Characterization of three new snRNAs from *Saccharomyces cerevisiae*: snR34, snR35 and snR36

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ABSTRACT

Genes for three novel snRNAs of *Saccharomyces cerevisiae* have been isolated, sequenced and tested for essentiality. The RNAs encoded by these genes are designated snR34, snR35 and snR36 respectively and contain 203, 204 and 182 nucleotides. Each RNA is derived from a single copy gene and all three RNAs are believed to be nucleolar, i.e. snoRNAs, based on extraction properties and association with fibrillarin. SnR34 and snR35 contain a trimethylguanosine cap, but this feature is absent from snR36. The novel RNAs lack elements conserved among several other snoRNAs, including box C, box D and long sequence complementarities with rRNA. Genetic disruption analyses showed each of the RNAs to be dispensable and a haploid strain lacking all three RNAs and a previously characterized fourth snoRNA (snR33) is also viable. No differences in the levels of precursors or mature rRNAs were apparent in the four gene knock-out strain. Possible roles for the new RNAs in ribosome biogenesis are discussed.

INTRODUCTION

It has become clear in the past few years that eukaryotic nuclei contain complex populations of small stable RNAs (snRNAs). At least a dozen of these RNAs play vital roles in RNA synthesis. Of the snRNAs characterized thus far a small subset occurs in the nucleoplasm, whereas the majority are associated with the nucleolus. The nucleoplasmic species include the spliceosomal snRNAs and a few other RNAs known or suspected of being involved in mRNA synthesis or tRNA processing (reviewed in 1–9). Because of their location the small nucleolar RNAs (snoRNAs) have long been expected to participate in ribosome biogenesis. This prediction has been borne out thus far for six snoRNAs shown to be required for normal processing of ribosomal RNA (2,3,6,10).

The total number of nuclear small RNAs is not yet known for any organism. Early estimates were low, especially for mammalian cells, due primarily to lack of detection of low abundance species. As detection and fractionation procedures improved, estimates of snRNA pool size increased. The past few years have

seen good progress in characterizing new snRNAs. Most of the snRNAs analyzed are from vertebrates and yeast, with humans, rodents and *Xenopus* receiving special attention among the vertebrates. Nearly 25 snRNAs have been identified from vertebrates and somewhat more than that number have been characterized from the yeast *Saccharomyces cerevisiae*. SnRNAs have also been described from plant sources, trypanosomes, slime molds and protozoa, but the number of species analyzed from these sources is still small (2).

Only a few snRNAs are known to be conserved between vertebrates and yeast or even among vertebrates, however, it is still too early to predict the number of snRNAs that are universal. snRNAs known to be conserved between animals, plants and yeast include: the five splicing snRNAs, RNase P RNA and the three snoRNAs U3, U14 and 7-2/MRP RNA (1–4,6–8). Interestingly, a small RNA with structural similarities to U3 has been found in the archaeobacterium *Sulfolobus acidocaldarius*, suggesting that snRNA functions are not confined to eukaryotes (11).

We estimate that *S.cerevisiae* may contain upwards of 50–60 snRNAs, based on high resolution electrophoretic analysis of RNA prepared from isolated nuclei (12; A. Balakin and M. J. Fournier, unpublished results; see also below). Equivalent analyses of nuclear RNA do not appear to be available for other cell sources. If each band of yeast RNA detected corresponds to an authentic snRNA, then perhaps only 40–50% of the snRNAs from this organism have been described, leaving another 25–35 species (or more) to be characterized. We report here structures and essentiality results for three new snRNAs from *S.cerevisiae*. All three are snoRNAs.

MATERIALS AND METHODS

Strains and media

Escherichia coli DH5aF' [F'/supE44 ΔlacU169 (φ80 lacZ-ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] was used for all cloning procedures. *E.coli* strain HB101 [supE44 hsdS20(r-m⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1] was used as a source of bacterial DNA for hybridization analyses. Both strains were grown on liquid or solid LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl).

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Yeast snRNA for direct sequencing and DNA for a subgenomic library were prepared from the protease-deficient strain BJ2407 (*a/a prb1-1122/prb1-1122 prc1-407/prc1-407 pep4-3/pep4-3 leu2/leu2 trp1/trp1 ura3-52/ura3-52*; 13,14). Gene disruptions and other genetic manipulations were carried out with yeast strain MH2 (*a/a ade2-101/ade2-101 his3/his3 trp1-Δ1/trp1-Δ1 ura3-52/ura3-52 leu2-3,112/leu2-3,112*; M. Fitzgerald-Hayes, unpublished results). Cells were grown on liquid or solid YEPD (1% yeast extract, 2% peptone, 2% glucose) or YNBD (0.67% yeast nitrogen base, 2% glucose), supplemented with appropriate amino acids and nucleic acid bases, at 30°C except where indicated. Diploid cells were sporulated at room temperature in liquid medium containing 1% potassium acetate and asci were dissected after treatment with zymolyase (ICN Biochemicals Inc.).

Preparation and analysis of RNA

A nuclear RNA fraction enriched in known nucleolar RNAs was prepared as described earlier (12). Partial sequences of gel-purified snRNAs were obtained using a RNA sequencing kit from Pharmacia, according to instructions supplied by the manufacturer. Reverse transcription was done essentially as described by others (15) using oligonucleotide primers complementary to the 3'-terminal sequences of the new RNAs (5'-CTTTCTTCGACATCCCAATTC for snR34, 5'-CCTGCCGGCCCTTGCATGGGCGTCTT for snR35 and 5'-CGTTCTAGTTACAGTACGATACC for snR36). The extreme 3'-terminal nucleotides of each RNA were determined by direct chemical sequencing (16). RNA for Northern and primer extension analyses was prepared by a hot phenol/glass beads procedure (17). The occurrence of 5'-trimethylguanosine caps was tested by probing blots of small RNA precipitated with TMG-specific antibodies. Association with fibrillarin (NOP1) was examined by electrophoretic analysis of RNA precipitated with fibrillarin antibodies, after labeling with [³²P]pCp. The antibodies used were rabbit R1131 anti-TMG and anti-fibrillarin monoclonal 72B9, kindly provided by R. Luhrmann and K. M. Pollard and E. M. Tan. Analyses of yeast rRNA and rRNA precursor levels were performed as described earlier (18,19). RNA was prepared from cultures in logarithmic growth.

Isolation and analysis of DNA

Large and small scale preparations of yeast DNA were carried out as described previously (20,21, respectively). Plasmids were isolated from *E. coli* by a boiling miniprep procedure (22). *E. coli* chromosomal DNA used in dot-blot hybridization assays with snRNA probes was purified as described (23).

DNA sequencing was accomplished with pUC18 or pBluescript II SK(-) vectors carrying 150–400 bp fragments of snRNA coding sequences and flanking DNA. Sequence data were generated for both strands using snRNA-specific primers, forward and reverse M13 primers and SK and KS pBluescript-specific primers. The protocol and assay materials were from a dsDNA Cycle Sequencing System kit (BRL Life Technologies Inc.). Computer DNA and RNA sequence analyses were carried out with programs developed by the Genetics Computer Group from the University of Wisconsin (24).

Preparation of labeled probes and hybridization procedures

The cDNAs corresponding to snR34, snR35 and snR36 were synthesized by one-sided (anchored) PCR (25). Oligonucleotide primers complementary to the 3' portions of each RNA (identified above) were used for reverse transcription. Poly(A) tails were added to single-stranded cDNAs with terminal deoxynucleotidyl transferase (Gibco BRL Life Technologies). Double-stranded cDNAs were produced by PCR using the same snRNA-specific oligonucleotides and oligo(dT). Restriction fragments derived from the amplified cDNAs were cloned into the pBluescript IISK(-) vector, fused to the T7 RNA polymerase promoter in reverse orientation to permit production of anti-sense RNAs. Transcription was carried out *in vitro* as described earlier (26), using plasmids linearized with appropriate restriction enzymes, [α -³²P]UTP (800 Ci/mmol; DuPont NEN) and T7 RNA polymerase (kindly provided by C. Martin). Gel-purified radioactive transcripts were used as probes in hybridization analyses. A radiolabeled DNA probe used in the *SNR35* gene replacement analysis was prepared with a NEBlot Kit (New England Biolabs) and [α -³²P]dCTP (800 Ci/mmol; DuPont NEN).

Oligonucleotides used to identify control snRNAs included: (i) 5'-CGCCTTCCGCGCCGTATGTGTGTGTGACC-3', for U1; (ii) 5'-CATAGGATGGGTCAAGATCATCGCGCC-3', for U3; (iii) 5'-CGATGGGTTTCGTAAGCGTACTCTACCGTGG-3', for U14.

Three oligonucleotides complementary to different regions of ³⁵S pre-rRNA were labeled with [γ -³²P]ATP (6000 Ci/mmol; DuPont NEN) and T4 polynucleotide kinase (New England Biolabs) and used to analyze patterns of pre-rRNA processing. The probes were: 5'-TTATTAAATTTCTTTAATGATCCTTCCGCA, complementary to 18S RNA and ITS1 at the junction region; 5'-CACCTAGCGACTCTCTCCACCG, complementary to the ETS1 region 543 nt upstream of the 5'-end of 18S RNA; 5'-CGCCTAGACGCTCTCTTCTTATCG, complementary to the ITS2 region, 40 nt upstream of the 5'-end of 25S RNA.

Hybridizations with labeled DNA, RNA or oligonucleotide probes were carried out as described previously (12).

Cloning

A subgenomic DNA library was prepared from yeast DNA digested with *Hind*III and fractionated on an 0.8% agarose gel. Based on results of Southern hybridization assays, fragments ranging in size from 6 to 8 kb were selected, electroeluted from the gel and ligated with *Hind*III-digested pUC18 vector. *E. coli* cells were transformed and clones with *SNR* genes were identified by colony hybridization using snRNA-specific antisense RNA probes. Shorter DNA fragments containing the *SNR* genes were subcloned in plasmid pUC18 and used for gene disruption analyses. The fragments were: (i) a 1.3 kb *Sac*I-*Pst*I fragment (*SNR34*); (ii) a 1.2 kb *Pst*I fragment (*SNR35*); (iii) a 1.0 kb *Bam*HI fragment (*SNR36*).

Construction of null alleles

The *SNR34* and *SNR36* genes were disrupted in pUC18 with *Bam*HI restriction fragments containing the yeast *LEU2* and *HIS3* genes respectively. The marker genes were derived from plasmids YDp-L and YDp-H (27). Unique restriction sites located within *SNR* gene coding sequences were used for the

disruptions. These were *Bbs*I for *SNR34* and *Eco*RV for *SNR36*. A *Pst*I fragment containing the *SNR35* gene was transferred from pUC18 to pBR322, where an *Acl*I site had been eliminated prior to the cloning operation. The *SNR35* gene was disrupted by replacing an *Ase*I fragment (380 bp) containing a portion of the snR35 coding sequence and 3'-flanking DNA with a *Bam*HI fragment containing the yeast *TRP1* gene derived from plasmid YDp-W (27).

Construction of strains with inactive SNR genes

Plasmids containing disrupted *SNR* genes were restricted with *Sac*I and *Pst*I to obtain a *snr34::LEU2* fragment; *Pst*I to obtain a *snr35::HIS3* fragment and *Bam*HI to obtain a *snr36::TRP1* fragment. The plasmid digests were used directly to transform diploid yeast MH2 cells by a lithium acetate procedure (28). Transformants were screened on selective media and disruption of chromosomal alleles was verified by Southern analysis. Haploid segregants were obtained by tetrad dissection.

The *snr34* and *snr36* strains were crossed (YS604 × YS603) and the resulting diploid dissected to yield the segregant YS606 containing both null alleles. Similarly, a *snr33/snr35* double mutant (YS607) was constructed by crossing *snr33* and *snr35* strains (YS591 × YS605; see 12 for the YS591 strain). Double mutants YS606 and YS607 were crossed, diploids were dissected and segregants containing mutations in three (YS608) or four (YS609) *SNR* genes were isolated. The parental and new strains have the following genotypes: YS591 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 his3 snr33::URA3*); YS603 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 his3 snr36::HIS3*); YS604 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 his3 snr34::LEU2*); YS605 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 his3 snr35::TRP1*); YS606 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 snr34::LEU2 snr36::HIS3*); YS607 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 his3 snr33::URA3 snr35::TRP1*); YS608 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 his3 snr34::LEU2 snr35::TRP1 snr36::HIS3*); YS609 (α *ade2-101 trp1-Δ1 ura3-52 leu2-3,112 his3 snr33::URA3 snr34::LEU2 snr35::TRP1 snr36::HIS3*).

RESULTS

Identification of new snRNAs and gene cloning

The snRNAs featured in this study were selected for characterization based on success in obtaining direct sequence data in each case. The individual RNAs were purified from a preparation of nuclear RNA. The purification procedure involved: (i) salt extraction of isolated nuclei, using conditions which enrich known nucleolar snRNAs; (ii) phenol extraction; (iii) labeling RNA with [³²P]pCp; (iv) fractionation of individual snRNAs by high resolution gel electrophoresis. A portion of the gel pattern obtained is shown in Figure 1. Partial sequences were developed for each purified RNA by direct enzymatic sequencing. Searches of the GenBank database showed each of the three snRNAs to be novel.

Our cloning strategy involved hybridization screening of genomic DNA clones with labeled anti-sense RNA transcripts. The cDNAs were synthesized from DNA primers complementary to the 3'-ends of the RNAs. A poly(A) tail was added to each reverse transcript and double-stranded cDNA was prepared by PCR amplification. This material was sequenced to identify

Figure 1. Fractionation of snR34, snR35 and snR36 RNAs. Nuclei were prepared from yeast spheroplasts, purified by centrifugation through a 0.5 M sucrose cushion and extracted with 25 mM HEPES-KOH, pH 7.6, 5 mM magnesium acetate, 0.25 M sucrose and NP-40. RNAs isolated by phenol extraction were labeled with [³²P]pCp and fractionated by electrophoresis in 8% polyacrylamide. RNA bands containing snR34, snR35 and snR36 were subjected to a second cycle of electrophoresis to obtain individual species. Bands corresponding to novel and several previously characterized snRNAs are marked. The electrophoretic pattern shown is for snRNAs in the size range 158–222 nt.

useful restriction sites and then cloned into a T7 transcription vector.

Results from Southern analyses showed each snRNA to be derived from a single genomic locus and revealed a cloning strategy (Fig. 2). All of the coding sequences occur in *Hind*III fragments of ~6–8 kb. This fraction of genomic DNA was then used to develop a library enriched for all three coding sequences. Each of the *SNR* genes was isolated from this library using anti-sense RNA probes. The sequences of the novel snRNA genes are shown in Figure 3.

Properties of the novel snRNAs and genes

The 5'-ends were defined by primer extension and the 3'-ends by chemical sequencing. The sizes of the new RNAs were revealed to be: (i) snR34, 203 bases; (ii) snR35, 204 bases; (iii) snR36, 182 bases. The cDNA and genomic DNA sequences agreed, indicating that the genes do not contain introns and that the transcripts are not edited. None of the RNAs contain the box C or D elements conserved among several other snRNAs or the box A or B sequences that are unique thus far to the U3 RNAs (reviewed in 2,4,6).

Figure 2. Southern analysis of genomic DNA. Yeast genomic DNA was digested with a number of restriction enzymes, fractionated on 0.8% agarose gels and electroblotted onto nylon membranes. The blots were probed with ^{32}P -labeled antisense snRNA transcripts. The three panels correspond to genes for *SNR34* (A), *SNR35* (B) and *SNR36* (C). Sizes were estimated with a *HindIII* digest of λ DNA labeled with ^{32}P .

Northern assays of total cell RNA yielded single bands in each case: no precursors or other variants were detected (related data are shown below). Immunoprecipitation assays with antibodies specific for trimethylguanosine yielded signals for snR34 and snR35, but not for snR36, indicating that snR36 lacks this modification (Fig. 4). All three RNAs were precipitated with a fibrillarin-specific antibody, indicating an association with NOP1 (data not shown). Precipitation efficiencies differed, as seen for other snoRNAs (2), but positive signals were evident in each case. Patterns of the fibrillarin-positive snRNAs will be presented elsewhere, as part of a more comprehensive study (A. Balakin and M. Fournier, in preparation). Based on their snoRNA-like extraction properties (salt-resistant) and association with fibrillarin, each of the new RNAs is believed to be nucleolar.

Screening of the GenBank database with the complete gene sequences did not reveal any significant relatedness to known genes. Negative Northern and Southern hybridization results were obtained with total RNA and DNA from *E. coli* and the fission yeast *Schizosaccharomyces pombe* and genomic DNA from *Arabidopsis thaliana* and mice (data not shown). Complementarity with precursor ribosomal RNA was also examined to assess the potential for direct interaction with these RNAs. Several segments of up to 10 complementary base pairs were identified, however, none is seen as especially promising, as similar extents of complementarity exist for some spliceosomal snRNAs and pre-rRNA.

TATA-like elements occur in the 5'-flanking segments of each new gene, at positions -81 for *SNR34*, -92 for *SNR35* and -108 for *SNR36*. The sequences and spacings are in good agreement with consensus patterns reported earlier for the first several snRNA genes sequenced from *S. cerevisiae* (29). This consensus sequence, TATAAARRGNR, is located 84-100 nt from the coding sequence. A more recent search revealed the gene for snR36 to be adjacent to the 3' non-coding region of a newly discovered gene for 3-phosphoserine aminotransferase (P. Belhumeur, N. Fortin and M. W. Clark, unpublished results). The two genes are in a tail-to-tail orientation and the coding regions are separated by 140 nt.

All three snRNAs are dispensable

The essentiality of the snRNAs was examined by replacing the normal genes with disrupted alleles. The null-alleles were constructed as in Figure 5. Hybridization data for five snR35 gene disruptions are shown in Figure 6A. Interestingly, transformation with the *snr35::TRP1* cassette resulted in disruption of both chromosomal *SNR35* alleles in one of the analyzed clones. (Fig. 6A, lane 4). Northern analysis of total RNA from this clone showed snR35 to be absent (Fig. 6B), indicating that the *SNR35* gene and its product are dispensable.

Haploids containing disrupted alleles for the *SNR34* and *SNR36* genes were isolated by tetrad dissection and identified by screening for the selectable markers. The disruption of each gene was verified by Southern and Northern analyses; the snRNA patterns are shown in Figure 7. Cells lacking the individual snRNAs were viable on YEPD medium at 30°C and no significant deviation in growth rate was evident in comparison with the wild-type strain.

The possibility that simultaneous loss of several snRNAs might have a synergistic effect on growth was also examined. Two strains containing multiple *SNR* gene knock-outs were constructed by a two-step cross-and-dissection procedure (see Materials and Methods for details). One strain (YS608) lacked all three of the new snRNAs. The second strain (YS609) lacked these three and a fourth non-essential species, snR33 described earlier (12). No growth rate differences were apparent for mutant and wild-type cells. Comparisons were made on YEP and YNB solid media with different carbon sources at different temperatures. The conditions examined were: 2% glucose, 2% galactose, 2% glycerol and 2% potassium acetate and 15, 25, 32 and 37°C (data not shown). Cell shape and bud formation also appeared normal for mutant cells grown in liquid YEP and YNB media with various carbon sources and at different temperatures.

No differences in the levels of precursor or mature rRNAs were apparent in the four gene knock-out strain YS609, relative to a control (MH2) haploid strain. The pre-rRNA patterns were analyzed with hybridization probes complementary to the ETS1,

SNR34

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      10      20      30      40      50
1  GTCTCTTCCT TGTATTCGCT TGCTGATATT CCGTACCTTA GATTTCTTAC
51 TTTTCGAAATA CATTACCCGG ATCAATTCCA TTATTTTCGC CTTTCAAAAA
101 ATTTCCCGGT TGTATAAAAA GGCAGGACTT CGACAGTCAA CAACAATTTT
151 GAGTATATTT ACCACATTTT TTCTGATTTT TTTTAAAGAG GGTGAATCAA
201 AAATTTATTT TTTACACGGA AACGATGCCA CAGTTGACTG AACTGTCTTT
251 CTAACAGTAG TAAATTGCCA GTATTTCAAA CAAGATTGGA AATACGAGTT
301 TCCCAGAATA ATTTATTTGG ACAGGATAGG AAGTCCGATT TCTGTGTTGT
351 CTCAAACGAG GCGATAGAAT TGGGATGTCG AAGAAAAGTCT ACATTACTCA
401 TATATTTACG TACTGTCTCT TGCAGGTAAA AAGTCTTGAA GATCAGTTTC
451 AATATTGAAA CTAACAAATT TAGTTAGTTT CTGTACTTAG TCCGAGGTAT
501 GTATGCTAAG ATTAGAGGAT GGTAAAAAAA TCAGCTATGA AAATACATGT

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SNR35

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      10      20      30      40      50
1  ACAAGCGTCT TCTTTAATAA CTCAAGGGAC CCTAATACGC CGTTCCTTAT
51 ATTCAACTCC GGGTAATGGA GATAGTATAT ATATATTTTT TTTTCTCCCC
101 AGTGCAACTA TAAAAAGAGA TGAAGAATTT CTAAAGAGTA CTACCTCGGT
151 TTTACTTCTT GTATATTTAC TCTTTTATT TCAAATTTTA AAAAAAACC
201 ATACAAAAAT AATCGTGCGG ATTAAATAAT CAGGACTATA AAACCGTGT
251 GTATATATCG AGTCTCTTTT GGTATAAGCG TCAAGTCCAT CGGAGAGATC
301 ATCATTTTGT TCATCTTAAT CCCCTTTTGT GTAGGAAAT GAGGGAAGTT
351 TAGGCTTCTC AACATTTTAA GACGCCTATG CAAGGGCTGG TAGGACAGAC
401 ATGCTATTCT TATTTTGACG TAGAAATAAC GTAGTTAAGA TTGAAGAGGA
451 AAGAGGTATT CTATATACAA TAAATATCAG TGTCCTGACT TCAGATCCCT
501 GAAAAAGTTC ATCATACCGG TAAAAACCAC CAACATTAAT

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SNR36

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      10      20      30      40      50
1  TCGGAAACGA GTTAAGATAA AATATAAGAA GAGGAAAGTA TTCCAATTA
51 TGTTTTCATT TAATTGGTAG ACGCGACAAA AAAAAGTATA ATATATTGGG
101 AGAGATAGTT CAGTCTCACC CACATTCTCT TTGCCCTGTG CCTCGCTCGG
151 TTGTTAATTC CCAATATTAC GATCTTGAGC TGGATGACAA AAAAATAAAA
201 ATACATTTTA TTTTTTTGAT CAACGGGCTA AAACAATTAG ACTTCTTTGA
251 GTACGAGGAT ATCGCTATTT TTATCTCAGC GTATCGTATT GTAATTAGAA
301 CGTCTCATAT CATATCGTAT AATTTCGGCT TTATTTCAAG AAAAAGAAGA
351 GAAGT

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Figure 3. Sequences of the genes for snR34, snR35 and snR36. The sequences of the snRNA coding segments and flanking regions are shown. The 3'-ends of the coding regions were defined by direct chemical sequencing of individual snRNAs and the 5'-ends by primer extension analysis. The sequences corresponding to the snRNAs are highlighted by italics and underlining. Candidate TATA elements are identified with bold letters. The TATA elements conform to a consensus sequence proposed earlier for *S.cerevisiae* SNR genes (TATAAARRGNNR; 30).

ITS1 and ITS2 regions of 35S rRNA. The levels of mature 18S and 25S rRNA were assessed by visual inspection of RNA gels stained with ethidium bromide (data not shown).

Figure 4. Immunoprecipitation with TMG antibodies. Immunoprecipitated small RNAs were fractionated electrophoretically, transferred to filters and identified with radiolabeled oligonucleotide probes. Control RNAs included U1, U3 and U14. Two U14 variants were included, TMG-minus (wild-type) and TMG-plus, the latter from a RNA polymerase II, galactose-dependent allele (*Gal^d*). Lane 1, total RNA; lane 2, RNA precipitated with non-immune human serum; lane 3, RNA precipitated with TMG antibody.

DISCUSSION

The snRNAs described are currently unique to *S.cerevisiae*. No homologs were present in the GenBank database. Negative results were also obtained in hybridization assays with RNA and DNA from different animal, plant and other fungal species. Hybridization results could be misleading, however, as snRNA homologs often have modest homologies. For example, U14 is conserved among all organisms tested thus far, yet probes for *S.cerevisiae* U14 do not cross-hybridize with RNA from the yeast *S.pombe* under different hybridization and wash conditions. Nevertheless, we have determined that *S.pombe* contains U14 and the gene for this RNA has now been isolated and sequenced (D. Samarsky and M. Fournier, in preparation).

Of the snRNAs characterized thus far, most are believed to be nucleolar. Yeast nucleoli are not as well defined morphologically as those of higher eukaryotes and no generally accepted procedure for isolating this complex exists at present. For this reason, classification of nucleolar RNAs in yeast is based on association with rRNAs or nucleolar proteins, generally NOP1, which is the yeast counterpart of vertebrate fibrillarin. Co-purification with the nucleolus is used with metazoan organisms, but has not been adopted for yeast. Using an alternative approach, a nuclear RNA fraction enriched with snoRNAs can be prepared by differential extraction. The three new snRNAs described are believed to be nucleolar as they: (i) exhibit extraction properties like these of known nucleolar RNAs (U3 and U14; 12); (ii) are precipitable with antibodies against fibrillarin. While these are accepted criteria, we note that they are somewhat tenuous, as both are indirect.

Yeast nucleolar RNAs are believed to be transcribed by RNA polymerase II. Most contain TMG caps, which are presumed to be formed by hypermethylation of m⁷G capped transcripts. Most coding units have TATA-like elements, however, none of these

Figure 5. Construction of null alleles for the snR34, snR35 and snR36 genes. Plasmids containing inactive snRNA genes were prepared by disrupting the RNA coding sequences with selectable yeast marker genes. The structures of the relevant DNA segments and restriction sites are shown.

Figure 6. Analysis of DNA and RNA from cells containing disrupted snR35 gene alleles. Diploid cells were transformed with *Pst*I restriction fragments from the *snr35* plasmid shown in Figure 5. Transformants were identified by growth on selective medium and subjected to Southern analysis, following digestion with *Pst*I. The Southern data (A) indicate that one allele had been disrupted in most isolates, but both alleles were damaged in one strain (lane 4). No snR35 RNA was detected in a Northern analysis of this diploid (B).

Figure 7. Southern and Northern data for strains containing null alleles of the snR34 and snR36 genes. Diploid cells transformed with *snR34* and *snR36* gene fragments (*Pst*I–*Sac*I and *Bam*HI respectively; Fig. 5A and C) were identified by growth on selective media and the genomic DNAs subjected to Southern analysis after treatment with *Pst*I and *Bam*HI respectively. Haploids containing disrupted *snr* alleles were prepared by tetrad dissection and analyzed for growth on selective media and for snRNA content. Southern (A) and Northern (B) assay results are shown for cells derived from spores of single tetrads. The data presented in both panels are for the same sets of clones.

have yet been shown to be involved in expression (2). The 5' non-coding regions of the new genes also have these elements and the positions and sequences of the elements are consistent with previously characterized yeast *SNR* genes. This feature implies that expression and regulation of the related snRNAs may occur by common mechanisms. The absence of a TMG cap in snR36 RNA suggests that this RNA results from processing. Two classes of cap-free nucleolar snRNAs are known to exist in yeast, both derived by processing. One class consists of species processed from pre-mRNA introns, e.g. U18 (A. Balakin and M. J. Fournier, in preparation) and snR38 and snR39 (J. Ni and M. J. Fournier in preparation). The second corresponds to RNAs processed from transcripts that do not appear to be intronic (U14 and snR190; 30). It is also possible that snR36 is transcribed by RNA polymerase III. In plants, for example, U3 and MRP RNAs have been shown to be transcribed by RNA polymerase III (31,32).

The gene disruption analyses did not provide any clues about the functions of the new RNAs. None of the genes is essential for cell viability and loss of the RNAs had no detectable effect on growth rate at several temperatures on different media. The

absence of any obvious phenotype in the triple and quadruple mutants lacking the new snRNAs and snR33 eliminates the possibility of a strong synergistic effect on growth. A similar situation has been described by Parker *et al.* for the snRNA species snR3, snR4, snR5, snR8 and snR9 (29), none of which are splicing snRNAs. A strain lacking all five RNAs had no apparent growth phenotype and a mutant disrupted for these snRNAs and snR10 RNA had the same phenotype as strains lacking only snR10; snR10 is not essential, but its loss yields a *cs* phenotype and impairment of rRNA processing (33). Of the non-spliceosomal yeast snRNAs characterized to date, including the RNAs described here, <30% are essential or exhibit a detectable growth phenotype (2). Dispensability is not limited to snRNA coding units. Studies of random disruptions suggest that up to 70% of the yeast genomic sequences are dispensable (34). In addition, of 45 putative protein coding regions identified in the sequence of chromosome III, 62% were shown to be non-essential (35).

The four yeast nucleolar snRNAs known to be essential are all required for processing of pre-rRNA, although precise functions have not yet been established (reviewed in 2–4,10). U3, U14 and snR30 are all essential for early processing steps, whereas the MRP species is needed for a later cleavage upstream of 5.8S RNA. The roles of the non-essential snRNAs remain an enigma. No function is a formal possibility, but it seems more likely that the dispensable RNAs are either functionally redundant or mediate processes that are beneficial, but not critical. Consistent with the latter view is the finding that loss of at least one non-essential snoRNA, snR31, does not affect growth, but does have a minor effect on rRNA processing (36). Non-essential snRNAs can be imagined to function at any stage of ribosome synthesis, from transcription of rDNA to export of ribosomal subunits. SnoRNAs could participate in any of these events, providing, for example, kinetic assists in RNA folding and rRNP assembly and minimizing flow along less productive pathways (2,37).

The fact that no phenotypic effects were observed with loss of the new snRNAs rules out the possibility of using simple genetic approaches to assess function. However, clues about their roles might be derived by identifying genes that encode interacting components, through characterization of synthetic lethal mutants. Insight might also come from biochemical analysis, such as cross-linking and sub-cellular fractionation studies. The large preponderance of non-essential snRNAs argues that these species are not simply superfluous 'selfish' RNAs. Although evidence is lacking, we favor the view that the large class of non-essential snoRNAs is relevant and likely to be involved in facilitating unusually complicated processes.

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