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Cloning of *Schizosaccharomyces pombe* genes encoding the U1, U2, U3 and U4 snRNAs

(Recombinant DNA; yeast; ribonucleoproteins; splicing; chromosomal localization; analogues of vertebrate; immunoprecipitation)

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SUMMARY

Schizosaccharomyces pombe contains a group of five relatively abundant small nuclear RNAs (snRNAs) which are immunoprecipitated by human autoimmune antibodies of Sm serotype. The *S. pombe* RNAs hybridise to probes specific for human U1, U2, U4, U5 and U6 and in each case are similar in size to the human species. A further group of snRNAs from *S. pombe* are precipitated by antibodies against U3 containing ribonucleoprotein; the most abundant of these species hybridises to a probe specific for human U3. We have cloned the genes encoding U1, U2, U3 and U4 from *S. pombe*, together with that encoding another abundant snRNA, previously designated SPU43. U2 and U4 are encoded by single-copy genes, while two genes encode U3. The latter are not clustered, since a chromosomal Southern transfer shows them to lie on different chromosomes.

INTRODUCTION

The snRNAs are a group of small stable RNA species found in all eukaryotes examined to date, which play roles in a variety of RNA processing reactions. The most abundant snRNAs in higher eukaryotes are designated U1-U6; U1, 2, 4, 5 and 6 are required for pre-mRNA splicing (reviewed by Maniatis and Reed, 1987). U3 is found hydrogen-bonded to pre-rRNA in the nucleolus and is believed to play some role in pre-rRNA processing or ribosome assembly (Prestayko et al., 1970; Zieve and Penman, 1976).

Characterisation of snRNPs has been greatly aided by the identification of a number of human autoimmune sera containing antibodies directed

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Abbreviations: anti-Sm, human autoimmune antibodies with serotype Sm; anti-(U3) RNP, human autoimmune antibodies against U3-containing RNP; bp, base pair(s); buffer A, see MATERIALS AND METHODS, section **b**; DTT, dithiothreitol; EtdBr, ethidium bromide; kb, 1000 bp; m_3G, 2,2,7-trimethyl-guanosine; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PMSF, phenylmethylsulfonyl fluoride; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; RNP ribonucleoprotein; snRNA, small nuclear RNA; snRNP, small nuclear RNP; TBE, see legend to Fig. 1; TE, see MATERIALS AND METHODS, section **c**.

against the protein component of snRNPs. Anti-Sm sera recognise proteins (frequently B, B′ and D) (Petterson et al., 1984) common to many snRNAs which contain the highly conserved Sm-binding site (Branlant et al., 1982; Mattaj and De Robertis, 1985), while scleroderma anti-(U3)RNP sera contain antibodies which recognise the 36-kDa U3-specific protein, 'fibrillarin' (Lischwe et al., 1985; Parker and Steitz, 1987).

To better characterise the role of snRNAs in vivo, we sought to clone the genes encoding them from the fission yeast *S. pombe*, for which powerful genetic techniques exist allowing the replacement of chromosomal genes by copies manipulated in vitro.

MATERIALS AND METHODS

(a) RNA, snRNPs and immunoprecipitation

RNA and snRNPs were extracted from *S. pombe* and immunoprecipitated as described by Tollervey and Mattaj (1987). Human autoimmune sera and anti-m_3G antibodies were generously provided by Reinhard Lührmann. The methods for the isolation and characterisation of these antibodies are described by Tessars (1988).

(b) Nuclear fractionation

Nuclei were prepared by modifications of the techniques of Ide and Saunders (1981) and Hughes et al. (1987). Exponentially growing cells were harvested and spheroplasted in buffer containing 1 M sorbitol/25 mM Na · phosphate pH 6.5/15 mM DTT with zymolase and novozyme 234. Spheroplasts were pelleted by centrifugation and resuspended in buffer A (10 mM KCl/10 mM Hepes pH 7.9/5 mM $MgCl_2$/0.5 mM $CaCl_2$/10 mM DTT/0.01% Triton X-100/0.4 mM PMSF) containing 18% ficoll. The suspension was left on ice for 10 min, during which time the spheroplasts lyse osmotically. Nuclei were pelleted by centrifugation for 25 min at 15000 rev./min in an SS34 rotor. RNA was prepared from the supernatant and taken as the cytoplasmic fraction. The nuclear pellet was resuspended in 2 ml buffer A, containing 8% ficoll, and layered onto a density gradient, which had been preformed by centrifugation of 30 ml buffer A containing 1 M sorbitol and 30% (v/v) Percol (Pharmacia) for 50 min at 16000 rev./min in an SS34 rotor. Nuclei were sedimented in the gradient for 15 min at 7000 rev./min in an HB4 rotor. RNA was extracted from the band of nuclei and taken as the nuclear fraction.

(c) Chromosomal DNA

DNA was prepared by a modification of the technique of Holm et al. (1986). Crude nuclei pelleted after the first centrifugation were used to prepare chromosomal DNA. Nuclei were resuspended in 2.2 ml 100 mM NaCl/20 mM Tris · HCl pH 8/50 mM EDTA, to this was added 0.24 ml 20% *n*-lauryl sarcosine and 10 mg proteinase K, and the tubes were incubated at 37 °C for 1 h. The lysate was cleared by centrifugation in an eppendorf centrifuge and the supernatant was added to 7.5 ml of saturated CsCl in TE buffer (10 mM Tris/1 mM EDTA) and centrifuged in an SW41 rotor. Fractions containing chromosomal DNA, identified by their high viscosity, were pooled and dialysed against TE buffer. The Southern transfer of *S. pombe* chromosomes, separated by pulsed-field gel electrophoresis, was generously provided by Denise Barlow (ICRF, London).

(d) Genomic banks

A genomic bank from *S. pombe* was constructed by partially digesting genomic DNA with *Sau*3A. Fragments between 1 kb and 4 kb were separated by sucrose gradient centrifugation. The cohesive ends left by *Sau*3A were partially filled in with PolIk and the fragments were ligated into a partially filled *Xho*I site in bluescript (KS+). 200000 transformants were obtained, over 95% with inserts. Clones carrying *snu2* were identified from this bank.

(e) Hybridisation and probes

Probes specific for vertebrate snRNAs were: *U1* and *U4* probes: human *U1* and *U4* genes cloned in pSP6 and generously provided by Douglas Black; *U2* probe: oligo L15 (Ares, 1986; Black et al., 1985) complementary to the loop region conserved between human *U2* and *SNR20* from *S. cerevisiae*; *U3* probe: oligo (5′–CTATAGAAATGATCC) complementary to 15-nt *boxA* region conserved

between human *U3* and *SNR17* (Hughes et al., 1987); *U5* probe: oligo (5′–CTGGTAAAAGG-CAAG) complementary to the loop region of *SNR7* from *S. cerevisiae*, identified by Patterson and Guthrie (1987) as being highly conserved with respect to human U5; *U6* probe: *Xenopus U6* gene (Krol et al., 1987).

Human *U1* and *U4* probes were prepared by transcription with SP6 RNA polymerase in the presence of [α-^{32}P]UTP, probes from other plasmids and from purified RNAs were prepared by extending random primers (New England Biolabs) with PolIk or reverse transcriptase, respectively, in the presence of [α-^{32}P]dCTP. Oligos were labelled with [γ-^{32}P]ATP and polynucleotide kinase, in the buffers recommended by the manufacturers.

Northern and Southern transfers were carried out using Hybond N (Amersham), as described by the manufacturer.

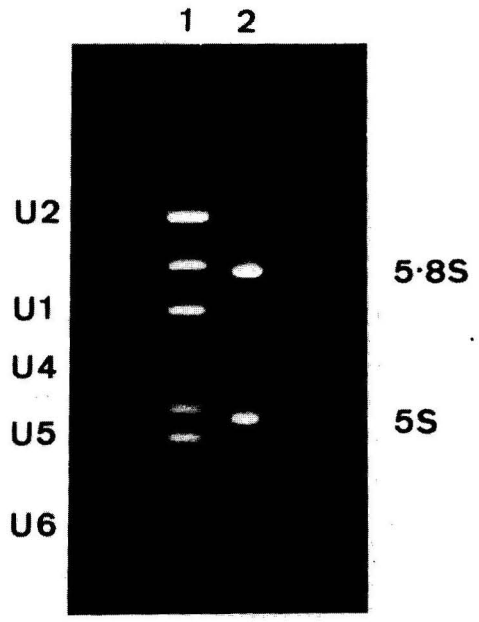

Fig. 1. *S. pombe* RNA immunoprecipitated by anti-Sm. Lanes: 1, RNA species precipitated from a lysate of *S. pombe* by anti-Sm serum; 2, total RNA from *S. pombe*. RNA was separated on an 8% polyacrylamide gel containing 8.3 M urea and 1 × TBE buffer (0.089 M Tris · base/0.089 M boric acid/0.002 M EDTA), run for 16 h at 4.5 V/cm and visualised by staining with EtdBr. The positions of U1, U2, U4, U5 and U6 are indicated, as are those of contaminating 5S (120 nt and 5.8S (158 nt) rRNA. A slight difference in the mobility of 5S and 5.8S rRNAs, when lanes 1 and 2 are compared, is probably due to the much higher purity of the immunoprecipitated RNA, as compared to the total RNA.

RESULTS

(a) Identification of analogues of vertebrate snRNAs

A unique feature found up until now only in snRNAs, is the presence of a 5′ cap structure which resembles the cap structure of mRNAs but contains m_3G rather than 7-methyl guanosine. Antibodies directed against m_3G were therefore used to specifically immunoprecipitate snRNPs from a lysate of *S. pombe*. Amongst the abundant snRNAs detectable by 3′ labelling or EtdBr staining of the precipitate, five major snRNAs previously designated SPU1 (approx. 149/151 nt), SPU2, (187 nt), SPU4 (128 nt), SPU5 (approx. 111 nt) and SPU6 (approx. 100 nt) were detected (Tollervey and Mattaj, 1987), which are also specifically precipitated by human anti-Sm sera (Fig. 1) and are similar in size to vertebrate U1, 2, 4, 5 and 6, respectively. Vertebrate U6 lacks a m_3G cap structure, but is recovered in immunoprecipitations of snRNPs by virtue of its association with U4 (Hashimoto and Steitz, 1984; Bringmann et al., 1984). Probes specific for vertebrate U1–6 each hybridise to a small RNA species from *S. pombe*. To confirm the relationship between the snRNAs detected following immunoprecipitation and those which hybridise to human snRNA probes, Northern transfers of immunoprecipitated RNAs were hybridised to probes for human U1-6 (Figs. 2, and 3, A and C). These demonstrate that the RNAs labelled U1, 2, 4, 5 and 6 in Fig. 1, hybridise to the appropriate vertebrate probes. In addition, partial 3′ sequence data indicate that *S. pombe* U1, U2 and U5 share primary sequence homology with the vertebrate RNAs (not shown).

A number of *S. pombe* snRNAs are specifically precipitated by anti-(U3)RNP sera, the most abundant of these was previously designated SPU41 (Tollervey, 1987); this RNA hybridises to a U3-specific probe (Figs. 2 and 3C) and will hereafter be referred to as U3.

In the immunoprecipitated U3 lane (Fig. 3C, lane 4) a second band is visible. This band is not seen with the total or anti-m_3G precipitated RNA and is therefore presumably due to a specific cleavage close to

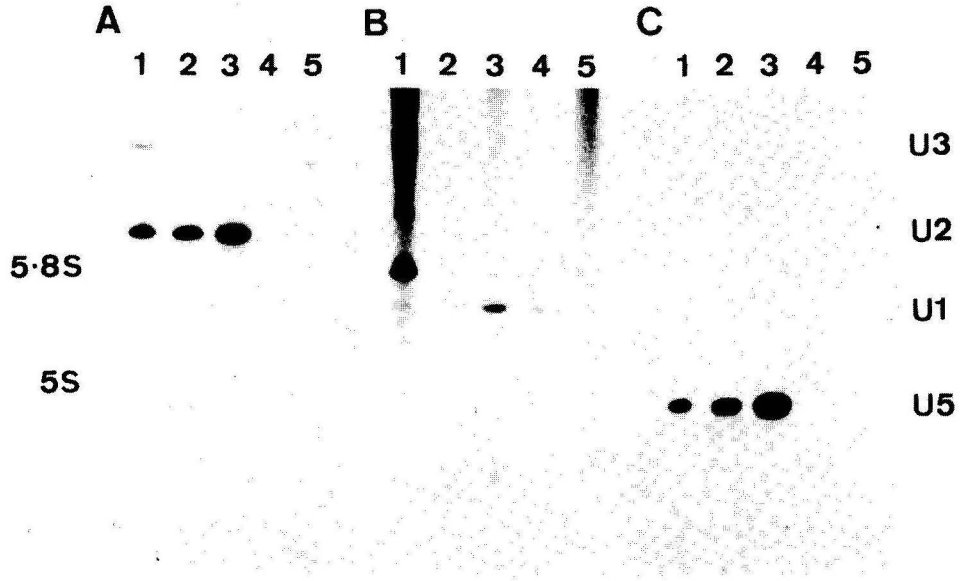

Fig. 2. *S. pombe* RNA immunoprecipitates hybridised with human snRNA probes. Probed with: (Panel **A**) human U2 (lower band) and human U3 (upper band); (panel **B**) human U1; (panel **C**) an oligo complementary to human U5. Lanes: 1, total RNA from *S. pombe*; 2, RNA immunoprecipitated by anti-m_3G serum; 3, RNA precipitated by anti-Sm serum; 4, RNA precipitated by anti-(U1)RNP serum; 5, RNA precipitated by control human serum, RNA was separated as described for Fig. 1 and electroblotted to Hybond N. The filter was hybridised successively with probes specific for the human snRNAs indicated. The gels and positions are as in Fig. 1.

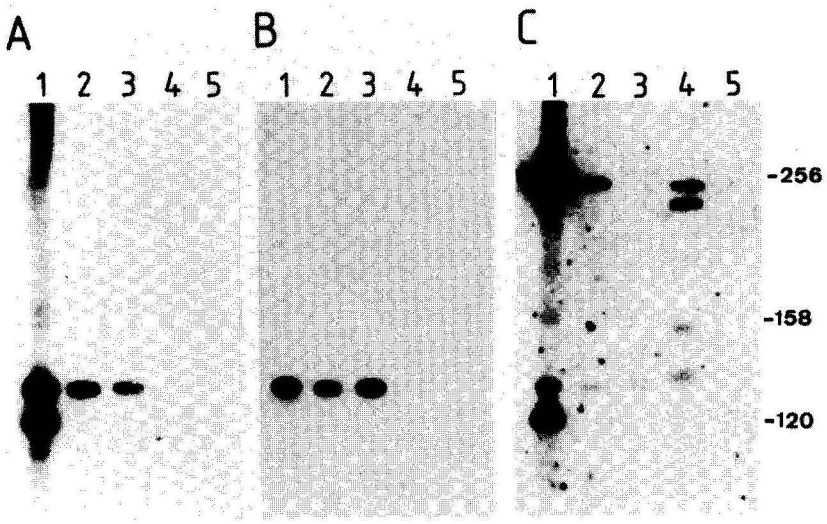

Fig. 3. *S. pombe* RNA immunoprecipitates hybridised with probes for human and *S. pombe* snRNAs. Probed with: (Panel **A**) cloned human U4; (Panel **B**) cloned *S. pombe snu4*; (panel **C**) an oligo specific for human U3. Lanes: 1, total RNA from *S. pombe*. RNA immunoprecipitated by: anti-m_3G serum (lane 2); anti-Sm serum (lane 3); anti-(U3)RNP serum (lane 4); control human serum (lane 5); RNA was separated as described for Fig. 1 and electroblotted to Hybond N. The filter was hybridised successively with the probes indicated. The positions of 5S rRNA (120 nt), 5.8S rRNA (158 nt) and 7SL RNA (256 nt) are given.

the 5′ end of the RNA which has occurred during the incubation of the cell extract with the antibodies. Specific cleavage of U1 snRNA close to the 5′ end has previously been reported (Lerner et al., 1980).

(b) Cloning of snRNA-encoding genes

snRNPs were preparatively fractionated over a protein A Sepharose column to which anti-m_3G antibodies had been bound. RNA was recovered from the eluted snRNPs and size fractions from a polyacrylamide gel were used to probe a *Hin*dIII genomic bank of *S. pombe* (Beach et al., 1982). Positive colonies were counterscreened with ^{32}P-labelled probe to rRNA, to eliminate those lit by residual rRNA in the snRNA preparation. Plasmid DNA was extracted from the remaining colonies and used to prepare probe for Northern transfers of total RNA and snRNAs immunoprecipitated by anti-m_3G antibodies. Clones which gave strong signals were further analysed. Clones containing *snu1*, *snu3A*, *snu3B* and *snu4* were obtained in this way, as well as *snu43*, which encodes another abundant snRNA (Tollervey, 1987). The gene encoding *S. pombe* U2 was not obtained from the *Hin*dIII bank; to clone this gene a *Sau*3A partial bank was constructed in a Bluescript (KS+) vector. This bank was probed with the L15 oligo, which hybridises to an evolutionarily conserved region of U2 (Ares, 1986; Black et al., 1985). Three characterised positive clones each carry overlapping fragments containing the *snu2* gene.

The RNA species encoded by all of the cloned genes are present in RNA immunoprecipitated by anti-m_3G antibodies. To identify the snRNA species encoded by each cloned gene, the size and immunoprecipitation pattern of the cloned snRNA was compared to the known snRNAs of *S. pombe* by Northern hybridisation.

The identity and size of the RNA encoded by *snu4* and that which hybridises to cloned human U4 is clearly shown in Fig. 3,A and B; similar experiments have been performed for *snu1*, *snu2*, *snu3A* and *snu3B*. Moreover, the greatly simplified pattern of snRNAs obtained following immunoprecipitation allows an unambiguous assignment of the abundant cloned snRNAs. The RNA encoded by *snu1* hybridises to an RNA species precipitated by anti-(U1)RNP serum and human serum G1, previously

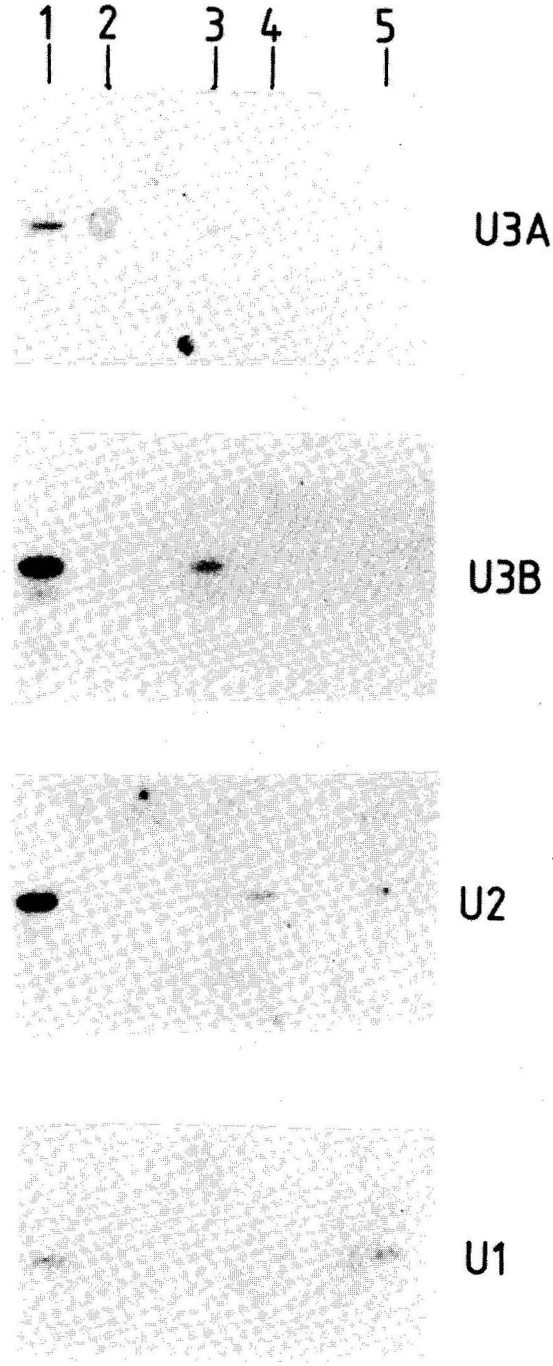

Fig. 4. *S. pombe* RNA immunoprecipitates hybridised with cloned snRNAs. Lanes: 1, total RNA from *S. pombe*; 2, control human serum; 3, mouse anti-(U3)RNP serum; 4, human serum G2 specific for U2, U4 and U5 from *S. pombe* (D.T., G. Tessars and R. Lührmann, submitted; 5, human serum G1 specific for U1 from *S. pombe* (D.T., G. Tessars and R. Lührmann, submitted). RNA was processed as described for Fig. 3.

shown (D.T., G. Tessars and R. Lührmann, submitted) to strongly precipitate U1-snRNPs from *S. pombe* (Fig. 4, lane 5), but not to human serum G2, specific for U2, U4 and U5 snRNPs and inactive against U1 (Fig. 4, lane 4). The *snu2* and *snu4* clones hybridise to RNAs precipitated strongly by anti-Sm G2 but only weakly by serum G1 (Fig. 4, lane 5) or anti-(U1)RNP serum.

Two genes encoding U3 were isolated; both hybridise to identically sized RNA species, which are precipitated by anti-(U3)RNP sera (Fig. 4) and both hybridise to a probe to a region of U3 conserved from humans to *S. cerevisiae* (Hughes et al., 1987). This oligo probe was also found to hybridise to similarly sized RNAs from other unrelated fungi (Fig. 5), and it may be of general use in cloning U3-like snRNAs. Two species, U3A and U3B, are resolved on two-dimensional gels (Tollervey, 1987), both of which are specifically precipitated by anti-(U3)RNP sera. From the genomic bank, two clones encoding U3A and three clones encoding U3B were isolated. The U3-specific oligo hybridises to two genomic fragments of appropriate size (not shown), making it very probable that *S. pombe* U3 is encoded by two genes. The cloning of U3A has recently been independently reported by Porter et al. (1988), who also found a U3 specific probe to hybridise to two genomic fragments.

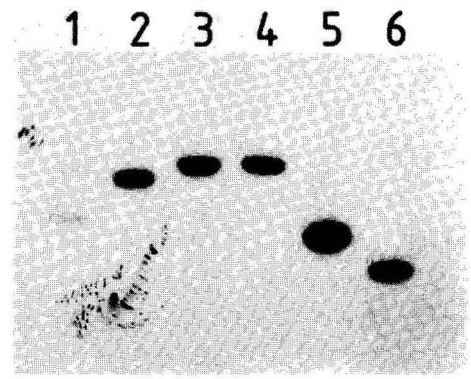

Fig. 5. Hybridisation of U3-specific oligo to RNA from a variety of fungi. An oligo complementary to the region conserved between vertebrate U3 and *S. cerevisiae* snR17 (Hughes et al., 1987) was found to hybridise to similarly sized RNAs from a range of fungi. Lanes: 1, *Aspergillus nidulans*; 2, *Neurospora crassa*; 3, *S. pombe*; 4, *Candida albicans*; 5, *Saccharomyces diastaticus*; 6, human. RNA was processed as described for Fig. 3.

Another clone carries the gene *snu43*, which encodes the abundant snRNA species of approx. 600 nt, previously designated SPU43 (Tollervey, 1987). Additional clones were isolated which encode lower-abundance snRNAs; these have not yet been further characterised.

Consistent with their identification as snRNAs, the RNA species encoded by *snu1*, *snu2*, *snu3A*, *snu3B*, *snu4* and *snu43* have all been shown to be

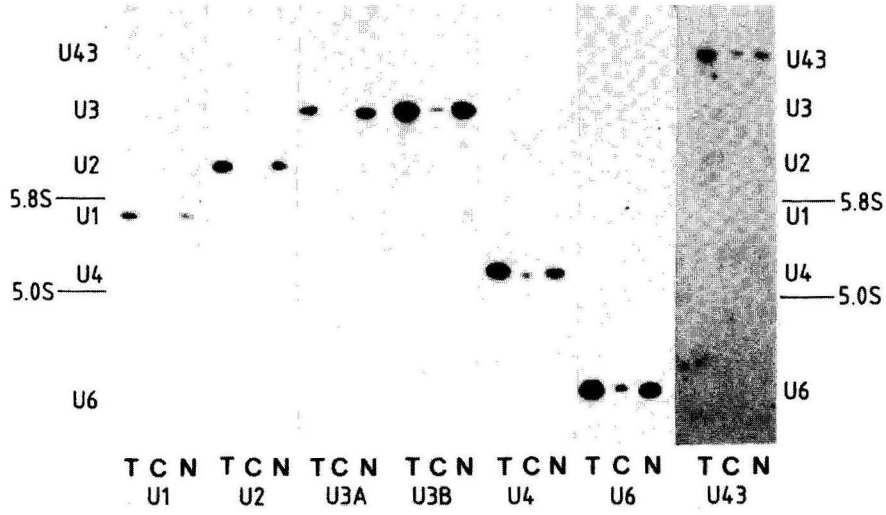

Fig. 6. Nuclear localisation of cloned *S. pombe* RNAs. The panels were probed separately with the genes encoding the *S. pombe* snRNAs indicated or with the *Xenopus* U6 clone. Lanes in each panel: T, total RNA from *S. pombe*; C, RNA from cytoplasmic fraction; N, RNA from nuclear fraction. RNA was processed as described for Fig. 3. The position of the 5S (120-nt) and 5.8S (158-nt) rRNAs are indicated as size markers. The same number of cell equivalents were loaded in each lane.

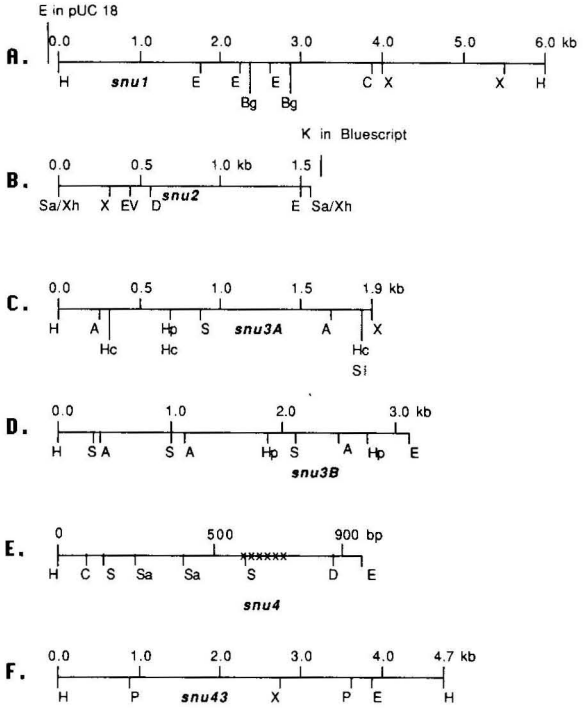

Fig. 7. Restriction maps of regions encoding *S. pombe* snRNAs. Sites are: A, *Acc*I; Bg, *Bgl*II; C, *Cla*I; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; S, *Ssp*I; Sa, *Sau*3A; Sl, *Sal*I; X, *Xba*I; Xh, *Xho*I. (Map **A**) A 6.0-kb genomic *Hin*dIII fragment containing *snu1*. The coding region lies within the 1.75-kb *Hin*dIII-*Eco*RI fragment. No sites were found for *Bam*HI, *Kpn*I, *Sma*I. (Map **B**) A 1.55-kb *Sau*3A partial fragment containing *snu2*. The coding region lies within 0.4 kb of the *Dra*I site, as defined by the end-point of one of the *Sau*3A fragments obtained from the genomic bank. No sites were found from *Bam*HI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I or *Xho*I. (Map **C**) A 1.9-kb genomic *Hin*dIII-*Xba*I fragment containing *snu3A*. The coding region lies within the 850-bp *Ssp*I-*Acc*I fragment. No sites were found for *Bam*HI, *Bcl*I, *Eco*RI, *Pst*I, *Sma*I or *Xho*I. (Map **D**) A 3.1-kb genomic *Hin*dIII-*Eco*RI fragment containing *snu3B*. The coding region lies within the 400-bp *Ssp*I-*Acc*I fragment. No sites were found for *Aat*II, *Bcl*I, *Bam*HI, *Cla*I, *Pst*I, *Sal*I, *Xba*I, *Xho*I. (Map **E**) A 0.9-kb genomic *Hin*dIII-*Eco*RI fragment containing *snu4*. The coding region includes the *Ssp*I site and is marked (× × × ×). No sites were found for *Pst*I, *Bam*HI, *Sal*I, *Xba*I or *Xho*I. (Map **F**) A 4.7-kb genomic *Hin*dIII fragment containing *snu43*. The coding region lies within the 1.9-kb *Pst*I-*Xba*I fragment. No sites were found for *Bam*HI or *Xho*I.

localised in the nucleus as has *S. pombe* U6 (Fig. 6). Crude restriction maps of the genes *snu1*, *snu2*, *snu3A*, *snu3B*, *snu4* and *snu43* are shown in Fig. 7. To assess the gene copy numbers, genomic Southern transfers were probed with small ^{32}P-labelled fragments encoding U2 and U4 (Fig. 8); in both cases single bands are lit in genomic DNA cut with a variety of enzymes. For *snu2* the same result was obtained using an ^{32}P-labelled oligo to an evolutionarily conserved region of U2.

In *S. cerevisiae* all snRNAs cloned to data are encoded by single-copy genes, with the exception of U3. This snRNA is more abundant than other snRNAs cloned from *S. cerevisiae* and is encoded by two genes, which are functionally redundant at least at the level of viability (Hughes et al., 1987). However, *S. pombe* U3 is not significantly more abundant than U1, U2 or U4 and the finding of the same genetic arrangement in *S. pombe* suggests that the two copies of U3 have functionally distinct roles, at least under some circumstances.

(c) Chromosomal localisation

To determine whether the cloned genes are clustered, and as an initial step in genetic mapping, the chromosomal locations of the loci were determined by Southern hybridisation of intact *S. pombe* chromosomes separated by pulse-field gel electrophoresis (Fig. 9) *snu2*, *snu3A*, *snu43* are located on chromosome I, while *snu1*, *snu3B* and *snu4* are on chromosome II. There is thus no evidence of clustering and, indeed, the two genes encoding U3 are located on different chromosomes.

(d) Conclusions

We have identified and cloned a number of genes encoding abundant snRNAs from *S. pombe*. It is very probable that amongst the cloned *S. pombe* snRNAs, are the analogues of U1, U2, U3 and U4. These snRNAs are abundant, localised in the nucleus, possess the 5′ m_3G cap structure characteristic of snRNAs and hybridise to probes specific for the corresponding vertebrate snRNA. U1, 2 and 4 are associated with analogues of the conserved 'Sm-proteins', which form components of the U1, 2 and 4 snRNPs in vertebrates (Lerner and Steitz, 1979), while *S. pombe* U3A and B are associated with the analogue of the vertebrate 36-kDa RNP carried by U3 (Lischwe et al., 1985; Parker and Steitz, 1987).

The cloning of single copy genes encoding the analogues of the major vertebrate snRNAs from

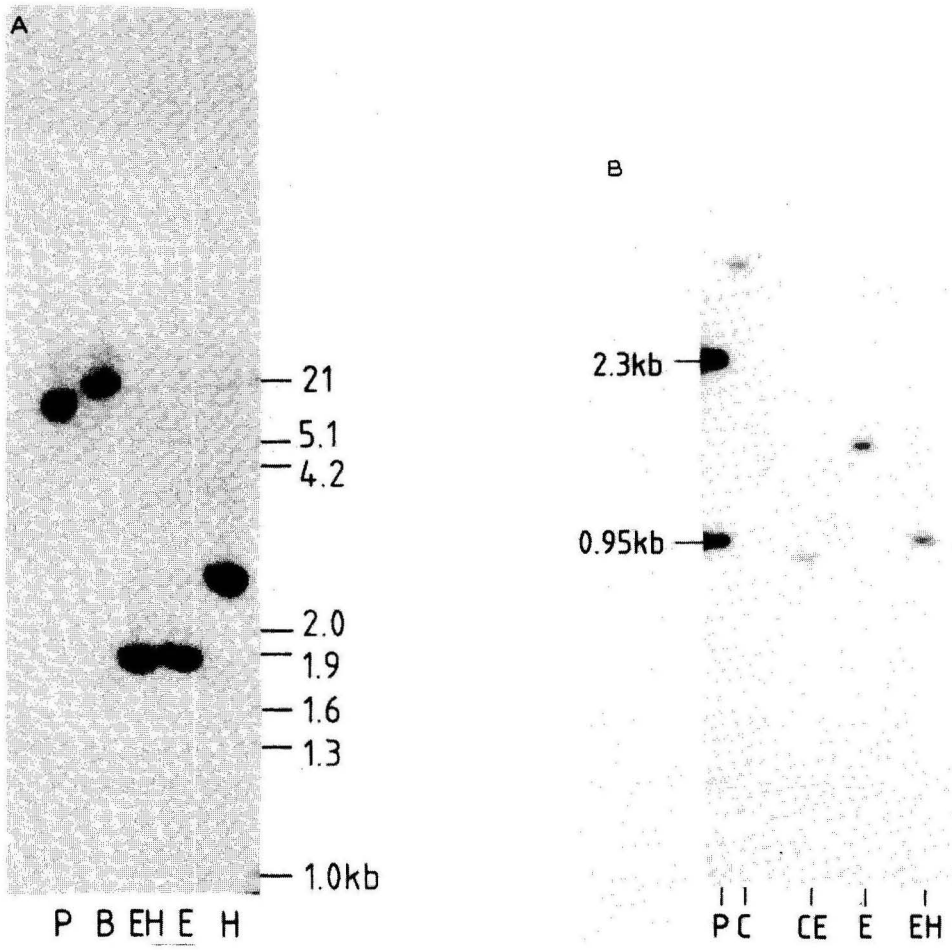

Fig. 8. Genomic Southern transfers (Panel A) Probed with *snu2*. Genomic DNA (cut with P, *Pst*I; B, *Bam*HI, EH, *Eco*RI + *Hin*dIII; E, *Eco*RI; H, *Hin*dIII) was separated on an 0.9% agarose gel in 1 × TBE, run for 15 h at 1.5 V/cm and transferred to a Hybond N filter. The filter was probed with a 400-bp fragment containing *snu2*, extending from the *Dra*I site to the end of one of the clones obtained from the *Sau*3A partial genomic bank. (Panel B) Probed with *snu4*. Lanes: P, plasmid encoding *snu4* cut with *Eco*RI + *Hin*dIII; C, CE, E, EH, genomic DNA cut with *Cla*I, *Cla*I + *Eco*RI, *Eco*RI and *Eco*RI + *Hin*dIII, respectively. Gel conditions as in panel A. A plasmid carrying the 950-bp *Eco*RI-*Hin*dIII fragment containing *snu4* was cut at the *Ssp*I site within the gene and probe synthesis was specifically primed by an oligo carrying the sequence of the 5′ 15 nt of the RNA, to generate a probe entirely within the coding region of the gene.

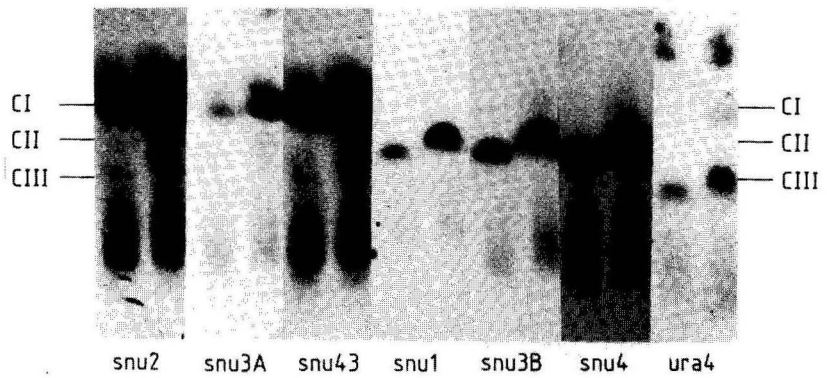

Fig. 9. Southern transfer of *S. pombe* chromosomes separated by pulsed-field gel electrophoresis. The filter (Hybond N, Amersham) was hybridised successively with ^{32}P-labelled clones encoding the genes indicated. The positions of CI, CII and CIII were identified by EtdBr staining. Chromosomes were separated on an 0.7% agarose gel containing 0.5 × TBE run for seven days at 15°C with an orthogonal field of 3.5 V/cm and pulse intervals of 55 min.

S. pombe now offers the possibility of directly analysing their function in vivo using genetic techniques in *S. pombe*.

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