



## Short communication

## A 5'–3' terminal stem in small non-coding RNAs extends their lifetime

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## ABSTRACT

4.5SI and 4.5SH are two non-coding RNAs about 100 nt long, synthesized by RNA polymerase III in cells of various rodents including mice, rats, and hamsters. The first RNA is long-lived whereas the half-life of the second is only 20 min. We previously found that the 16 bp double-stranded structure (stem), formed by 4.5SI RNA termini, contributes essentially to the long lifetime of this RNA (Koval et al., 2012). The rapid decay of 4.5SH RNA seems to be related to the lack of a similar structure in this RNA. The aim of this work was to verify whether the lifetime of any other short-lived non-coding RNA can be prolonged following creation of the double-stranded structure with its terminal regions. Here RNAs transcribed by RNA polymerase III from short interspersed elements (SINEs) B2 and Rhin-1 from the genomes of mouse and horseshoe bat, respectively, were used. Replacement of 16 nt at the 3'-terminal region by the sequence complementary to the 5' end region of B2 and Rhin-1 RNA increased their half-life more than 4 fold. In addition, we demonstrated that shortening of the terminal stem from 16 to 8 bp decreased only slightly the 4.5SI RNA lifetime. Finally, we showed that the disruption of an internal (non-terminal) stem in 4.5SI RNA did not accelerate its decay in cells. Possible mechanisms of the small non-coding RNA lifetime extension are discussed.

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## 1. Introduction

The intensity of RNA synthesis and rate of RNA decay, as well as regulation of these two processes, define RNA level in cell. RNA metabolism rate can be directly related to the RNA function in the cell. For example, mRNAs of many proteins involved in regulation of cell proliferation are intensively synthesized and rapidly decayed. Rapid decay of mRNA is usually due to AU- and GU-rich elements in 3'-untranslated regions (Chen and Shyu, 1995; Khabar, 2010; Vlasova-St Louis and Bohjanen, 2011). On the contrary, many long-living mRNAs contain stabilizing elements, for instance, HNRPA2B1 protein-binding ones (Goodarzi et al., 2012). A multi-protein complex, exosome, degrades mRNAs starting at the 3' end, as well as cleaves RNAs at sites within the molecule (Schmid and Jensen, 2008). In another way of mRNA decay, exoribonucleases (XRN) are involved in 5' to 3' mRNA degradation (Parker, 2012).

The same degradation machineries are involved in the non-coding RNA decay. These processes were mainly studied for ncRNA molecules with defects (nucleotide substitutions, deficiency of nucleotide modifications, and misfolding) (Wichtowska et al., 2013). The defects result in decreased stability of RNA secondary and tertiary structures that can be the signal for marking of the aberrant RNA molecules. For example, the TRAMP complex added poly(A) at the 3' end of various defective

ncRNAs for targeting to the nuclear exosome (Schmidt and Butler, 2013). There is one more marking mechanism for some aberrant tRNAs: synthesis of an additional CCA at the 3' end leading to degradation of tRNA (Wilusz et al., 2011).

Unlike aberrant ncRNAs, regulation of degradation of normal ncRNA remains poorly studied. Furthermore, until recently structural features of normal small ncRNAs that determine their lifetime remain uninvestigated. 4.5SI and 4.5SH RNAs comprise a convenient model for study of this problem, because they have many similar features but differ dramatically in their lifetime. 4.5SI RNA is a long-living transcript, whereas 4.5SH RNA half-life is only 20 min (Koval et al., 2012). Both these abundant nuclear RNAs are about 100 nt in length, synthesized by RNA-polymerase III, transcribed in different tissues, and typical only for Muridae-related rodent families (Ro-Choi et al., 1972; Harada and Kato, 1980; Gogolevskaya and Kramerov, 2002; Gogolevskaya et al., 2005). The role of these enigmatic RNAs remains largely unclear. Study of the rapid decay of 4.5SH RNA and stability of 4.5SI RNA could be a key to the elucidation of their cellular functions. The absence of these two RNAs in other mammals makes them a convenient object for the study of decay processes when their genes are introduced into heterologous cells. For example, after transfection of human cells, the mouse 4.5SI and 4.5SH RNAs can be easily monitored in these cells because they lack the corresponding endogenous RNAs.

Recently, using transfection of HeLa cells by original, chimeric or modified 4.5SH/4.5SI RNA genes we showed that the complementarity of 16-nt end regions of 4.5SI RNA proved to contribute to its stability in cells, whereas the lack of such complementarity in 4.5SH RNA caused its rapid decay (Koval et al., 2012). On the other hand, it was found that

Abbreviations: SINEs, short interspersed elements; pol III, RNA polymerase III; ncRNA, non-coding RNA; TRAMP complex, Trf4/Air2/Mtr4p Polyadenylation complex.

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tRNAs and tRNA-like small RNAs with unstable acceptor and T-stems were selectively marked by cell for their degradation (Whipple et al., 2011; Wilusz et al., 2011). Here we studied: (i) whether the terminal stem formed by complementary ends of the RNA influences the lifetime of other small RNAs (SINE transcripts), (ii) whether a short (8 bp) terminal stem can prevent the fast degradation of 4.5SI RNA in cell and (iii) if other (internal) stems contribute to the 4.5SI RNA lifetime.

## 2. Materials and methods

### 2.1. Cells cultivation, transfection, inhibition of transcription, RNA isolation

HeLa cells were grown to 80% confluency in 60 mm Petri dishes using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. Cells were transiently transfected with 4.5 µg of plasmid DNA using TurboFect Transfection Reagent (Thermo Scientific) following the manufacturer's protocol. Actinomycin D (5 µg/ml) was added to cells 20 h post-transfection. Then total cellular RNA was isolated in different time periods (0 to 180 min) using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Following ethanol precipitation, RNA was treated with 100 µg/ml RNase-free DNase I at 37 °C for 30 min and purified by chloroform extraction. Ethanol precipitated RNA was dissolved in 40 µl of 0.1% SDS and its concentration was measured by NanoDrop 1000 Spectrophotometer.

### 2.2. Plasmid constructs

Mouse 4.5SI RNA gene Mmu1' construct (Gogolevskaya and Kramerov, 2010) and its derivatives had an 87 bp genomic upstream sequence. Nucleotide sequences of 4.5SI RNA gene were changed by PCR. In the case of constructs L, M, and N two-round PCR was used. The 4.5SI RNA constructs (L, M, N, and O) were named according to the nomenclature used in Koval et al., 2012.

Construct B2-pA<sub>0</sub> contained a mouse B2 SINE copy Mmu14 (Acc. No: K00132.1) with two substitutions (T → C at 3' region) that were introduced to inactivate poly(A) signals (Borodulina and Kramerov, 2008). Rhin1-pA<sub>0</sub> construct carried a horseshoe bat Rhin1 SINE copy (Acc. No: K1165832.1; positions: 16,504–16,739) with an inactivated poly(A) signal. In both cases poly(A) signals were affected to prevent polyadenylation of the SINE transcripts (the polyadenylated transcripts have a long lifetime and are heterogeneous in length). B2-pA<sub>0</sub> and Rhin1-pA<sub>0</sub> plasmids were kindly provided by Olga Borodulina. In constructs B2-compl and Rhin1-compl, 3' end regions of SINEs were replaced by sequences complementary to their 5'-end regions using PCR.

The list of PCR primers is shown in Supplementary Table 1. PCR products were cloned into pGEM-T plasmid (Promega) and constructs obtained were subsequently used for transfection experiments. All constructs were sequenced in order to avoid nucleotide substitutions introduced during PCR. Plasmid DNA for cell transfection was isolated with NucleoBond kit (Macherey-Nagel).

### 2.3. Northern blot analysis

Equal amounts of cellular RNA were separated by electrophoresis in denaturing 6% polyacrylamide gel and transferred onto Hybond-XL membrane (GE Healthcare UK Ltd., Buckinghamshire, England) by semidry electroblotting at 5 V for 1.5 h. The B2, Rhin-1, 4.5SI RNA and their derivatives were detected by hybridization with <sup>32</sup>P-labeled probes obtained by PCR (Gogolevskaya and Kramerov, 2002). Nucleotide sequences of the probes and the primers used are presented in Supplementary Fig. 1. The blot was incubated overnight with a probe in the solution containing 50% formamide, 5× Denhardt solution, 4× SSC, 1% SDS, and 0.1 mg/mL salmon sperm DNA at 42 °C. The membrane was washed in 0.1× SSC–0.1% SDS at 42 °C for 1 h, exposed against an X-ray film, and scanned by Cyclone phosphorimager.

### 2.4. Computer analysis

Secondary structure was predicted for small RNAs using the mFold 3.2 web server with default parameters (Zuker, 2003). The best prediction was used.

## 3. Results

### 3.1. Generation of 5'–3' terminal stem in SINE transcripts significantly increases their lifetime

SINEs are non-autonomous mobile genetic elements 100–500 bp long (Okada, 1991; Kramerov and Vassetzky, 2011; Vassetzky and Kramerov, 2013). Mammalian genomes contain multiple (10<sup>4</sup>–10<sup>6</sup>) copies of a SINE species. These genetic elements are transcribed by RNA polymerase III due to an internal bipartite promoter (Paolella et al., 1983; Kramerov et al., 1990; Borodulina and Kramerov, 2008). We transfected HeLa cells with plasmids containing an individual copy of SINE B2 from the mouse (*Mus musculus*) genome (Krayev et al., 1982) or SINE Rhin-1 from the horseshoe bat (*Rhinolophus ferrumequinum*) genome (Borodulina and Kramerov, 2005) and found that the lifetime of their pol III transcripts is quite short:  $t_{1/2} \sim 22$  min (Fig. 1). Constructs of B2 and Rhin-1 SINEs with 3' end regions replaced by 16-nt sequences complementary to their 5' ends were generated and transfected into HeLa cells. Half-lives of transcripts of these B2 and Rhin-1 SINE derivatives in the cells were estimated at 120 and 90 min, respectively (Fig. 1). Thus, a terminal stem can prolong lifetime of different small RNAs.

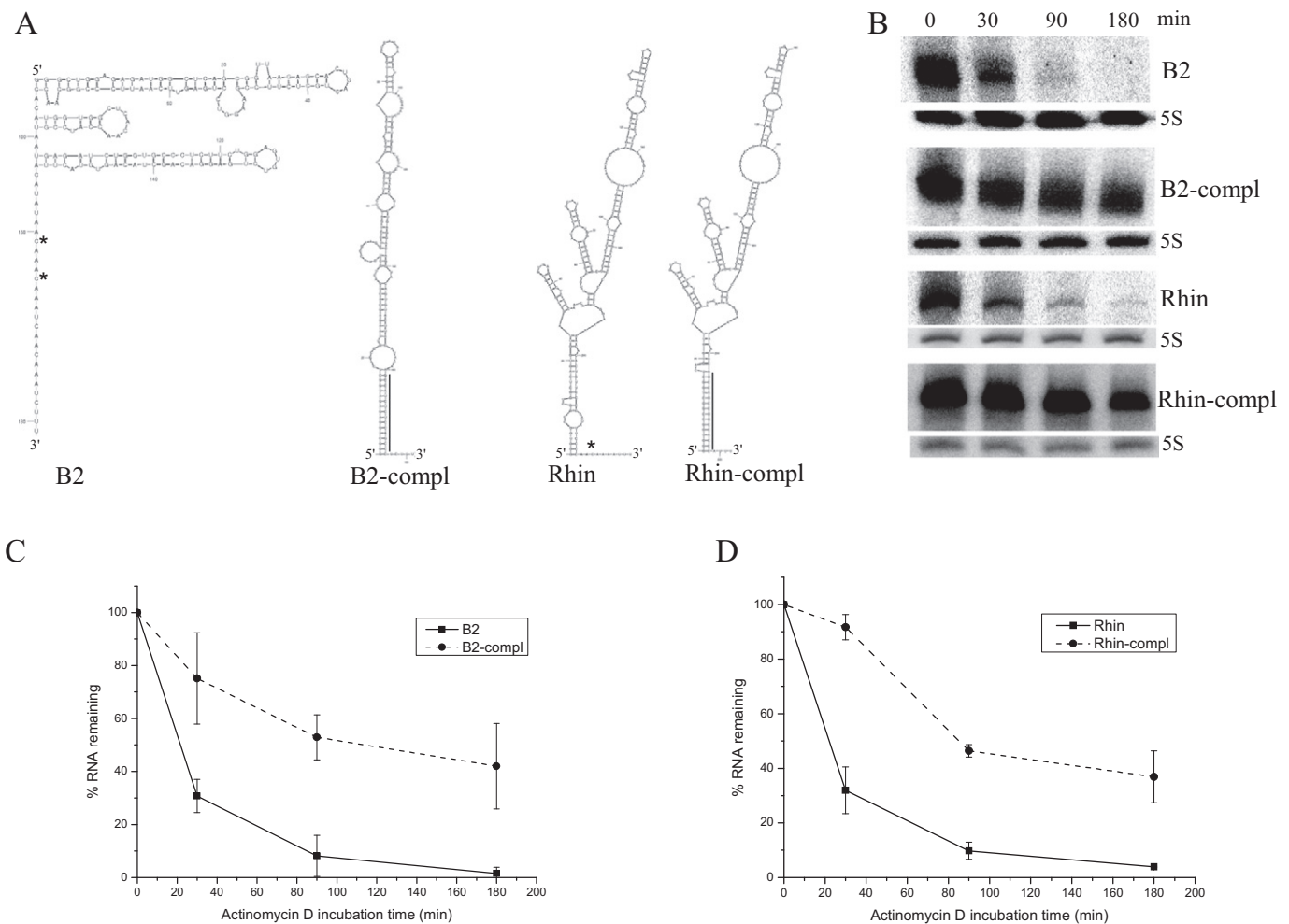
### 3.2. Shortening of 5'–3' terminal stem decreases slightly 4.5SI RNA lifetime

Some types of small RNAs are known to have 5'–3' terminal stems: tRNAs (Juhling et al., 2009), 5S RNA (Szymanski et al., 2003), Y RNAs (Hall et al., 2013), and C/D box snoRNA (Deschamps-Francoeur et al., 2014). However, these stems are usually quite short (7–12 bp). This prompted us to investigate whether shortening the terminal stem in 4.5SI RNA from 16 to 8 bp (Fig. 2) could affect its lifespan. An appropriate construct was obtained: the octanucleotide GGCTGTCT was replaced by CCGACAGA in the 3' region of 4.5SI RNA gene; the construct was introduced into HeLa cells. We found that the modified 4.5SI RNA had  $t_{1/2} = 1.5$  h. Although this value is lower than the half-life of the original 4.5SI RNA (3 h), it is essentially higher than that of 4.5SI RNA with disrupted end complementarity (20 min) (Fig. 2). Thus, the short 5'–3' terminal stem also can protect small RNA from rapid decay in HeLa cells.

### 3.3. Internal stem does not influence 4.5SI RNA lifetime

Besides the long terminal stem, 4.5SI RNA forms two short hairpins in its central region (Fig. 2). Stem II was chosen for this study (Fig. 2), and we generated three constructs by replacing stem-forming tetranucleotides by other sequences. One of the two tetranucleotides was replaced in constructs L and M, respectively, that resulted in the disruption of stem II (Fig. 2A). Both these tetranucleotides were replaced in construct N that recovered the hairpin. These constructs, as well as the original 4.5SI RNA gene, were introduced into HeLa cells and lifetimes of their transcripts were measured (Fig. 2). It was found that the difference between half-life times of analyzed transcripts (approx. 2–3 h) was statistically insignificant. Thus, stem II apparently was not important for 4.5SI RNA lifespan.

It should be noted that we earlier presumed that original 4.5SI RNA had the same stability in human cells (HeLa) as in mouse cells (Koval et al., 2012). However, the experiments described above showed that half-life time of original 4.5SI RNA in transfected HeLa cells was about 3 h. It is considerably lower than the lifespan of endogenous 4.5SI RNA in the mouse cells ( $t_{1/2} \sim 22$  h) (Koval et al., 2012). This distinction between 4.5SI RNA lifetimes in human and mouse cells might be explained



**Fig. 1.** 5'-3' terminal stem extended SINE transcript lifetime. (A) Predicted secondary structures of RNA transcribed from SINE B2, SINE Rhin-1, and their derivatives. Bold vertical lines nearby mark the changed sequences. Inactivated polyadenylation signals at 3' region of SINEs are marked by asterisks. See Supplementary Figs. 2–3 for RNA nucleotide sequences and detailed secondary structures. (B) Northern blot detection of RNA transcribed from the constructs B2, B2-compl, Rhin, and Rhin-compl in HeLa cells treated with actinomycin D. 5S rRNA was used as a loading control. (C) Decay kinetics of RNA transcribed from constructs B2 and B2-compl. Each graph is based on data from three transfection experiments (error bars, s.d.). (D) Decay kinetics of RNA transcribed from constructs Rhin and Rhin-compl. Each graph is based on data from three transfection experiments (error bars, s.d.).

by: (i) interaction with some mouse-specific proteins or (ii) difference in the activity of RNA-degradation machinery.

#### 4. Discussion

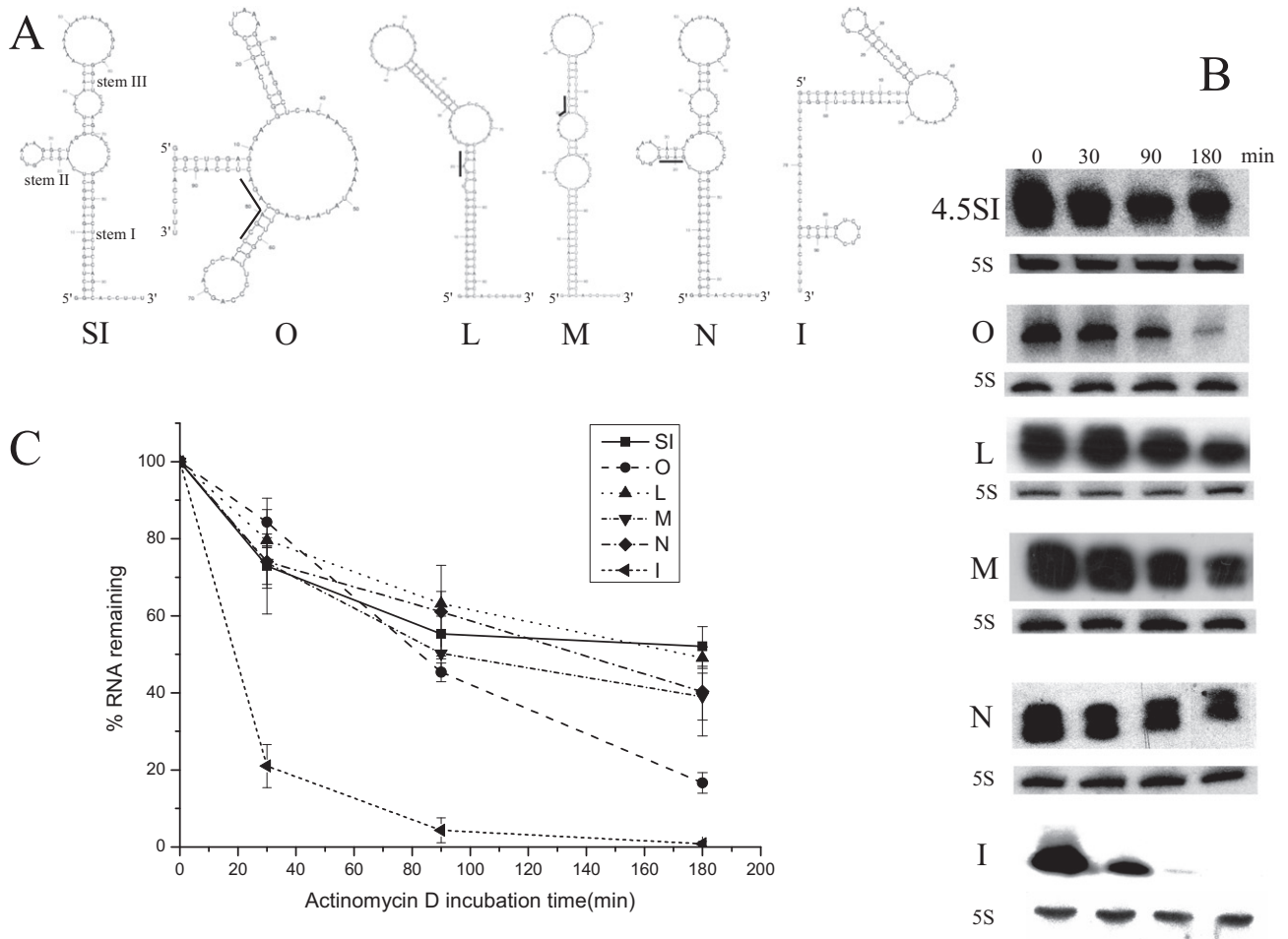
This study extends the previous work (Koval et al., 2012), where we showed that a stem formed by terminal regions of 4.5SI RNA significantly contributed to its lifetime in cells. Lack of a similar stem in 4.5SH RNA determined its short lifetime ( $t_{1/2} \sim 20$  min). It remained unclear whether this is a unique phenomenon or whether the described way of lifetime prolongation can be observed in other RNAs. It should be noted that abundant non-coding RNAs with short lifespan are very rare, although many non-abundant non-coding transcripts are short-lived (Tani et al., 2012; Clark et al., 2012). For our experiments, we chose RNAs transcribed by RNA polymerase III from SINEs B2 and Rhin-1. Previously we found that B2 RNA with inactivated polyadenylation signals decayed rapidly in cells (Borodulina and Kramerov, 2008). The same was true for Rhin-1 RNA (Fig. 1). It was demonstrated that the replacement of 16 nucleotides in the 3'-end region of B2 or Rhin-1 RNA by the nucleotide sequence complementary to 5'-end region of these RNAs resulted in the 4.1–4.5 fold prolongation of their half-life (Fig. 1). The length of B2 RNA (180 nt) and Rhin-1 RNA (230 nt) is significantly greater than that of 4.5SI (98 nt) and 4.5SH (94 nt) RNAs. These results showed that a 5'-3' terminal stem significantly increased the lifetime of these rather long RNAs. Thus,

the described phenomenon apparently is limited not only to 4.5SI RNA. We carried out the experiments only with pol III transcripts, however, we believe that a 5'-3' terminal stem could also expand the lifetime of pol II transcripts because of the significant similarity between mechanisms of pol II and pol III transcript degradation.

We also addressed another question: how long does a 5'-3' terminal stem need to be to sufficiently stabilize RNA in a cell? Here we showed that shortening the terminal stem from 16 to 8 bp also prevented the rapid decay of 4.5SI RNA in the cell ( $t_{1/2} = 90$  min). As there are various small non-coding RNAs containing short (about 8 bp) 5'-3' terminal stems (tRNA, 5S RNA, Y RNAs etc.), our data allow us to speculate that RNAs with terminal stems contributing to the elongation of their lifetime are not very rare.

Finally, it remained unclear whether non-terminal stems of 4.5SI RNA could influence its lifetime. It turned out that destruction of one of the two internal hairpins did not significantly change 4.5SI RNA half-life (Fig. 2). The result suggests the major role of the terminal stem rather than the internal one in the determining of 4.5SI RNA lifetime.

At least two mechanisms could determine the stability of non-defective small ncRNA in cells. The majority of non-defective small RNA is long-lived, probably, because they are included in complex RNPs (Noonberg et al., 1996; Kuglstatler et al., 2002; Gantier et al., 2011; Will and Luhrmann, 2011) where the RNA can be effectively protected by proteins (Labbe et al., 1999). Some other small RNAs



**Fig. 2.** The impact of the structural changes of 4.5SI RNA on its lifetime. (A) Predicted secondary structures of RNA transcribed from 4.5SI RNA gene and its derivatives (details in the text). The changed tetranucleotides are marked by bold lines nearby sequences. See Supplementary Figs. 4–5 for RNA nucleotide sequences and detailed secondary structures. (B) Northern blot detection of RNA transcribed from the 4.5SI RNA gene and its derivatives in HeLa cells treated with actinomycin D. 5S rRNA was used as a loading control. (C) Decay kinetics of RNA transcribed from the constructs. Each graph is based on data from three transfection experiments (error bars, s.d.). The construct I data are from Koval et al., 2012.

apparently are not permanently bound to proteins (e.g. tRNAs) or are part of small and simple RNPs, where RNA is poorly protected from degradation machine. The secondary structure of these RNAs could play an important role in their degradation rate. We believe that rapidly decayed 4.5SH, B2, and Rhin-1 RNAs belong to that type of transcripts. It is likely that if HeLa cells are transfected with an artificial scrambled RNA-expressed construct, the generated transcript would decay rapidly ( $t_{1/2} = 20$  min). However, we showed for 4.5SH and 4.5SI RNA (Koval et al., 2012), as well as for B2 and Rhin-1 RNA, that terminal stem can significantly prolong the lifetime of these RNAs.

Two different mechanisms of RNA lifetime prolongation mediated by the 5'–3' terminal stem can be suggested: (i) the stem is low-sensitive to nucleases; and (ii) the stem interacts with specific protein(s) protecting RNA molecule from degradation, which makes study of 4.5SI RNA-binding proteins rather important.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gene.2014.10.061>.

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