The *Schizosaccharomyces pombe* mgU6-47 gene is required for 2′-O-methylation of U6 snRNA at A41

Hui Zhou, Yue-Qin Chen, Yan-Ping Du and Liang-Hu Qu*

Key Laboratory of Gene Engineering of the Ministry of Education, Biotechnology Research Center, Zhongshan University, Guangzhou 510275, People’s Republic of China

Received November 9, 2001; Revised and Accepted December 19, 2001

ABSTRACT

Through a computer search of DNA databases, we have identified the homologs of the mgU6-47 snoRNA gene from the yeast *Schizosaccharomyces pombe*, the fly *Drosophila melanogaster* and human. The three box C/D-containing snoRNA genes showed no significant similarity in their sequences except for an 11 nt long complementarity to U6 snRNA, suggesting that the mechanism of snoRNA guided snRNA methylation is conserved from mammals to yeast. The corresponding snoRNAs have been positively detected by reverse transcription and northern blotting. Taking advantage of the fission yeast system, we have disrupted the yeast mgU6-47 gene and demonstrated that it is absolutely required for site-specific 2′-O-methylation of U6 at position A41. No growth differences between mgU6-47 gene-disrupted and wild-type cells were observed, suggesting that the mgU6-47 gene, as for most rRNA methylation guides, is dispensable in yeast. Nevertheless, it was revealed by temperature shift assay that abolition of A41 methylation in yeast U6 snRNA might cause a small decrease in mRNA splicing efficiency. The timing of *S. pombe* U6 pre-RNA transport in the nucleus for splicing and methylation was also analyzed and is described.

INTRODUCTION

The spliceosomal small nuclear RNAs (snRNAs) contain a number of modified nucleotides (1), most of which occur in or around phylogenetically conserved regions (2). This implies radical roles in common required in various organisms. Post-transcriptional modification is an essential process for snRNA maturation and a large cellular machinery is devoted to introduce different kinds of modified nucleotides into snRNAs. The biosynthetic pathway of the different snRNAs is complicated and may differ from one snRNA to another. Transit to the cytoplasm is necessary for N2, N2, 7-trimethylguanosine (TMG) capping of U1, U2, U4 and U5 snRNAs (3,4). However, it is likely that internal modifications are introduced into some snRNAs only after return to the nucleus (5,6).

Conversely, U6 snRNA, the only snRNA transcribed by RNA polymerase III, is not transported to the cytoplasm (7), but transits the nucleoli for post-transcriptional modification (8–10). It has been shown that the highly modified regions in snRNAs correspond to functionally important regions, which participate in hydrogen bonding with pre-mRNA or interact with other snRNAs (2,11). This is also reflected in the conservation of some modified nucleotides in phylogenetically diverse organisms. Nevertheless, apart from the conserved modified nucleotides, a greater number of modified nucleotides are observed in mammals than in yeast (2,12). It is possible that modified nucleotides may be more important in higher organisms than single cell organisms such as yeast. The requirement for modified nucleotides for snRNA function in small nuclear ribonucleoprotein particle (snRNP) biogenesis has been assayed in several reconstitution systems. Clearly the modifications of mammalian U2 snRNA are required for snRNP assembly and pre-mRNA splicing in HeLa splicing extract and *Xenopus* oocytes (5,13,14). However, in the case of yeast, *in vitro* synthesized U2 snRNA can restore splicing (15,16). This is probably because mammalian U2 snRNA is more extensively modified than its counterpart in the yeast *Saccharomyces cerevisiae*, and yeast is well known to differ from other eukaryotes in some aspects of RNA splicing (17). Moreover, although various *in vitro* synthesized snRNAs have been shown to be functional in reconstitution systems, the influence of modified nucleotides on overall splicing efficiency *in vivo* remains to be evaluated. Information about modified nucleotides in snRNAs are well documented from mammalian cells, plants and yeast (12), however, no specific function has been assigned to particular site-specific modifications in snRNAs except for the TMG cap in nuclear import of Sm-antigen binding snRNP (3,4).

Recent progress on 2′-O-methylation of animal U6 snRNA (10) has led to a breakthrough in the understanding of the mechanism of snRNA modification. 2′-O-Methylation of U6 snRNA is guided by snoRNAs that were initially thought to be specific for rRNA modification (18,19). Recently, characterization of brain-specific and imprinted snoRNAs that display the hallmark of the two guide RNA families point to a potential role in mRNA processing (20). The nucleolus has been shown to play an essential role in post-transcriptional modification and processing of nuclear components and other cellular RNAs (21). The finding of snoRNA guides for internal modification

*To whom correspondence should be addressed. Tel: +86 20 84112399; Fax: +86 20 84036551; Email: lsbre04@zsu.edu.cn

*AJ007733, AJ007735 and AJ007736*
of U6 snRNA and for other cellular RNAs also shows a new way to investigate in vivo the function of a particular site-specific modification in RNA molecules using snoRNA gene disruption. Thus, the fission yeast is an attractive model system for molecular genetics studies.

In this work we report the identification of three homologs of mouse mgU6-47 RNA from human, *Drosophila melanogaster* and the fission yeast *Schizosaccharomyces pombe*. Using the fission yeast system, we have demonstrated that the mgU6-47 gene is required for site-specific 2′-O-methylation of *S. pombe* U6 snRNA. We also report the effects on cell growth and mRNA splicing of disruption of the snoRNA gene. Our results also provide a glimpse into the timing of splicing and methylation.

**MATERIALS AND METHODS**

All techniques used for manipulation of *Escherichia coli*, DNA, RNA and oligonucleotides were performed essentially as described by Sambrook *et al.* (22). Molecular genetics analysis of the fission yeast *S. pombe* was carried out according to a standard protocol (23).

**Computer search of the nucleic acids databases**

The nucleic acids databases GenBank and EMBL were screened using the BLAST (24) and Fasta (25) programs. Searches for perfect 10 nt complementarity to snoRNA ribose-methylated sequences immediately followed by the sequence NCUGA were carried out as previously described (26). Sequences exhibiting snoRNA gene features were selected and further analyzed using the Pseqne 6.0 package.

**Strains and media**

The *S. pombe* wild-type haploid strain sp972 was used for transformations and all RNA and DNA analyses. This strain was grown in rich (YPD) medium (1% yeast extract, 1% Bacto yeast extract, 0.5% NaCl) liquid or solid medium for 24 h or 3 days, then 37°C or 25°C for 2 days, respectively. Yeast were transformed by the lithium acetate method. Transformants were screened on selective plates with 200 mg/l G418 and the chromosomal allele was checked by PCR. *Escherichia coli* strains TG1 [F′supE, hsdA5, thiA(proAB)] and DH5α [F′, endA1, lacIΔ17 (rk-mk+), supE44, thi-1, recA1, gyrA (Nalr), relA1, Δ(lacZΔM15)] grown on 2YT (1.6% Bacto tryptone, 0.5% NaCl) liquid or solid medium were used for all cloning procedures.

**RNA extraction and analyses**

RNA from HeLa cells, mouse liver, *D. melanogaster* and *S. pombe* cells were isolated by guanidinium thiocyanate/phenol-chloroform extraction as described by Chomczynski and Sacchi (27). Temperature shift assays were done by heat shock treatment of yeast cells grown at 35°C for 2 days, then 37°C for 30 min, 2 h and 7 h, before isolating the RNA. For northern analysis, 50 μg total RNA was fractionated on 10% acrylamide–7 M urea gels, electroblotted onto a nylon membrane (Amersham), hybridized with 5′-end-labeled probes and washed as described (28). Reverse transcription was carried out in a 20 μl reaction mixture containing 25 μg total RNA, 20 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 MLV reverse transcriptase (Promega) was added and extension carried out at 42°C for 30 min. The cDNAs were then analyzed on 10% acrylamide–7 M urea gels.

**Detection of ribose-methylated nucleotides**

Ribose-methylated nucleotides in U6 snRNA were detected by reverse transcription at low dNTP concentrations with oligonucleotide U6sp. In brief, primer extension was carried out in parallel on two aliquots in the presence of either 0.1 or 1.5 mmol/l dNTPs (26). To remove the intron sequence, a U6 snRNA sequence ladder was prepared from the full-length U6 cDNA. After adding a 3′ poly(G) tail using terminal transferase, U6 cDNA was amplified by PCR with the primers poly(C) and U6sp. The PCR product was purified and cloned into the Smal site of plasmid pTZ18. The recombinant plasmid was sequenced with 5′-end-labeled primer U6sp and run in parallel with reverse transcription of U6 snRNA as a molecular weight marker. The U6 gene sequence ladder used in U6 snRNA precursor analysis was prepared by cloning a PCR fragment of the U6 snRNA gene with primers U6sp-Fw and U6sp.

**Construction of plasmids for the snoRNA gene disruption**

For *S. pombe* mgU6-47 gene disruption an ~1.2 kb fragment of *S. pombe* genomic DNA encompassing the mgU6-47 snoRNA gene and flanking sequences was PCR amplified with the Fu6sp and Ru6sp primer pair. After digestion with EcoRI and *KpnI*, the amplified fragment was cloned into the corresponding restriction site of pTZ19. The EcoRV–SalI region in the amplified fragment was replaced by a 1.4 kb selectable marker module from pFA6a-kammx4 (29), which permits efficient selection of transformants resistant to geneticin (G418), and the SalI–BglII fragment encompassing the 5′-region of the mgU6-47 snoRNA gene was substituted by a 18 bp linker produced from sal-link and bgl-link, giving rise to plasmid pU6sp3. pU6sp3 was linearized with *KpnI* and used to transform the wild-type haploid strain sp972 of *S. pombe* by the lithium acetate procedure. Transformants were screened on selective plates with 200 mg/l G418. Disruption of the chromosomal alleles of the mgU6-47 snoRNA gene was verified by PCR with the Fu6sp and Ru6sp primer pair. Deletion of expression of mgU6-47 snoRNA was analyzed by reverse transcription and northern analysis using oligonucleotide Pz30sp.

**Oligonucleotides**

Oligonucleotides were synthesized and purified by Sangon Co. (Shanhai, China). The sequences of oligonucleotides used for northern and reverse transcription analyses of mgU6-47 snoRNAs were as follows: Pz30hs, 5′-AGCTCCAGAGAAGATTAAAGRG-3′; Pz30dm, 5′-GGCTGAGGAGAAAGATTGG-3′; Pz30sp, 5′-GGATCAGAGAAGATTAGCT-3′; These oligonucleotides are complementary to the 3′-end of the mgU6-47 snoRNAs from human, *D. melanogaster* and *S. pombe*, respectively. The following oligonucleotides were used to disrupt the *S. pombe* mgU6-47 gene: Fu6sp, 5′-CATGAGCAAAGATGACTCTC-3′; Ru6sp, 5′-CACAGAGTATTAAAGTCTCA-CCG-3′; sal-link, 5′-TGACCCAAAGGTCCGAA-3′; bgl-link, 5′-GATCTGGGAGATCTTGGG-3′. The probe and primer used for northern and reverse transcription analyses of *S. pombe* U6 snRNA and its precursor were as follows: U6sp, 5′-AATGGGTTTTTCTCTCAATGTCGAG-3′; U6sp-In, 5′-CTAA-
ACAA CGGAT TAGT TAGT GACTCG-3'. The poly(C) primer used for PCR amplification of oligo(G)-tailed U6 cDNAs was 5'-GGAATTCC GATC-3'. The sequence of U6sp-Fw, used for PCR amplification of the U6 snRNA gene, was 5'-TGCTT CAGTGT TTTGAT GTCT-3'. The oligonucleotides were 5',32P-end-labeled as described previously (28) and used directly as probes for northern hybridization or submitted to purification by electrophoresis on a 10% acrylamide-7 M urea gel before utilization as reverse transcription primers.

Nucleotide sequence accession numbers

The S.pombe, D.melanogaster and human mgU6-47 sequences have been deposited in the EMBL database under accession nos AJ007736, AJ007735 and AJ007733, respectively.

RESULTS

mgU6-47 is a conserved snoRNA between S.pombe and human

It has been shown that most, if not all, sites of rRNA ribose-methylation are specified through a process that involves many snoRNAs (30,31). This mechanism of methylation also seems applicable for some snRNAs, such as U6 snRNA (10). Knowing the modification pattern of snRNAs from various eukaryotes (12), we performed a search of the DNA databases to find novel snoRNA guides for snoRNA modification with complementarity to the sequence flanking the methylated nucleotide together with the conserved elements of snoRNAs, i.e. box C/D snRNAs. A number of DNA sequences coding for potential snoRNAs were identified. Here we focus our attention to three candidate mgU6-47 snoRNA homologs (initially termed Z30), which correspond to the methylation guides for U6 snRNA from S.pombe, D.melanogaster and human (Fig. 1).

Schizosaccharomyces pombe mgU6-47 was found as an independent transcript gene located on a 1.4 kb genomic DNA spacer fragment of chromosome II (accession no. AL022299), which is between two protein coding genes. A promoter element (TATAAA box) was found 95 bp upstream of the snoRNA coding region. Drosophila melanogaster mgU6-47 was identified as an intronic RNA encoded in the first intron of a RNA helicase gene (accession no. AF017777). Human mgU6-47 was from an unidentified EST sequence (accession no. AA552905). All three mgU6-47 sequences are box C/D-containing snoRNAs with 11–13 nt long complementarity to U6 snRNA. According to the relationship of antisense snoRNA structure and function (32–34), this sequence complementarity precisely targets a conserved methylated nucleotide of U6 snRNA. According to the relationship of antisense snoRNA structure and function (32–34), this sequence complementarity precisely targets a conserved methylated nucleotide of U6 snRNA.

Mouse mgU6-47 RNA was also analyzed as a reference. In addition, northern analysis of RNA isolated from purified HeLa cell and S.pombe nuclei showed that mgU6-47 was enriched in the nucleus (data not shown). Reverse transcription was carried out to map the 5'-end of the snoRNAs (Fig. 2B), followed by cloning and sequencing of the cDNAs. As expected, all cDNA sequences perfectly matched the sequences of the snoRNA coding regions, as shown in Figure 1.

Although mgU6-47 appears to be a well-conserved snoRNA, our attempt to search for a mgU6-47 gene in the DNA database of the yeast S.cerevisiae failed to give any significant result. 2'-O-ribose methylation analysis of U6 snRNA by reverse transcription at low dNTP concentrations confirmed that the four
methylated sites in *S. pombe* were not methylated in *S. cerevisiae* (L.-H. Qu and H. Zhou, unpublished results).

**Disruption of the yeast mgU6-47 gene abolishes 2′-O-methylation of A41 in U6 snRNA**

Although an analysis of mouse mgU6-47 sequence complementarity to U6 snRNA has suggested that it could direct 2′-O-methylation of A47 in U6 snRNA (10), identification of the *S. pombe* mgU6-47 gene provides us with a useful genetic system to prove this function and to evaluate the effects on cell growth of disruption of the mgU6-47 gene in *S. pombe*.

Disruption of the mgU6-47 gene was carried out in a wild-type haploid strain of *S. pombe*. We first disrupted the mgU6-47 gene by inserting a 1.4 kb long Kan selectable marker module between the promoter TATA box element and the mgU6-47 RNA coding region, resulting in the construct Pu6sp2 (Fig. 3A).

Unexpectedly, this disruption was insufficient to suppress expression of the mgU6-47 gene after transformation of the haploid wild-type strain by the disrupted allele (data not shown). Making use of the endonuclease sites at the 5′-end of the mgU6-47 gene, direct modification in the gene was carried out by replacing the 36 bp fragment of Pu6sp1 with a synthetic 18 bp *Sal*-BglII linker (Fig. 3A). This manipulation deleted the 5′-end TGATGA (box C), a key sequence for snoRNA stability and nucleolar localization (38,39), of the mgU6-47 gene (plasmid pU6sp3). After transformation of *S. pombe* with delineated pU6sp3, suppression of expression of the mgU6-47 gene by the disrupted allele was observed in Kan+ colonies. PCR analysis of genomic DNA with a specific primer demonstrated that the mgU6-47 locus was disrupted and modified in the Kan+ cells. An expected 2.5 kb PCR product, instead of the 1.1 kb band, was amplified with the Fu6sp and Ru6sp primer pair (Fig. 3B). Disruption of the mgU6-47 gene in the Kan+ cells was further confirmed by primer extension and northern blot analysis (Fig. 3B). In both cases no signal was detected with the specific primer for mgU6-47 RNA when probing total RNA from the Kan+ cells, in contrast to what was observed for the parental wild-type strain RNA.
different dNTP concentrations. A47 gene was analyzed using primer extension at low dNTP concentrations which became less evident at 1.5 mmol/l dNTPs when RNA from either the wild-type or a disrupted strain was used. The unusual behavior of A47, which was very different from the other three 2′-O-methylated nucleotides, made the methylation at this site suspect. Conversely, our experiment indicated another possible 2′-O-methylation site, A64, which had the same repressive effect on reverse transcription as the three 2′-O-methylated nucleotides, and this effect largely depended on dNTP concentration (Fig. 4).

Abolition of methylation of U6 snRNA slightly affects the efficiency of splicing

Although no phenotypic differences were observed under various growth conditions, an investigation of the impact at the mRNA precursor level of abolition of A41 methylation of U6 snRNA was carried out. The S. pombe U6 snRNA gene is split by a mRNA-type intron (41,42), which could be spliced by the same machinery as carries out mRNA processing in the cell. U6 pre-RNA was easily detected in the wild-type strain by temperature shift assay (43). Therefore, the experiment analyzed U6 snRNA and its precursor from mgU6-47 gene-disrupted and wild-type cells grown under normal and restrictive conditions.

Total RNA was extracted from two mgU6-47 gene-disrupted strains, sp5m and sp14m, and the parental wild-type strain. Primer extension was performed with the exon-specific oligonucleotide U6sp, complementary to the 3′-end of S. pombe U6 snRNA, and intron-specific oligonucleotide U6sp-In, complementary to the intron sequence of S. pombe U6 snRNA (Fig. 4). The latter was also used to detect the U6 precursor by northern hybridization. There were no evident differences in the levels of mature U6 snRNA and its precursor among disrupted and wild-type cells grown at 30°C (data not shown). However, when the cells were shifted from 25 to 37°C, slightly more U6 snRNA was extracted from U6sp3 disrupted strain RNA; lane 4, primer extension at 1.5 mmol/l dNTPs on disrupted strain RNA; lane 3, control reaction at 1.5 mmol/l dNTPs on wild-type strain RNA; lane 2, primer extension at 0.1 mmol/l dNTPs on disrupted strain was analyzed. The latter was also used to detect the U6 precursor by northern hybridization. There were no evident differences in the levels of mature U6 snRNA and its precursor among disrupted and wild-type cells grown at 30°C (data not shown). Therefore, abolition of A41 methylation resulted directly from instability of the truncated mgU6-47 RNA, whose 5′-end box C sequence was completely deleted.

Abolition of methylation of U6 snRNA slightly affects the efficiency of splicing

Although no phenotypic differences were observed under various growth conditions, an investigation of the impact at the mRNA precursor level of abolition of A41 methylation of U6 snRNA was carried out. The S. pombe U6 snRNA gene is split by a mRNA-type intron (41,42), which could be spliced by the same machinery as carries out mRNA processing in the cell. U6 pre-RNA was easily detected in the wild-type strain by temperature shift assay (43). Therefore, the experiment analyzed U6 snRNA and its precursor from mgU6-47 gene-disrupted and wild-type cells grown under normal and restrictive conditions.

Total RNA was extracted from two mgU6-47 gene-disrupted strains, sp5m and sp14m, and the parental wild-type strain. Primer extension was performed with the exon-specific oligonucleotide U6sp, complementary to the 3′-end of S. pombe U6 snRNA, and intron-specific oligonucleotide U6sp-In, complementary to the intron sequence of S. pombe U6 snRNA (Fig. 4). The latter was also used to detect the U6 precursor by northern hybridization. There were no evident differences in the levels of mature U6 snRNA and its precursor among disrupted and wild-type cells grown at 30°C (data not shown). However, when the cells were shifted from 25 to 37°C, slightly more U6 snRNA was extracted from U6sp3 disrupted strain RNA; lane 4, primer extension at 1.5 mmol/l dNTPs on disrupted strain RNA; lane 3, control reaction at 1.5 mmol/l dNTPs on wild-type strain RNA; lane 2, primer extension at 0.1 mmol/l dNTPs on disrupted strain was analyzed. The latter was also used to detect the U6 precursor by northern hybridization. There were no evident differences in the levels of mature U6 snRNA and its precursor among disrupted and wild-type cells grown at 30°C (data not shown). Therefore, abolition of A41 methylation resulted directly from instability of the truncated mgU6-47 RNA, whose 5′-end box C sequence was completely deleted.
DISCUSSION

The fission yeast is an attractive organism for studying post-transcriptional modification of snRNA

We have identified the homologs of mgU6-47 RNA from *S. pombe, D. melanogaster* and human. The three C/D box snoRNAs show no significant similarity in their sequences of primary transcripts. As discussed in the next section, the fission yeast is an attractive organism for studying the timing of U6 snRNA splicing and methylation.
except for the 11 nt long complementarity to U6 snRNA at the 3'-end, which is strictly conserved among human, fly and yeast. The conservation of mgU6-47 RNA, which guides a conserved site-specific methylated nucleotide in U6 snRNA, implies a biological significance in common throughout evolution. However, failure to determine the counterpart of mgU6-47 RNA and its related methylated nucleotides in S. cerevisiae suggests that mgU6-47 RNA is not ubiquitous in eukaryotes. It would be interesting to know why the conservation of mgU6-47 RNA is so strictly maintained between mammals and S. pombe, but not between the two yeasts, which both belong to the Ascomycetes. The fission and budding yeasts probably diverged from a common ancestor around one billion years ago (2). One distinct difference between the two yeasts, with respect to snRNA processing, is the scarcity of introns in the S. cerevisiae genome. There are only 235 introns within 6275 hypothetical protein coding ORFs (45). It is reasonable to suppose that a higher efficiency of mRNA splicing is required in mammals and fission yeast, which possess many more introns as compared to budding yeast. Taking into account the fact that there are nine 2'-O-methylated nucleotides in mammalian U6, seven in a plant and only four in S. pombe, with possibly none in S. cerevisiae (12; L.-H. Qu and H. Zhou, unpublished results), the role played by 2'-O-methylated nucleotides in mRNA splicing can only be speculated at. Being more closely related to mammalian cells with respect to snRNA methylation pattern as compared to budding yeast, S. pombe is an attractive organism in which to study the occurrence and function of post-transcriptional modifications of snRNA. Characterization of the homolog of mgU6-47 RNA from the fission yeast S. pombe provides the first example in the field.

The mgU6-47 gene is required for S. pombe U6 snRNA methylation

The internal modification of U6 snRNA specified by the snoRNA guide process was first revealed by identification of mgU6-47 and mgU6-77 RNA (10). mgU6-77 RNA, which possesses an unusual sequence complementarity to both U6 snRNA and 28S rRNA, was suggested to function in the methylation of two different kinds of RNA. This suggestion was proved by microinjection of antisense RNA into Xenopus laevis oocytes. In contrast to mgU6-77 RNA, preliminary data suggested that mgU6-47 RNA functions as a RNA guide for site-specific methylation of U6 snRNA. However, there is no direct evidence for this yet. In our experiment, disruption of the S. pombe mgU6-47 gene resulted in complete abolition of methylation at A41 of U6 snRNA. This result is consistent with the sequence comparison between U6 snRNA and mgU6-47 RNA (10). The absolute effect of the gene disruption on methylation of A47 in U6 snRNA indicates that the S. pombe mgU6-47 gene is a single copy gene and that its function in site-specific methylation of U6 snRNA cannot be fulfilled in an alternative way in S. pombe. However, the other methylated nucleotides in U6 snRNA were unaltered by this operation. Therefore, formation of the 2'-O-methylated nucleotides in U6 snRNA proceeds independently. In addition to the previous analysis (10), our results provide genetic evidence for a clear relationship between site-specific methylation of U6 snRNA and a
snoRNA gene from \textit{S. pombe}, supporting the function of the nucleolus in the modification of U6 snRNA.

**Regulation of splicing at the post-transcriptional level**

Although many snRNAs require 2'-O-methylation as a post-transcriptional modification, the function of this modification remains largely unknown. 2'-O-methylation introduces a positive charge and increases the hydrophobicity. This effect may prevent the 2'-hydroxyl group from forming hydrogen bonds with phosphodiester bridges in RNA and/or with peptide bonds in proteins (46). Recently, a conserved pseudouridine modification in U2 snRNA has been reported to induce a change in the structure and stability of the branch site sequence (47). It is likely that conserved modifications in snRNAs confer a slight advantage to the organism by strengthening or weakening the interactions through hydrogen bonding between RNA and protein. Among all snRNAs, U6 snRNA is the most highly conserved. U6 snRNA is very sensitive to mutation and chemical modification, probably because it is directly involved in the catalysis of splicing (48, 49). In this work we have demonstrated that the abolition of U6 pre-RNA methylation at A41 by disrupting the mgU6-47 gene does not necessarily result in the inhibition of U6 pre-RNA maturation and snRNP assembly in yeast. Normal growth of the disrupted strain was observed at different temperatures, revealing that mgU6-47 RNA is dispensable in fission yeast. However, a slight accumulation of RNA precursors was observed when cell cultures were shifted from 23 to 37°C, suggesting that fine tuning by site-specific 2'-O-methylation is involved in the regulation of splicing. This tuning is sensitive to the temperature shift. Our results imply that the efficiency of splicing may be regulated at the post-transcriptional level, although a low importance of U snRNA post-transcriptional modification for the yeast splicing machinery has been suggested (15). It is worth noting that the effect was produced by deletion of only one of four methylations normally present in \textit{S. pombe} U6 snRNA. A more significant effect would possibly be evident if all four methylations in U6 snRNA were abolished.

**How U6 snRNA trafficks through the nucleolus**

The existence of an intron sequence in \textit{S. pombe} U6 pre-RNA makes its biogenesis complicated. It appears that the spliced U6 RNA does not immediately integrate into the spliceosome, although it must first pass into the spliceosome for intron processing. A further trafficking through the nucleolus is necessary for the modification of U6 snRNA. It would be of interest to know how the spliced U6 snRNA trafficks through the nucleolus for modification. In addition to the individual U6 and U4 snRNPs, the U4 and U6 snRNAs can interact by base paring and organize as a U4/U6 snRNP in the nucleus. It has been reported that pseudouridine formation in U6 snRNA is dependent on its interaction with U4 snRNA (50) and that some internal methylations occur in mammalian U4 snRNA and maximal pseudouridine formation in mammalian U4 snRNA requires nuclear factors (6). It is very likely that unmodified U6 snRNA is accompanied by U4 snRNA in trafficking through the nucleolus and that U4 snRNA also requires a snoRNA for internal modification. The recent identification of a C/D box snoRNA predicted to direct U4 methylation (51) provides further evidence for this speculation.

**ACKNOWLEDGEMENTS**

We thank Jean-Pierre Bachellerie and Yves Henry for helpful suggestions on our experiments. We are particularly grateful to Xin-Jie Chen and Marlene Faubladier for their experimental help and helpful discussions. We thank Wei-Xin Zhou and Jin Zhao for their assistance in the analysis of \textit{D.melanogaster} RNA. We also thank Dr Henri Grosjean for revising the text of the manuscript. This research was supported by the National Natural Science Foundation of China (key projects 39730300, 39970171 and 30170216), by a Fund for Distinguished Young Scholar from the Education Ministry of China and the Natural Science Foundation of Guangdong province (990242).

**REFERENCES**


