Sequence and genetic analysis of a dispensible 189 nucleotide snRNA from *Saccharomyces cerevisiae*

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ABSTRACT

The structure of a *Saccharomyces cerevisiae* gene that encodes a small nuclear RNA (snRNA) of 189 nucleotides is described. This gene, designated *SNR189*, is located 400 base pairs upstream of the *CRY1* gene on yeast chromosome III. Gene replacement analysis revealed the *SNR189* gene to be dispensable for growth under a variety of culture conditions. The snR189 sequence lacks homology with other sequenced yeast or metazoan snRNAs.

INTRODUCTION

The small nuclear RNAs (snRNAs) are a class of metabolically stable RNA found in the nuclei of all eukaryotic cells examined to date (1). Several early lines of evidence implicated these molecules in the process of mRNA maturation (2,3). This hypothesis has now been firmly established and the metazoan U1, U2, U4, U5 and U6 snRNAs shown to be involved in mRNA splicing (4-10). The U7 snRNA is required for 3' processing of histone pre-mRNA (11,12) and the U3 snRNA is implicated in processing of pre-rRNA (13-16).

Genetic characterization of the metazoan snRNAs will be complicated because of redundancy at the DNA level, some of which appears to be due to pseudogenes (17). This and other limitations provide strong impetus for analyzing snRNA genes in genetically more tractable organisms, such as the yeast *Saccharomyces cerevisiae*. The occurrence of snRNAs in yeast has been well established (18,19). Evidence for their existence was based initially on the detection in bulk small RNA of trimethylguanosine capped RNAs, a common feature of snRNAs (18). Demonstrations of nuclear enrichment came with characterization of cloned genes. To date, about a dozen yeast snRNA genes (*SNR*) have been isolated, all of which are present as single-copy sequences. At least six *SNR* genes are essential for cell viability (20-25; Zagorski *et al.*, submitted) while several others are dispensible (26,27, and see ref.28 for a summary). In the present report we describe the structure of a novel snRNA gene and show that this locus is not essential for cell growth.

MATERIALS AND METHODS

Strains and culture conditions.

Yeast strain A364A (*a*, *adel*, *ade2*, *ural*, *his7*, *lys2*, *tyr1*, *gal1*) from the Yeast Genetic Stock Center was used as the source of nuclear and total cell RNA. The strain SB9882-4CR (*a/a*, *trp1-289/trp1-289*, *ura3-52/ura3-52*, *leu2-3,112/LEU2*, *his4-519/HIS4*) was used for disruption of the *SNR189* gene and subsequent genetic analysis. Cells were grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose), or on supplemented SD minimal media (0.67% yeast nitrogen base, 2% dextrose; ref. 29). Transformations were done using the lithium acetate procedure developed by Ito *et al* (30). Yeast diploids were sporulated and analyzed by tetrad analysis as described by Sherman *et al* (29).

Isolation of yeast nuclei.

All manipulations were performed at 5°C. Cells were harvested from early to mid-log cultures (100 to 150 klett units: green filter) by centrifugation, washed once with 0.1 M EDTA, 0.5% β -mercaptoethanol and once with distilled water. The pellet was resuspended in S buffer (1.35 M sorbitol, 20 mM Tris-HCl pH 7.4, 5 mM CaCl₂) plus 1 mM PMSF at 5 ml per gram wet weight of cells. Spheroplasts were prepared by digestion with Zymolyase 60K (Kirin; 1 mg per gm wet cells) at room temperature and washed twice in cold S buffer to remove the Zymolyase. To isolate nuclei, the spheroplasts were resuspended in nuclei buffer (20 mM HEPES, pH 6.8, 7% Ficoll, 2 mM CaCl₂, 0.5% diethylpyrocarbonate, and 1 mM PMSF; 2 ml buffer per gram wet weight of cells). Lysis was achieved by addition of 10% Triton X100 to a final concentration of 0.2%. The suspension was diluted with an equal volume of nuclei buffer plus 40% glycerol and 2 M sorbitol. Nuclei were then isolated by the differential centrifugation scheme of Halvorson *et al* (31,32). Whole cells and cell debris were removed by centrifugation at 6500 rpm (Sorvall SS34 rotor) for 7 minutes. The nuclei in the supernatant were pelleted at 13,000 rpm (SS34 rotor) for 20 min. The pellet was resuspended in nuclei buffer plus 20% glycerol and 1 M sorbitol and recentrifuged as before.

The yield and quality of the nuclear preparation was assessed by both microscopic and biochemical methods. The isolation procedure was monitored at each stage by phase contrast microscopy and the final preparation evaluated by this means and by determining the relative abundances of DNA (33), RNA (34) and protein (35). Under phase contrast microscopy the organelles appeared uniform and free of endoplasmic reticulum. When

normalized to DNA mass, the DNA:RNA:protein ratios were 1:2.4:15. These values are in excellent agreement with others reported for highly purified intact nuclei (1:2.5:16; refs. 31,32). Based on DNA recovery, the overall yield of nuclei was approx. 25%.

Isolation of whole cell and nuclear RNA.

Total yeast RNA was isolated from spheroplasts lysed by the addition of 20% sodium dodecylsulfate (SDS) to a final concentration of 1%. Following extractions with phenol and then chloroform bulk RNA was recovered by ethanol precipitation. RNA from purified nuclei was isolated by first resuspending nuclear pellets in 100 mM NaCl, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA (10 ml per 1 liter of culture) and adding SDS to a final concentration of 1%. Protein was removed by extensive extraction with phenol followed by chloroform. The resulting RNA was precipitated with ethanol. Fractionation of the small RNA species was by electrophoresis through gels of 7% polyacrylamide containing 7M urea and Tris-borate buffer (89 mM Tris-borate, pH 8.3, 4mM EDTA).

S1 nuclease mapping.

S1 nuclease transcript mapping was performed by a modification of the procedure of Berk and Sharp (36). Restriction fragments originating in the snR189 coding region were 3' end-labeled using the Klenow fragment of *E.coli* DNA polymerase I (New England Biolabs) or at the 5' end by polynucleotide kinase (Boehringer; ref. 37) and hybridized to 60 µg of total yeast RNA. The annealed samples were divided and portions digested at 25°C or 37°C for 30 minutes with 100-2000 units of S1 nuclease (Boehringer). The nuclease-protected fragments were analyzed by electrophoresis in an 8% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 8 M urea and Tris-borate buffer (89 mM Tris-borate, pH 8.3, 4 mM EDTA).

Immunoprecipitation of snRNAs with trimethyl guanosine antibodies.

Capped RNAs were precipitated using the anti-trimethyl guanosine antibody described by Luhrmann et al (38). RNAs and antibody were combined in PBS (10 mM sodium phosphate, pH 7.5, 150 mM sodium chloride) at a ratio of 1 ug antibody per ug RNA and incubated on ice for 60 min. RNA-antibody complexes were collected with PBS-washed Pansorbin (Calbiochem; 1 ul per 1 ug antibody). After 30 min on ice, complexes were collected by centrifugation and washed three times with low salt NET (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 5 mM EDTA) and twice with high salt NET (containing 400 mM sodium chloride). The final pellet was resuspended in 100 ul of high salt NET, phenol extracted twice, chloroform extracted once and ethanol precipitated.

Figure 1: Electrophoretic analysis of nuclear and whole-cell small RNA. [32 P] pCp-labeled RNAs were fractionated through gels of 7% polyacrylamide containing 7M urea and Tris-borate buffer (89 mM Tris-Borate, pH 8.3, 4mM EDTA). The samples correspond to: lane 1, whole-cell RNA; lane 2, nuclear RNA; lane 3, capped RNA prepared by immunoprecipitation of cell RNA with trimethyl G cap antibody. The 5S and 5.8S ribosomal RNAs are identified along with the estimated sizes of co-migrating capped and nuclear RNAs.

RESULTS AND DISCUSSION

Isolation of snRNAs.

The population of small RNAs associated with purified nuclei was evaluated by comparing the electrophoretic patterns of nuclear and whole-cell RNA. Comparisons included pattern analysis of total RNA from each source and

assays of immunoprecipitated 5' trimethyl G capped species (38) in each preparation. The patterns of pCp-labeled (39) total cell and nuclear RNA are shown in lanes 1 and 2 of Figure 1; lane 3 is trimethyl guanosine capped cell RNA. When compared with total cell RNA approximately 14 of the two-dozen or so bands in the size range of 128 to 343 nucleotides (estimated) appear to be enriched in the nuclear fraction. Nearly all of the RNA species exhibiting nuclear enrichment also show electrophoretic correspondance to capped whole-cell RNAs. Conversely, all of the capped species in whole-cell RNA correspond in mobility to nuclear enriched species. Further, the patterns of capped RNA from nuclear and whole-cell RNA preparations show good correspondance (results not shown; see ref.40 for pattern of nuclear capped RNA in this same preparation). The estimated number of small RNA species present in the purified nuclei used here (about two dozen) is consistent with an earlier estimate of snRNA number based on 2D-gel analysis of capped whole-cell RNA (28). The current results reinforce the earlier conclusion that *Saccharomyces cerevisiae* contains a more complex set of snRNAs than has been reported for metazoans.

Identification of a snRNA gene linked to the *CRY1* gene.

Several metazoan snRNAs are known or suspected of interacting with mRNA precursors by direct base pairing (9,10). We hypothesized that a similar interaction between yeast snRNA and introns might be detected by hybridization. Dot-blot hybridization assays were carried out with several cloned yeast genes and a mixed probe of pCp-labeled gel-purified nuclear RNAs. The nuclear RNAs were the 12 most abundant nuclear-enriched RNAs observed in side-by-side comparisons of cell and nuclear RNAs. Six genes containing introns and several intron-less genes were examined. No intron-specific hybridization was observed with the mix of snRNAs. However, the mixed probe did specifically hybridize to a plasmid encoding the ribosomal protein 59 gene (rp59; ref.41), revealing the presence of a snRNA gene fortuitously associated with the rp59 locus. The gene for rp59 is designated *CRY1* as mutant variants can confer resistance to the translation elongation inhibitor cryptopleurine (42). The hybridizing RNA, identified by hybrid-selection as an approximately 190 nucleotide (nt) species, was used to probe Southern blots (43) of a *CRY1* plasmid. Results from this analysis (not shown) placed the region of snRNA homology roughly 400 base pairs upstream from the rp59 coding sequence. Northern hybridization assays were also carried out with whole-cell and nuclear RNA - to confirm that the product of the small RNA gene was indeed a snRNA. As shown in Figure 2, a DNA probe

Figure 2: Verification that snR189 is a nuclear RNA. Preparations of whole cell and nuclear small RNA were fractionated in 7% polyacrylamide containing 7 M urea, transferred electrophoretically to DBM paper (Schleicher and Schuell) and probed with DNA 5' to the *CRY1* gene. Lanes 1 and 2 correspond to 5 ug each of whole cell and nuclear RNA respectively. The enrichment of the 189 nt species in lane 2 confirms its identity as an authentic nuclear RNA.

from the region 5' to *CRY1* gives a positive signal and shows the small RNA to be enriched in RNA from isolated nuclei. The presence of a 5' cap was subsequently shown by electrophoretic analysis of hybrid-selected RNA immunoprecipitated with cap antibody (results not shown).

S1 nuclease mapping.

Results from the Southern analysis of the initial *CRY1* plasmid revealed that the snRNA coding sequence is bisected by a *Hind III* site ~450 base pairs upstream from the rp59 coding region. This site was used to define the RNA coding sequence by S1 nuclease transcript mapping. Here, radiolabeled DNA fragments originating at the internal *Hind III* site were purified from an appropriate *CRY1* subclone (pJL37; ref.41), 5'- or 3'-labelled with [γ -³²P]-

Figure 3: S1 transcription map of the *SNR189* gene. The position of the *snR189* coding sequence in pJL37 was determined by S1 transcription mapping from a *Hind III* site internal to the RNA coding region. Lanes 3 and 6 show the S1 nuclease protected DNA fragments corresponding to the 5' and 3' ends of the *SNR189* gene respectively. Lane 1 contains a set of Φ X174 *Hae III* DNA standards, lane 2 is a DNA ladder; lane 4 is a S1-minus control of the 3' fragment, and lane 5 is the same as lane 6 except S1 digestion was at 37°C instead of 25°C.

ATP or [α -³²P]-dATP, hybridized to total yeast RNA and treated with S1 nuclease. The signals obtained from the 5'- and 3'- end labelled DNAs indicated that *SNR189* is transcribed in the same direction as the *CRY1* gene located downstream. The electrophoretic patterns obtained for the nuclease-protected DNA fragments are shown in Figure 3 (lanes 3 and 6 correspond to

Figure 4: Relative positions of the *SNR189* and *CRY1* loci in chromosome III. The central portion of chromosome III is shown along with an expanded view of a 1.6 kb segment containing the *SNR189* and *CRY1* loci. The direction of transcription is shown with overhead arrows and the intron of *CRY1* is designated by an insert in the identifying arrow.

the 5' and 3' protected fragments of the *snRNA* coding region). In each case, a single tight cluster of protected fragments was produced. After correcting for 4 nucleotides added by the 3' end-labelling procedure, the combined lengths of these segments gives a total size for the mature *snRNA* sequence of 189 nt. We refer to this species as *snR189* and the corresponding gene as *SNR189*. Because the size of the S1 protected sequence closely matches the size of the mature RNA, it seems likely that the coding sequence is contiguous at the DNA level. The mapping results derived from these analyses are summarized in Figure 4. Also shown are the relative positions of the linked *CRY1* and *SNR189* loci on chromosome III.

Sequence of the *SNR189* gene.

The DNA sequence of the *SNR189* gene region is shown in Figure 5. Several features commonly found in sequences flanking other yeast genes are present around the *SNR189* gene. The canonical "TATAAA" sequence characteristic of RNA polymerase II transcription units is present 72 nucleotides upstream from the position specifying the 5' end of the *snR189* coding segment. For yeast genes this element typically occurs in the region -30 to -150 (45). A 20 base pair segment that is 80% T occurs immediately upstream from the candidate "TATAAA" box and a similar segment follows the

10	20	30	40	50	60	
GCTTTGCAAC	GCAAAATTTT	GGTGAGCTTT	TGCTTCAGCA	ATGTATTTTC	TTTAAGCCAT	60
GCATGAATCA	CATTGTTACC	TGGCAAAATT	ATTGAAAAAT	ATTATCAAGG	GCCGGGTAAT	120
	*****	*****	**	*****	**	
ATACCCAGAT	ATACATTTT	TTTTCTTC	TTTCTTTAT	AAAGTGAGAT	CCTTCTTTTA	180
TTGATTCACT	GTGACCGTTG	TTTTTCAAC	TGTGAACTTA	TAAGAAAATT	<u>AACAAAATAA</u>	240
		SNR189				
<u>TTTACATTCC</u>	<u>AAGGACCGGT</u>	<u>ATTATTGTAG</u>	<u>GGGATTGTG</u>	<u>ACTTTCAAGG</u>	<u>CAACGTCCTC</u>	300
		-				
<u>TCTACAACCG</u>	<u>AGTTCGAGAA</u>	<u>TAAGTACAAA</u>	<u>TGGCTCTTTT</u>	<u>TGTTATTGCA</u>	<u>AAGCTTACAA</u>	360
<u>GTTCTGGTAT</u>	<u>ATTCTATATA</u>	<u>CTCACTTATT</u>	<u>ACTTTCAAGT</u>	<u>ACTTCACAGG</u>	<u>GGCCTGACAT</u>	420
CTCTATTCTT	GTTACTCACT	GATTATCGTT	CTTGTTCATA	CTTGTATTGT	ATCTTCTTTC	480
TCCCTATTTA	AAATGTAATA	GAGACTGCCT	TGGAGTACTT	ACGTGCGGTG	TACGGACTTA	540
	****	* * ****	*****	**	*****	
ATAAACATCT	GTACATTTA	CTACTCTTTT	TTTTTGCGTT	CTTTTTTTCA	CCTTCAGCAC	600

Figure 5: Sequence of the *SNR189* gene region. The sequence shown is for 600 nucleotides encompassing *SNR189*. Sequencing was by the method of Maxam and Gilbert, with data derived from both strands (44). The coding region delineated by S1 nuclease mapping is shown in italics and underlined. A candidate 'TATAAA' box and elements of a potential termination sequence are marked by overlines. T-rich regions on both sides of the *SNR* sequence are marked with overhead asterisks.

SNR sequence. Such T-rich regions are common near yeast protein genes (46). The presence of these sequence features suggests that *snR189* is a product of RNA polymerase II transcription as has been demonstrated for the *snRNAs* of higher eukaryotes (47).

Several different sequence elements have been implicated in transcriptional termination in yeast. Zaret and Sherman proposed a consensus termination sequence of TAG...TATGT (or TAGT)...TTT with T-rich and AT-rich segments preceding the first and third elements respectively (48). Henikoff and Cohen have identified the sequence TTTTATA as a candidate (49). Two elements of the former class occur some 46 nucleotides downstream from the segment specifying the mature *snRNA*, suggesting the possibility that *snR189* may be synthesized as a larger precursor. *In vivo* evidence that the *SNR* gene region contains a functional Pol II terminator has been obtained by assessing the effect of inserting this segment between a plasmid-encoded *GAL10* promoter and centromere element (M. Fitzgerald-Hayes, pers. comm.). The segment tested included the 3' half of the *SNR189* sequence, the intergenic region between *SNR189* and *CRY1*, and a 5' piece of the *CRY1* gene. The presence of this region in either orientation conferred plasmid stability during growth on galactose. This finding suggests that the insert does contain a Pol II

Figure 6: Hypothetical secondary structure of snR189. A minimum free energy structure predicted by a computer program of M. Zuker is shown. The program used (55) predicts structure based on parameters compiled by Salser (56). The proposed structure is 60% base paired and has a predicted free energy of -47.8 kcal/mol.

terminator, since transcription through a centromere interferes with its function (M. Fitzgerald-Hayes, pers. commun.).

Comparison of the snR189 sequence with other snRNAs.

The possibility that snR189 is evolutionarily related to other snRNAs was examined by comparing its sequence with those of 14 other small nuclear RNAs. The matrix homology programs of Pustell and Kafatos were employed for this comparison (50). No significant relatedness (>40%) was detected when snR189 was compared with the human U1-U8 molecules (1,51,52), two *Dictyostelium* snRNAs (53,54), and four other yeast snRNAs, ie. snR3, snR10 (26,27) and species snR190 and snR128 from our group (Zagorski *et al*, submitted).

Hypothetical secondary structure of snR189.

An interesting secondary structure model for snR189 is predicted by the computer program of Zuker (55). This program uses the thermodynamic

Figure 7: Structure of the *SNR189* locus and gene replacement strategy. Plasmid segments corresponding to wild-type and disrupted alleles of *SNR189* are shown. Also depicted are the 2.1 kb *Eco RI* fragment used in the gene replacement and the genotype of the yeast diploid transformed with the *snr189::URA3* fragment.

parameters compiled by Salser (56) to select hypothetical structures with the lowest free energy values. These parameters allow GU base pairs except at the end of a stem. The structure predicted for snR189, presented in Figure 6, consists of two adjoining hairpin domains each of which is interrupted by several small unpaired regions. The proposed structure has a predicted free energy of -47.8 kcal/mol resulting from pairing that involves 60% of the component bases. Confidence in the double-hairpin motif is reasonably high as all other alternatives with ΔG values within 20% of the structure shown are minor variations of this basic configuration. Interestingly, a secondary structure strikingly similar to that presented here has been proposed for the Dd8 snRNA from *Dictyostelium discoideum* (53). As already noted, however, no clear sequence relationship is evident for these two RNAs.

The *SNR189* gene is dispensible for cell viability.

The essentiality of the *SNR189* gene was tested by transplacement of a non-functional allele into the chromosome of a diploid host and characterization of the sporulation products. The mutant allele was

Figure 8: Identification of *SNR189/snr189::URA3* heterozygotes by Southern blot analysis. Results are shown for *Eco* RI digests of seven *ura*⁺ transformants. The hybridization probe was the 1.4 kb *Eco* RI fragment from pJT6 (see Fig. 4) nick-translated with [α -³²P]-dATP. Lane M is a 3' end-labeled *Hind* III digest of λ DNA; lanes 1 through 8, DNA from transformants SB(*URA*)17 through 24. Hybridizing fragments of the size expected for the *snr189::URA3* allele are indicated with asterisks.

constructed by replacing a 340 bp *Hind* III fragment containing the 5' two-thirds of the snRNA coding sequence and probable promoter with a 1.1 kb fragment containing the *URA3* gene. Figure 7 shows the details of this construction. A wild type allele was replaced with the mutant allele by the one-step transplacement procedure of Rothstein (57). The 2.1 kb *Eco* RI fragment from pJZ13 containing the *snr189::URA3* mutant allele was then used to transform a *ura*⁻ diploid strain (SB9882-4CR) to uracil prototrophy. Replacement at the *SNR189* locus was verified by Southern blot analysis (Figure 8). Of seven transformants examined, two (lanes 4 and 5) yielded hybridizing fragments of the sizes predicted for both the *SNR189* allele (1.4 kb) and the *snr189::URA3* allele (2.1 kb).

To test whether the snR189 RNA is required for cell growth, the two *SNR189/snr189::URA3* heterozygotes and the parent strain SB9882-4CR were examined by tetrad analysis. For both heterozygotes, all tetrads dissected gave rise to four viable spores, indicating that the snR189 locus is not essential for growth (data not shown). Segregation of the *ura* phenotype was 2⁺:2⁻ as expected. To verify that disruption of *SNR189* abolished

Figure 9: Northern blot screening for snR189 in cells containing a disrupted *SNR189* gene. RNA from control and test cells was fractionated in a 7% polyacrylamide gel containing 7M urea, transferred electrophoretically to a Zeta-Probe nylon membrane (Bio-Rad) and probed for snR189. Lane N, 3' end-labeled nuclear RNA; lanes 1-4 and 6-9, total RNA from haploids derived from two *SNR189/snr189::URA3* tetrads, SB(*URA*)20-1 and SB(*URA*)21-1; lanes 5 and 10, control RNA from strains SB9882-4CR and A364A.

accumulation of the product RNA, northern blot assays were performed on eight haploids derived from two tetrads. As shown in Figure 9 (lanes 1, 2, 7 and 8), four of the eight spores, all phenotypically *ura*⁺, lacked detectable snR189. Growth of cells lacking snR189 was also evaluated under other culture conditions but none was found where differences were apparent. The conditions tested included other temperatures (23° and 37°), a non-fermentable carbon source (glycerol-ethanol) and hypertonic media (YPD with 1.5 M KCl or 2 M sorbitol). Evaluation of *rp51* mRNA splicing in *snR189* cells by northern blot analysis also indicated that splicing is not impaired in these strains.

Thus, we have been unable to identify any phenotype associated with the loss of snR189 expression. Of course, as noted for other dispensible snRNAs, this negative result does not mean that snR189 lacks a cellular function. Phenotypic effects associated with snR189 loss might be observed under other growth conditions. It is also possible that snR189 function can be mediated by another, functionally redundant molecule, perhaps another snRNA. The absence of hybridizing snR189-like species in *snR189*⁻ cells argues that any functionally related snRNA cannot share substantial sequence relatedness with snR189. Still, the greater diversity of snRNAs in *Saccharomyces cerevisiae*,

compared with metazoan cells, would seem to enhance the possibility of functional redundancy. On the other hand, the yeast snRNAs could perform strictly unique functions, but participate in a greater number of functions (many dispensible) than do the apparently smaller number of metazoan snRNAs. It will be interesting to learn if metazoan cells have homologs to the non-essential snRNAs of *Saccharomyces cerevisiae*.

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