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Mutational Analysis of *Schizosaccharomyces pombe* U4 snRNA by Plasmid Exchange

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We have developed a system for testing mutations by plasmid exchange in the fission yeast *Schizosaccharomyces pombe*. This system has been used to test the requirement for different regions of the small nuclear RNA U4 in *S. pombe*. Surprisingly, five of seven deletion and substitution mutations tested in different regions of U4 prevent the accumulation of the mutant RNA. Substitution of the U4 sequence in stem I of the U4/U6 interaction domain allows accumulation of the mutant U4, but does not support viability. Two sequences with homology to the Sm binding site are found in the 3′ region of *S.pombe* U4; substitution of the 3′ sequence of the two does not interfere with accumulation or function of U4, indicating that the 5′ sequence is the functional Sm-binding site.

KEYWORDS — *Schizosaccharomyces pombe*; snRNA; snRNP; U4.

INTRODUCTION

The small nuclear RNAs (snRNAs), a group of stable, low molecular weight RNA species, have been found in the nuclei of all eukaryotes examined to date. The most studied species, U1, U2, U4, U5 and U6, are all required for pre-mRNA splicing *in vivo* (reviewed by Maniatis and Reed, 1987) and *in vitro* (reviewed by Guthrie, 1987; Woolford, 1989). A number of studies have shown that while U1, U2 and U5 exist as distinct ribonucleoprotein particles (snRNPs), U4 and U6 are associated by base-pairing in a single snRNP (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Brow and Guthrie, 1988). The role of the snRNAs in pre-mRNA splicing has been studied in *in vitro* systems derived from vertebrate tissue culture cells, and in the budding yeast *Saccharomyces cerevisiae*. However, there are clearly significant differences between *S. cerevisiae* and higher eukaryotes and we wished to extend this comparison to another distantly related organism.

We, and others, have reported the cloning of snRNAs from the fission yeast, *Schizosaccharomyces pombe* (Brennwald *et al.*, 1988; Dandekar and Tollervey, 1989; Porter *et al.*, 1990). Sequencing of the gene for U4 from *S. pombe* showed it to have good homology to the vertebrate snRNA, and gene disruption showed the single copy gene *snu4* to be essential for viability (Dandekar *et al.*, 1989). Here we report the analysis of the regions of U4 required for function of the snRNA as judged by viability in *S. pombe*.

MATERIALS AND METHODS

Strains and media

Growth and handling of *S. pombe* were as described by Gutz *et al.* (1974). The haploid *S. pombe* strain used carries h^+, *leu1.32*, *ura4⁻ D18*, *ade6.704* (kindly provided by J. Kohli).

Mutagenesis

Mutations were made using a site-directed mutagenesis kit (Amersham) and confirmed by sequencing. The mutagenic oligonucleotides used are given in brackets (the wild-type sequences of the respective regions are shown in Figure 1):

Deletion mutations

snu4.1 (GCAATAACGCCATCTAGTACTGAT AAGCGTAATACCC)

snu4.2 (CCTCACTGATAAGCGTAATCGATG GGGAATGGTAAACTC)

snu4.3 (GAGAGTTGGAGCGGTTAACTAGCA ATAACGCCATC)

Replacement mutations

snu4.4 (GGTTTCCAAATATTCCGACGTCTCT TAACGAGAGTTGG)

0749–503X 92/080647–07 $08.50

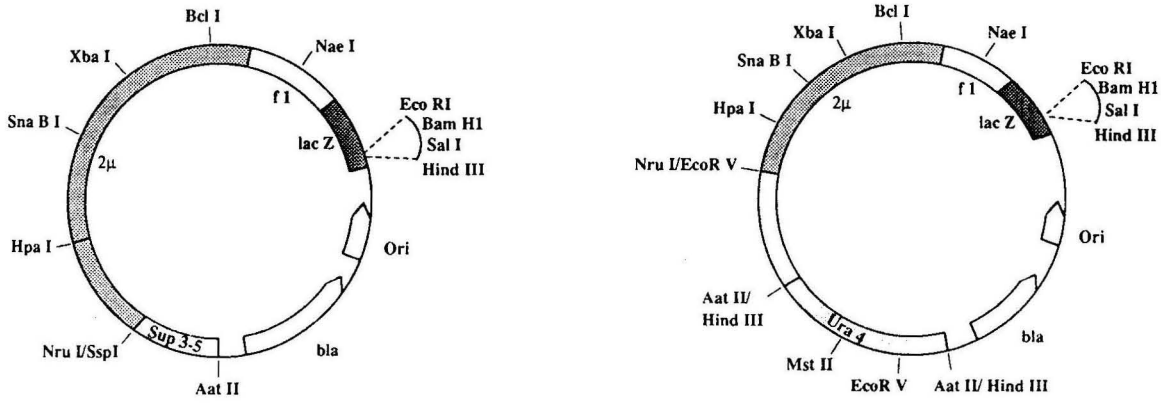

Figure 1. Structure of the plasmids used for exchange. The left panel shows the map of YEpSUP3-5, the right panel the map of YEpU4 (Dandekar *et al.*, 1989). Useful restriction sites are indicated.

snu4.5 (GCAATAAGTTGGTTTCCAAACGCG TCCAAAACTCTTAACG)
snu4.6 (GGAGCGGTCAGGGTAATCCCGGGC AACTAGCAATAACGCC)
snu4.7 (GGGTAATAGTTTTCACGATCGAAT AACGCCATCTAGG)

Transformation

S. pombe was transformed using the protocol described for *S. cerevisiae* by Ito *et al.* (1983).

RNA analysis

RNA extraction and Northern analysis were as previously described (Maniatis *et al.*, 1982; Tollervey and Mattaj, 1987).

DNA sequencing

DNA was sequenced using a Sequenase kit (USB) according to the manufacturer's instructions.

Construction of YEpSUP3-5

The plasmid was constructed by cloning the *Ssp*I-*Aat*II fragment containing the *sup3.5* gene (Hottinger *et al.*, 1982) into the *Nru*I-*Aat*II fragment of pEMBL YEp23 (Cesareni and Murray, 1987), which contains sequences for replication in *Escherichia coli* and the *S. cerevisiae* 2μ replication origin (Figure 1). The *sup3.5* gene (previously *sup3-e*) encodes $tRNA^{ser}_{UGA}$ (Hottinger *et al.*, 1982) which, among other mutations, suppresses *ade6.704* (J. Kohli, personal communication) conferring adenine prototrophy. The plasmid has unique sites for *Eco*R1, *Bam*H1, *Sal*I and *Hin*dIII within the *lacZ* α-complementing fragment, allowing blue/white color testing for insertions in *E. coli*, and can be recovered as single strands following infection with F1 helper phage (Cesareni and Murray, 1987). To make psnu4.1-psnu4.7, the *snu4* gene was cloned as an *Eco*R1-*Hin*dIII fragment into M13 mp8 and mutations were made and confirmed by sequencing. The mutant *snu4* genes were then recloned into the *Eco*R1-*Hin*dIII sites of YEpSUP3-5.

RESULTS

Initial experiments using native gels indicated that, as in other eukaryotes, U4/U6 form a complex in *S. pombe* (T. Dandekar and D. Tollervey, unpublished). Moreover, *S. pombe* U6 can be precipitated by Sm-antibodies via its interaction with U4 (Dandekar and Tollervey, 1989). The available data for *S. cerevisiae* and higher eukaryotes support the presence of two domains of interaction between U4 and U6, giving a 'Y'-shaped structure (Brow and Guthrie, 1988). U4 and U6 from *S. pombe* can also be drawn in such a configuration (Figure 2). To test for regions of *S. pombe* U4 important for interaction with *S. pombe* U6, we made a deletion of the U4 sequence in stem I (*snu4.2*) and a substitution of the U4 sequence in stem II (*snu4.7*) of the U4/U6

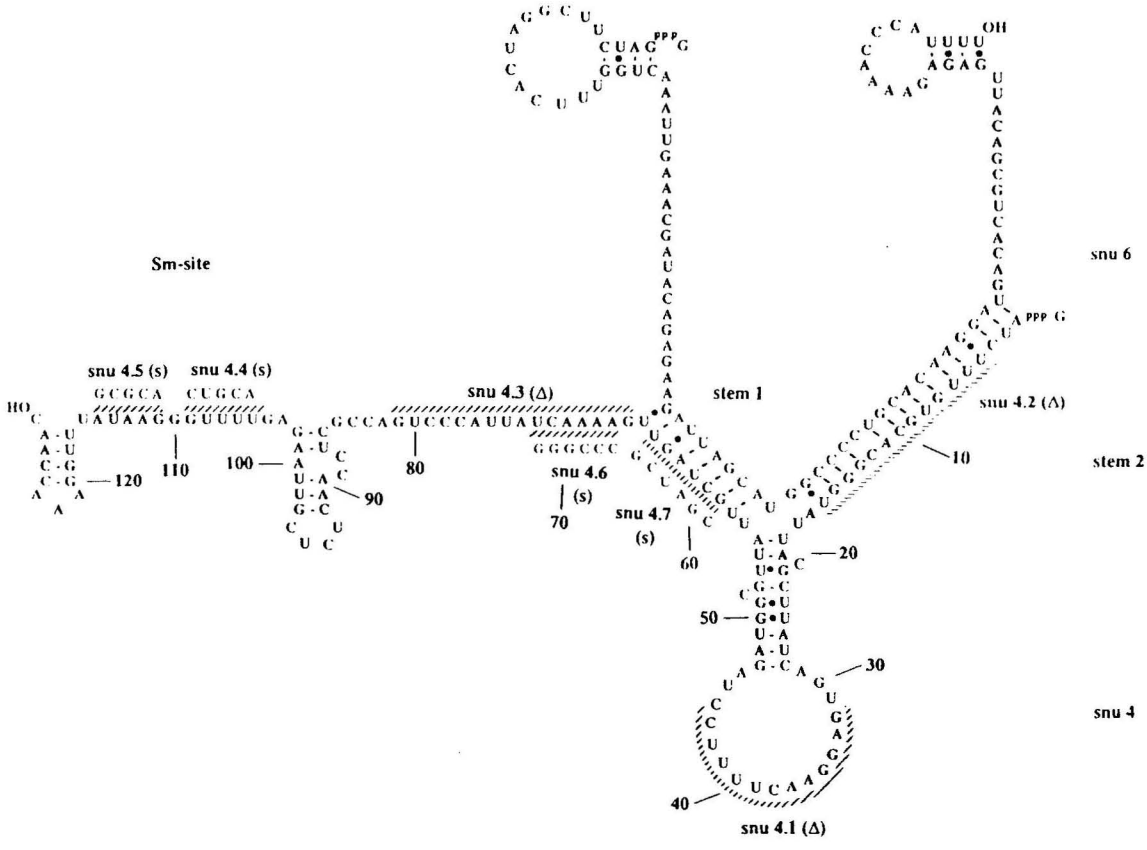

Figure 2. U4 and U6 RNA from *S. pombe*. The wild-type sequences are shown and the substituted or deleted sequences are marked by shaded lines (the mutated sequences are given in Materials and Methods). Mutations which result in deletions are indicated by 'Δ'; substitution mutations are indicated by 's'; letters in smaller size indicate the replaced nucleotides.

interaction domain. In addition, a deletion of the central loop region of the Y model was made (*snu4.1*). The region of highest primary sequence homology between U4 from vertebrates and *S. pombe* (nucleotides 56 to 72) (Dandekar *et al.*, 1989) is drawn as single stranded in the Y model, but forms a large loop in a previously proposed structure (Rinke *et al.*, 1985). Two mutations were made to test the importance of this region; replacement of a six-nucleotide sequence with an unrelated sequence containing an *XmaI* restriction site (*snu4.6*), and a large deletion (*snu4.3*) of the evolutionarily conserved loop region of the structural model of Rinke *et al.* (1985). U4, in common with other nucleoplasmic snRNAs, has the conserved binding site for the Sm class of common snRNP proteins. Previous work showed that two potential Sm-binding sites could be drawn close to the 3′ end of the molecule (Dandekar *et al.*, 1989). As drawn in Figure 2, the 3′-most site is partially base-paired. However, an alternative folding can be drawn with the 5′ Sm site included in stem-loop 2 and the 3′ Sm site single stranded, in a structure resembling the Sm site in the *S. cerevisiae* U5 snRNA (Patterson and Guthrie, 1987). To test which of these potential sites is the functional Sm site, two replacement mutations were made (*snu4.4* and *snu4.5*) which were expected to prevent the function of the 5′ or 3′ potential Sm sites respectively. These replacements were chosen so as not to impair the folding of the flanking loops of either potential Sm site.

Mutagenesis of the *snu4* gene was performed for each of these regions separately, using site-directed mutagenesis. Mutations were checked by sequencing and the mutated *snu4* genes were cloned into the *S. pombe* replicating vector YEpSUP3-5 to give plasmids psnu4.1-psnu4.7, and transformed in *S. pombe*. This vector carries the *sup3-5* suppressor tRNA allele (Hottinger *et al.*, 1982), which suppresses the *ade6-704* mutation present in the

Table 1. Loss of pSNU4 and psnu4.1-psnu4.7 during non-selective growth.

Mutation	psnu4 only (ADE$^+$ ura$^-$)	pSNU4 only (ade$^-$ URA$^+$)	Both (ADE$^+$ URA$^+$)	Class
snu4.1	0	50	76	Lethal
	0	45	145	
snu4.2	0	30	188	Lethal
	0	24	63	
snu4.3	0	20	142	Lethal
	0	18	143	
snu4.4	0	24	16	Lethal
	0	26	17	
snu4.5	18	19	32	Non-lethal
	24	9	13	
snu4.6	0	50	0	Lethal
	0	90	5	
snu4.7	0	10	158	Lethal
	0	7	162	

Independent transformants were grown on non-selective medium for approximately 12 generations and cultures were diluted and plated on non-selective medium. Plasmid loss was tested by replica plating to selective media. Numbers of colonies which had retained only the pSNU4 plasmid, only the psnu4 plasmid or both plasmids, are indicated. Figures for plasmid loss from two independent experiments are given.

host strain (Figure 1, left). The recipient for transformation carried a chromosomal gene disruption of *snu4* (*snu4::LEU2*) (Dandekar *et al.*, 1989). This had been complemented by pSNU4, which contains a wild-type copy of *snu4* on the plasmid YEpU4 (Dandekar *et al.*, 1989), which also carries the *URA4* selective marker (Figure 1, right). For transformation, selection for both plasmids was maintained. To determine whether the mutated copy of the *snu4* gene was able to supply *snu4* function, the strain with both plasmids was then grown without selection, in rich YEPD medium. Following growth in YEPD liquid culture, the strains were plated for single colonies on solid YEPD medium. Colonies were then replicated onto solid minimal medium, lacking either adenine or uracil, to determine whether either the psnu4 or pSNU4 plasmids had been lost. Since *snu4* is essential, it should not be possible to recover strains lacking both plasmids (i.e. ade$^-$, ura$^-$ strains). If the mutated *snu4* gene remains functional, either plasmid can be lost and both ade$^-$ and ura$^-$ strains should be recovered. In contrast, if the mutated *snu4* gene is non-functional, ade$^-$ strains (i.e. strains lacking the psnu4 plasmids) should be recovered, but no ura$^-$ strains (i.e. strains lacking the pSNU4 plasmid) should be found. For each mutation, at least three independent transformants were tested to determine whether colonies complemented by the mutated *snu4* gene alone could be obtained. From the data presented in Table 1 it can be seen that only in the strain carrying psnu4.5 could the mutant plasmid be recovered without pSNU4. From this it can be concluded that *snu4.5* is still able to provide *snu4* function, but all other mutations tested prevent snRNA activity or synthesis. The level of plasmid loss was reproducibly different for different psnu4 plasmids. This might indicate that some plasmids are negatively selected due to inhibition by the mutant snRNAs, although no clear differences in the growth rates of the strains were observed under selective conditions.

Northern analysis was performed for the strain complemented by psnu4.5, which had lost the pSNU4 plasmid. Figure 3A shows that the RNA is expressed stably and is hybridized by an oligonucleotide specific for the *snu4.5* mutation (Figure 3A, lane 1). The control lane shows that U4 RNA from a strain complemented by a plasmid with wild-type *snu4* is not hybridized by the oligonucleotide specific for *snu4.5* mutation (Figure 3A, lane 2). Figure 3B shows that the wild-type U4 RNA is hybridized by a probe against the entire *snu4* gene.

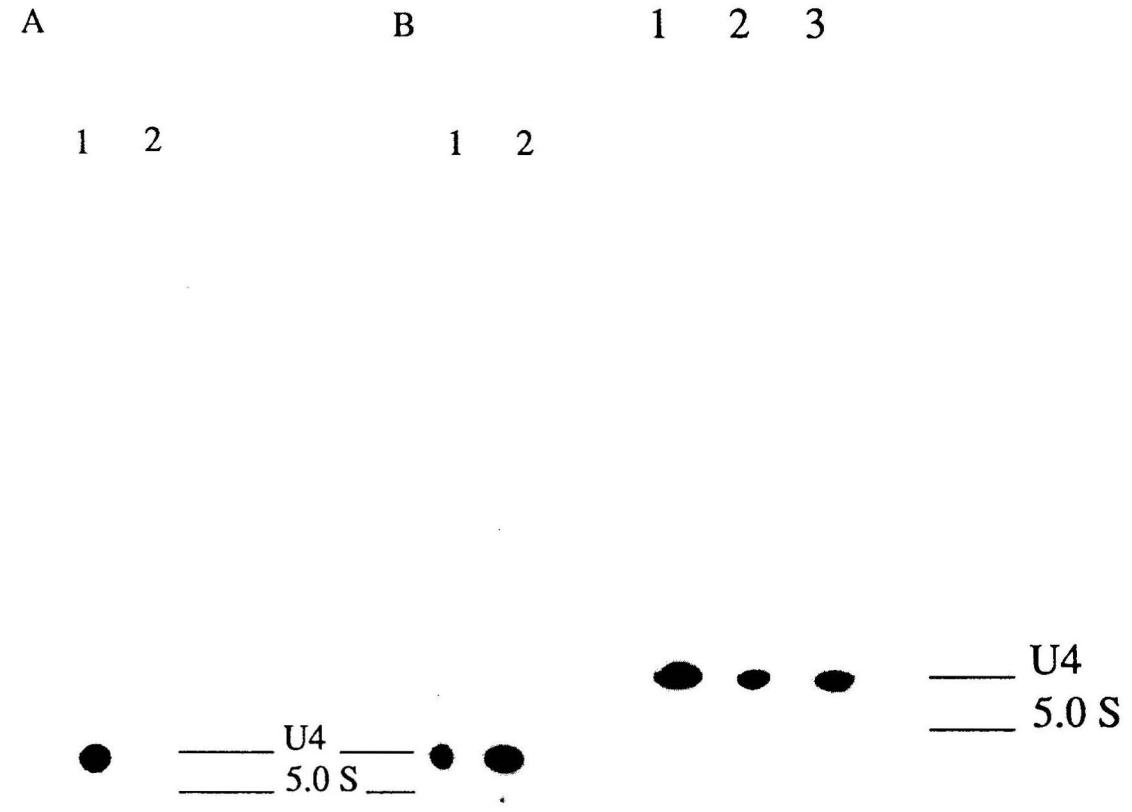

Figure 3. Northern analysis of a strain complemented by psnu4.5. (A) Northern filter probed with the oligonucleotide specific for the *snu4.5* mutation. (B) The same Northern filter hybridized by random-primed labelled *snu4* probe. Lane 1, RNA extracted from a strain carrying psnu4.5; lane 2, RNA extracted from a strain carrying pSNU4.

Figure 4. Northern analysis of a strain carrying pSNU4 and psnu4.1. Lane 1, RNA from a strain carrying only pSNU4; lanes 2 and 3, RNA extracted from two strains carrying both psnu4.1 and pSNU4. The filter was hybridized with random-primed labelled *snu4* probe. If stable, U4 RNA transcribed from the *snu4.1* gene should have been visible as a band smaller than wild-type U4.

For the essential mutations, the stability of the mutant RNA was tested in strains carrying both plasmids. For *snu4.1*, *snu4.2*, *snu4.3*, *snu4.4* and *snu4.6*, the RNA was not stable. Figure 4 shows this for RNA extracted from a strain carrying both pSNU4 and psnu4.1. U4 RNA from the *snu4.1* gene would appear as a band shorter than wild-type U4 on Northern analysis and is not detected. In contrast, U4 RNA from *snu4.7* is stable in the strain carrying pSNU4 and psnu4.7 (Figure 5). Figure 5A shows that U4 RNA from this strain is hybridized by the oligonucleotide specific for the *snu4.7*

Figure 5. Northern analysis of a strain carrying pSNU4 and psnu4.7. (A) Northern hybridization using an oligonucleotide specific for the *snu4.7* mutation. (B) The same Northern filter probed with random-primed labelled *snu4* probe. Lane 1, RNA extracted from strain carrying pSNU4; lane 2, RNA extracted from strain carrying both psnu4.7 and pSNU4.

mutation, whereas U4 from the control strain complemented by wild-type plasmid is not. Figure 5B shows that the wild-type U4 RNA is present in both strains.

DISCUSSION

We have developed and used a plasmid exchange system for *S. pombe* which may be of general use for the analysis of mutations in essential genes. This makes use of plasmids carrying the *ura4* and *sup3.5* genes. Plasmids carrying *ura4* can be strongly negatively selected on media containing 5-fluoro-orotic acid (Grimm *et al.*, 1988). Strains carrying the *ade6.704* allele accumulate a red pigment; suppression by the *sup3.5* marker (Hottinger *et al.*, 1982) not only allows growth on medium lacking adenine but also results in white colonies, allowing visual screening for plasmid loss.

This system was applied to the analysis of mutations in the essential gene *snu4*, which encodes the snRNA U4. The conclusions that can be drawn from this analysis are limited, however, by the fact that of seven mutations tested, five prevent the accumulation of the snRNA. Not surprisingly, all of these mutations also prevent *snu4* function. At present there is no reason to think that any of these mutations would interfere with the transcription of U4, and we attribute the absence of the mutant snRNAs to instability. This was somewhat surprising since mutation of equivalent sequences does not prevent U4 accumulation in *Xenopus* oocytes (Vankan *et al.*, 1990). In *S. cerevisiae*, deletion of the 5′ loop region, equivalent to mutation *snu4.1*, prevents U4 accumulation (Bordonné *et al.*, 1990). The testing of mutations in *S. cerevisiae* U4 which are equivalent to the other *snu4* mutations has not yet been reported, but deletions affecting the Sm site of U5 prevent snRNA accumulation (Jones and Guthrie, 1990).

Two mutations in *snu4* did not prevent accumulation of the snRNA; substitution of a potential Sm site near the 3′ end of the molecule does not interfere with U4 function (*snu4.4*), while substitution of the U4 sequence in stem I of the U4/U6 interaction domain prevents U4 function (*snu4.7*). In *Xenopus* functional analyses of U4 have shown a requirement for the U4 sequence in stem I, whereas the 3′ region of U4 could be replaced by a hairpin of unrelated sequence (Vankan *et al.*, 1990). Reconstitution studies *in vitro* show a requirement for stem I for the U4/U6 interaction (Hamm and Mattaj, 1989; Pikielny *et al.*, 1989; Bindereif *et al.*, 1990; Vankan *et al.*, 1990) and psoralen cross-linking confirms the presence of this base-pairing in intact U4/U6 (Rinke *et al.*, 1985). It is possible that the disruption of this interaction is responsible for the lethality of the *snu4.7* mutation.

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REFERENCES

Bindereif, A., Wolff, T. and Green, M. R. (1990). Discrete domains of human U6 snRNA required for the assembly of U4/U6 snRNP and splicing complexes. *EMBO J.* **9,** 251–255.

Bordonné, R., Banroques, J., Abelson, J. and Guthrie, C. (1990). Domains of yeast U4 spliceosomal RNA required for PRP4 protein binding, snRNP-snRNP interactions, and pre-mRNA splicing *in vivo*. *Genes & Dev.* **4,** 1185–1196.

Brennwald, P., Porter, G. and Wise, J. A. (1988). U2 small nuclear RNA is remarkably conserved between *Schizosaccharomyces pombe* and mammals. *Mol. Cell. Biol.* **8,** 5575–5580.

Bringmann, P., Appel, B., Rinke, J., Reuter, R., Theissen, H. and Lührmann, R. (1984). Evidence for the existence of snRNAs U4 and U6 in a single ribonucleoprotein complex and for their association by intermolecular base pairing. *EMBO J.* **3,** 1357–1363.

Brow, D. and Guthrie, C. (1988). Spliceosomal RNA U6 is remarkably conserved from yeast to mammals. *Nature* **334,** 213–218.

Cesareni, G. and Murray, J. A. H. (1987). Plasmid vectors carrying the replication origin of filamentous single stranded phages. In Setlow, J. K. (Ed.), *Genetic Engineering*, vol. 9. Plenum Publ. Corp., New York. pp. 135–154.

Dandekar, T., Ribes, V. and Tollervey, D. (1989). *Schizosaccharomyces pombe* U4 small nuclear RNA closely resembles vertebrate U4 and is required for growth. *J. Mol. Biol.* **208,** 371–379.

Dandekar, T. and Tollervey, D. (1989). Cloning of *Schizosaccharomyces pombe* genes encoding the U1, U2, U3 and U4 snRNAs. *Gene* **81,** 227–235.

Grimm, C., Kohli, J., Murray, J. and Maundrell, K. (1988). Genetic engineering in *Schizosaccharomyces pombe*: A system for gene disruption and replacement using the *ura4* gene as a selectable marker. *Mol. Gen. Genet.* **215,** 81–86.

Guthrie, C. (1987). Genetic analysis of yeast snRNAs. In Birnstiel, M. L. (Ed.), *Structure and Function of Major and Minor Small Ribonucleoprotein Particles*. Springer-Verlag, Heidelberg. pp. 196–212.

Gutz, H., Heslot, H., Leupold, U. and Loprieno, N. (1974). *Schizosaccharomyces pombe*. In King, R. C. (Ed.), *Handbook of Genetics*, vol. 1. Plenum Press, New York. pp. 395–446.

Hamm, J. and Mattaj, I. W. (1989). An abundant U6 snRNP found in germ cells and embryos of *Xenopus laevis*. *EMBO J.* **8,** 4179–4187.

Hashimoto, C. and Steitz, J. A. (1984). U4 and U6 RNAs co-exist in a single ribonucleoprotein particle. *Nucl. Acids Res.* **12,** 3283–3293.

Hottinger, H., Pearson, D., Yamao, F., Gamulin, V., Cooley, L., Cooper, T. and Söll, D. (1982). Nonsense suppression in *Schizosaccharomyces pombe*: the *S. pombe* sup3-e $tRNA^{ser}$-UGA gene is active in *S. cerevisiae*. *Mol. Gen. Genet.* **188,** 219–224.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153,** 163–168.

Jones, M. H. and Guthrie, C. (1990). Unexpected flexibility in an evolutionarily conserved protein-RNA interaction: Genetic analysis of the Sm binding site. *EMBO J.* **9,** 2555–2561.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Maniatis, T. and Reed, R. (1987). The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. *Nature* **325,** 673–678.

Patterson, B. and Guthrie, C. (1987). An essential yeast snRNA with a U5-like domain is required for splicing *in vivo*. *Cell* **49,** 613–624.

Pikielny, C. W., Bindereif, A. and Green, M. R. (1989). *In vitro* reconstitution of snRNPs: A reconstituted U4/U6 snRNP participates in splicing complex formation. *Genes & Dev.* **3,** 479–487.

Porter, G., Brennwald, P. and Wise, J. A. (1990). U1 small nuclear RNA from *Schizosaccharomyces pombe* has unique and conserved features and is encoded by an essential single copy gene. *Mol. Cell. Biol.* **10,** 2874–2881.

Rinke, J., Appel, B., Digweed, M. and Lührmann, R. (1985). Localization of a base-paired interaction between small nuclear RNAs U4 and U6 in intact U4/U6 ribonucleoproteins by psoralen cross-linking. *J. Mol. Biol.* **185,** 721–731.

Tollervey, D. and Mattaj, I. W. (1987). Fungal small nuclear ribonucleoproteins share properties with plant and vertebrate U-snRNPs. *EMBO J.* **6,** 469–476.

Vankan, P., McGuigan C. and Mattaj, I. W. (1990). Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus oocytes*. *EMBO J.* **9,** 3397–3404.

Woolford, J. L. (1989). Nuclear pre-mRNA splicing in yeast. *Yeast* **5,** 439–57.