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Identification and functional analysis of a novel yeast small nucleolar RNA

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ABSTRACT

snR31 is a RNA species of 225 nt. which has the trimethyl guanosine cap structure typical of small nuclear RNAs (snRNAs) and yeast small nucleolar RNAs (snoRNAs), and is associated with the nucleolar proteins fibrillarin (NOP1) and GAR1. On sub-nuclear fractionation, snR31 behaves like other snoRNAs, and is enriched in a nucleolar fraction. The *SNR31* genomic locus is close to the *SNR5* locus, which encodes another snoRNA. The two genes are divergently transcribed with 217 bp separating the transcription start sites. Disruption of the *SNR31* gene does not detectably impair growth in a haploid strain. Analyses of pre-rRNA processing in wild-type and *snr31*⁻ strains shows some accumulation of the 35S primary transcript in the mutant, indicating a mild impairment of the initial steps in pre-rRNA processing.

INTRODUCTION

The transcription of pre-ribosomal RNA (pre-rRNA), and most post-transcriptional steps in ribosome synthesis, take place in the nucleolus, a specialized sub-nuclear structure. In recent years it has become clear that the processing of pre-ribosomal RNA, and perhaps other steps in ribosome synthesis, requires the action of a class of small RNA species localized to the nucleolus, the small nucleolar RNAs (snoRNAs). The cloning and characterization of 11 yeast snoRNAs has previously been reported. Of these, three species, U3, U14 and snR30, are essential for cell viability, while mutants lacking snR10 are impaired in growth (1–4). The absence of any of these snoRNAs results in the accumulation of the 35S primary transcript of pre-rRNA, and inhibition of the processing steps which normally lead to the synthesis of the small subunit rRNA (18S in yeast). Synthesis of the large subunit rRNA (25S in yeast) is little affected. Disruption of the genes encoding the other cloned snoRNAs (snR3, snR4, snR5, snR8, snR9, snR189 and snR190) (5–8) does not detectably impair growth. Even a sextuple mutant lacking simultaneously snR3, snR4, snR5, snR8, snR9 and snR10, is not more impaired in growth than an snR10 single mutant strain (5). The snoRNAs are associated with proteins in small nucleolar ribonucleoprotein particles (snoRNPs). The cloning of four yeast snoRNP proteins, NOP1, GAR1, SOF1 and SSB1, has been reported. Of these, NOP1 (the yeast homologue of the vertebrate nucleolar protein fibrillarin) appears to be associated with all yeast snoRNAs, while the others are associated with subsets of the snoRNAs (9–12)

A large number of snoRNA species are also likely to be present in higher eukaryotes. At the time of writing, the cloning of 10 human snoRNAs (U3, U8, U13, U14, U15, U16, U17, E1, E2, E3) has been reported (13–18), and the number is rising rapidly. Of these, functional analyses *in vivo* using *Xenopus* oocytes have been reported for U3 and U8 (19, 20). In contrast to the results of genetic analyses in yeast, destruction of these RNAs by microinjection of complementary DNA oligonucleotides, interferes with synthesis of the large subunit rRNA, but not the small subunit rRNA. In addition, analyses *in vitro* indicate that U3 is also required for a cleavage event in the 5′ external transcribed spacer (21, and references therein).

Here we report the sequence, characterization and initial functional analysis of a novel yeast snoRNA, which is non-essential but has a mild effect on the processing of pre-rRNA.

MATERIALS AND METHODS

Strains and media

Standard *S.cerevisiae* growth and handling techniques were employed. Transformation was by the Li Cl method (22). The diploid strain used for gene disruption carries *MAT***a**/α, *ade8*/+, *his4*/+, *his3*/+, *ade2/ade2*, *leu2/leu2*, *lys1/lys1*, *ura3/ura3* (strain JU42×JR26-19B; kindly provided by Eduard Hurt). The haploid *SNR31*⁺ strain carries, *MAT***a**, *ura3-52*, *leu2-3.112*, *ade1-100*, *his4-519* (strain BWG1-7A; kindly provided by L.Guarente). The strain used for analysis of the effects of the absence of snR31 is isogenic except that it has the *snr31::URA3* construct integrated at the *SNR31* locus.

Cloning

For gene disruption, the XhoI–BglII genomic fragment carrying *SNR31* was subcloned into the XhoI–BamHI sites of pBS (KS+). The EcoRI–XmnI region of *SNR31* was replaced by the EcoRI–SmaI fragment of YIp5, which includes the *TET*ᴿ and *URA3* genes. This deletes 9 nucleotides of 5′ flanking sequence and 159 nucleotides of *SNR31* coding sequence. The linear fragment, *SNR31* 5′ flanking sequence-*TET*ᴿ-*URA3*-*SNR31* 3′

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flanking sequence, was used to transform a diploid strain of yeast, with selection for *psURA3*$^+$. Following sporulation, *URA3*$^+$ haploid progeny could be recovered, demonstrating that *SNR31* is not essential for spore germination or growth. The same construct was subsequently used to transform a haploid strain of yeast (BWG1-7A) to produce otherwise isogenic *SNR31* and *snr31::URA3* strains.

RNA extraction and analysis

RNA extraction, polyacrylamide and agarose-formaldehyde gel electrophoresis and hybridization were performed as described (4), except that Hybond-N+ (Amersham) was used for all Northern hybridizations. The vectors previously described (23) were used to synthesize riboprobes for U3, U14, snR10, snR30, snR190, U4, U5 and U6 hybridizations. The EcoRI−XmnI restriction fragment including the *SNR31* coding sequence, was cloned into the riboprobe vector pT3/T7a-19 (BRL). This vector was then transcribed using T7 RNA polymerase to generate riboprobes for hybridization to snR31. The hybridization probe to the 5′ region of ITS1 was an oligonucleotide of sequence, GCTCTTTGCTCTTGCC. The 3′ region of ITS1 was amplified by PCR using primers which insert EcoRI and HindIII sites at cleavage sites A_2 and B_1 respectively. This fragment was cloned into pT3/T7a-18 (BRL) and used to prepare riboprobes.

The primer extension to determine the 5′ end of snR31 was performed in molar excess of primer as described (24) except that Actinomycin D was omitted from the extension buffer. Annealing and extension were carried out at 46°C. After extension with AMV reverse transcriptase, the RNA was hydrolyzed and the DNA was precipitated and run on a sequencing gel. The same oligonucleotide was used to obtain a sequence ladder and was first kinased with unlabeled ATP so as to ensure identical migration. The oligonucleotide used (GCACTGAAGCAACGCCC) is complementary to nucleotides 76−92 of snR31.

RNase protection to determine the 5′ and 3′ ends of snR31 was performed as described (25), except that 0.2 u of RNase H was added per reaction. RNases A + T1, were used with antisense RNA transcribed from the genomic XmnI−EcoRI and PvuII−XmnI fragments (see Figure 1) subcloned in to pT3/T7α-19.

Immunoprecipitation

Lysate was prepared from strain BWG1-7A following growth in glucose minimal medium. Lysis and immunoprecipitation were as previously described (9), except that the cell lysis buffer for immunoprecipitation contained 150 mM KAc, 20 mM Tris Ac pH 7.5, 5 mM MgAc, 5 mM VRC (BRL), 0.2% Triton X-100 and 2 mM PMSF. The same buffer, without VRC and PMSF, was used for antibody binding and washing. Antibodies against NOP1 and GAR1 were affinity purified as previously described (9, 10). Immunoprecipitation was for 2 hours at 4°C using lysate prepared from the equivalent of 5 OD_{600} unit of cells (about 10^8 cells). Under these conditions the lysate is present in excess. Precipitated RNAs were recovered and analyzed as previously described (9).

Sub-cellular fractionation

Cell fractionation and subnuclear fractionation were carried out as described (9, 26).

RESULTS

The cloning of *SNR5* has previously been reported (5). When we used the genomic clone as a hybridization probe, we observed, in addition to snR5, a second, larger RNA which we designated snR31. Subcloning of the region showed that the second RNA is encoded by sequences 5′ to the *SNR5* gene. Riboprobes prepared to this region showed that snR31 is transcribed on the opposite strand from snR5 with a divergent transcription pattern

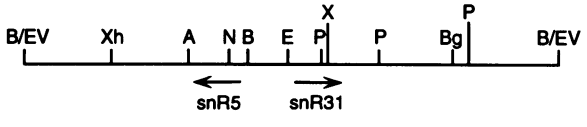

Figure 1. Restriction map of the *SNR5−SNR31* region. The restriction map is shown for a 2.3 kb genomic BamHI partial fragment in plasmid pES3, in which the BamHI sites have been ligated to vector EcoRV sites (E.Schuster and C.Guthrie, personal communication). Sites shown are A = AccI, B = BamHI, Bg = BglII, E = EcoRI, EV = EcoRV, N = NdeI, P = PvuI, X = XmnI, Xh = XhoI. No sites were detected for BclI, HindIII, HpaI, KpnI, NcoI, NotI, NruI, PstI, SacI, SalI, SphI, StuI, XbaI or XmaI in this region. *SNR31* and *SNR5* are divergently transcribed as shown.

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-600 TCCTTATCCT TTGACAGTAA AATTCAAAAA ACTCACTAGA AAGTTCACTA -551
-550 AAGCGCCTTA GCTGACTACA GCACAACCCA ACCCCAAAAA CTAGTGAACT -501
-500 ACACACTAAG AGAATTTATG GTATTTCCAT AATTGAAGTA TATGTACGAT -451

                                 OHUC ACUUCUAUAC AUGUGGAUCU snR5
-450 GTATACAATG TAAACAAGAG AGTAATGAAG TGAAGATATG TACACCTAGA -401

     CGCUUGGUUA CUAUUAAACA AAAGAAUUAA UUCUUUUUGG UAAUUUUGAC snR5
-400 GCGAACCAAT GATAATTTGT TTTCTTAATT AAGAAAAACC ATTAAAACTG -351

     CUAAUCUAAA GUUAUUACUU UUCGAUCCUU GUUUUUUUUA ACCAGAUGAA snR5
-350 GATTAGATTT CAATAATGAA AAGCTAGGAA CAAAAAAAAT TGGTCTACTT -301

     GGUCGGUAAA CGCGAAAUAU AUCUGUAUAC CUCCGCACUA CAGAAUUCGU snR5
-300 CCAGCCATTT GCGCTTTATA TAGACATATG GAGGCGTGAT GTCTTAAGCA -251

     ACCCAUUAGG CCUUCUAGUC AAAUAACUUA CUA'5                 snR5
-250 TGGGTAATCC GGAAGATCAG TTTATTGAAT GATGGTTTTC TTATCCTAAG -201

-200 GATCCTGCAA ATGAAGTAAG TTCAAAGAAT TGAAATGAAA TCGCCTTTTA -151

-150 TACCCTCGCC GCACCAAGGA GCGCGCTGTA AAATTTTTCG CTTTATCTCT -101

-100 TAGGGCTCCT ACTGTCGGTA GAGAAAAGTT GAGTATATTT AAAGCATGAG  -51

 -50 GTAACTTTTC TGCCCATTTT TACTTCTATT TCATTTGAAT TCAGAAAAGT   -1

  +1 GAAGCAAAAT TACACCATGA GTTCCTATTA ACGTCAGCCT CTTTTGTACT  +50
snR31 GAAGCAAAAU UACACCAUGA GUUCCUAUUA ACGUCAGCCU CUUUUGUACU

 +51 CATAATGTGG CTTCGGATGT TTGATGGGCG TTGCTTCAGT GCGTACGGCT +100
snR31 CAUAAUGUGG CUUCGGAUGU UUGAUGGGCG UUGCUUCAGU GCGUACGGCU

+101 CATGGTAGAT TAATTATTAG AAAGATGTAT CTCCAGCTGT TGATATTAGA +150
snR31 CAUGGUAGAU UAAUUAUUAG AAAGAUGUAU CUCCAGCUGU UGAUAUUAGA

+151 GGGGCAAGCC TTTCTCTTTC ACCTCGCCTT TTTAAACACC TGATACAGTT +200
snR31 GGGGGAAGCC UUUCUCUUUC ACCUCGCCUU UUUAAACACC UGAUACAGUU

+201 GGTCATGATT CGTTCTACAT TTTAATTTCT TTATGTAACC ACGTTGTATA +250
snR31 GGUCAUGAUU CGUUCUACAU UUUAAOH

+251 TTTTCAATCT GGAAAGTAAC CTAAAAGAGT AAAAACTGTT TATTAAAAAA +300
+301 AATGTTATTC AAAATTTATA CATAGTACAC GTACAGCTGC CTGAAGAAAG +350
+351 CATGAGCGAA TGACTTATGC TTCGTCGTCT TCGTCTTCAC TTTCCAAAGC +400
+401 AGCAAATGCG TTGAAA                                     +416
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Figure 2. Sequence of the *SNR5−SNR31* region. The sequence of *SNR31* is shown, starting at +1. The inferred RNA sequence of snR31 (225 nt.) is shown below the DNA sequence. The 5′ flanking sequence is shown to −600. This region includes the *SNR5* locus (5); the sequence of snR5 is shown (in the 3′→5′ orientation) above the DNA sequence (−217 is the 5′ end, and −422 is the 3′ end of snR5). Regions homologous to the box B and box C sequences previously described in other snoRNAs, are shown in bold in the snR31 sequence (box B is the more 5′). A line above nucleotides −148 to −155 denotes a potential TATA box region for *SNR5* on the opposite strand. A potential TATA box region for *SNR31* (from −67 to −57) is underlined.

(Figure 1). This region was sequenced on both strands (Figure 2). The DNA sequence of this region has been submitted to the EMBL database under accession number X72299. Primer extension from within the presumed coding region gave a unique stop 217 bp from the 5′ end of snR5 (Figure 2). RNase protection using a riboprobe transcribed from the subcloned EcoRI−XmnI fragment (see Figure 1) confirmed that this is the correct 5′ end of snR31. The 3′ end was identified by RNase protection using a riboprobe transcribed from the subcloned XmnI−PvuII fragment (see Figure 1). This gave a single protected fragment. The size of the RNA predicted from the sequence, 225 nt., is in good agreement with the size predicted from the gel mobility of snR31. During the course of this work we learned that the *SNR31* gene had been independently cloned by Balakin,A.G., Schneider,G.S., Corbett,M.S., Ni,J. and Fournier,M.J. (manuscript submitted for publication).

To investigate whether snR31 is localized in the nucleolus, subnuclear fractionation was performed (Figure 3A). snR31 is retained in purified nuclei (Figure 3A, lane 2) as are the snoRNAs U3 and snR10, and the snRNA U5. Comparison with a sample of whole cell RNA (Figure 3A, lane 1), shows that snR31 is recovered in the nuclear fraction with similar efficiency to that of the other snoRNAs. Extraction of nuclei with 2% Triton-X114 efficiently releases nucleoplasmic components (9, 26). Nucleolar components, including snoRNAs, are retained, but can be released by extraction with a mixture of the ionic detergents sodium cholate and sodium deoxycholate (9, 23). Triton extraction efficiently releases U5 (Figure 3A, lane 3) but U3, snR10 and snR31 are largely retained, and are released only by extraction with cholate/deoxycholate (Figure 3A, lane 4).

Association of snR31 with nucleolar proteins was assessed by immunoprecipitation. Anti-NOP1 antibodies precipitate all known yeast snoRNAs (9), and precipitate snR31 with an efficiency similar to that of snR10 (Figure 3B, lane 5 and Figure 3C, lane 3). Anti-GAR1 antibodies precipitate snR30 and snR10, but only weakly precipitate U3 and U14 (10). snR31 is precipitated by two different anti-GAR1 sera with an efficiency similar to that of snR30 or snR10 (Figure 3B, lanes 3 and 4). The nucleoplasmic snRNA U6 is not detectably precipitated by anti-NOP1 or anti-GAR1 antibodies.

Immunoprecipitation was also used to determine whether snR31 has the 5′ tri-methyl guanosine (TMG) cap structure, which is present on all other yeast snoRNAs characterized to date except U14 and snR190. Monoclonal antibodies against TMG immunoprecipitate snR31 with an efficiency similar to that of snR10 (Figure 3C, lane 2), which was shown by 2-dimensional thin layer chromatography to be quantitatively capped with TMG (27), or U3 (data not shown).

To analyze its function, the *SNR31* gene was deleted by a gene replacement technique (see Materials and Methods), in which the 5′ 159nt. of the *SNR31* coding region was replaced by the selective marker *URA3*. One copy of the *SNR31* gene was initially replaced in a diploid strain. On sporulation, both the *snr31::URA3* and *SNR31* haploid progeny could be recovered, showing that snR31 is not required for spore germination or cell growth. The *SNR31* gene was disrupted in a haploid strain, allowing the direct comparison of otherwise isogenic *snr31::URA3* and *SNR31* strains. The absence of snR31 from the *snr31::URA3* strain was shown by Northern hybridization (Figure 4). The steady-state levels of other snoRNAs tested (snR10, U3 and U14) were not altered by the absence of snR31 (Figure 4). Haploid *snr31::URA3* strains have a growth rate indistinguishable from that of isogenic *SNR31* strains at a range of growth temperatures from 15° to 37°C, on minimal and complex media and in the presence of elevated salt concentrations (data not shown).

RNA was extracted from two independent *snR31::URA3* strains and the isogenic *SNR31* strain. To determine whether deletion of *SNR31* has any effect on the processing of pre-rRNA, Northern analyses were performed using probes specific for the transcribed pre-rRNA spacers (Figure 5). The only consistent difference is that the *snr31::URA3* strain accumulates an elevated level of the 35S primary transcript. This effect was seen for four different

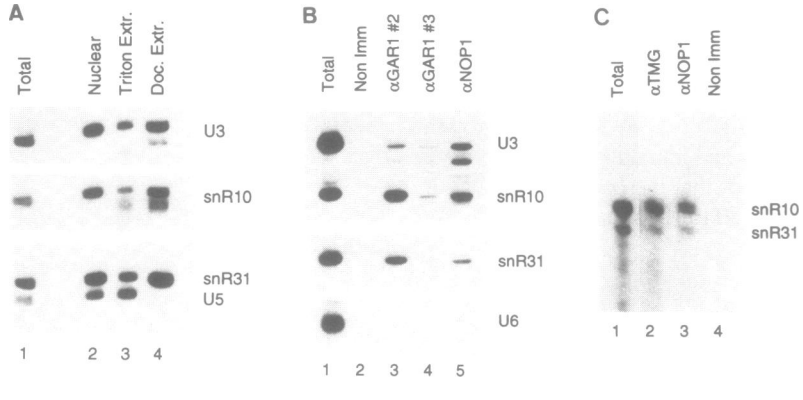

Figure 3. snR31 is a small nucleolar RNA. **A.** Sub-nuclear fractionation. Lane 1, Total RNA. Lane 2, RNA recovered in the total nuclear fraction. Lane 3, RNA released by extraction with Triton X-114. Lane 4, RNA released by extraction with sodium cholate plus sodium deoxycholate. **B.** Immunoprecipitation with αGAR1 and αNOP1 antibodies. Lane 1, Total RNA from the lysate used for immunoprecipitation. Lane 2, RNA recovered following immunoprecipitation with non-immune rabbit serum. Lanes 3−4, RNA recovered following immunoprecipitation with antibodies affinity purified against GAR1 from serum #2 (lane 3), or from serum #3 (lane 4). Lane 5, RNA recovered following immunoprecipitation with antibodies affinity purified against NOP1 from serum EC1. RNA was recovered following immunoprecipitation, separated by PAGE, transferred to for Northern hybridization and hybridized with anti-sense riboprobes for the snoRNAs and snRNAs indicated. **C.** Immunoprecipitation with αTMG antibodies. Lane 1, Total RNA from lysate used for immunoprecipitation. Lane 2, RNA recovered following immunoprecipitation with monoclonal antibodies against tri-methyl guanosine. Lane 3, RNA recovered following immunoprecipitation with antibodies affinity purified against NOP1 from serum EC1. Lane 4, RNA recovered following immunoprecipitation with non-immune rabbit serum.

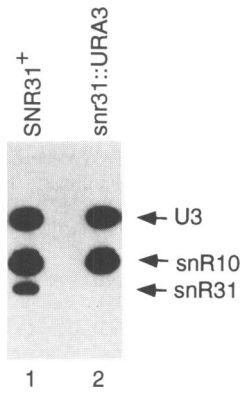

Figure 4. The *snr31::URA3* mutation prevents synthesis of snR31. Lane 1, RNA extracted from an *SNR31* strain. Lane 2, RNA extracted from an otherwise isogenic *snr31::URA3* strain. Total RNA from each strain was separated by PAGE, transferred for Northern hybridization, and hybridized for the snoRNAs indicated.

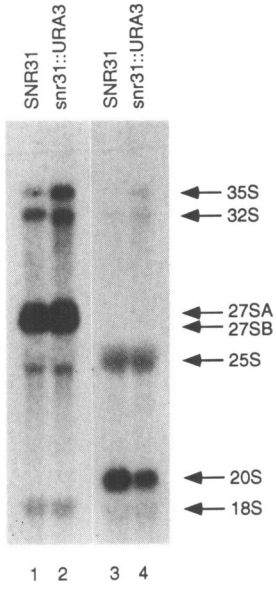

Figure 5. Processing of pre-rRNA in *snr31::URA3* strains. Total RNA was extracted from an *SNR31* strain (lanes 1 and 3) and an otherwise isogenic *snr31::URA3* strain (lanes 2 and 4). The RNA was separated by electrophoresis on an agarose gel containing formaldehyde and transferred for Northern hybridization. Lanes 1 and 2 were hybridized with a probe specific for the 3′ region of internal transcribed spacer 1. Lanes 3 and 4 were hybridized with a probe specific for the 5′ region of internal transcribed spacer 1.

snr31::URA3 strains in five independent experiments. Phosphoimager scanning of other Northern hybridizations shows that ratio of 35S pre-rRNA to its immediate product 32S pre-rRNA in the *snr31::URA3* strains is 1.5 fold that of *SNR31* strains (data not shown). This would be consistent with a mild reduction in the rate of processing of the 35S primary transcript of pre-rRNA. Other pre-rRNA species are not reproducibly altered by the absence of snR31. The absence of several other snoRNP components, the snoRNA U3, U14, snR10 and snR30 or the NOP1, GAR1 and SOF1 proteins (1, 2, 4, 10, 11, 23, 28), results in the accumulation of 35S, but in all of these cases this is associated with other alterations in the pathway of processing leading to the synthesis of 18S rRNA. All of these mutations accumulate an aberrant 23S pre-rRNA species. Accumulation of 23S is not seen in the *snr31::URA3* strain; both of the probes shown in Figure 5 have the potential to hybridize to 23S pre-rRNA, but no signal is detected by Northern hybridization. In addition, primer extension shows that the absence of any of the other above mentioned snoRNP components inhibits cleavage of the pre-rRNA at processing sites designated A_1, which lies at the 5′ end of the 18S rRNA coding region, and A_2, which lies within internal transcribed spacer 1 (D.T., unpublished). In addition, genetic depletion of U3, SOF1 or NOP1 inhibits cleavage at site A_0, which lies within the 5′ external transcribed spacer (1 and D.T., unpublished). Primer extension analyses show that cleavage at none of these sites is altered by deletion of *SNR31* (data not shown).

The sequence of snR31 shows modest homology to that of another snoRNA, snR10. Deletion of the *SNR10* gene is not lethal, but results in an impairment of pre-rRNA processing (4), suggesting the possibility that snR10 and snR31 might have redundant functions. To test this, *snr10*$^-$ and *snr31*$^-$ strains were mated and sporulated, and the resulting tetrads were analyzed. The effects of deletion of *SNR10* on pre-rRNA processing are greater than those of deletion of *SNR31* and strains lacking both snR10 and snR31 were not detectably more impaired in growth or rRNA processing than sister strains lacking only snR10 (data not shown). This may indicate that snR10 is epistatic to snR31; the effects of disruption of *SNR10* may mask the effects of disruption of *SNR31*, because it acts earlier in the processing pathway.

DISCUSSION

The sub-nuclear localization of snR31 and it's association with the nucleolar proteins NOP1 and GAR1 clearly establish snR31 as a novel snoRNA species. In higher eukaryotes, U14 and several other snoRNAs are excised from intronic sequences of larger transcripts (15−17, 29). In yeast, the genes encoding U14 and snR190 are very closely located (8). These two species also differ from all other yeast snoRNAs in that they lack both 5′ cap structures and obvious transcription signals. This suggests that yeast U14 and snR190 may also be excised from a larger transcript. In contrast, snR31 possesses a 5′ cap structure, and a putative TATA element is located in the 5′ flanking sequence of *SNR31* (see Figure 2), making it likely that snR31 is directly transcribed by RNA polymerase II.

One of the most surprising results to emerge from the study of the yeast snoRNAs, is the number of non-essential species. Of 12 snoRNAs characterized, 8 (including snR31) have no detectable effects on cell growth when their genes are disrupted. These RNAs are presumably not without function, particularly as at least one of them, snR3, shows quite strong conservation between yeast and the very distantly related filamentous fungus, *Neurospora crassa* (6). Moreover, recent reports indicate that comparably large numbers of snoRNAs are also present in higher eukaryotes. For two snoRNAs, U3 and U14, homologous RNAs have been identified from yeast and vertebrates; whether all snoRNAs will show a 1:1 correspondence between yeast and vertebrates is not yet clear. The results presented here show that snR31, although not necessary for the maturation of pre-rRNA, does appear to be required for optimum efficiency of the process. Several other yeast snoRNP components, the snoRNAs U3, U14, snR10 and snR30 and the snoRNP proteins NOP1, GAR1 and

SOF1, have been shown to be required for normal processing of the pre-rRNA (1, 2, 4, 10, 11, 23, 28). Genetic depletion of any of these components impairs the pathway leading to the synthesis of 18S rRNA, resulting in the absence of the normal processing intermediates, 32S, 27SA and 20S, and the appearance of an aberrant 23S pre-rRNA. In contrast, the absence of snR31 results in the accumulation of the 35S primary transcript, consistent with a reduced rate of processing, but does not otherwise alter the processing pathway.

By analogy to the roles of the nucleoplasmic snRNAs in mRNA splicing and processing, snoRNA mutants have been analyzed primarily for effects on pre-rRNA processing. It is, however, possible that they also play roles in other post-transcriptional steps in ribosome synthesis, such as modification of the pre-rRNA and ribosome assembly. Mutations in the nucleolar protein fibrillarin, which in yeast is encoded by the *NOP1* gene and is associated with all known snoRNAs, interfere with the modification of the pre-rRNA and ribosome assembly in addition to pre-rRNA processing (30). This would be consistent with snoRNAs playing roles in all of these processes. The large number of snoRNAs present in both yeast and higher eukaryotes, would also be consistent with their having a range of functions. It is therefore possible that snR31 plays a role in some other aspect of ribosome synthesis, and that the observed, mild, processing defect is a secondary consequence of this impairment.

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