Schizosaccharomyces pombe U4 Small Nuclear RNA Closely Resembles Vertebrate U4 and is Required for Growth

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The single-copy gene snu4, which encodes the small nuclear RNA (snRNA) U4, has been cloned and sequenced. Schizosaccharomyces pombe U4 is 128 nucleotides in length, similar in size to vertebrate U4 and shows substantial primary and secondary structure homology. The gene lacks sequences closely resembling vertebrate snRNA transcription signals, but has a TATA box at -33 to -30; TATA sequences flanked by several additional conserved nucleotides are found in the same position in the 5' regions of other snRNA genes from Schiz. pombe.

The cloned snu4 gene was disrupted by transposon mutagenesis and used to replace one chromosomal copy of snu4 in a diploid strain. On sporulation snu4 haploid strains could not be recovered, demonstrating that U4 is required, at least for spore germination. Haploid snu4 strains are viable if they also carry snu4+ on a replicating plasmid but are unable to loose the plasmid under non-selective growth, demonstrating a continuous requirement for U4 for viability.

1. Introduction

The small nuclear RNAs (snRNAs†) are a group of stable low molecular weight RNA species found in the nuclei of all eukaryotes examined to date, which are believed to play roles in a variety of RNA processing reactions. The most studied species, designated U1, U2, U4, U5 and U6, are all required for pre-messenger RNA (pre-mRNA) splicing in vitro (for a review, see Maniatis & Reed, 1987). Splicing of pre-mRNA occurs within a complex structure termed a spliceosome. Major events in spliceosome assembly include the association of U1 and U2 small nuclear ribonucleoproteins (snRNPs) with the 5' splice junction and site of branch formation, respectively. U4 and U6 snRNA are associated by hydrogen-bonding and are found in a single snRNP particle (Bringmann et al., 1984; Hashimoto & Steitz, 1984) and may associate with the spliceosome as a U4/U5/U6 complex; U4 is released from the spliceosome before intron cleavage (Pikielny et al., 1986; Cheng & Abelson, 1987; Lamond et al., 1988). The site and mechanism of action of U4 snRNP remains obscure but it is essential for splicing activity in vitro. We wished therefore to use molecular genetic techniques to study the function of U4 snRNPs in vivo.

Genetic analyses in the budding yeast Saccharomyces cerevisiae (S. cerevisiae) have shown that the major spliceosome-associated snRNAs, including U4, are all essential for viability (Siliciano et al., 1987; for a review, see Guthrie & Patterson, 1988). However, it is clear that, while many features are common between pre-mRNA splicing in S. cerevisiae and higher eukaryotes, there are also distinctive differences in the system of S. cerevisiae. We therefore considered it to be useful to analyse genetically the role of snRNPs in the fission yeast Schizosaccharomyces pombe (Schiz. pombe), in which both the RNA and protein components of the snRNPs as well as signals in pre-mRNA introns, are rather closer to those of higher eukaryotes (Tollervey & Mattaj, 1987; Käuffer et al., 1985; Brennwald et al., 1988; D. Tollervey, G. Tessarz & R. Lührmann, unpublished results).

We have previously reported the identification of snRNAs from Schiz. pombe including the analogues of vertebrate U1-U6 and the cloning of the genes encoding several such snRNAs, including the single-copy gene snu4 (Tollervey & Mattaj, 1987; Tollervey, 1987; Dandekar & Tollervey, 1989). Schiz. pombe U4 is similar in size to vertebrate U4 and has many features characteristic of snRNAs: (1) it is localized in the nucleus; (2) it carries the distinctive 2,2,7-trimethylguanosine cap structure

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‡ Abbreviations used: snRNAs, small nuclear RNAs; snRNPs, small nuclear ribonucleoproteins; bp, base-pairs; kb, 10^3 bases or base-pairs.
unique to snRNAs; (3) it is in a ribonucleoprotein particle, associated with proteins that are immunologically related to the vertebrate "Sm-proteins" common to U1, 2, 4 and 5; and (4) it retains a conserved, functional binding site for such proteins since Schiz. pombe U4 is able to associate with Xenopus Sm-proteins following microinjection into Xenopus oocytes.

Since both the RNA and protein components of Schiz. pombe U4 snRNPs appear to have been highly conserved with respect to those of vertebrates, it is likely to be of value in analysing the function of this snRNP in vivo. As a first step in such an analysis, we have determined whether U4 is required for viability.

2. Materials and Methods

(a) Strains and media

Growth and handling of Schiz. pombe have been described by Gutz et al. (1974). The diploid strain used for gene disruption carries h', leu1-32; h', leu1-32; ura4-18/ura4-18; ade6-1014/ade6-1014 (P. Nurse, personal communication).

(b) Chromosomal DNA

DNA was prepared by a modification of the technique of Holm et al. (1986). Crude nuclei fractionated as described by Dandekar & Tollervey (1989) were used to prepare chromosomal DNA. Nuclei were resuspended in 2:2 ml of 100 mM-NaCl, 20 mM-Tris-HCl (pH 8), 50 mM-EDTA. To this was added 0-24 ml of 20%, N-lauryl sarcosyl and 10 mg of 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 loaded onto 7-5 ml of saturated CsCl in TE buffer (10 mM-Tris (pH 8), 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.

(c) Sequence analysis

The sequence analysis was carried out using the assistance of the GCG software package (Devereux et al., 1984). The alignments for the primary structure were obtained using the program "gap" of the above package for the pairwise comparisons to Schiz. pombe (gap weight 5-0, gap length weight 0-3) before the sequences were aligned together and the common alignment was further optimized.

(d) RNA

RNA was prepared by the hot phenol/guanidinium method (Maniatis et al., 1982) as modified by Tollervey & Mattaj (1987). Immunoprecipitations of RNA were carried out as described by Tollervey & Mattaj (1987). The U4 RNA 3' end was determined enzymatically (Donis-Keller et al., 1977) using gel-purified immunoprecipitated RNA. The 5' RNA sequence was determined by primer extension as described by Geliebter (1987), using an oligonucleotide complementary to nucleotides 56 to 72 of Schiz. pombe U4.

(e) DNA sequencing

The sequence of the snu4 gene was determined byideoxynucleotide chain termination (Sanger et al., 1977). The region shown was sequenced on both strands.

(f) Transposon mutagenesis and transformation

The method of transposon mutagenesis was as described by Seifert et al. (1986). The transposon, m-Tn3 (LEU2), carries the S. cerevisiae selective marker LEU2, which complements the U4-1 mutation of Schiz. pombe (Beach & Nurse, 1981). Schiz. pombe was transformed using the protocol described for S. cerevisiae by Ito et al. (1983).

3. Results

(a) Localization and sequence of snu4

The cloning of the gene snu4 and several other snRNA genes from Schiz. pombe has been reported (Dandekar & Tollervey, 1989). Southern hybridization using probe prepared from purified U4 was used to localize the gene to and within the 940 bp genomic HindIII–EcoRI fragment shown in Figure 1. This region was sequenced using deoxy nucleotides. The 3' end of the coding region was identified by comparison with the 3' end RNA sequence obtained from purified U4 snRNA. The 5' end of the coding region was identified by primer extension with an oligonucleotide complementary to nucleotides 56 to 72 of the RNA. These gave a single 3' and a single 5' end-point at the positions indicated. The length of the RNA given by these end-points is very close to our estimates of the size of U4 based on gel mobility. However, the slight possibility that the RNA might be a few nucleotides longer and that the stop in the primer extension reaction might be due to reasons other than the true end of the RNA cannot be completely excluded. The 128-nucleotide RNA sequence and the DNA sequence extending to 208 nucleotides 5' and 463 nucleotides 3' of the coding region are shown in Figure 2.

No sequences with high homology to the consens us vertebrate snRNA gene transcription signals were detected. However, a TATA sequence (underlined in Fig. 2) is present at −33 to −30, a position similar to that of TATA sequences of other genes of Schiz. pombe transcribed by polymerase II (Russel, 1983). The snu4 gene carried by YEpsnu4 has only 220 nucleotides of 5' flanking sequence. Northern hybridization shows that this is sufficient for full expression of the snu4 gene (data not shown). Comparison of the 3' flanking region of snu4 with those of snu2 (T. Dandekar & D. Tollervey, unpublished results; J. A. Wise, personal communication) and snu3A (Porter et al., 1988) reveals a region of moderate sequence conservation (underlined in Fig. 2) at a position similar to that of the transcription termination signals of vertebrate U snRNA genes (for a review, see Dahlberg & Lund, 1988).
(b) U4 structure

Comparison of the primary structure of U4 from Schiz. pombe and a number of other eukaryotes is shown in Figure 3. These RNAs are clearly homologous. The number of matched nucleotides is 77 for human, 72 for rat, 81 for chicken, 79 for Drosophila, 73 for bean and 82 for S. cerevisiae.

Figure 4 shows a comparison of a proposed secondary structure for human U4 (Rinke et al., 1985) and a possible secondary structure for Schiz. pombe U4. Blocks of identical nucleotides are enclosed in boxes, and a potential Sm-binding site (AGUUUGGG) is drawn in bold type. Another sequence with homology to a consensus Sm-binding site (AAUAUUUGG) is present at positions 112 to 120 (underlined in Fig. 4), and in fact this sequence is aligned against the Sm-binding sites of other organisms in Figure 3. This sequence, however, has an A residue within the poly(U) tract, in contrast to
Sm-binding sites from other snRNAs (Reddy, 1988) including Schiz. pombe U2 (Brennwald et al., 1988). If the sequence at nucleotides 112 to 120 is indeed the Sm-binding site, then the predicted secondary structure would more closely resemble that of S. cerevisiae, with stem/loop III extended and stem/loop IV absent. An alternative secondary structure for U4, proposed by Myslinski et al. (1984) on the basis of comparison with the Drosophila U4 sequence, is modified from that shown in Figure 4 by an extension of stem 1 and reduction of loop II. The structure derived is not compatible with the sequence of Schiz. pombe U4.

The loop II region is highly conserved from human to Schiz. pombe U4. To determine whether this region is also conserved in U4 snRNAs from other fungi an oligonucleotide complementary to nucleotides 56-72 of Schiz. pombe U4 was synthesized. This oligonucleotide hybridizes strongly to U4-sized RNAs from Aspergillus nidulans, Neurospora crassa, Candida albicans, Saccharomyces diastaticus and S. cerevisiae (data not shown).

Phylogenetic comparisons have led to the proposition of the "Y"-model for the structure of the U4/U6 interaction (Brow & Guthrie, 1988). The sequence of Schiz. pombe U6 has been reported
Figure 4. Comparison of predicted secondary structures of Schiz. pombe and human U4. Human U4 is drawn according to the secondary structure of Rinke et al. (1985). Schiz. pombe U4 is drawn to resemble this structure. Boxed regions contain identical sequences. The putative Sm-binding site is shown in bold type and a second sequence with homology to the Sm-binding site is underlined (see the text).

U4 snRNA from Schiz. pombe

(c) Disruption of snu4

The gene snu4 has been shown to be present in a single copy in haploid cells (Dandekar & Tollervey, 1989). To determine whether this gene is required for viability, a gene replacement experiment was performed. Cloned snu4 was disrupted by insertion of a Tn3 transposon carrying AmpR and the LEU2 gene from S. cerevisiae. Plasmids in which the transposon is inserted into the coding region were identified by Southern hybridization with cDNA probe prepared from purified U4. The disrupted copy of snu4 was used to replace one chromosomal copy of snu4 in a diploid strain homozygous for leu1.32, which is complemented by the LEU2 gene carried by the transposon (Beach & Nurse, 1981; Seifert et al., 1986; Ribes et al., 1988). Of four transformed diploids analysed by genomic Southern blots, three gave the pattern of bands predicted for
Figure 5. Proposed interaction of U4 and U6. *Schiz. pombe* U4 and U6 (upper panel) and human U4 and U6 (lower panel) are drawn in the Y-structure proposed by Brow & Guthrie (1988). The site of the intron in *Schiz. pombe* U6 (Tani & Ohshima, 1989) is indicated by an arrow. Two potential stem/loop structures, conserved in position between both U6 sequences are drawn at the 5' and 3' ends of human and *Schiz. pombe* U6. The positions of Watson-Crick base-pairs (−) and G·U base-pairs (●) are indicated.
correct gene replacement at the snu4 locus (Fig. 6). Digestion with EcoRI and ClaI gives a 800 nucleotide genomic DNA fragment (Fig. 1), which is visible in Figure 6, lanes 1 and 2. In the transformed diploid (Fig. 6, lane 1) additional bands of 1·3 and 1·7 kb are expected, due to cleavage at the EcoRI sites within the transposon (Seifert et al., 1986). The hybridization probe is to a region entirely within the coding sequence of snu4, demonstrating that this region has indeed been interrupted by the insertion. The growth of the diploid carrying one disrupted copy of snu4 is indistinguishable from that of the wild-type parent. Densitometer tracing of Northern hybridizations demonstrate that the ratio of U2/U4 in these strains is identical, indicating that the regulation of snu4 is such as to provide complete dosage compensation.

The transformed diploid is homozygous h\(^+\)/h\(^+\) at the mating-type locus. To obtain haploid spores, rare h\(^{-}\)/h\(^{-}\) revertants were identified (Gutz et al., 1974), which switch mating type to h\(^+\)/h\(^+\) and sporulate. Amongst 2700 haploid spores, none was found to carry the inserted LEU\(^+\) marker, demonstrating that snu4 is essential, at least for spore germination.

(d) Genetic complementation with snu4

To determine whether snu4 is also required for vegetative growth, and to confirm fully that the lack of U4 is responsible for the lack of growth of the LEU\(^+\) haploids, a complementation experiment was performed.

A Schiz. pombe replicating plasmid, YEpU4, has been constructed (Fig. 7) that carries the S. cerevisiae 2 µ origin of replication and the ura4 gene from Schiz. pombe, together with the phage F1 replication origin, which permits the isolation of single-stranded DNA molecules suitable for DNA sequencing or in vitro mutagenesis. The 940 bp EcoRI–HindIII region containing snu4 (Fig. 1) was subcloned into YEpU4 to generate YEpsn4. The sequence of the snu4 fragment is not compatible with it containing an intact protein gene, and this region does not hybridize to other small RNA species. The snu4::Tn3(LEU2)/snu4\(^+\) diploids also carry ura4-D18 and ura4-D18 (Grimm et al., 1988), and YEpsn4 was transformed into such a strain. Spores from the diploid carrying YEpsn4 were plated on medium lacking uracil to select for those that had retained the plasmid during meiosis, and replicated onto medium with and without leucine. Of 90 spores tested, 53\% carried the inserted LEU2 marker. Southern hybridization of two separate LEU2\(^+\) strains confirmed that they lack an intact genomic copy of snu4 (Fig. 6). The transformed diploid (Fig. 6, lane 3) and the snu4\(^-\) haploid (Fig. 6, lane 4) give rise to HindIII fragments of 2·0 and 6·3 kb, due to the disruption of the 4·7 kb
genomic HindIII fragment carrying snu4 by the transposon (3-6 kb) which contains a single HindIII site (Seifert et al., 1986). In the diploid strain (lane 3), but not in the haploid strain (lane 4), there is an intact chromosomal copy of the snu4 gene, which gives rise to the 4-7 kb genomic HindIII fragment. In the haploid strain (lane 4) the 9-5 kb band is the complementing YEpsnu4 plasmid, which carries the intact snu4 allele, linearized by cleavage at the unique HindIII site (Fig. 7). The snu4− strain shown in Figure 6 (lane 4) and a snu4+ sister strain were grown without selection for the YEpsnu4 plasmid for approximately 12 generations. Cells from each culture were plated on non-selective medium and replicated onto media with and without uracil, to test for the presence of the plasmid. From 900 snu4− colonies, 150 (17%) were found to retain the plasmid, a value in good agreement with our previous estimates of the stability of this plasmid. From 900 snu4− colonies, 100% retained the YEpsnu4 plasmid. The same result was obtained in three independent experiments.

4. Discussion

U4 snRNA from Schiz. pombe shows high primary sequence homology to U4 snRNAs from vertebrates, insects, and the budding yeast S. cerevisiae. Perhaps surprisingly, overall homology of Schiz. pombe U4 compared with any of the other species is similar, with generally around 60 to 63% matched nucleotides. U4 from Schiz. pombe can be drawn in secondary structure very similar to the structure of “free” U4 (i.e. not associated with U6) proposed by Rinke et al. (1985). In addition, Schiz. pombe U4 and U6 can be drawn in a Y structure as proposed for the U4/U6 interaction (Brow & Guthrie, 1988), which also very closely resembles that of human U4/U6.

In current models for spliceosome function, the active enzymic components are RNA molecules, the snRNAs and the pre-mRNA. The high evolutionary conservation of U6 and the release (or less-tight association) of U4 from the spliceosome prior to intron cleavage (Pikiehny et al., 1986; Cheng & Abelson, 1987; Lamond et al., 1988) has led to the further suggestion that U6 is the enzymically active component, and that the role of U4 is to act as a carrier for U6 and to block its activity until the appropriate moment. However, it is notable that only a part of the region of highest primary sequence conservation between U4 from Schiz. pombe and vertebrates corresponds to the U4/U6 interaction domain. The very high evolutionary conservation of the whole of the loop II region of U4 strongly suggests that U4 plays some more active role in spliceosome function.

Following microinjection into Xenopus oocytes, Schiz. pombe U4 becomes associated with Xenopus snRNP proteins that are stockpiled in the cytoplasm (Zeller et al., 1983; Tollervey & Mattaj, 1987). This demonstrates that Schiz. pombe U4 retains a functional “Sm-binding site”: a short nucleotide sequence, which is apparently sufficient to permit the association of RNA species with the “Sm-proteins” that are common between U1, 2, 4 and 5 (Branlant et al., 1982; Mattaj & De Robertis, 1985). A sequence (AGUUUUG) similar to a consensus Sm-binding site is found in a single-stranded region of Schiz. pombe U4.

Neither the Xenopus U2 nor S. cerevisiae U5 genes are detectably expressed in Schiz. pombe (D. Tollervey, unpublished results). Consistent with this, we detect no sequences with good homology to the vertebrate snRNA gene consensus distal sequence element (DSE), which acts as an enhancer, or to the proximal sequence element (PSE), which fulfils the role of a TATA box. Genes encoding snRNAs from S. cerevisiae also lack sequences homologous to PSE and DSE elements, but have well-conserved TATA sequences located between −80 and −100 (for a compilation of such sequences, see Bally et al., 1988), a position similar to that of TATA boxes for other genes transcribed by RNA polymerase II in S. cerevisiae. The Schiz. pombe snu4 gene does not have a TATA box in this region but does have a TATA sequence at −33 to −30, a position similar to that of TATA sequences of other polymerase II genes from Schiz. pombe (Russel, 1983). It is striking that a TATA sequence is found in identical positions in the Schiz. pombe snu2 gene (D. Tollervey & T. Dandekar, unpublished results; J. A. Wise, personal communication) and, at a similar position in the snu3A gene (Porter et al., 1988); several additional nucleotides around the TATA box are also conserved between these genes, lending increased significance to the conservation of this region.

Comparison of six genes encoding U2 from a plant, Arabidopsis, reveals that these too have a conserved TATA sequence, surrounded by a number of other completely conserved nucleotides, situated at a distance from the initiation site identical with that of the Schiz. pombe TATA sequences (Vankan & Filipowicz, 1988).

As the first step in our genetic analysis of the function of U4 in vivo, we have demonstrated that it is essential for viability. The failure to recover snu4− haploids from a diploid carrying only one intact copy of the snu4 gene demonstrates that the snu4 gene is at least required for spore viability or germination. Haploid snu4− strains can be recovered if they also carry an intact copy of snu4 on a replicating plasmid, demonstrating that the failure of snu4− strains to grow is due to a lack of U4 snRNA. These strains are unable to lose the complementing plasmid over many generations of unselected growth, demonstrating that U4 is continuously required for vegetative growth. These experiments also provide the genetic proof that snu4 is the only gene encoding U4 in Schiz. pombe and that the gene-disruption experiments have indeed resulted in replacement at the snu4 locus.

The techniques used here should be of general use in analysing the structure and function of genes in...
Schiz. pombe. The transposon mutagenesis system, developed for S. cerevisiae (Seifert et al., 1986) enables essentially random insertions to be made in any gene, even if the sequence is not known. This is particularly useful for making insertions into snRNA genes, which, owing to their small size, frequently lack convenient restriction sites. The plasmid we have constructed permits the complementation of the disrupted gene in haploid cells. Since it carries the phage F1 replication origin, this plasmid can be used directly to construct and sequence in vitro mutations for complementation testing.

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References


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