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# Characterization and functional analysis of a novel double-guide C/D box snoRNA in the fission yeast

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**Abstract**

Ribose methylation of eukaryotic rRNA is directed by box C/D small nucleolar RNAs (snoRNAs), which pinpoint the nucleotide to be methylated in specific position within the rRNA sequence. Here, we report the identification of a novel double-guide C/D box snoRNA termed snR88 that directs methylation of two previously undetermined sites in 25S rRNA from the fission yeast. Knockout of the predicted TATA box of the snR88 gene resulted in the complete blocking of its expression, showing that snR88 is an independently transcribed gene and dispensable for yeast viability. The depletion of snR88 abolished 25S rRNA methylation at U2304 and U2497 simultaneously. Interestingly, an unusual pause of reverse transcription at U2495 was observed, which implies an unknown structure of 25S rRNA related to ribose methylation at U2497 in the fission yeast.


*Keywords:* snR88; snoRNA; Methylation; *Schizosaccharomyces pombe*

The ribosomal RNAs (rRNAs) of eukaryotic organisms contain extensive post-transcriptional modifications. The vast majority of these are methylation of ribose (2′-*O*-methylation) and pseudouridylation, mainly situated within the most conserved and functionally important domains of rRNA [1,2]. The two prevalent types of nucleotide modification are directed by two major families of small nucleolar RNAs, box C/D and box H/ACA snoRNAs, respectively. In both cases modification occurs through the formation of a specific snoRNA–rRNA duplex [2]. To date a large number of snoRNAs have been found in animals [3–7], plants [8], yeast [9], and also in archaea [10], wherein a specific antisense snoRNA is associated with between one and at most four (snR49 in the budding yeast) modified rRNA residues, with the exception of orphan-guide snoRNAs whose function remains an enigma [11] and a handful of them being involved in pre-rRNA cleavage. Additionally, an exciting breakthrough has revealed that HBII-52 functions in orchestrating the alternative splicing of serotonin receptor 2C, and the defective pre-mRNA processing contributes to the Prader–Willi syndrome [12].

The structural determinants of methylation guiding snoRNA have been analyzed in detail. They are defined by the phylogenetically conserved C (consensus 5′-RUGA UGA-3′ where R is often a purine) and D (consensus 5′-CUGA-3′) boxes close to the 5′- and 3′-termini of the RNA, respectively. Additional sequence-degenerate, internal copies of these elements, designated C′ and D′, are also usually present in these RNAs [13]. The guide elements that specify the methylation sites are sequences located upstream of either the internal D′ box (upstream antisense element, UAE) or the 3′-terminal D box (downstream antisense element, DAE) [13]. A box C/D methylation-guiding RNA is designated a double-guide RNA if it contains and uses both UAE and DAE to target two different methylation sites [10].

It has been experimentally established that the position of 2′-*O*-methylation of rRNA is within the helix formed by the antisense element of box C/D snoRNA and precisely five nucleotides upstream of box D/D′ [14]. It is termed the "D/D′ + 5" rule. For all species examined so far, this rule

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always holds true. Although the "D/D′ + 5" rule makes it feasible to predict the position of rRNA 2′-*O*-methylation computationally even on a genome-wide level [15], the need has arisen to precisely localize the 2′-*O*-methyl groups in rRNAs of eukaryotes to check for their presence or absence. The methods of primer extension with low dNTP concentrations or partially hydrolyzed rRNAs [14] and RNase H cleavage [16], have been applied for such positioning objectives, in which the RT-based approaches were broadly exploited to map most, if not all, known or candidate 2′-*O*-methylated sites in the rRNAs of several organisms [1].

Here, we report the identification of a novel box C/D snoRNA displaying dual antisense tracks in the fission yeast. By knocking out the promoter element of the novel snoRNA in the fission yeast system, we critically assessed its function and effects on yeast cell growth. In addition, the conservation of these methylated sites across phyla was examined.

## Materials and methods

*Strains and media*. The *Schizosaccharomyces pombe* wild-type haploid strain sp972 was grown in rich YPD medium at 30 °C. The *Escherichia coli* strain TG1[F_/supE, hsd,5, thi,(lac-proAB)] grown in 2YT liquid or solid medium was used for cloning procedures.

*cDNA cloning and sequencing*. Reverse transcription was performed as described below to produce first strand of cDNA. cDNAs of the predicted size were gel-purified and tailed with poly(dG) at the 3′ ends using terminal deoxynucleotidyl transferase (Takara), then amplified by PCR with primers used for Northern blot and *Bam*HI$(C)_{16}$, and finally cloned into pMD18-T plasmid. The cDNA library was screened by PCR with the P47 and P48 universal primer pair. Only the recombinant plasmids bearing fragments of the right size were chosen for sequencing which was performed with an automatic DNA sequencer (Applied Biosystems, 377) using the Big Dye Deoxy Terminator cycle-sequencing kit.

*Plasmid construction and gene deletion*. The flanking sequences of the snR88 gene were amplified with specific primers, digested by *Sac*I/*Kpn*I and *Sal*I/*Sph*I, and cloned into the corresponding restriction sites of plasmid pTZ18, which contains a 1.4-kb selectable marker module from pFA6-kanmx4 [17] allowing an efficient selection of transformants resistant against geneticin (G418). As there was a *Sph*I restriction site in the 5′ flanking sequence, the plasmid was digested with *Sph*I to obtain the 2-kb fragment composed of the flanking sequences and the selectable marker and it was used for yeast transformation by the lithium acetate procedure [18]. Transformants were screened on selective plates with 200 μg/μl G418. The deletion of the fragment of interest was examined by PCR using yeast genomic DNA as template. The depletion of snoRNA was analyzed by Northern blot.

*RNA analyses and rRNA methylated site mapping*. Total cellular RNA of *S. pombe* at exponential growing phase was isolated as described previously [19]. For Northern blot, 15 μg of total cellular RNA was separated by electrophoresis on 8% polyacrylamide, 8 M urea gels, electrotransferred onto nylon membrane (Hybond-N+; Amersham Biosciences), followed by UV light irradiation for 3 min. The probe was labeled with 5′ end [$\gamma$-$^{32}$P]ATP. After hybridization in high-SDS concentration hybridization buffer at 42 °C over 8 h, the nylon membrane was washed in 2× SSPE solution twice for 10 min at room temperature. The membranes were exposed to a phosphor screen and analyzed by the Typhoon 8600 variable mode imager. For partial alkaline hydrolysis, the concentration of total RNA from the fission yeast, rice, and mouse was adjusted to 0.5 μg/μl in 50 mM $Na_2CO_3$ (pH 9.0). The hydrolysis was carried out by water incubation for 10 min at 90 °C and the limitedly degraded RNA was recovered by ethanol precipitation [14]. Reverse transcription was performed in a 20 μl reaction mixture containing 15 μg of total cellular RNA, 10 ng 5′-end-labeled primer and appropriate concentrations of dNTPs as required. After denaturation at 65 °C for 5 min and cooling to 42 °C, 200 U of M-MLV reverse transcriptase (Promega) was added and extension carried out at 42 °C for 1 h. The cDNAs were separated on 8% polyacrylamide, 8 M urea gels, and then analyzed by the imager. The preparation of the *S. pombe* 25s rDNA plasmid and sequence ladder was performed as described previously [9].

*Yeast growth assay*. The wild-type and △snR88 strains of *S. pombe* were cultured to mid-log phase in liquid YPD medium, spotted at dilutions of $10^{-1}$–$10^{-4}$ on YPD agar with (200 μg/ml ) or without G418 and grown for 3 days at 23, 30, and 37 °C, respectively. Plates were photographed with the scanmaker 3830 at 1000 dpi (Microtek).

*Computational analysis*. Novel RNA sequences were confirmed by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). The secondary structures of the snoRNA were analyzed by an mfold program (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) [20]. New candidate box C/D snoRNAs were predicted by "snoscan" (http://lowelab.ucsc.edu/snoscan/) [21]. Fission yeast snoRNAs data can be found at http://www.genedb.org/genedb/pombe/index.jsp. The sequence of snR88 has been deposited in GenBank with the Accession No. EF165542.

*Oligonucleotides*. The following oligonucleotides, synthesized and purified by Sangon Co. (Shanghai, China), were used in this study. 5′-CTCAGAAAGTGAAGAAATAG-3′ for Northern blot and reverse transcription; 5′-AAAGAGCTCAGGTATCGCCTACTGTGGT-3′(LF), 5′-TGTGGTACCTACCCTGAAGAAAACG-3′(LR), 5′-GGAGTCGACTATGTATAATTTTTGTATATC-3′ (RF), 5′-ATTGCATGCAGAGTATAAGCGGTAATGGT-3′ (RR), for plasmid construction (the restriction sites are underlined); 5′-TGACGAGGCATTTGGCTACC-3′ and 5′-CTC CCA CTT AT*Y* CTA CAC C-3′ were used in the primer extension assays; 5′-CTATCTGAACTACCGACCCACA-3′ for internal control Sp20; P48, 5′-GAGCGGATAACAATTTCACACAGG-3′ and P47, 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ as universal sequencing primer; *Bam*HI$(C)_{16}$, 5′-GGAATTCGGAT$(C)_{16}$-3′ for cDNA cloning.

## Results

### *SnR88 is a novel box C/D snoRNA predicted for two previously undetermined methylation sites of 25S rRNA in S. pombe*

Following screening of the dataset from a specialized cDNA library of small RNAs, a novel box C/D snoRNA, snR88 (named after the current repertoire of box C/D snoRNA in *S. pombe*), was further identified by Northern blot and reverse transcription analysis (Fig. 1A). SnR88, 84 nt in length, exhibits the typical features of the box C/D snoRNA, and possesses two 13-nt-long antisense stretches complementary to large subunit rRNA (25S rRNA) of *S. pombe* (Fig. 1B). According to the D/D′ + 5 rule, the DAE and UAE of snR88 were predicted to direct 2′-*O*-methylations of the fission yeast 25S rRNA at U2304 and U2497, respectively. The two predicted methylated nucleotides have not been mapped by prior work, demonstrating that they are novel to *S. pombe*.

### *SnR88 is independently transcribed from an upstream promoter*

The snR88 gene was located at a 533 bp genomic spacer in chromosome I (Accession No. Z98853.1), which is flanked by the COPI-coated vesicle associated protein

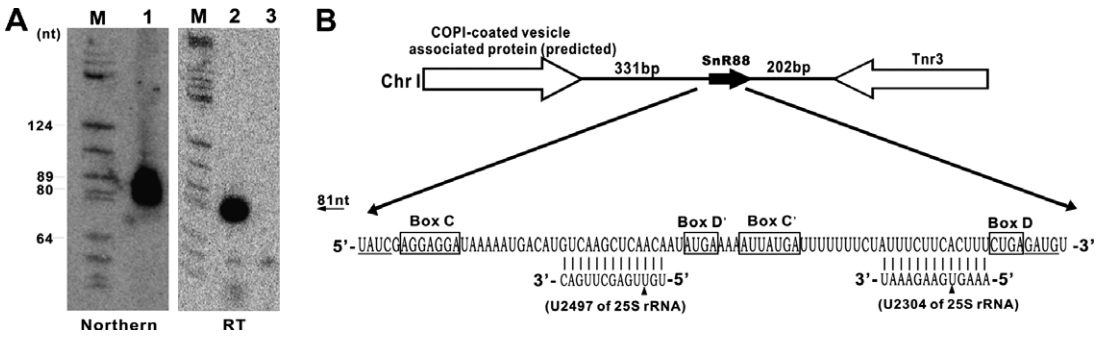


Fig. 1. Identification and sequence context of snR88. (A) Identification of snR88 by Northern blot and RT analysis. Lane 1 is the Northern blot identifying snR88. Lane 2 shows reverse transcription of snR88 with specific primer and lane 3 is the negative control (distilled water as template). M is pBR322 marker (Vector pBR322 digested with *Hae*III, and 5′-end-labeled with [$\gamma$-$^{32}$P]ATP). Size of the bands is shown. (B) Gene organization, location and hallmark motif of snR88 in fission yeast genome. The 5′ and 3′ genes of snR88 are indicated and the distances are shown. Conserved motifs C, C′, D, and D′ are boxed. Nucleotides comprising the terminal stem are underlined. The antisense stretches complementary to fission yeast 25S rRNA are aligned and the targeting methylation sites are denoted by arrows.

(upstream) and tnr3 (downstream) (Fig. 1B). There is a classical promoter element (TATA box) 136 bp upstream of the snoRNA coding region, implying that snR88 appears to be independently transcribed. To rule out the possibility that snR88 is polycistronic, the ~2000 bp sequence downstream of snR88 was examined using "snoscan" to search for new box C/D snoRNAs [21]. The search failed to show any candidate box C/D snoRNAs in this region, indicating that snR88 is a singlet. Furthermore, we did not find typical 5′ donor and 3′ acceptor signal around the coding sequence of snR88, implying that snR88 is not likely to be processed from an intron of a host gene [22].

Taking advantage of homologus recombination in the yeast, we deleted a 73-bp fragment encompassing the putative promoter TATA box with the 1.45-kb-long Kan$^R$ selectable marker module in the genome of the fission yeast (Fig. 2A and B). PCR analysis of genomic DNA of Kan$^+$ transformants with a pair of specific primers showed that an expected 2 kb product, instead of a 0.63 kb product,

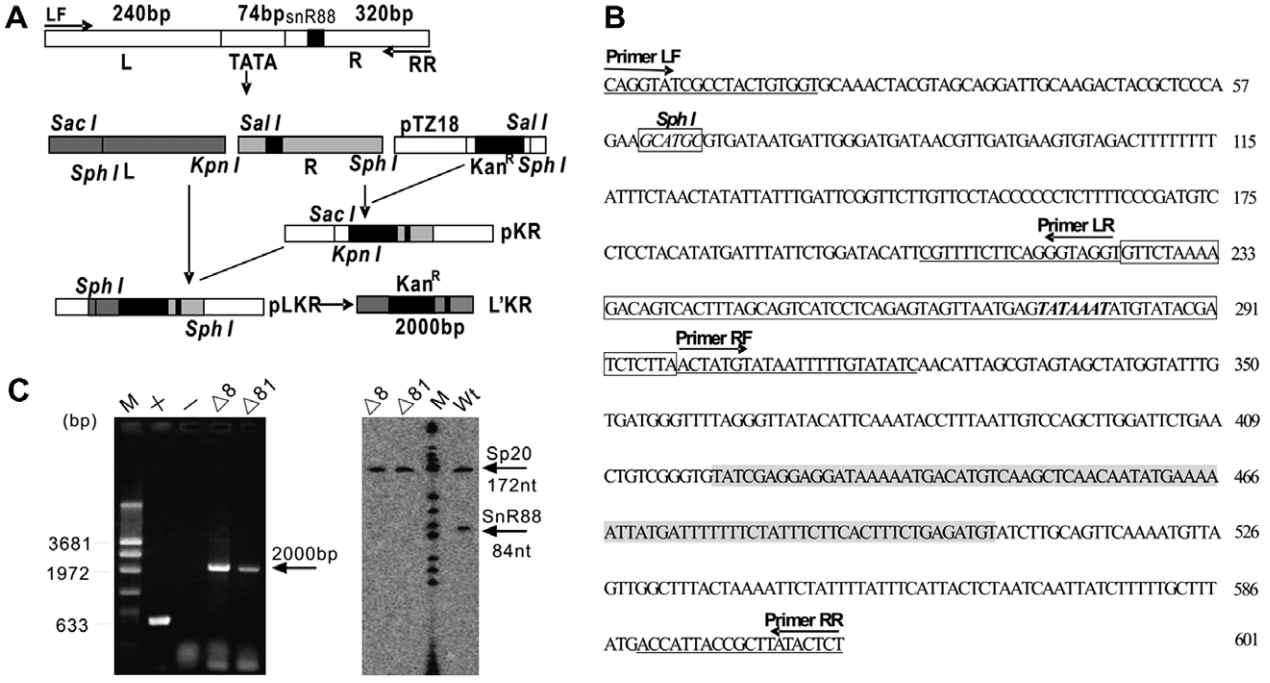


Fig. 2. snR88 disruption. (A) Strategy for plasmid construction. The 5′ and 3′ fragments encompassing the potential promoter TATA box of snR88 gene were amplified and inserted into pTZ18 vector containing G418-resistant marker gene. The construct pLKR was digested with *Sph*I to obtain a 2-kb fragment for transformation; the restriction site, name and length of fragment and construct are shown. (B) Location of primers. The TATA box is blacked in italics; the 74-bp fragment to be deleted are boxed; the primers LF, LR, RF, and RR are in italics with arrows for orientation; the coding sequence of snR86 is shaded; the natural *Sph*I restriction site is boxed in italics. (C) Analyses of transformants. The left picture shows the result of PCR amplification of genomic DNA from two Kan$^+$ transformants, △8 and △81, and wild-type strain ("+") with primers LF and RR. "–" is negative control with distilled water as template. M is pBR322 marker digested with *Hae*III. The right picture is the result of Northern blot using total RNA from the two Kan$^+$ transformants and the wild-type strain with snR88-specific probe. Sp20 is the internal marker. The sizes of bands are denoted.

was amplified (Fig. 2C, left). Moreover, no signal was detected with the specific primer for snR88 by Northern blot when probing total RNA from the $Kan^+$ transformants, in contrast to what was observed for the wild-type fission yeast strain (Fig. 2C, right). Altogether, expression of snR88 was completely suppressed by deleting the TATA box and this manipulation lent support to the assumption that snR88 is independently transcribed.

*SnR88 is required for the 2′-O-methylation of U2304 and U2497 in 25S rRNA of the fission yeast*

To test whether snR88 is required for the site-specific methylation of 25S rRNA at U2304 and U2497, we compared the methylation pattern of 25s rRNA obtained from the wild-type and △snR88 yeast strain by primer extension assays. In primer extension reactions performed at low dNTP concentrations, the 2′-*O*-methylated nucleotides in the template interfere with the normal passage of reverse transcriptase (RTase) and give rise to pauses one nucleotide immediately preceding the methylation sites, and sometimes ''stuttering'' opposite ribose-methylated nucleotides [23]. One band was detected due to the 2′-*O*-methylation of U2304 in 25S rRNA obtained from the wild-type yeast strain (Fig. 3A, lanes 5 and 8). In contrast, no blockage of transcription was observed under the same conditions with 25S rRNA of $Kan^+$ origin (Fig. 3A, lanes 6, 7, 9, and 10). However, detection of the presence of 2′-*O*-methylation of U2497 of 25S rRNA in the fission yeast predicted by snR88 yielded a surprising result. Three stops

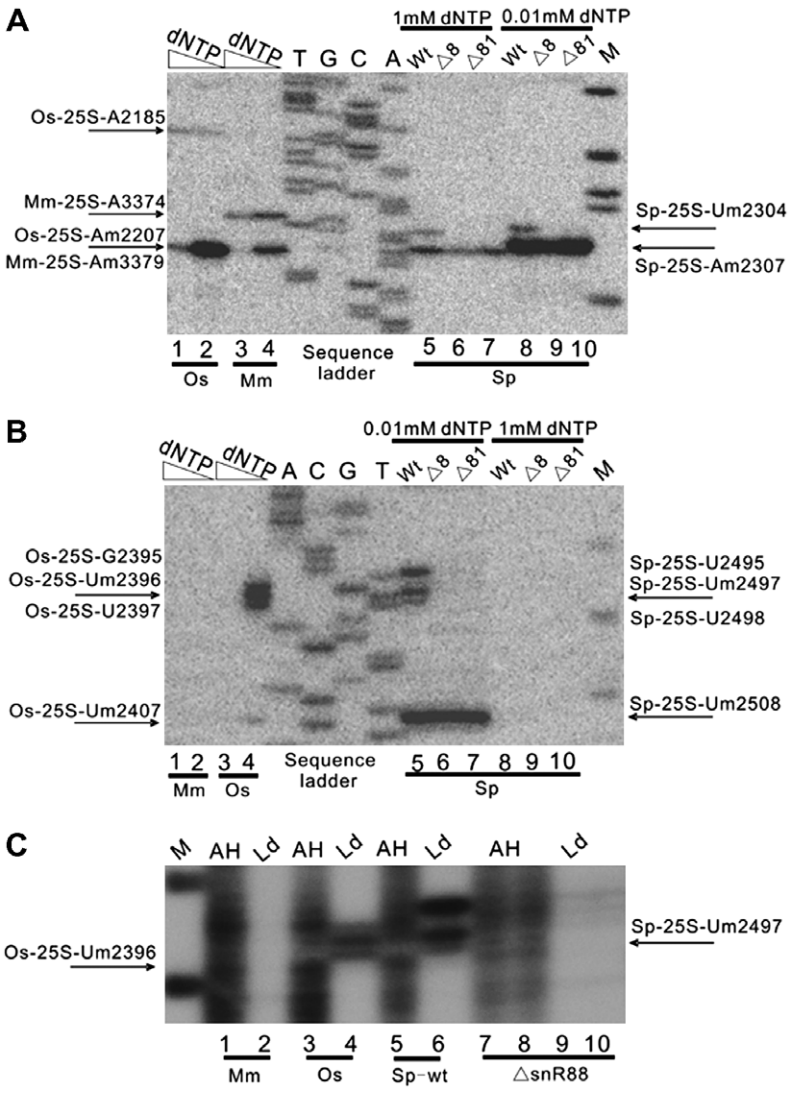


Fig. 3. Experimental validation of methylation guiding function for snR88 at U2304 and U2497 of fission yeast large subunit ribosome (25S rRNA). Reverse transcription was performed with 10 μg total RNA (low dNTP assay) or 20 μg partially hydrolyzed RNA from yeast, rice, and mouse as templates. The fission yeast 25S rDNA molecular size ladder was generated using T7 sequencing Kit from USB. (A and B) The detection for Um2304 and Um2497 in the presence of decreasing dNTP concentrations (1 and 0.01 mM) with total RNA from mouse, rice, and fission yeast. (C) Represents the methylation patterns of Um2497 (yeast numbering) derived from partially hydrolyzing the total RNA of mouse, rice, and fission yeast. M is pBR322 marker. The coordinates of methylation sites are indicated. Triangles stand for the trend of gradient concentrations of dNTPs. Os, *Oryza sativa*; Mm, *Mus musculus*; Sp, *Schizosaccharomyces pombe*; AH, alkaline hydrolysis; Ld, low dNTP concentration.

were detected in the wild-type fission yeast at U2498, U2497, and U2495 (Fig. 3B, lane 5) and were uniformly missing in the △snR88 yeast strains (Fig. 3B, lanes 6 and 7). To determine whether these three stops resulted from the pentose methylation of corresponding nucleotides, reverse transcription was carried out with partially hydrolyzed rRNAs as template. The principle of the hydrolysis method is that the ribose 2′-*O*-methylation stabilizes the RNA internucleotide linkage to alkaline hydrolysis and thus a gap should appear in the ladder at the position in the nascent transcripts immediately preceding any 2′-*O*-methyl in the template [1]. As shown in Fig. 3C (lanes 5 and 6), only the gap corresponding to U2498 was present, implying that U2497 is the *bona fide* 2′-*O*-methylated residue. Similarly, U2396, rather than U2395 and G2394, is 2′-*O*-methylated in rice (Fig. 3C, lanes 3 and 4). Note that the methylation of 25S rRNA in the △snR88 yeast strains was not affected at earlier determined sites, such as Am2307 (Fig. 3A, lanes 5–10) and Um2508 (Fig. 3B, lanes 5–7) of 25S rRNA, indicating that the ribose methylation of U2304 and U2497 are site-specifically guided by snR88. In addition, a parallel extension assay using total RNA from rice and mouse revealed that Um2304 is specific to the fission yeast (Fig. 3A, lanes 1–4). The rice counterpart to Um2497 (Um2396) were also mapped, whereas the murine counterpart was absent, suggesting a conserved rRNA methylation between plants and *S. pombe* (Fig. 3C, lanes 1–4).

*Impact of snR88 disruption on yeast growth*

To investigate the essentiality of snR88, we performed growth assay by gradient dilution to assess the phenotypic difference between △snR88 strain and wild-type yeast (Fig. 4). The growth rates of the △snR88 strain and wild-type strain on YPD medium without G418 displayed no apparent difference at the same temperature, but it was faster at 30 and 37 °C than that at 23 °C. The △snR88 strain was completely unable to grow at 37 °C on YPD medium with G418, whereas it grew at the same rate at 23 and 30 °C on the medium containing G418. These results indicate that the growth of yeast recombinants on YPD medium with G418 was temperature-sensitive. It also implies that snR88 gene is dispensable for the fission yeast, as are most rRNA methylation guides.

## Discussion

It has been recognized that in the low-concentration dNTP assay for localizing the 2′-*O*-methyl groups in eukaryotic ribosomal RNA, the intensity of cross-band deriving from the pause of reverse transcriptase at some methylated sites was strong enough to cause a ‘‘stuttering’’ effect [1]. For example, the methylation pattern of U24, a box C/D snoRNA in the budding yeast directing the 2′-*O*-methylation of C1436 and the doublet A1448-G1449, exhibited the ‘‘stuttering’’ effect, in which the RTase

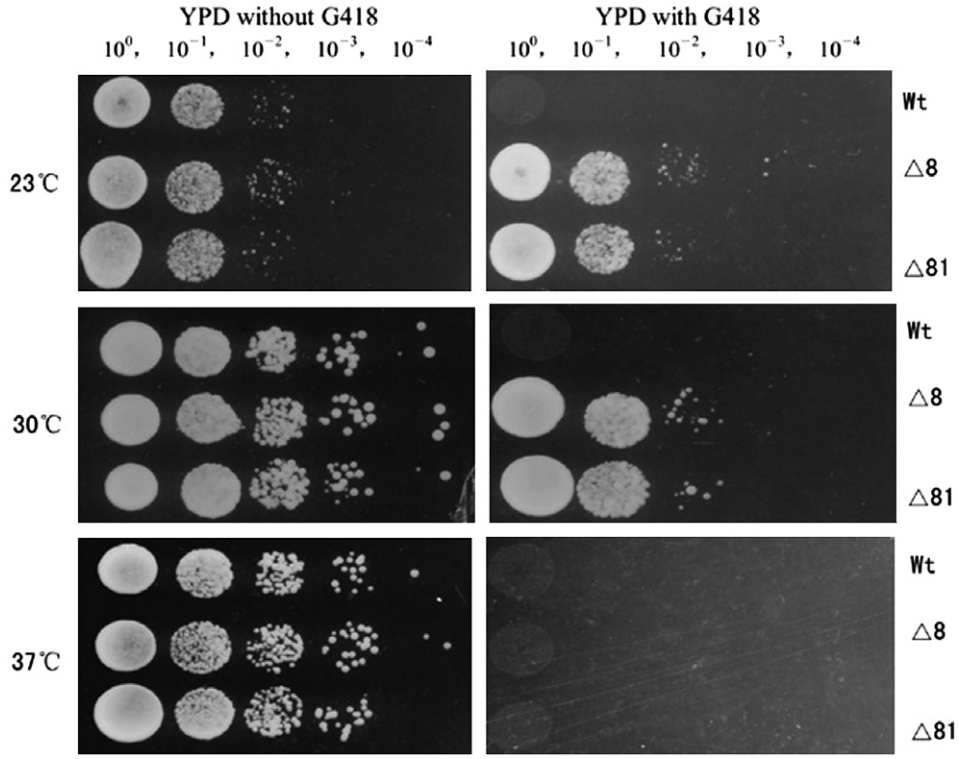


Fig. 4. Fission yeast growth assay. △snR88 strain and wild-type strain were cultured to mid-log phase at 30 °C in a shaking incubator, spotted at dilutions of $10^0$–$10^{-4}$ on YPD agar with (right) or without (left) G418, and grown for 3 days at 23, 30, and 37 °C, respectively. WT, wild-type yeast strain; △8 and △81 are two yeast strains with depletion of snR88.

stuttered over two nucleotides giving rise to successive stops in analytic gels [13,14]. Generally speaking, the RTase is inclined to stutter over two nucleotides, i.e. the +6 and +5 site upstream from the functional box D/D′ of the methylation guide snoRNAs, for an individual 2′-*O*-methylated residue in rRNA. Interestingly, in this study, we showed for the first time that one 2′-*O*-methylated nucleotide Um2497 specified by snR88 in the fission yeast can bring about three reverse transcription pauses, that is, +6, +5, and +3 site upstream from the box D′ of the snoRNAs, at the presence of low dNTP concentrations. It is noteworthy that not only the RTase leaped over the nucleotide at the +4 site but the pause at the +3 site is much more intense than the additional two normal ones. Furthermore, by the alkaline hydrolysis method, we certified that this pause did not originate from another sugar-methylated nucleotide directed by snR88. An attractive scenario to explain the unusual pausing is that the secondary and tertiary structure of the substrate RNA molecule may play a role in affecting the activity of the RTase. A recent influx of information has revealed that the ribose methylation enhances the base stacking that plays a major role for the stabilization of RNA structure [24]. Moreover, it has been reported that the 2′-*O*-methylated residue serves to affect the local conformational properties of loop folding and thus indirectly mediates the rRNA tertiary packing [25]. Finally, several lines of evidence have suggested that the RTase tends to be "stalled" by complex configurations in RNA such as the stem-loop structure giving rise to a prominent stop [26,27]. In fact, the appearance or loss of the reverse transcription pause at U2495 was coupled with the methylation and demethylation of U2497, implying the possible role of ribose methylation in facilitating the folding of rRNA and thus affecting the steady-state working of RTase. Accordingly, it is tempting to speculate that the pause of reverse transcription at U2495 was likely to result from the sterical structure of 25S rRNA associated with the ribose methylation at U2497 in the fission yeast.

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