The EMBO Journal vol.6 no.7 pp.2145–2155, 1987

The yeast homologue of U3 snRNA

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Communicated by G.Cesareni

snR17, one of the most abundant capped small nuclear RNAs of *Saccharomyces cerevisiae*, is equivalent to U3 snRNA of other eukaryotes. It is 328 nucleotides in length, 1.5 times as long as other U3 RNAs, but shares significant homology both in nucleotide sequence and in predicted secondary structure. Human scleroderma antiserum specific to nucleolar U3 RNP can enrich snR17 from sonicated yeast nuclear extracts. Unlike other yeast snRNAs which are encoded by single copy genes, snR17 is encoded by two genetically unlinked genes: *SNR17A* and *SNR17B*. The RNA snR17A is more abundant than snR17B. Deleting one or other of the genes has no obvious phenotypic effect, except that the steady-state level of snR17B is increased in *snr17a*⁻ strains. Haploid strains with both genes deleted are inviable, therefore yeast U3 is essential.

Key words: autoantibodies/gene disruption/RNA processing/*Saccharomyces cerevisiae*/U3 snRNA

Introduction

U small nuclear RNAs (U snRNAs) are a class of structural RNAs, distinct from messenger, ribosomal and transfer RNA, present in all eukaryotic organisms. They are so named because of their high uridylic acid content and distinguished by their unique 5′ cap structure, nuclear location and metabolic stability (Zieve and Penman, 1976). Because of their association in the nucleus with precursor messenger and ribosomal RNA (pre-mRNA and pre-rRNA) in the form of ribonucleoprotein (RNP), snRNPs have been implicated in the processing of these precursor molecules to their mature forms (Prestayko *et al.*, 1970; Lerner *et al.*, 1980; for reviews see Busch *et al.*, 1982; Steitz *et al.*, 1982; Reddy and Busch, 1983; Jacob *et al.*, 1984; Maniatis and Reed, 1987). Good evidence is now available showing, indeed, that U1, U2 (Yang *et al.*, 1981; Padgett *et al.*, 1983; Krämer *et al.*, 1984; Krainer and Maniatis, 1985; Black *et al.*, 1985; Zhuang and Weiner, 1986), U4, U6 (Black and Steitz, 1986) and possibly U5 (Chabot *et al.*, 1985) are required for the removal of introns and the splicing of exons of eukaryotic pre-mRNA. U7 is required for 3′ end maturation of histone mRNA (Galli *et al.* 1983; Strub *et al.*, 1984) and an unidentified snRNP has been implicated recently in 3′ end cleavage and polyadenylation of SV40 and adenovirus mRNA by HeLa cell extracts (Hashimoto and Steitz, 1986).

U3 snRNA has been a relatively neglected member of the snRNAs. It is unique among the abundant series U1–U6 in that it is located in the nucleolus (Weinberg and Penman, 1968) and no cross-reactivity has been described between U3 snRNP and anti-sera of the Sm serotype from patients with the autoimmune, rheumatic disease lupus erythematosus (Lerner and Steitz, 1979; Lerner *et al.*, 1980). U3 was the first snRNA for which a role in pre-RNA processing was proposed (Prestayko *et al.*, 1970) but the evidence for this has remained circumstantial: namely that U3 resides in the nucleolus, the site of rRNA gene transcription, pre-rRNA processing and ribosome assembly (Hadjiolov, 1985; Sommerville, 1986), and that after removal of protein from nucleolar extracts, it remains hydrogen bonded to large RNA fractions containing pre-rRNA (Prestayko *et al.*, 1970; Calvet and Pederson, 1981; Epstein *et al.*, 1984).

In this paper we describe the characterization of U3 snRNA from the yeast *Saccharomyces cerevisiae* and the cloning of its genes. At least 24 capped snRNAs have been identified in yeast (Guthrie, 1986; Guthrie *et al.*, 1986), as opposed to only 10 so far in mammalian cells (Reddy *et al.*, 1985). Other fungi and plants, such as *Aspergillus, Neurospora, Schizosaccharomyces pombe, Dictyostelium discoideum*, pea and broad bean appear to have retained a complement of snRNAs much more similar to mammalian snRNAs in terms of relative abundance of the major species, size and sequence homology (Wise and Weiner, 1980; Takeishi and Kaneda, 1981; Kiss *et al.*, 1985; Tollervey and Mattaj, 1987). *S. cerevisiae* snRNAs, however, have diverged to the extent that none are present in the same amounts as the abundant mammalian U1–U6 (100–1000 molecules per cell for yeast, as opposed to $0.2-1.0 \times 10^6$ for mammals), and only one of these, LSR1, has been shown to have significant sequence homology to any of the mammalian species (Ares, 1986). This poses the interesting question of whether the functions of *S. cerevisiae* snRNAs have been conserved. Evidence accumulated so far suggests that they have: splicing of pre-mRNA in yeast proceeds in a manner similar to mammalian systems (Brody and Abelson, 1985; Pikielny and Rosbash, 1985; Lin *et al.*, 1985; Parker and Guthrie, 1985); purified yeast 40S RNP splicing complexes ('spliceosomes') contain snRNAs (Pikielny and Rosbash, 1986) identified as LSR1 (snR20 by the nomenclature of Riedel *et al.*, 1986), snR7 and snR14 which have been shown to be precipitable by Sm anti-sera (Tollervey and Mattaj, 1987; Riedel *et al.*, 1987) just as mammalian snRNPs involved in splicing are Sm precipitable. The 5′ end of LSR1 has 80% sequence homology to mammalian U2 snRNA, although it is much longer at the 3′ end (Ares, 1986). Its association with the yeast spliceosome would suggest it is the functional equivalent of U2.

We propose that U3 snRNA has its yeast equivalent in snR17. Like U2 snRNA, yeast U3 is significantly larger than its mammalian counterpart and, unlike any other yeast snRNA described so far, it is encoded by two genes per haploid genome instead of one.

Results

The RNA under study was one of several different RNAs originally isolated in order to discover new and hitherto uncharacterized RNAs possibly playing important regulatory roles in eukaryotic cells (M.Bally *et al.*, in preparation). Having cloned the genes for a molecule originally named 'R3', comparison by hybridization of clones exchanged between our laboratories con-

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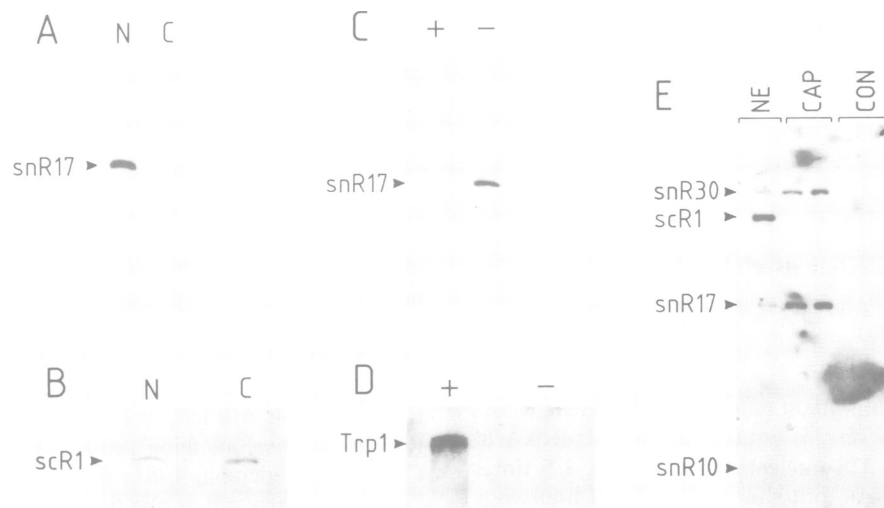

Fig. 1. snR17 is similar to other snRNAs. Yeast RNA blots from polyacrylamide/urea gels, equal quantities of RNA were loaded in each lane: **A** and **B**, nuclear (N) and cytoplasmic (C) RNA fractions were probed with mixed *SNR17A* and *SNR17B* or *SCR1* DNA; **C** and **D**, polyadenylated (+) and non-polyadenylated (−) RNA probed with *SRN17* or *TRP1* DNA; **E**, nuclear extract RNA before (NE), and after, immunoprecipitation by protein A−Sepharose beads with anti-m_3G serum (CAP) or beads without anti-serum as control (CON), probed with clones of different RNA genes (duplicated samples).

firmed the identity of R3 with a RNA designated snR17 by C.Guthrie's laboratory (Riedel *et al.*, 1986); for the sake of consistency, and to avoid confusion, we have adopted this name.

snR17 is one of the three most abundant RNA bands to be seen within the range from 5.8S to 18S rRNA on an ethidium-bromide-stained polyacrylamide gel (see the right-hand lanes of Figure 5). By hybridizing tritiated, total RNA to filters containing the cloned gene, we estimate that it constitutes 0.06% of total yeast RNA, corresponding to ~400−1000 molecules/cell. The genes for snR17 were isolated by excising the RNA band from a gel, labelling it, and using it as a probe to screen a yeast genomic library.

snR17 shares features with other snRNAs

Figure 1 illustrates that several features common to other snRNAs, namely nuclear location, lack of polyadenylation and possession of a specific 2,2,7-trimethyl guanosine (m_3G) cap at their 5′ end, are shared by snR17. Figure 1A shows a strong enrichment of snR17 in the nuclear RNA fraction when equal amounts of nuclear and cytoplasmic RNA were probed on filters with DNA clones. When the amounts of nuclear and cytoplasmic RNA loaded on the gel were proportional to the yields per cell, snR17 was still enriched in the nuclear fraction (data not shown). For comparison, Figure 1B shows the distribution of a cytoplasmic RNA species, scR1, whose gene we have also isolated (F.Felici *et al.*, in preparation). No trace of snR17 could be detected in purified polyadenylated yeast RNA, whereas a strong signal was seen when this fraction was probed with *TRP1*, a gene for a known polyadenylated mRNA (Figure 1C and D). Rabbit anti-serum specific to the m_3G cap structure unique to snRNAs, was used to immunoprecipitate capped RNA species from a deproteinized, total yeast RNA extract. Figure 1E shows equal amounts of RNA in each lane probed with different genes: snR17 is strongly enriched in the immunoprecipitated samples as is the snRNA snR10 (Tollervey and Guthrie, 1985); a third, as yet undescribed RNA species (snR30), for which we have isolated a gene (M.Bally *et al.*, in preparation), is also precipitated with anti-m_3G serum, whereas the cytoplasmic RNA scR1 is not. Pulse-chase labelling experiments indicate that the half-life of snR17 is much longer than 30 min (data not shown).

snR17 is encoded by two genes

All the *S. cerevisiae* snRNA genes that have been characterized so far are present as single copies in haploid genomes (Wise *et al.*, 1983; Ares, 1986). We were therefore surprised to discover that two genes coding for snR17 exist: two bands were always visible on genomic Southern blots using DNA digested with a variety of different restriction endonucleases. The second gene was found to be on a separate 20-kb cloned fragment in the λEMBL4 library and was sub-cloned into pEMBL8 (see Materials and methods). We have called the two loci *SNR17A* and *SNR17B*. The DNA sequences of the two genes are compared in Figure 2B.

To establish whether both genes were transcribed, two oligonucleotides were synthesized: oligo 49 and oligo 50, complementary to the RNA coding sequences of *SNR17A* and *SNR17B* respectively, in a region where there are five out of 18 nucleotide mismatches between the two genes. When labelled and used as probes on filters of total yeast RNA, both oligonucleotides hybridized to RNA species of exactly the same size, at stringencies which did not allow non-specific annealing. The fact that both genes were transcribed was confirmed later when we deleted one or other of the two, and could show that the transcript from the deleted gene was absent whereas the transcript from the intact gene was still present (see below).

The 5′ end of snR17A was mapped by reverse transcription of total RNA using 5′-end-labelled oligo 49 as primer, the 3′ end by S1 nuclease protection. The products of reverse transcription were resolved on a sequence gel, and a sequence ladder primed with the same oligonucleotide on a single-stranded *SNR17A* DNA template was used as a size marker (data not shown). Two

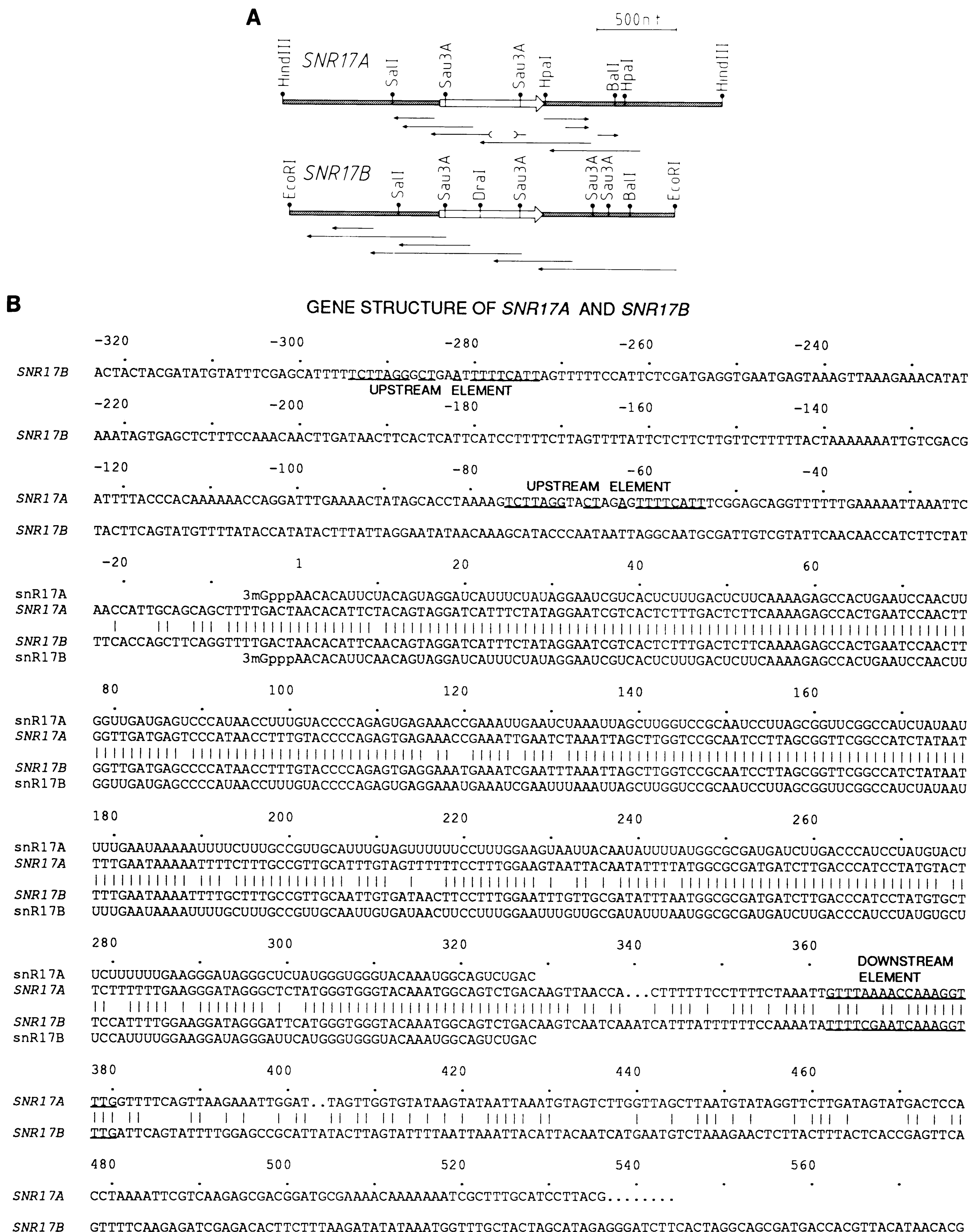

Fig. 2. Structure of SNR17 genes. (**A**) Restriction map and sequencing strategy for *SNR17A* and *SNR17B*; arrows (5′−3′) indicate the lengths of sequence derived from each deletion. (**B**) Nucleotide sequence comparison of *SNR17A* and *SNR17B* genes; the upstream and downstream flanking elements are indicated (see text).

U3 snRNA SEQUENCE ALIGNMENT

```
                10          BOX A  30                 50                        70
                                  HAIRPIN 1
Human          AAGACUAUACUUUCAGGGAUCAUUUCUAUAGUGU..GUUACUAGAGAAGUUUCUCUGAACGUGUAGAGCACC...GAAAACCACGAGGAA
Rat U3A        AAGACUAUACUUUCAGGGAUCAUUUCUAUAGUUC..GUUACUAGAGAAGUUUCUCUGACUGUGUAGAGCACC...CGAAACCACGAGGAG
Rat U3B        AAGACUAUACUUUCAGGGAUCAUUUCUAUAGUUC..GUUACUAGAGAAGUUUCUCUGACUGUGUAGAGCACC...CGAAACCACGAGGAC
Dictyos. D2    AUGACCAAACUCUUAGGGAUCAUUUCUAGAGUAU..CGU.CUAUUAAAAUUAUUCAUCAAUAAUUUUUCCUCUUUCAUAGCUAGGAUGAU
Yeast snR17A   AACACAUUCUACAGUAGGAUCAUUUCUAUAGGAAUCGUCACUCUUUGACUCUUCAAAAGAGCCACUGAAUCC......AACUUGGUUGAU
Yeast snR17B   AACACAUUCAACAGUAGGAUCAUUUCUAUAGGAAUCGUCACUCUUUGACUCUUCAAAAGAGCCACUGAAUCC......AACUUGGUUGAU
                                  HAIRPIN 1

               90    CENTRAL  110  BOX B      130              150                170
                     STEM
Human          GAGAGGUA....GCGUUUUCUCCUGAGCGUGAAGCCG.....................................................
Rat U3A        GAGACGUA....GCGUUCUCUCCUGAGCGUGAAGCCG.....................................................
Rat U3B        GAGACAUA....GCGUCCCCUCCUGAGCGUGAAGCCG.....................................................
Dictyos. D2    GAUACAUA....CUCACUAUACGAAAGCGUGAAACCG.....................................................
Yeast snR17A   GAGUCCCAUAACCUUUGUACCCCAGAGUGAGAAACCGAAAUUGAAUCUAAAUUAGCUUGGUCCGCAAUCCUUAGCGGUUCGGCCAUCUAU
Yeast snR17B   GAGCCCCAUAACCUUUGUACCCCAGAGUGAGGAAAUGAAAUCGAAUUUAAAUUAGCUUGGUCCGCAAUCCUUAGCGGUUCGGCCAUCUAU
                                                                                  HAIRPIN 4

                     190              210          230                  250    BOX C
                                              HAIRPIN 2
Human          ............................GCUUUCUGGCGUUGCUUGGCUGCAACUGCCGUCAGCCAUU........GAUGAUCGU...
Rat U3A        ............................GCUCU.UAGUGUUGCUUCCGUGCAACUGCUAUUGGCCAUU........GAUGAUCGU...
Rat U3B        ............................GCUCU.AGGUGCUGCUUCUGUGCAGCUGCCUCUUGCCAUU........GAUGAUCGU...
Dictyos. D2    ............................UUAUUAUCGAAUGAUUCAUUUAU...UUGUUAUUAACAUU........GAUGACCGU...
Yeast snR17A   AAUUUUGAAUAAAAAUUUUCUUUGCCGUUGCAUUUGUAGUUUUUUCCUUUGGAAGUAAUUACAAUAUUUUAUGGCGCGAUGAUCUUGACC
Yeast snR17B   AAUUUUGAAUAAAAAUUUUGCUUUGCCGUUGCAAUUGUGAUAACUUCCUUUGGAAUUUGUUGCGAUAUUUAAUGGCGCGAUGAUCUUGACC
                                                         HAIRPIN 2

               270             290                     CENTRAL    BOX D
                       HAIRPIN 3                        .STEM
Human          ..UCUUCUCUCCGU....AUUGGGGAGUGAGA...........GGGAGAGAAGGCGGUCUGAGUGGU    217 Nucleotides
Rat U3A        ..UCUCGGUCCUCU....CUGAGGG.GCGAGA...........GGGAGAGAAGGCAGUCUGAGUGGU    215 Nucleotides
Rat U3B        ..UCUUCUCUCCUU....CGGGGGG.GUAAGA...........GGGAGGGAAGGCAGUCUGAGUGGA    215 Nucleotides
Dictyos. D2    ....CUAAUUCAGGGAUGAAUUGG...................UUGUGUGGUGGGAUUCGUACUGGCU   210 Nucleotides
Yeast snR17B   CAUCCUAUGUACUUCUUUUUUGAAGGGAUAGGGCUCUAUGGGUGGGUACAAAUGGCAGUCUGAC       328 Nucleotides
Yeast snR17B   CAUCCUAUGUGCUUCCAUUUUGGAAGGAUAGGGAUUCAUGGGUGGGUACAAAUGGCAGUCUGAC       328 Nucleotides
                         HAIRPIN 3
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Fig. 3. Alignment of U3 snRNA sequences. Boxes A, B, C and D are regions of highly conserved nucleotide sequence. The features of the secondary structure models in Figure 4 are marked: hairpins 1, 2, 3 and 4 are indicated by lines above and below the sequences and the two complementary sides of the central stem are underlined.

bands were visible: an upper fainter band, which indicated the first 'A' of AACACA . . . (see Figure 2B) to be the first transcribed nucleotide; and a stronger band one nucleotide shorter. The lower stronger band might indicate a second initiation site at the second 'A' of AACACA . . ., or it might be the result of premature termination or degradation of the reverse transcript, or degradation of the RNA. In Figure 2B we have assigned the transcription initiation site according to the position of the upper, larger band; other yeast snRNAs also start with AAC . . (Tollervey *et al.*, 1983; Tollervey and Guthrie, 1985; M.Bally *et al.*, in preparation). The initiation site indicated by reverse transcription was a few nucleotides further upstream to that indicated by S1 protection; therefore we cannot discount the possibility that the 3′ end of the RNA is also slightly extended, because this was mapped only by S1 protection (data not shown). The 5′ and 3′ ends of snR17B were not determined experimentally.

The two *SNR17* genes appear to be separated in the yeast

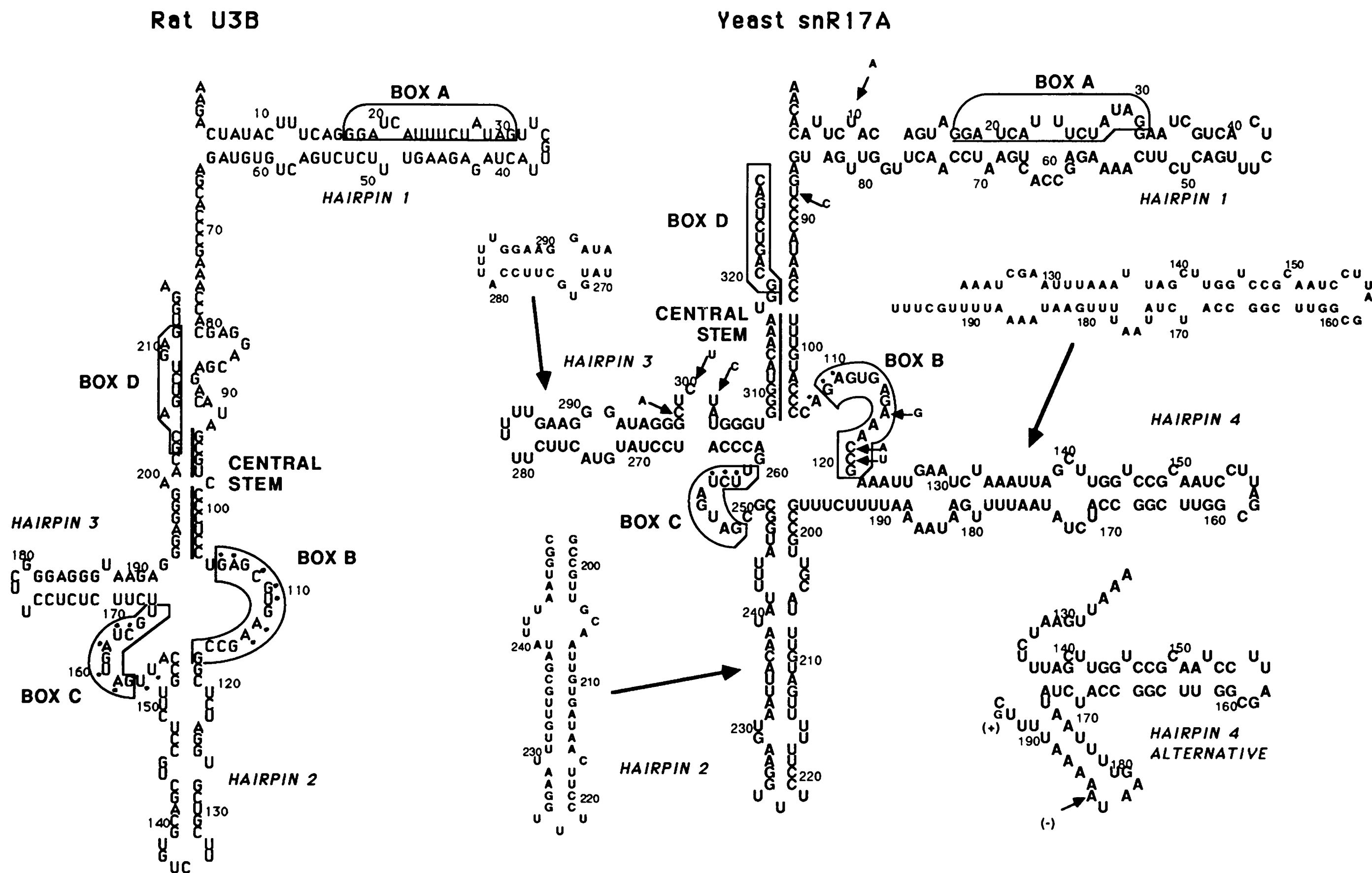

Fig. 4. Secondary structure models. The rat U3B model represents the consensus for all other U3 RNAs. The yeast snR17A model is shown; differences in snR17B are indicated by smaller characters and arrows. Boxes A, B, C and D are the conserved regions of Figure 3. The very stable central stem is marked with a line. The sites of possible, additional, complementary base-pairing interactions (variable between species) between nucleotides of box B and box C are indicated by dots.

genome: they were isolated from different 20-kb DNA fragments and are not genetically linked. The RNA coding sequences are 328 nucleotides long and share 96% homology (see Figure 2B); weaker homology (61%) extends 100 nucleotides downstream of the coding sequence. Thirty-four nucleotides downstream of the coding sequence is a stretch of 19 nucleotides sharing 84% homology, which shows some similarity to the conserved element GTTT(N)$_{0-3}$AAA$^{G}_{A}$NNAGA known to be important for efficient 3′ end formation of other snRNAs (Hernandez, 1985; Ciliberto *et al.*, 1986). The 5′ flanking sequences of *SNR17A* and *SNR17B* show little similarity either with each other, or with any other snRNA genes: however, both contain long stretches of 'T's and 'A's (from −43 to −25 in *SNR17A* and from −147 to −131 in *SNR17B*), and we have noticed an element conserved between *SNR17A* and *SNR17B*, TCTTAGG$^{G}_{TA}$CT$^{GA}_{AG}$A$^{T}_{G}$TTT-TCATT (from −76 to −54 in *SNR17A* and from −294 to −273 in SNR17B), which could be homologous in function in the 'U3 box' of Stroke and Weiner (1985), although dissimilar in nucleotide sequence.

snR17 is homologous to U3 snRNA in primary and potential secondary structure

The discovery of a perfectly matched stretch of 15 nucleotides between snR17 and rat U3B near their 5′ ends (Box A in Figure 3) led us to look more closely for similarities between snR17 and other U3 RNAs. The complete sequences of U3 snRNAs from rat, *Dictyostelium* (Reddy, 1985) and human (Suh *et al.*, 1986) aligned with snR17 are shown in Figure 3 (see Materials and methods). Notable in the alignment is the close matching of four highly conserved regions: boxes A, B, C and D. The potential functional importance of these primary structure elements is highlighted by the inclusion of the more divergent yeast sequence in the comparison. Whereas boxes A, B and C have been already noted as conserved regions between rat and *Dictyostelium* U3 RNAs (Wise and Weiner, 1980) box D has not been previously emphasized. snR17 is over 100 nucleotides longer than the other U3 snRNAs: the alignment shows that the extra length is mainly taken up in a major 'insertion' between nucleotides 122 and 204. Over the first 120 nucleotides snR17 is 45−47% homologous to the other U3 RNAs, whereas *Dictyostelium* and human are 54% homologous in the same region.

Having observed significant sequence homology between snR17 and the known U3 snRNAs, we asked whether the two yeast sequences could form a similar secondary structure: if regions of conserved primary structure also formed conserved features of secondary structure, a functional equivalence would be suggested. Figure 4 shows secondary structure models for the rat U3B RNA and the yeast snR17A RNA. The main structural features of both models are also indicated in the alignment in Figure 3. The model for the rat U3B also holds for the rat U3A(D), human, *Dictyostelium* and the partial bean U3 (Kiss *et al.*, 1985) sequences; although the 'hairpin 1' of *Dictyostelium* is relatively unstable (−1.6 kcal). The very stable 'central stem', the 'hairpin 1' and the 'hairpin 3' are the same as the 'stem E',

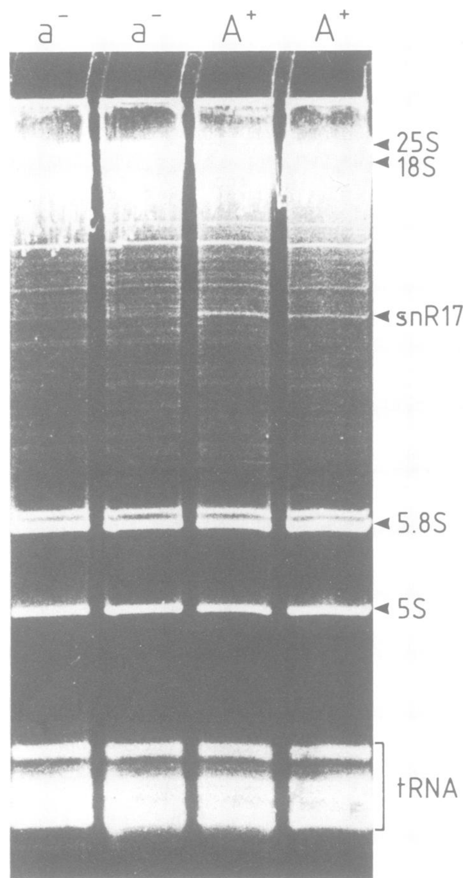

Fig. 5. Deletion of SNR17A. Total RNA extracted from four haploid strains, the meiotic products of an *a/A B/B* diploid, stained with ethidium bromide on a 6% polyacrylamide/urea gel (20 mg RNA/lane). A^+:*SNR17A SNR17B*; a^-.*Δsnr17a::URA3 SNR17B.'*

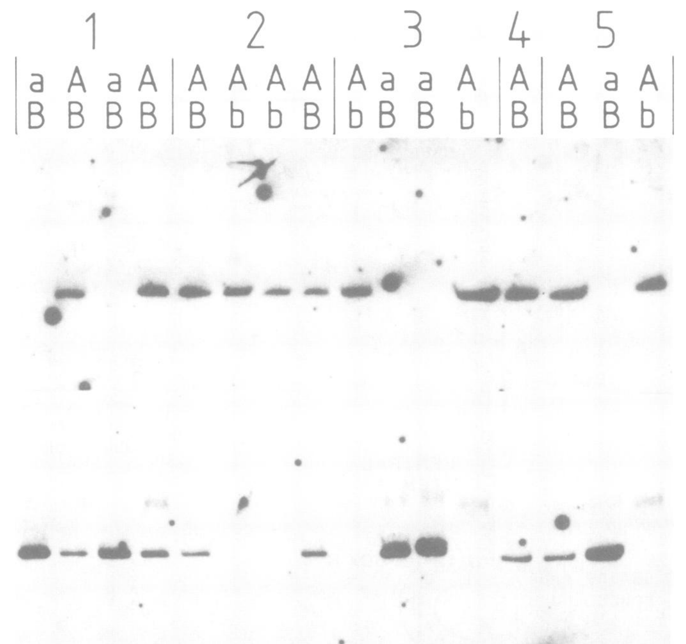

Fig. 6. Probing for snR17A and snR17B in $\Delta snr17a^-$ and $\Delta snr17b^-$ strains. RNA segregation pattern in tetrads from **1**, *a/A B/B* diploid; **2**, *A/A b/B* diploid; **3**, *a/A B/b* diploid, parental ditype; **4**, *a/A B/b* diploid, non-parental ditype (only two out of four spores were viable, and only one of the identical, viable spores is shown); and **5**, *a/A B/b* diploid, tetratype (only three out of four spores were viable). The samples are blots of whole cell RNA extracts (equal quantities of RNA in each lane, extracted from cultures grown to the same optical density), probed wth snR17A-specific (**upper panel**) or snR17B-specific (**lower panel**) oligonucleotides.

'stem A' and the 'stem C' respectively of the structure of Bernstein *et al.* (1983). The 'hairpin 2' is essentially the same as that of Stroke and Weiner (1985) except that it has been extended basally, thus disrupting their 'stem D' and opening out a single-stranded loop containing the conserved box B, our structure at this point has a lower free energy, and furthermore, the suggested stem D for *Dictyostelium*, rat and bean is not formed by homologous nucleotides. Our model is the same as a model derived for human U3, based on an analysis of sites susceptible to chemical modification and RNase protection (K.Parker, in preparation). Apart from an extra hairpin (hairpin 4) following the conserved box B in the yeast, the models in Figure 4 are very similar: hairpins 1, 2, 3 and the central stem are each formed by the same regions shown to be aligned by nucleotide sequence homology in Figure 3; the highly conserved box A forms part of hairpin 1; boxes B and C are single stranded (although additional base-pairing interactions in this region are possible, the exact positions of which vary between species, as indicated by dots in Figure 4 for rat and yeast), while box D forms the 3′ end. Box D is partially base paired to nucleotides 76–95 in the rat U3B model; there is experimental evidence that the 3′ end of vertebrate U3 is hydrogen bonded to this region in so far as reverse transcription can be primed from the 3′ terminus with the complementary side of the stem acting as template (Bernstein *et al.*, 1983). Box D of snR17A could from a similar interaction by the pairing of the terminal GAC (nucleotides 326–328) with CUG (nucleotides 89–87), but the U to C base change at 88 would disrupt this in snR17B, so we have left the yeast box D open. Most of the extra nucleotides of snR17 are taken up in the additional hairpin 4: an alternative structure for this, of approximately equal stability, is also shown in Figure 4.

snR17 is an essential RNA

Deletion mutants were created using the gene replacement technique described by Rothstein (1983) in order to discover something about the function and importance of snR17 *in vivo*. By replacing deleted sections of *SNR17A* and *SNR17B* with the selectable marker genes *URA3* and *LEU2* respectively and selecting for Ura^+ and Leu^+ phenotypes, we isolated *Δsnr17a::URA3/SNR17A SNR17B/SNR17B* and *SNR17A/SNR17A Δsnr17b::LEU2/SNR17B* diploid strains (*a/A B/B* and *A/A b/B*) (see Materials and methods). After sporulation, both the *a/A B/B* and *A/A b/B* strains produced tetrads in which all four spores were viable and showed no obvious differences in growth rates. Ura^+/leu^- and ura^-/Leu^+ phenotypes segregated 2:2 (see Figure 7); Ura^+ cosegregated with the *Δsnr17a::URA3* band pattern, and Leu^+ with the *Δsnr17b::LEU2* band pattern on Southern blots. Figure 5 shows total RNA isolated from four spores of a tetrad from the *a/A B/B* diploid. The lack of snR17A has segregated 2:2 between the spores: snR17 is seen clearly as one of the most prominent bands between 5.8S and 18S rRNA in the two right-hand lanes, but is markedly reduced in the left-hand lanes.

We confirmed the presence or absence of snR17A and snR17B in strains derived from the sporulation of *a/A B/B* and *A/A b/B* by probing the RNA with oligos 49 and 50, specific to snR17A and snR17B respectively. Figure 6, lanes 1–4, shows that snR17A is lacking from two out of four spores from *a/A B/B*,

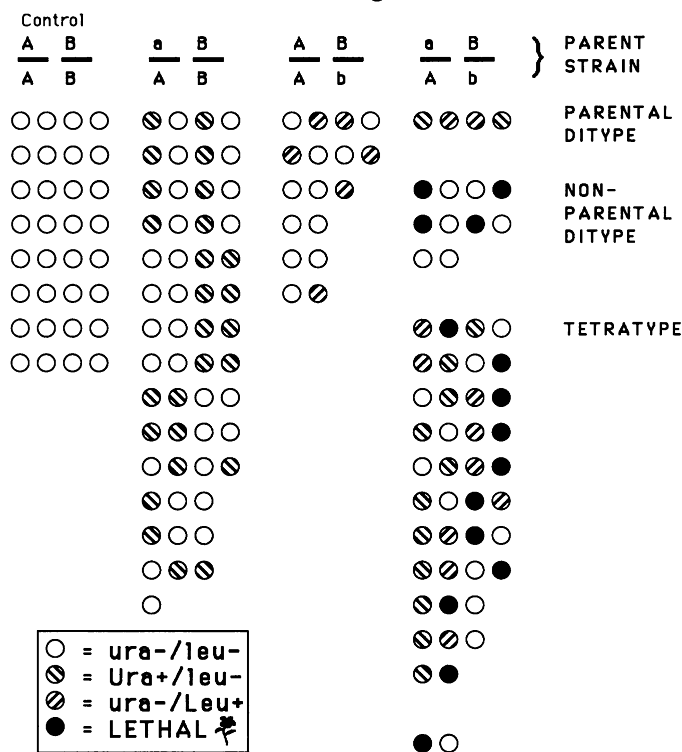

Fig. 7. Tetrad analysis. Germinated spores are marked as discs, arranged in rows of four corresponding to tetrads. Spores which did not germinate are not marked: only those spores which germinated but failed to go through more than three or four cell divisions are marked 'LETHAL'. A = *SNR17A*; a = *Δsnr17a::URA3;* B = *SNR17B*; b = *Δsnr17b::LEU2*.

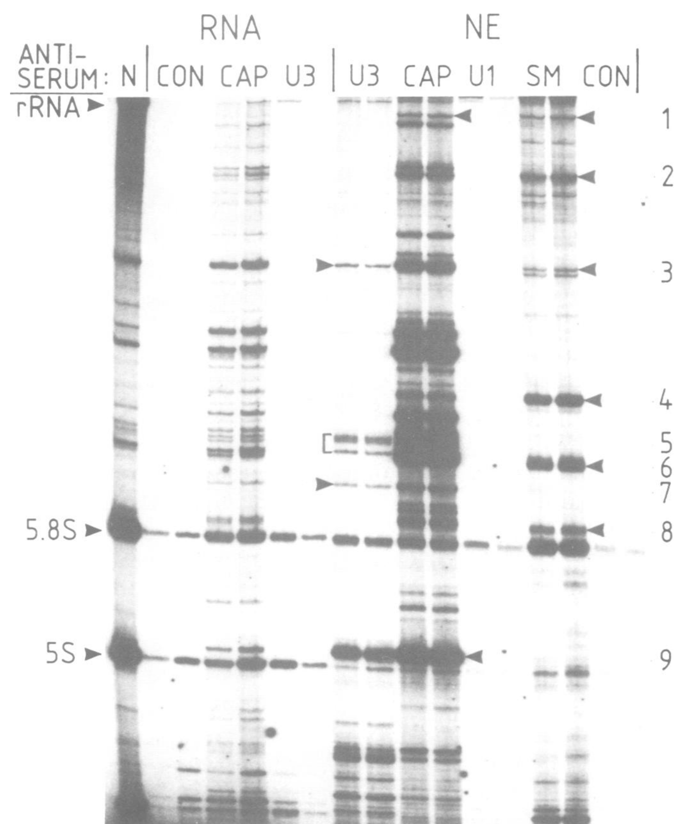

Fig. 8. Immunoprecipitation of RNA from yeast nuclear extracts. RNA (on 6% polyacrylamide/urea gel) 3' end labelled after immunoprecipitation from sonicated nuclear extract (NE) or form deproteinized nuclear extract (RNA), with different anti-sera bound to protein A–Sepharose beads. Precipitations (in duplicate) were with control of beads without anti-serum (CON); rabbit anti-m_3G (CAP); scleroderma anti-U3 RNP (U3); lupus anti-U1 RNP (U1); and lupus anti-Sm (Sm). N, complete nuclear extract RNA. Identification of RNA species (by probing with DNA clone): **3**, snR17; (by comigration): **1**, LSR1 (1175 nt); **2**, snR19 (~600 nt); **4**, snR71 (213 nt); **6**, snR7s (178 nt); **7**, snR15 (160 nt); **8**, snR14 (160 nt); and 9, snR13 (124 nt).

whereas snR17B is present in all four; the levels of snR17B have increased in the strains lacking snR17A. Figure 6, lanes 5–8, shows that snR17B is present in two out of four spores from *A/A b/B* and that snR17A remains present in all four; the levels of snR17A do not change detectably when snR17B is absent. We estimate a 2- to 5-fold increase in the steady-state level of snR17B when snR17A is absent; this phenomenon was reproducible when snR17A was deleted in a different yeast strain. snR17A is normally about 5- to 10-fold more abundant than snR17B, and as can be seen in Figure 5, the corresponding increase in snR17B in *Δsnr17a* strains is not sufficient to restore the total amount of snR17 to normal levels.

A strain was constructed heterozygous for deletions in both snR17 loci (*Δsnr17a::URA3/SNR17A SNR17B/Δsnr17b::LEU2*) by mating two appropriate haploids and selecting for Ura^+/Leu^+. After sporulation, the products were analysed by Southern and Northern blotting, and by scoring the phenotypes. Figure 7 shows the results: the observed ratio of parental ditype:non-parental ditype:tetratype tetrads of 1:3:11 is consistent with the expected ratio of 1:1:4 for the random segregation of two unlinked genes (Mortimer and Hawthorne, 1969). The coincidence of the Ura^+ and Leu^+ phenotypes with the absence of snR17A and snR17B respectively was confirmed by northern analysis (see Figure 6, lanes 9–14). No viable haploids inherited the double deletion *Δsnr17a::URA3 Δ2snr17b::Leu2*: where this genotype was predicted from the segregation pattern of mutant and wild-type alelles among the other spores of the same tetrad, microscopic examination of the agar plates revealed that, either the planted spore had not germinated, or the spore had germinated and undergone three or four cycles of cell division before the colony died. In Figure 7, spores which failed to germinate have not been marked; a certain percentage of spore inviability was apparent also in the controls. Only the abortive colonies which failed to grow after germination have been marked as 'lethal', and these were only observed where the *Δsnr17a::URA3 Δsnr17b::LEU2* genotype was predicted.

snR17 is immunoprecipitable with human antiserum species for U3 snRNP

We acquired human antiserum, which reacts specifically with a 34-kd protein of the U3 snRNP, from a patient with the autoimmune disease scleroderma (K.Parker, in preparation). snR17 is enriched from yeast nuclear extracts by immunoprecipitation with this serum. Figure 8 shows the RNA species found in immunoprecipitates from yeast nuclear extracts using different antisera: lupus erythematosus anti-Sm and anti-U1 RNP, rabbit anti-m_3G, and the scleroderma anti-U3 RNP. The precipitations were performed by incubating the nuclear extract with antisera bound to protein A–Sepharose beads, and as controls, beads without antisera were used. As a further control to show that anti-U3 serum was reacting specifically with protein components in the nuclear extract, antisera were incubated with deproteinized RNA ('nak-

ed' RNA). The pattern of snRNAs precipitated by anti-cap serum is essentially the same whether from nuclear extract or naked RNA, and corresponds well to the pattern presented by Riedel *et al.* (1986), by comparison with whose data we have tentatively identified the major species.

At least eight prominent RNA bands appear to be enriched specifically by anti-U3 RNP serum: snR17, 328 nt long, at '3' in Figure 8, which we have positively identified by hybridization with a DNA probe; a cluster of unidentified bands at '5' between 180 and 190 nt (we have resolved at least five on other gels); a band of ~169 nt at '7' comigrating with snR15, and a band at '9' of ~124 nt comigrating with snR13. In the anti-Sm precipitate we see three very prominent, specifically enriched bands, at '4', '6' and '8' which we assume correspond to the 'large' (213 nt) and 'small' (178 nt) forms of snR7 and snR14 (160 nt) respectively (Tollervey and Mattaj, 1987; Riedel *et al.*, 1987). Several other bands of lesser intensity are visible in the anti-Sm precipitates: among these, we tentatively identify LSR1 (1175 nt) at '1', snR19 (~600 nt) at 2, and positively identify the yeast U3, snR17 (328 nt) at '3'. No strong, specific bands appear in the anti-U1 precipitate.

Discussion

snR17 is one of the most abundant RNA species intermediate in size between 18S and 5.8S rRNA in *S. cerevisiae*. It shares characteristics with other snRNAs, such as nuclear location, lack of polyadenylation and possession of a m_3G cap; it shows specific homology to U3 snRNA of other eukaryotes in terms of primary and predicted secondary structure, and furthermore, associates with a protein that shares immunological properties with a vertebrate U3-associated protein. snR17 differs from U3 of other organisms in its relative abundance: we estimate a copy number of 400–1000/cell as opposed to an estimate for rat of 2×10^5 (Weinberg and Penman, 1968); it differs also in its greater size: 328 nt compared with 210 nt (*Dictyostelium*), 215 nt (rat) and 217 nt (human).

On aligning the nucleotide sequence of snR17 with other U3 sequences, four regions of strong conservation were observed, referred to as boxes A–D. Box A near the 5′ end, with the exception of one mismatched nucleotide in the *Dictyostelium* sequence, is perfectly conserved over 15 nucleotides in all species, and first drew our attention to the similarity of snR17 with U3. This pentadecamer is unique to U3 and neither it nor its complement is to be found in any other published sequence in either the EMBL or the Genbank data libraries. In the secondary structure models of U3, box A always forms one side of the stem of hairpin 1. Because no complementary, conserved sequence is observed on the other side of the stem, its importance probably lies, not in the features of secondary structure it could maintain, but in its specific nucleotide sequence: the conservation of stems is usually reflected by the occurrence of compensatory base changes, and not of strongly conserved nucleotide sequence (compare the conservation of nucleotides forming the central stem in Figure 3). The conserved boxes B and C form single-stranded regions in our secondary structure models. They might be conserved because of their particular tertiary folding properties, allowing the formation of tertiary base–base hydrogen bonds as in the case of tRNAs (Kim, 1979; Sundaralingam, 1979); they could be protein binding sites or regions available for complementary base pairing with other RNAs. Box C is the region proposed to interact with the processing site at the junction of the internal transcribed spacer 2 (ITS2) and 28S rRNA of pre-rRNA, resulting in correct excision of the ITS2 and leaving 5.8S rRNA hydrogen bonded to the 5′ end of 28S rRNA (Bachellerie *et al.*, 1983): however, no experimental support for this hypothesis is available and we detect no equivalent complementarity on the yeast pre-rRNA. Box D could be an important feature of the mature RNA or its position at the 3′ end might suggest a role as a transcription termination or 3′ end processing signal.

U3 is known to be hydrogen bonded in mammalian cell extracts to high molecular weight, nucleolar RNA fractions containing rRNA precursors (Prestayko *et al.*, 1970; Calvet and Pederson, 1981; Epstein *et al.*, 1984). We know now that snR17 remains hydrogen bonded to what appears to be the yeast 35S initial pre-rRNA transcript after protease digestion of nuclear extracts (D.Tollervey, in preparation). We have found several potential base-pairing interactions between snR17 and different regions of the yeast initial pre-rRNA transcript, many of them theoretically more stable than, for example, the interaction of the 5′ end of U1 with the 5′ splice site of pre-mRNA (Mount *et al.*, 1983). The question whether any of these interactions exist *in vivo* must be resolved experimentally.

snR17 is immunoprecipitated from yeast nuclear extracts with human scleroderma antiserum specific for a 34-kd protein of the U3 RNP (K.Parker, in preparation); in HeLa cells this protein is specifically located in the fibrillar region of nucleoli (Lischwe *et al.*, 1985). We have observed at least seven prominent RNA bands which coprecipitate with snR17 using this serum, two of which show the same electrophoretic mobilities as snR13 and snR15, while the remaining five comigrate with a dense cluster of closely spaced snRNAs which we have not identified. The fact that these snRNA species copurify with a protein which is presumably homologous to the 34-kd scleroderma antigen of HeLA cell nucleoli, suggests they may all play roles in some aspect of yeast ribosome biogenesis. It remains to be determined whether they exist in independent particles, each binding the same antigen, or whether they are all part of a single complex.

Sm antiserum reacts with several snRNP proteins, the binding of which to a snRNA depends on the presence of an Sm binding site: $A(U)_{3-6}G$ on the snRNAs U1, U2, U4, U5 and U6 (Branlant *et al.*, 1982; Bringmann *et al.*, 1983; Mattaj and De Robertis, 1985; Mattaj, 1986; Mattaj *et al.*, 1986). This human antiserum has been shown to immunoprecipitate yeast snRNPs both directly from yeast extracts and after injection of yeast snRNAs into *Xenopus* oocytes (Tollervey and Mattaj, 1987) and eggs (Riedel *et al.*, 1987). The main species of yeast snRNAs identified in anti-Sm precipitates, snR14, snR7s, snR71 and LSR1 appear to be those associated with the yeast spliceosome (Pikielny and Rosbash, 1986). U3 RNP has been shown neither to be Sm precipitable from mammalian cells, nor to be involved in pre-mRNA splicing. In our Sm precipitates of yeast nuclear extracts, we detect a relatively weak, but distinct band which we identify as snR17; Riedel *et al.* (1987) have observed weak Sm precipitation of snR17 after injection into *Xenopus* eggs. Why is yeast U3 apparently Sm-precipitable while mammalian U3 is not? The coprecipitation of snR17 may be due to a weak association with the yeast splicing complex or direct binding of the Sm antigen to the RNA. The main difference between the secondary structure models for snR17 and for other U3 RNAs in Figure 4 is the extra 'hairpin 4' of snR17, which contains in its nucleotide sequence a potential Sm binding site, AAUUUUGA (nucleotides 175–182): this may account for the precipitation of snR17 with anti-Sm.

snR17 is unique among yeast snRNAs in being encoded by two genes; other yeast snRNAs have single copy genes (Wise *et al.*, 1983; Ares, 1986). According to the criteria we have tested, i.e. nuclear location, anti-U3 RNP precipitation and hydrogen bonding to 35S pre-rRNA (D.Tollervey, personal communication, snR17A and snR17B behave identically; furthermore, snR17A can compensate for the loss of snR17B, and *vice versa*: if the two genes have diverged in function at all, they have done so in subtle ways. snR17B exhibits dosage compensation in the absence of snR17A: it would be interesting to find out whether this control operates at the transcriptional or post-transcriptional level.

Sufficient evidence has been presented to argue convincingly that yeast snR17 is functionally equivalent to U3 in other eukaryotes. Several characteristics of snR17 and U3, namely their structures and associations with other molecules, have been highly conserved, which is to be expected of a molecule whose function is of vital importance to the cell. Further study of snR17 should help elucidate the function of mammalian U3 RNA.

Materials and methods

Strains

Escherichia coli strain 71-18 Δ(*lac-proAB*), *thi*, *supE*, [F',*proAB*$^+$, *lacI* ^{q}ZΔM15] (Yanisch-Perron *et al.*, 1985) was the bacterial host for all cloning vectors. *SNR17A* and *SNR17B* genes were isolated from the wild-type *S. cerevisiae* strain ATCC 25657 obtained from G.Schatz. Δ*snr17a::URA3* and Δ*snr17b::LEU2* strains were constructed from GY87:α/a *ura3-52/ura3-52 leu 2-3,112/leu2-3, 112 lys1-1/LYS1 ade2/ade2 Δhis3/HIS3 HIS4/his4 TRP1/trp1 can1-100/can1-100*, a cross between JR26-19B and JU4-7 from O.Fasano.

Hybridization and labelling techniques

RNA gel electrophoresis was on 6% polyacrylamide (30:1 acrylamide:bisacrylamide)/8 M urea/TBE gels. Blotting was by electrotransfer to GeneScreen (Dupont) according to manufacturer's instructions. Oligonucleotides, synthesized by phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer and purified by h.p.l.c. were 5' end labelled and used as hybridization probes as described by Zoller and Smith (1984). DNA blots were prepared according to Church and Gilbert (1984); DNA probes were prepared according to Feinberg and Vogelstein (1983); RNA probes were prepared by creating 5'-OH ends by partial alkaline hydrolysis (100°C for 1 min in 0.1 M Tris−HCl pH 9.5) followed by labelling with [γ-^{32}P]ATP and polynucleotide kinase; and RNA 3' end-labelling was according to England and Uhlenbeck (1978). Radiochemicals were from Amersham.

Cloning, sequencing and mapping of genes

Genes encoding yeast RNAs were isolated by excising and eluting RNA bands between 5.8S and 28S rRNA (visualized by u.v. light after ethidium bromide staining) from polyacrylamide/urea gels, labelling them, and probing a yeast genomic DNA library of partial *Sau*3A fragments cloned into the bacteriophage vector λEMBL4 (Frischauf *et al.*, 1983). rRNA contaminating the probe gave rise to a background of positive rDNA clones which were identified by hybridizing replica filters to a purified rRNA probe: clones of the RNA genes of interest were selected as those not hybridizing to the latter. Yeast DNA from the λ clones was sub-cloned into the plasmid vectors pEMBL8 or pEMBL9 (Dente *et al.*, 1983). Plasmids containing DNA fragments complementary to the RNA probes were identified. These were shown to hybridize to unique bands on blots of total yeast RNA corresponding in size to the original, excised bands.

Dideoxynucleotide sequencing (Sanger *et al.*, 1977) was performed on series of deletions created by using restriction endonucleases, Bal31 exonuclease or random DNase I cleavage (Frischauf *et al.*, 1980); single-stranded DNA templates were prepared directly from the pEMBL plasmids (Dente *et al.*, 1983).

S1 mapping was performed as follows: single-strand specific probes, incorporating hot deoxynucleotides and complementary to the RNA, were generated on single-stranded DNA templates prepared from the same deleted plasmids used for sequencing; after annealing to total yeast RNA and digesting with S1 nuclease, a series of truncated, protected fragments were generated, whose lengths indicated the distance from the 5' end of the RNA to the edge of the deletion within the coding sequence; the 3' end of the RNA was placed relative to the 5' end according to the length of protected non-deleted probes, complementary to the whole length of the RNA. Reverse transcriptase for mapping of the 5' end was used as described by Goodman and MacDonald (1979).

Yeast methods

Growth of yeast at 30°C in YPD or selective media, preparation of protoplasts, mating and tetrad analysis were performed as described by Sherman *et al.* (1983). Sporulation was in 2% potassium acetate pH 7.0 (Haber and Halvorson, 1975). Yeast DNA was prepared as described by Baldari and Cesareni (1985). RNA was prepared from whole yeast cells in mid-exponential growth phase, or from sub-cellular fractions, by the hot phenol/SDS method (Rubin, 1975). Polyadenylated RNA was prepared according to Maniatis *et al.* (1982). Yeast nuclei were purified by centrifugation through Percoll (Pharmacia) gradients (Ide and Saunders, 1981) after hypotonic lysis of protoplasts in 18% Ficoll (Sigma: type 400) dissolved in 20 mM potassium phosphate pH 6.5, 0.5 mM calcium chloride, 0.25% PMSF.

Gene disruption

The principle of the gene disruption method has been explained by Rothstein (1983). Δ*snr17a::URA3* deletions were created by replacing 303 nucleotides from +74 to +483 downstream of the transcription initiation site in *SNR17A* with a 1330-nucleotide *Eco*RI/*Sal*I fragment taken from pLGD-312 (Guarente and Mason, 1983) bearing the *URA3* gene; Δ*snr17b::LEU2* by replacing 470 nucleotides (between two *Sau*3A sites) from +18 to +487 in *SNR17B* with a 2230-nucleotide *Sal*I/*Xho*I fragment taken from YEp13 (Broach *et al.*, 1979) bearing the *LEU2* gene. Both constructions were excised as linear fragments using unique *Sal*I and *Bal*I sites present in the flanking sequences of both genes, and used to transform GY87 (see 'Strains' above) by the lithium acetate method (Altherr *et al.*, 1983).

Analysis of primary structure homology

The UWGCG 'Wordsearch' program was used to screen the EMBL and Genbank sequence data libraries and first revealed box A (Figure 3) near the 5' ends of snR17 and rat U3B. The multiple sequence alignment method 'Trials' (Hogeweg and Hesper, 1984; Konings *et al.*, 1987) was used to align yeast snR17A and snR17B sequences with U3 RNA sequences of rat, *Dictyostelium* (Reddy, 1985), human (Suh *et al.*, 1986) and *Xenopus* (C.Jeppesen, personal communication, not shown in Figure 3). The algorithm is based on an integrated method for a tree construction, and, given the similarity (or phyletic) tree, a pair-wise alignment of sequences along the branches of the tree, via actual and intermode sequences. The penalty for gap formation was varied, and the gap length was not penalized: it turned out that a gap penalty of 3 generated the best overall alignment in terms of low number of gaps, and matched, conserved sequence blocks. The hypothetical tree relationships of yeast and *Dictyostelium* were not clearly defined: of yeast, owing to its large 'insertion', and of *Dictyostelium*, because it is the most divergent at the 3' end. By combining alignments based on slightly different trees, therefore, we produced the final alignment as shown in Figure 8. The major part of the alignment (1−250) was based on the tree in which the overall sequence similarities showed yeast to be the most dissimilar, whereas the last part (250−328) was based on the tree in which *Dictyostelium* was the most divergent, i.e. was the last sequence considered in the tree alignment procedure. Since the final alignment was used as a reference for the secondary structure modelling, it is important to note that the alignment was derived by a uniform procedure, and independently of secondary structure considerations.

Secondary structure modelling

Potential secondary structures of U3 RNAs were studied using the dynamic programming algorithm described by Williams and Tinoco (1984). The algorithm is able to generate all optimal free energy structures as well as defined, sub-optimal structures; knowledge of these various possible structures is important because the rules of RNA folding, and the destabilizing or stabilizing effects of other molecules such as proteins, are still imperfectly understood. The optimal (stable) structures for rat, human, *Xenopus* and *Dictyostelium* were investigated in order to derive a uniform secondary structure for these molecules, which could be used for comparison with the two yeast U3 RNAs. A secondary structure for the two yeast RNAs was derived by considering only those optimal free energy structures common to snR17A and snR17B, and by selecting the model from these in which the conformation of the four conserved sequence boxes was most similar to that of the model for the other U3 RNAs. It should be noted that the models proposed previously for rat and *Dictyostelium* (Bernstein *et al.*, 1983; Stroke and Weiner, 1985) were derived from different considerations; those of structure building based on consensus helix/loop units formed by aligned sequences ('consensus structure building') in contrast to applying thermodynamic rules. To take into account present uncertainties about thermodynamic free energy data, we analysed the U3 RNAs of human, rat and *Dictyostelium* primarily with two different sets of previously reported values, those of Zucker and Sandoff (1984), and those of Cech *et al.* (1983). The two sets of structures obtained were judged against the models independently derived by consensus structure building (Bernstein *et al.* 1983, Stroke and Weiner, 1985); the same evaluation was performed for the known U1 and U2 snRNAs as a control. The optimal structures generated using Cech's free energy values best represented the structures suggested by consensus structure building. The most important difference between the two sets

is the increased instability of 'bulges' using that of Cech; apparently this increase tends to discourage arbitrary bulge formation. Recently an experimentally improved set of free energy values was published by Freier *et al.* (1986). An identical analysis was used to evaluate these new values (which also show increased instability of bulges) and the models for rat and yeast U3 derived with Cech's energy values were supported.

Immunoprecipitation

Antisera used were 'LS' anti-U3 RNP, 'AG' anti-U1 RNP (K.Parker in preparation), 'DK' anti-Sm (Mattaj and De Robertis, 1985) and rabbit anti-m_3G (Lührmann *et al.*, 1982). The precipitation method was based on that of Matter *et al.* (1982). For the experiment described in Figure 8, all incubations and washes were performed at 4°C in IP (0.15 M NaCl, 40 mM Tris–HCl pH 7.4, 0.05% Triton X-100), except the removal of RNA from bead-bound antibodies which was at 37°C for 30 min in 0.3 M NaCl, 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 1.5% SDS, 2 mg/ml proteinase K (Sigma) followed by phenol/chloroform extraction and ethanol precipitation with 20 mg/ml glycogen as carrier. For each sample, 2 mg protein A–Sepharose beads (Pharmacia) were used to bind antisera: for LS anti-U3 and AG anti-U1, the amount used was sufficient to precipitate quantitatively U3 and U1 RNP from 10 ml and 5 ml of HeLa cells respectively. After washing, each sample was incubated with either 0.1 mg of yeast RNA or nuclear extract equivalent to 10^9 yeast cells, in 0.5 ml IP with 20 mg/ml heparin for 2 h. Nuclei (see 'Yeast methods') were sonicated in 5 ml IP (containing heparin) on ice for 4 × 10 s (with pauses) with a microprobe attached to a Branson Cell Disruptor B15, output setting 4, and clarified by 5 min centrifugation in an Eppendorf bench top centrifuge.

Acknowledgements

We are greatly indebted to Kathy Parker for providing scleroderma anti-U3 and lupus anti-U1 sera, to Rheinhardt Lührmann for rabbit anti-cap serum, to Iain Mattaj for lupus anti-Sm serum, to Cosima Baldari-Telford for the yeast genomic library, to David Tollervey for the snR10 clone and to CLaus Jeppesen for communicating unpublished material. We would also like to thank I.Mattaj, L.Philipson, P.Hogeweg and A.L.Williams,Jr for helpful discussion, and in particular, A.L.Williams, Jr for his hospitality shown to D.K.

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Received on April 1, 1987