea in which box H/ACA snoRNA homologs for rRNA pseudouridylation have been reported (29,30). It is worth noting that not all box H/ACA snoRNAs possess an exact ACA motif at 3′ end, e.g. ~7 and 3% of the snoRNAs exhibit variation of the triplet such as AUA or AGA in human and the fruit fly, respectively. For an overall analysis, therefore, it is feasible to design an additional AUA- and AGA-anchored primer for trapping the snoRNAs with the varied motifs.

Recently, computational analysis of the box H/ACA snoRNAs has made great progress. Two computational programs, i.e. MFE and snoGPS, which were based on the sequence and secondary structure of the snoRNAs with minimum free-energy structure stability or a deterministic search algorithm, were developed to screen the H/ACA snoRNAs in genomic databases (31,32). With the increasing snoRNA candidates predicted in silicon, highly efficient experiment methods will be required for their confirmation. On the other hand, owing to the diversity in structure and function of snoRNAs, the computational methods seem insufficient for identifying novel box H/ACA snoRNAs with unusual sequence motifs or structures, especially orphan or chimeric snoRNAs, while experimental method will provide a better solution for obtaining a variety of box H/ACA snoRNAs in eukaryotes. Thus, it would be important to combine the novel experimental approach with the computational analyses for the identification of the box H/ACA snoRNAs on genome-wide scale from novel organisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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