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The ribosomal protein rpl26 promoter is required for its 3′ sense terminus ncRNA transcription in *Schizosaccharomyces pombe*, implicating a new transcriptional mechanism for ncRNAs

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ABSTRACT

Transcriptome studies have revealed that many non-coding RNAs (ncRNAs) are located near the 3′ sense terminus of protein-coding genes. However, the transcription and function of these RNAs remain elusive. Here, we identify a 3′ sense termini-associated sRNA (TASR) downstream of rpl26 in *Schizosaccharomyces pombe* (*S. pombe*). Structure and function assays indicate that the TASR is an H/ACA box snoRNA required for 18S rRNA pseudouridylation at U121 and U305 sites and is therefore a cognate of snR49 from the budding yeast. Transcriptional studies show that pre-snR49 overlaps most of the coding sequence (CDS) of rpl26. Using scanning deletion analysis within promoter region, we show that the rpl26 promoter is required for the 3′ TASR transcription. Interestingly, chromosomal synteny of rpl26–snR49 is found in the *Schizosaccharomyces* groups. Taken together, we have revealed a new transcriptional mechanism for 3′ sense TASRs, which are transcribed by the same promoter as their upstream protein genes. These results further suggest that the origin and function of 3′ sense ncRNAs are associated with upstream genes in higher eukaryotes.

1. Introduction

Functional genomic strategies have revealed that eukaryotic genomes are almost entirely transcribed, generating abundant ncRNAs [1,2]. However, the origin and function of ncRNAs challenged previous views of basic gene expression [2]. The number of unexpected ncRNAs has led to the concept of 'pervasive' transcription [1–3]. A large number of ncRNAs were found to be located in different regions related to protein genes. Both sense and antisense promoter upstream transcripts (PROMPts) were produced and located approximately 0.5–2.5 kilobases upstream of active transcription start sites (TSSs) [4]. Transcription start site-associated RNAs (TSSa-RNAs) and promoter-associated sRNAs (PASRs) were generated within a short distance from the upstream and downstream TSSs [3,5–7]. ncRNAs were clustered at the 5′-untranslated region (UTR) boundaries of protein-coding genes and were also enriched at the 3′-UTR termini [3]. TASRs were abundant and less than 200 nt in length, and they were located at both the anti-sense and sense 3′ boundaries of annotated transcripts [3]. Recently, transcriptome studies in humans indicated that nearly 40% of these unannotated short RNAs were PASRs and TASRs [3,8]. Functional study of a 3′ sense terminus ncRNA indicated that it might be involved in regulation of gene transcription [9]. However, the function and transcription of 3′ sense TASRs remain largely unknown.

As a classical organism, *Schizosaccharomyces pombe* shares many similar features in molecular genetics with higher eukaryote cells, and it can be easily genetically manipulated [10]. Transcriptome and proteome studies of this organism suggested switch-like transcription of mRNAs during the cell cycle [11]. Single-nucleotide high-resolution transcriptomics in this species provided much condition-specific information regarding novel non-coding transcripts [12]. Comparative functional genomics of the *Schizosaccharomyces* groups identified 1097 putative ncRNA transcripts, of which 449 are intergenic and 648 are antisense [12–15]. Approximately 20% of these ncRNAs were hypothesized to be alternative UTRs for overlapping annotated UTRs on the same strand [13,16]. Currently, there are 1538 ncRNAs in PomBase which is a comprehensive database for *S. pombe* [16]. Among these ncRNAs, the majority have elusive functions and transcription, particularly the UTR overlapping transcripts.

In this study, we identify a functional H/ACA box snoRNA snR49, whose primary transcript overlaps most of the CDS of its upstream gene rpl26. Promoter deletion analyses prove that the rpl26 promoter is required for both itself and the downstream snR49 transcription. And computational analyses indicate that this

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mRNA-snoRNA organization is conserved in the *Schizosaccharomyces* species.

2. Materials and methods

The *S. pombe* sequences of snR49 and rpl26 have been deposited in GenBank under the accession numbers KF184994 and KF184995, respectively.

2.1. Strains and media

The *S. pombe* strain *h972*$^{-}$ was grown at 30 °C in rich YPD medium consisting of 2 g peptone, 1 g yeast extract and 2 g dextrose in 0.1 L distilled water. The *Escherichia coli* (*E. coli*) strain *TG1* was grown in either liquid or solid 2YT medium. It was then used for cloning procedures.

2.2. Northern blot and reverse transcription analyses

For the snoRNA northern blot, total cellular RNA was isolated by acid guanidinium thiocyanate [17]. Total cellular RNA (30 μg) was separated by electrophoresis on an 8% polyacrylamide/8 M urea gel, and then electrotransferred onto a nylon membrane (Hybond-N^{+}; Amersham Biosciences), followed by UV light irradiation for 5 min. Hybridization with 5′-end labeled probes was performed as previously described [18]. The membranes were exposed to a phosphor screen and analyzed by the Typhoon 8600 variable mode imager.

Reverse transcription was performed as previously reported [18]. The cDNA was excised from the gel and then tailed with dGTP using 7 U terminal deoxynucleotidyl transferase. The cDNAs were amplified using PCR with the poly(C) forward primer and reverse primer. The fragment was then cloned into the pTZ18 plasmid, and the resulting plasmids were used to transform *E. coli TG1*. After screening by filter hybridization, the sequences were determined.

2.3. Plasmid construction and gene deletion

The deletion of the snoRNA genes and promoter scanning deletion analyses were performed as previously described [18]. Briefly, the flanking sequences of the snoRNA genes were amplified with specific primers. After digestion with SacI/KpnI and SalI/SphI, the amplified fragments were cloned into the corresponding restriction sites of pTZ-19M. pTZ-19M is a pTZ19-derived plasmid and contains a 1.4-kb selectable marker module from pFA6-kanmx4 [19], which permits the efficient selection of transformants resistant to geneticin (G418). The plasmid was linearized and used to transform the haploid type yeast *h972*$^{-}$ using the lithium acetate procedure. Transformants were screened on selective plates with 200 mg/L G418. The deletion of chromosomal alleles was verified by PCR amplification of the genomic DNA with an appropriate primer pair. The snoRNA was detected by northern analysis with specific primers.

2.4. Pseudouridylation site mapping

Total cellular RNA was extracted using the hot acidic phenol method. The mapping of rRNA pseudouridylation sites was performed according to the CMC primer extension method [20]. Total cellular RNA (50 μg) was treated with 100 μl 0.17 μM CMC (N-cyclohexyl-N′-β-(4-methyl morpholinium) ethylcarbodiimide *p*-tosylate) at 37 °C for 20 min. Subsequently, the CMC-treated RNA was subjected to alkali hydrolysis in the presence of 50 mM Na_2CO_3 (pH 10.4) at 37 °C for 4 h. As a control, another 50 μg total RNA not treated with CMC was subjected to alkali hydrolysis under the same conditions as above.

Reverse transcription reactions were performed in the presence of 3–5 μg CMC-treated or untreated RNA and an appropriate primer labeled at the 5′-end with ^{32}P. The rDNA fragments from the *S. pombe* 18S rRNAs were inserted into the pMD18-T vector as described previously [18]. The plasmid DNA insert was directly sequenced with the same primer used for rRNA pseudouridylation mapping and run in parallel with the reverse transcription reactions on 8% polyacrylamide denaturing gels and then analyzed using the imager.

2.5. 3′ RACE, RT-PCR and real-time PCR

The 3′ RACE was performed according to the 3′ full RACE kit (Takara). The RT-PCR was performed after DNA contamination was removed with DNase I (Takara). Reverse transcription was performed according to the manufacturer's instructions (Promega), and the PCR was performed with a standard PCR reaction system (Takara). Reverse transcription for real-time PCR was performed with the RT Reagent Kit with gDNA Eraser (Takara). The PCR was then performed with MightyAmp® for Real Time (SYBR® Plus) (Takara).

2.6. Primer extension analysis for TSSs

Reverse transcription was performed with 1.5 mM dNTP concentration. Total cellular RNA (30 μg) and 20 ng primer labeled at the 5′-end with ^{32}P are required. After denaturation at 65 °C for 5 min and cooling to 42 °C, 200 U M-MLV reverse transcriptase (Promega) was added to the reactions (-RT: without reverse transcriptase) and extension was performed at 42 °C for 1 h. The DNA fragments across rpl26 and snR49 were inserted into the pMD18-T vector. The plasmid DNA insert was directly sequenced with the same primer used for the reverse transcription and run in parallel with the reverse transcription reactions on 8% polyacrylamide denaturing gels and then analyzed using the imager.

2.7. Primers and oligonucleotides

The sequences of the primers and oligonucleotides used in this study are listed in Table S1.

2.8. Computer analysis

Novel RNA sequences were confirmed by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). The secondary structures of the snoRNA were analyzed by RNA structure 4.3 [21]. The function of the H/ACA box snoRNA was predicted with the "snoGPS" [22]. Promoter motif analysis was aligned with the FMM learning and motif finder software (available at http://genie.weizmann.ac.il/) [23]. Sequences alignments were analyzed with clustalw2 software [24].

3. Results

3.1. Characterization of a novel 3′ sense termini ncRNA downstream of rpl26

The majority of ncRNAs in *S. pombe* are intergenic with unknown function. Interestingly, several ncRNAs are located near the 3′-UTR of protein-coding genes in the sense strand [16]. Among these, SPNCRNA.1444 is a predicted intergenic ncRNA with a length of 322 nt, located 131 nt downstream of the ribosomal protein gene rpl26 [13] (Fig. 1A). Using secondary structure analysis,

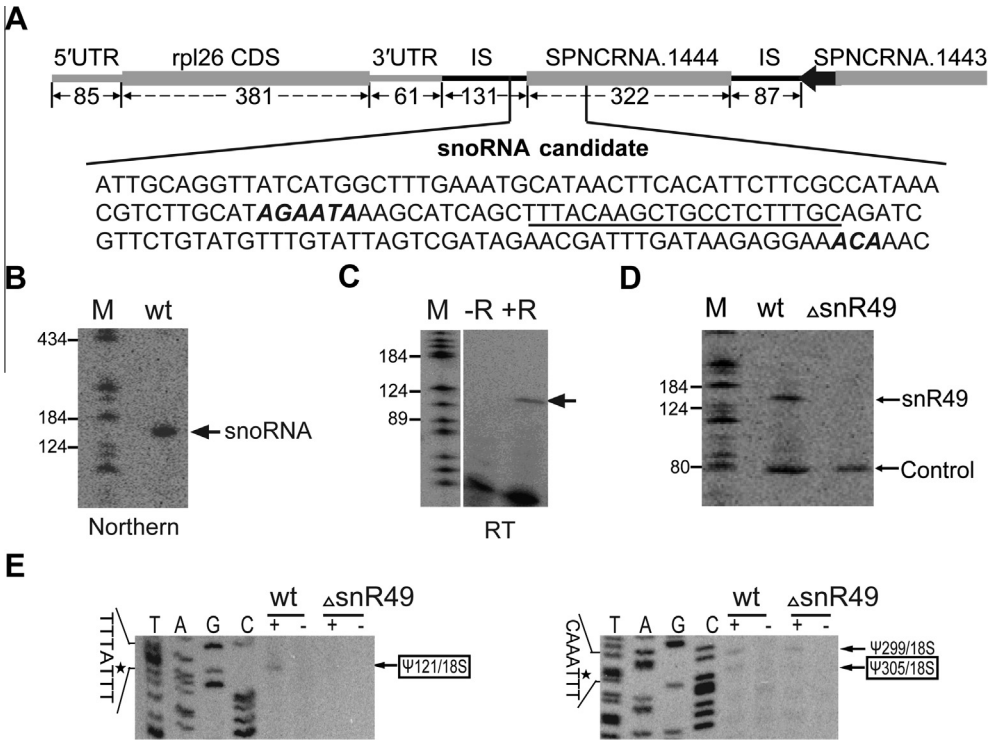

Fig. 1. Characterization of a novel 3′ sense termini ncRNA downstream of rpl26. (A) Gene loci and sequences of the rpl26 downstream ncRNA candidate. UTRs, IS, CDS and ncRNA are indicated with their lengths. IS: intergenic space. SPNCRNA.1444 represents a predicted ncRNA in PomBase. The novel predicted H/ACA box snoRNA is indicated with sequences. The H box and ACA box are indicated with italic capital letters. Primer used in northern blot and 5′-end labeled reverse transcription is underlined. (B) Positive detection of H/ACA box snoRNA candidate. M: DNA molecular weight marker is indicated with sizes. wt: wild-type strain. snoRNA: H/ACA box snoRNA candidate. (C) Sequence determination of the H/ACA box snoRNA candidate by 5′-end labeled reverse transcription. The cDNA of the snoRNA candidate is indicated with an arrow. Lane M: DNA molecular weight marker is indicated with sizes. +R: normal reverse transcription. −R: reverse transcription without reverse transcriptase. (D) Confirmation of snR49 deletion by northern blot. snR49 is indicated with its name and an arrow. Control: snR39. M: DNA marker with indicated lengths. wt: wild-type strain. △snR49: snR49 deletion strain. (E) rRNA pseudouridylation validation of snR49 by CMC primer extension assay. The *S. pombe* 18S rDNA molecular size ladder (T/A/G/C) was generated using the T7 Sequencing Kit from USB. wt: wild-type strain. △snR49: snR49 deletion strain. +: total RNA treated with CMC. −: total RNA treated without CMC. The modification sites guided by snR49 are indicated in both sequences with stars and arrows with boxes. A new modification site guided by unknown molecule is indicated with an arrow.

we found an H/ACA box snoRNA candidate that overlaps this ncRNA (Fig. 1A and Fig. S1).

To determine whether SPNCRNA.1444 and the H/ACA box snoRNA candidate exist, northern blot was performed with specific probe located within the common region of SPNCRNA.1444 and the H/ACA box snoRNA candidate. The result showed one sharp band, which length is between 124 and 184 nt, probably represents the H/ACA box snoRNA candidate. However, we did not detect the predicted SPNCRNA.1444 with a length of 322 nt (Fig. 1B).

Then we performed 5′-end labeled reverse transcription analysis to synthesize the cDNA of the snoRNA candidate (Fig. 1C). After cloning and sequencing the cDNA, we obtained the exact 5′ end sequence of this H/ACA box snoRNA candidate. For the 3′ end, the sequences were considered as ACA box plus 3 extra nucleotides. The results suggested that the snoRNA candidate is 156 nt long with 4 nt of its 5′ end hang out and the rest 152 nt overlaps SPNCRNA.1444 (Fig. 1A).

To determine whether this novel H/ACA box snoRNA is functional, we firstly predicted its function using snoGPS [22,25] and found that it can guide 18S rRNA pseudouridylation at U121 and U305. Pseudouridylation of those two sites are conserved and guided by snR49 in budding yeast (Fig. S2A) [26], so we named the newly identified H/ACA box snoRNA snR49 in *S. pombe* by homology.

Then we performed experiments to detect whether the modification sites guided by snR49 actually exist. We deleted the majority of snR49 sequences by homologous recombination, including both the H box and ACA box (Fig. S2B). Northern blot analysis demonstrated that snR49 was successfully deleted in △snR49 strain compared to the wild-type strain (Fig. 1D). Then we used primer extension assay to examine the rRNA pseudouridylation sites. The result showed the reverse transcription paused at U121 and U305 of 18S rRNA in the wild-type strain, while the corresponding modification sites disappeared in △snR49 strain (Fig. 1E). These results suggested that snR49 is a double-guide H/ACA box snoRNA which is required for pseudouridylation at U121 and U305 sites of 18S rRNA in *S. pombe*. Besides, we also identified a novel pseudouridylation site of 18S rRNA at U299, but no snoRNA was predicted for guiding pseudouridylation at this site (Fig. 1E).

Taken together, these results suggested that a novel functional H/ACA box snoRNA is located at the 3′ sense terminus of rpl26.

3.2. Analyses of rpl26 and snR49 transcripts

Previous studies of the H/ACA box snoRNAs in *S. pombe* indicated that the majority of snoRNAs are intergenic and independently transcribed [18]. Since snR49 is located at the 3′ sense terminus of rpl26 and no typical promoter between these two genes can be found, we hypothesize that the transcription of snR49 probably has connection with rpl26.

We used different pairs of primers in RT-PCR assay to define the boundaries of snR49 and rpl26 primary transcripts (Fig. 2A and Fig. S3). Firstly, the production of primers D5F5/D5R showed snR49 can be transcribed at least from CDS (D5F5 primer location) of rpl26. The slight band of RT-PCR with primers D3F8/D3R9 also

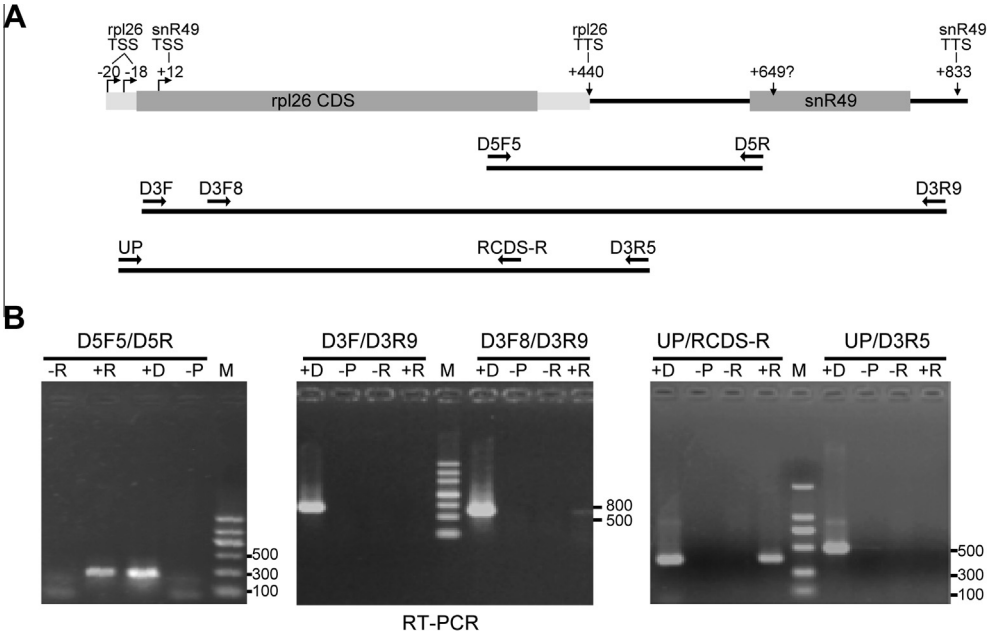

Fig. 2. Characterization of rpl26 and snR49 transcripts. (A) Schema of RT-PCR primers across rpl26–snR49. TSS: transcription start site. TTS: transcription terminating site. rpl26 TSSs: −20/−18. snR49 TSS: +12. rpl26 TTS: +440. snR49 TTS: +883. +649 is an unknown TTS. Amplified regions are indicated with lines and corresponding primer pairs. Detailed sequences are presented in Fig. S3. (B) Agarose gel analysis of RT-PCR products. cDNA was synthesized with oligodt18. D5F5/D5R, D3F/D3R9, D3F8/D3R9, UP/RCDS-R, UP/D3R5 represent different primer pairs used in PCR. −R: reverse transcription without reverse transcriptase. +R: normal reverse transcription. +D: PCR amplification with genomic DNA as template. −P: PCR amplification without template. M: DNA marker with indicated lengths.

suggested snR49 can be transcribed at least from CDS (D3F8 primer location) of rpl26, and stopped downstream of D3R9 primer location. However, there is not any comparable band observed when primers D3F/D3R9 were used (Fig. 2B), demonstrating that snR49 could not be transcribed with rpl26 from the upstream of rpl26 as a cluster, and probably the TSS of pre-snR49 is between D3F and D3F8 primer location. Meanwhile, a '+12' TSS site (located between D3F and D3F8) defined by 5′ primer extension analysis further suggested it might be the TSS of snR49 (Fig. S3 and Fig. S4A and B). Secondly, primers UP/RCDS-R and UP/D3R5 were used to detect how rpl26 transcribes from its own TSS. A clear band was showed with UP/RCDS-R while no band was showed with UP/D3R5 (Fig. 2B), indicating that rpl26 cannot transcribe bypass the D3R5 primer location. These results suggested that pre-snR49 and rpl26 have their own primary transcripts, although most of their sequences are overlapped.

We also used 5′ primer extension analysis and 3′ RACE to detect accurate boundaries of the rpl26 and snR49 primary transcripts (Fig. S4). Combined with the above RT-PCR assay, the results suggested the possible transcription start site (TSS) and transcription terminating site (TTS) of rpl26 are −18/−20 and +440, respectively; whereas those of pre-snR49 are +12 and +833, respectively (Fig. 2A and Fig. S3). Interestingly, a TTS (+649) within matured snR49 was found, which phenomenon has been reported but the mechanism is still unknown (Fig. S3 and Fig. S4C) [27].

3.3. The rpl26 promoter is essential for both itself and downstream snR49 transcription

Genome-wide studies of snoRNA promoters in fission yeast suggest that homol-D box is a conserved motif upstream of the majority of snoRNAs (unpublished data). Previous studies in fission yeast demonstrated that homol-D box is critical for both ribosomal proteins and U3 snoRNA transcription [28,29]. Given the TSSs of snR49 and rpl26 are different but closed and there are no evident homol-D motifs across rpl26–snR49, it's reasonable to suspect that the rpl26 promoter homol-D box might be responsible for snR49 transcription.

Promoter analysis suggested that there are three possible promoters upstream of rpl26: a TATA motif, a homol-D motif (TGTGACTG) and a homol-D-Like motif (CTGTCACA) (Fig. 3A). We deleted the upstream region of rpl26 from −226 to −31 using promoter scanning deletion. Real-time PCR analysis of rpl26 and snR49 in TATA box deleted strain (ΔH0) suggested that the TATA motif is not critical for their transcription. Whereas when the homol-D box was deleted (ΔH1), the transcriptional level of both rpl26 and snR49 significantly decreased. When the homol-D-Like motif was also deleted (ΔH2), both rpl26 and snR49 disappeared (Fig. 3B). Northern blot analyses of snR49 also suggested the homol-D motif and homol-D-Like motif are important for snR49 transcription, instead of TATA motif, which was consistent with the real-time PCR results (Fig. 3C). These results indicated that the rpl26 promoter is essential for both itself and snR49 transcription, implicating a novel transcriptional mechanism for ncRNAs.

3.4. Chromosomal synteny of rpl26–snR49 in Schizosaccharomyces groups

Genomic sequences and transcriptomics of the *Schizosaccharomyces* groups identified conserved and divergent features of gene structure and regulation in fission yeasts [13,30].

We found that snR49 was conserved in fission yeasts, despite several sequences missing between the H box and ACA box (Fig. 4A). Genomic organization of rpl26 and snR49 suggested they share synteny in fission yeasts. Analysis of promoter alignments upstream of rpl26 indicated that the homol-D sequences found in *S. pombe* are conserved in *Schizosaccharomyces* groups (Fig. 4B). We found that *S. japonica* has one more homol-D motif than other fission yeasts, and the homol-D-Like motif (CTGTCACA) was only found in *S. pombe*. Also, we found an unusual homol-D-Like motif (TGTGATTG) located between rpl26 and snR49 in *Schizosaccharomyces japonicus* (Fig. 4C).

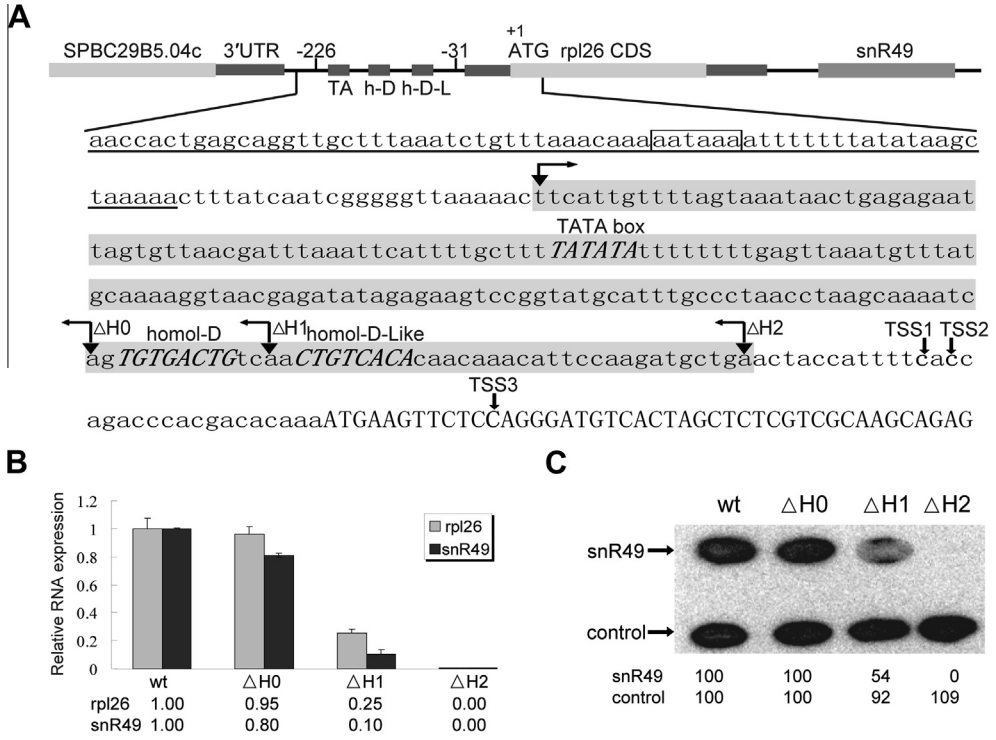

Fig. 3. Confirmation of snR49 promoter. (A) Promoter deletion schema. The deletion regions are shown with shadow (−226 to −31) and arrows of opposite directions. △H0 (TATA box deletion strain), △H1 (TATA box and homol-D box deletion strain), △H2 (TATA box, homol-D box and homol-D-Like box deletion strain). The TATA motif, homol-D motif, and homol-D-Like motif are indicated with italic capital letters. TA: TATA motif. h-D: homol-D motif. h-D-L: homol-D-Like motif. The TSSs are indicated with arrows. The SPBC29B5.04c's partial 3′ UTR is underlined, and its Poly(A) signal is boxed. The capital letters represent rpl26 CDS. (B) Real-time PCR of rpl26 and snR49. The relative expressions of rpl26 and snR49 from different promoter deletion strains are shown with percentages compared to wild-type strain. (C) Northern blot of snR49. snR39 was used as a control. wt: wild-type strain. △H0, △H1 and △H2 represent different promoter deletion strains.

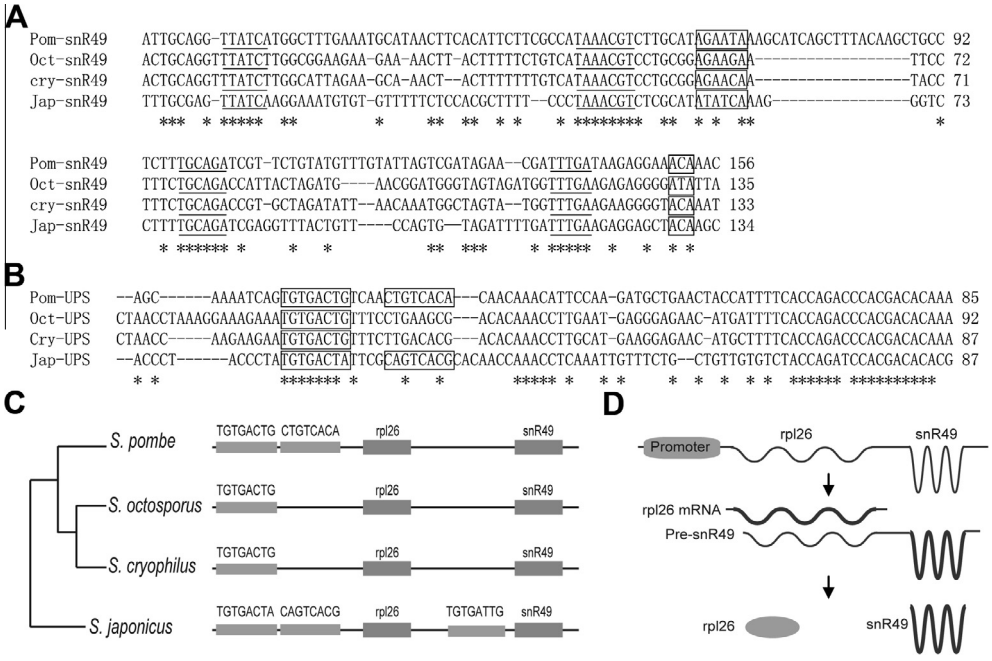

Fig. 4. Conservation of snR49 and the promoter motifs of rpl26 in *Schizosaccharomyces* groups. (A) Conservation of snR49. Base-pairing with rRNA are underlined. The H box and ACA box are indicated with boxes. The same nucleotide among fission yeasts is indicated with an asterisk. Pom-snR49: snR49 in *S. pombe*. Oct-snR49: snR49 in *S. octosporus*. Cry-snR49: snR49 in *S. cryophilus*. Jap-snR49: snR49 in *S. japonicus*. (B) Promoter conservation of rpl26 among *S. pombe*-related genera. Homol-D motifs and homol-D-Like motifs are indicated with boxes. The same nucleotide among fission yeasts is indicated with an asterisk. Pom-UPS: *S. pombe* promoter region. Oct-UPS: *S. octosporus* promoter region. Cry-UPS: *S. cryophilus* promoter region. Jap-UPS: *S. japonicus* promoter region. (C) Schema of chromosomal synteny of rpl26–snR49 in *Schizosaccharomyces* groups. The possible promoter motifs are indicated with sequences. rpl26 and snR49 are also indicated. (D) Potential model of snR49 transcription.

Chromosomal synteny of rpl26–snR49 is conserved in fission yeasts, suggesting the novel transcription mechanism of rpl26–snR49 is conserved in the *Schizosaccharomyces* groups. So we proposed a potential transcriptional model for snR49. In this model, the rpl26 promoter is required for snR49 transcription (Fig. 4D).

4. Discussion

In this study, we confirmed that the transcription of a 3′ sense TASR, snR49, is dependent on the promoter of its upstream gene, rpl26, in *S. pombe*. Our results about a novel transcriptional mechanism for 3′ sense ncRNAs provide new information regarding the expression of 3′ sense ncRNAs.

Interestingly, such novel transcriptional mechanism for 3′ sense ncRNAs is not only restricted to rpl26–snR49 expression. We found that snR93 is 78 nt downstream from rpl29 (Fig. S5A). RT-PCR assays showed that the transcription pattern of rpl29-snR93 is similar to that of rpl26–snR49 (Fig. S5B). Notably, chromosomal synteny of rpl29-snR93 was also found among the fission yeasts (Fig. S6), suggesting that this transcriptional mechanism may be universal. TASRs are abundant and stable in higher eukaryotes [3,8]. In the PomBase, there are 442 intergenic ncRNAs, about 50% of which overlapped to annotate UTRs [13,16]. It will be great interest to study whether their transcription is dependent on the promoters of their upstream protein-coding genes.

Synergic or antergic expression of 3′ sense ncRNAs and their upstream protein genes may suggest they were functionally linked. Two 3′ sense TASRs we found are both H/ACA box snoRNAs, which play important roles in ribosome biogenesis, and the corresponding upstream protein genes are both ribosome protein genes. As one of the most complex processes in eukaryotic cells, it is very important to coordinate the expression of ribosomal proteins, rRNAs and snoRNAs for ribosome biogenesis in cells [31,32]. Primarily, eukaryotic snoRNAs are found within the introns of protein-coding genes involved in ribosome biogenesis, and thus synergistically co-express with ribosome biogenesis related host-genes [33,34]. Our results showed that besides using-intron, synergistic transcriptional regulation of protein and non-coding genes can also be maintained by using a single promoter.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.018.

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