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Small nuclear RNAs from *Saccharomyces cerevisiae*: Unexpected diversity in abundance, size, and molecular complexity

(yeast/RNA processing)

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ABSTRACT **Previous work showed that the simple eukaryote *Saccharomyces cerevisiae* contains a group of RNAs with the general structural properties predicted for small nuclear RNAs (snRNAs), including possession of the characteristic trimethylguanosine 5′-terminal cap. It was also demonstrated that, unlike their metazoan counterparts, the yeast snRNAs are present in low abundance (200–500 molecules per haploid cell). We have now used antibody directed against the 5′ cap to investigate the total set size of snRNAs in this organism. We present evidence that the number of distinct yeast snRNAs is on the order of several dozen, that the length of the capped RNAs can exceed 1000 nucleotides, and that the relative abundance of a subset of these RNAs is 1/5th to 1/20th that of the class of snRNAs described previously. These findings suggest that the six highly abundant species of snRNAs (U1–U6) typically reported in metazoans may represent a serious underestimation of the total diversity of snRNAs in eukaryotes.**

Studies of small nuclear RNAs (snRNAs) have focused on the six so-called U-RNAs (U1–U6), first discovered some 15 years ago (for review, see ref. 1). In metazoans, these RNAs range in length from 90 to 216 nucleotides (nt), are extremely abundant (there are $\approx 10^6$ U1 molecules in a HeLa cell, for example) and are encoded by multigene families (2, 3). Although functional studies have lagged behind structural characterization, these RNAs are widely believed to mediate a spectrum of RNA-processing reactions in eukaryotic cells. Indeed, recent information from *in vitro* analyses directly implicates the involvement of U1 (4–6), U2 (7, 8), and probably U5 (9) in pre-mRNA splicing. Similarly, one or more U-RNAs appear to be required for 3′ end formation/polyadenylation *in vitro* (10, 11), although the specific suggestion that these are U4/U6 (12) has not been verified. Finally, the involvement of U3 in rRNA maturation is deemed likely because U3 is restricted to the nucleolus and appears to be hydrogen-bonded to 28S pre-rRNA (13, 14).

Several years ago, we initiated a search for snRNAs in the simple eukaryote *Saccharomyces cerevisiae*, with the goal of exploiting the powerful genetic tools available in this organism. As described (15, 16), a group of RNAs were identified that conformed to several predicted criteria. These included small size, nuclear localization, metabolic stability, and possession of the characteristic trimethylguanosine 5′ cap. The latter characteristic provides a particularly powerful diagnostic in that snRNAs are the only class of RNAs known to possess this terminus. However, it was also shown that the yeast snRNAs differ in several potentially important ways from U1–U6. A significant asset to our genetic strategy was the finding (15, 17, 18) that the six yeast RNAs tested (designated snR3, snR4, snR7, snR8, snR9, and snR10) are encoded by single-copy genes (designated *SNR3*, etc.). In keeping with this lack of genetic redundancy, the yeast RNAs are present in low abundance: 200–500 molecules per haploid cell (15).

A further surprise was the provisional estimation (15) that yeast contained at least 11 distinct small capped RNAs. Further studies aimed at determining the complete set size of yeast snRNAs, as well as their more detailed biochemical characterization, were severely hindered by the difficulty of obtaining pure preparations in sufficient yields. We have recently reported (16) that antibody to the trimethylguanosine cap is able to precipitate yeast snRNAs. Now we describe the extension of this approach to investigate the size of the snRNA family in *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Construction of Genomic Clone Bank. To clone yeast snRNAs without prior purification of individual species, we constructed a genomic clone bank by inserting yeast DNA, digested to completion with restriction endonuclease *Bam*HI into plasmid pUC9 and screened it with RNA of 160–1500 nt size-fractionated by 6% acrylamide gel electrophoresis. After eliminating previously cloned snRNAs by counter-screening with existing clones by colony hybridization, 18 positive recombinants remained, which had 8 distinct restriction maps. Plasmid DNAs were prepared by a modification of the procedure of Tanaka and Weisblum (19).

Preparation of Probes. Radioactive probes from cognate clones were prepared using the nick-translation kit N 5,000 (Amersham). ^{32}P-labeled cDNA was made from size-fractionated RNA (see below) by use of reverse transcriptase (Life Sciences, St. Petersburg, FL), random calf thymus primers, and [α-^{32}P]CTP.

Hybridization Techniques. Colony hybridization was performed according to Maniatis *et al.* (20). DNA hybridization and RNA hybridization from acrylamide gels was performed as described in Wise *et al.* (15).

RNA Preparation. Total RNA from yeast cells was obtained as described (15). Low molecular weight RNA was purified by batch elution from DEAE-Sephacel (Pharmacia; wet particle size 40–150 μm) which was equilibrated in 100 mM Tris·HCl (pH 7.5) and washed with 300 mM NaCl/10 mM Tris·HCl, pH 7.5. RNA was eluted with 1 M NaCl/10 mM Tris·HCl, pH 7.5. Preparation of nuclear RNA was as follows. Yeast cells were grown to OD_{600} 0.8; spheroblasts were washed in HMS [25 mM Hepes, pH 7.7/5 mM magnesium acetate/0.25 M sucrose/0.1% Nonidet P-40 (NP-40)] and Dounce-homogenized with six strokes, using a loose pestle. The lysate was centrifuged at 6000 × *g* for 5 min through a 2.5-ml cushion of 25 mM Hepes, pH 7.7/5 mM magnesium acetate/0.1% NP-40. The pellet comprised the nuclear fraction, and the top layer the cytoplasmic fraction.

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Abbreviations: snRNA, small nuclear RNA; nt, nucleotide(s).

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The nuclear fraction was resuspended in HMS; both fractions were extracted successively with equal volumes of phenol, phenol/chloroform (1:1, vol/vol), and chloroform; and the RNA was precipitated with ethanol.

Hybrid Selection. Plasmid DNA was denatured by boiling in H_2O for 10 min and successive incubation in 1 M NaOH for 20 min at room temperature. The solution was neutralized with an equal volume of 0.5 M Tris·HCl, pH 8.0/0.1 M NaCl/30 mM sodium citrate/8.6% HCl. Plasmid DNA was immobilized on nitrocellulose at 80 μg of DNA per cm^2. Prehybridization and hybridization was at 42°C in 50% formamide/5× SSC (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7). *Escherichia coli* tRNA (100 μg/ml) was added as carrier during prehybridization. Low molecular weight yeast RNA (1 mg) was hybridized to 80 μg of plasmid DNA bound to nitrocellulose. Filters were washed in 0.2× SSC at room temperature before the RNA was eluted by heating the filters to 90°C for 5 min in water.

Immunoprecipitation of Trimethylated RNA Species. Protein A-Sepharose (Pharmacia) was coupled with rabbit antibodies directed against trimethylguanosine; rabbit antiserum was kindly provided by D. Black and J. Steitz (Yale University). After extensive washes in 50 mM Tris·HCl, pH 7.5/150 mM NaCl/2 mM EDTA, total yeast RNA was added and incubated with antibody in the same buffer at 4°C for 1 hr. Nonradioactive 7-methylguanosine 5'-phosphate (P-L Biochemicals) was included (0.02 μg per μl of antiserum). The pellet was washed at least four times. *E. coli* tRNA carrier was added and the RNA was eluted by phenol/chloroform extraction.

Labeling of RNA. *In vivo* labeling of total yeast RNA was performed as described (15). 3'-End-labeling of total RNA *in vitro* was performed using cytidine 3'-phosphate 5'-[^{32}P]phosphate ([5'-^{32}P]pCp) and bacteriophage T4 RNA ligase (Pharmacia) as described by England and Uhlenbeck (22).

RESULTS

Fractionation of Immunoprecipitated ^{32}P-Labeled RNAs. Immunoprecipitation of ^{32}P-labeled *Saccharomyces* RNA with antibody to the trimethylguanosine cap results in a substantial enrichment for capped RNAs in the size range of 5–7 S (16). We have now subjected the immunoprecipitated species to fractionation by three sequential steps of polyacrylamide gel electrophoresis. Fig. 1 shows the two-dimensional pattern achieved by successive electrophoresis in 10% and 20% acrylamide, in the presence of 4 M urea; these semidenaturing conditions maximize the distribution of the RNAs off the diagonal. Some 15 prominent spots can be distinguished by this method.

To determine whether multiple species are comigrating under these conditions, individual spots were cut out, and the RNA was eluted and electrophoresed under fully denaturing conditions (6% acrylamide, 7 M urea). Fig. 2 shows the results of one such experiment. In the majority of cases, the spots can be seen each to comprise only a single species, although in some instances minor bands can also be detected (e.g., arrow in Fig. 2, lane k). In contrast, lane i reveals two species with distinctive mobilities, which we designated snR6 and snR7_l, respectively. Lane b also shows a complex pattern: three bands are generated when snR18 is reelectrophoresed; one of these appears to comigrate with snR17 (cf. lane c).

A final point is the unexpected size range of immunoprecipitable small RNAs. Our previous work (15) focused on RNAs that were capped by the criterion of enzymatic digestion patterns and, in keeping with the previously reported sizes of U-RNAs, were <300 nt long. As can be seen in Fig. 2, at least four species migrate considerably more slowly than snR10, which is 245 nt (18). By comparison with

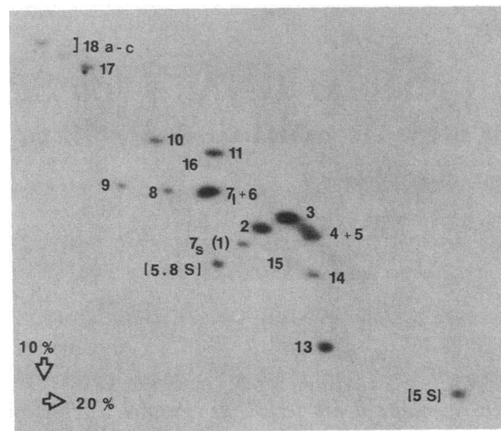

FIG. 1. Two-dimensional gel electrophoresis of ^{32}P-labeled RNA immunoprecipitated with anti-cap antibody. RNA was prepared from cells labeled *in vivo*, immunoprecipitated, and subjected to two-dimensional electrophoresis (10% acrylamide/4 M urea; 20% acrylamide/4 M urea). Minor amounts of 5S and 5.8S rRNAs precipitate nonspecifically. The snRNA species are numbered arbitrarily, as described in the text and Table 1. Mixed species are indicated (+); see Fig. 2. Two forms of snR7 are indicated as large (l) and small (s); the latter was originally (15) designated snR1. In other preparations, the spots bracketed here as 18 a–c migrate as a single band in the first dimension; the relationship of these species to one another is not known. Species snR19 and snR20 (see Fig. 2) were not included in the second dimension.

known size markers, we estimate these sizes as 311 (snR17), 350 (snR18), 625 (snR19), and ≈1300 nt (snR20). Enzymatic digestion has demonstrated the presence of trimethylguanosine caps on snR17 (16) and snR18 (data not shown). Although the variable yields of snR19 and snR20 have so far precluded cap analysis, we note that under the conditions we have employed, immunoprecipitation with this antibody appears to be highly specific for the di- or trimethyl derivative of guanosine (16). Moreover, we have isolated a presumptive clone for snR20 that hybridizes to an RNA band ≈1300 nt long that is enriched in nuclear fractions (see Fig. 3*e*) and is present exclusively in poly(A)$^-$ RNA fractions (Fig. 3*f*); from these observations, and the fact that snR20 can be labeled with [5'-^{32}P]pCp, we conclude that snR20 is neither a small mRNA nor a breakdown product of rRNA.

Cloning of Additional snRNAs. The foregoing experiments do not allow us to establish a reliable number for the total set size of snRNAs for (at least) two reasons. First, distinctive electrophoretic mobilities do not preclude a structural relationship between two species; conversely, two unrelated species might comigrate under the conditions we have tested. Second, the sensitivity of these analyses is insufficient to eliminate the possible existence of minor species; indeed, a number of faint spots can be observed upon longer autoradiographic exposure of gels such as that shown in Fig. 1. Definitive information will ultimately require cloning and structural analysis of the entire family.

Previously, six *SNR* genes (*SNR3*, *-4*, *-7*, *-8*, *-9*, and *-10*; ref. 15) were cloned by the use of cDNA probes to individual RNA species that had been purified by three rounds of gel electrophoresis. As an alternative approach, we constructed a *Bam*HI bank in pUC9 and screened it with cDNA prepared from bulk size-fractionated RNA. Positive colonies were then counter-screened against the existing *SNR* clones, which left us with eight apparently unique isolates. To determine the relationship between these clones and the RNAs that had previously been characterized, we performed "dot blot" analyses. For each of the RNA species purified by three-dimensional electrophoresis, cDNA probes were made using random primers and hybridized to filters containing

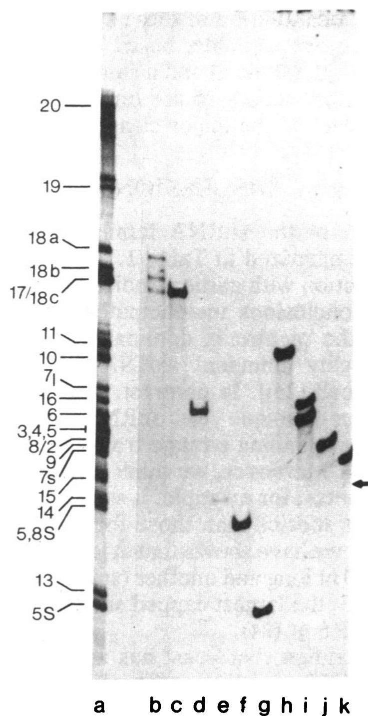

FIG. 2. One-dimensional gel electrophoresis of RNAs eluted from two-dimensional gel system. RNAs separated as in Fig. 1 were eluted, concentrated, and re-run in a 6% acrylamide/7 M urea sequencing gel. The reference lane (a) is total immunoprecipitated *in vivo*-labeled low molecular weight RNA. In some other preparations there is only a single band at the position of snR19; the relationship between the two species seen here is not known. Similarly, a series of fainter bands below snR20 show a variable pattern; some may be generated by breakdown of snR20 (see Fig. 3*e*). Other lanes: b, snR18(a–c); c, snR17; d, snR16; e, snR15; f, snR14; g, snR13; h, snR11; i, snR6 (lower band) + 7_l (upper band), taken from "snR7_l + 6"; j, snR2; k, snR1. The arrow in lane k indicates a minor RNA species, henceforth designated snR21 (see text).

DNA from both the new and the original sets of clones. The interpretation of these results was facilitated by blot hybridization analyses of electrophoretically fractionated total, nuclear, and cytoplasmic RNA. Selected examples of these analyses are shown in Fig. 3. The conclusions can be summarized as follows.

(*i*) An additional snRNA (henceforth called snR5) comigrates with snR4. Probe made from the "snR4" spot (cf. Fig. 1) hybridizes to the *SNR4* clone and, more faintly, to a clone (designated *SNR5*) from the *Bam*HI bank. The *SNR5* clone hybridizes to a band of the appropriate size (≈190 nt) on blots (Fig. 3, lane a). Moreover, the snR5 signal persists in cells containing an *SNR4* gene deletion (H.S. and C.G., unpublished data). We conclude that snR3, snR4, and snR5 are three unique snRNAs with similar electrophoretic mobilities.

(*ii*) Two structurally related forms of snR7 exist. Probes made from "snR1" spot (Fig. 1) and "snR7" spot (Fig. 2, lane i, upper band) both hybridize to the *SNR7* clone; by nuclease S1 and sequence analyses, the two forms of snR7 have been shown to differ from one another by 35 nucleotides at the 3′ terminus (B. Patterson and C.G., unpublished data).

(*iii*) Two different size forms of snR17 also exist. Probe made from highly purified "snR6" (Fig. 2, lane i, lower band), which is <200 nt long, hybridizes to a *Bam*HI clone that recognizes an RNA of >300 nt, the size of snR17, on RNA blot hybridization analysis (Fig. 3, lane b). We suspect that "snR6" is a degradation product of snR17 because this species is not labeled with [5′-^{32}P]pCp *in vitro* (Fig. 4, lane c; cf. Fig. 2, lane a), consistent with the presence of a 3′ phosphate generated by nucleolytic breakdown. Moreover, the yield of snR6 in preparations labeled *in vivo* is variable and we have been unable to detect a signal of the size of "snR6" on RNA blots (Fig. 3, lane b).

(*iv*) Probe made against the minor species indicated by the arrow in Fig. 2 (lane k) hybridizes to a *Bam*HI clone designated *SNR21*; RNA blot analysis (Fig. 3*c*) using the cloned DNA identifies two bands of equal intensity, one of which has a mobility consistent with the size of the RNA in Fig. 2 from which the cDNA was prepared (≈165 nt). It remains to be seen whether the larger band is a longer form of snR21 or corresponds to an unrelated RNA. [Both forms are enriched in the nucleus (Fig. 3*c*) and are immunoprecipitable (see below). We have thus provisionally designated the larger species as snR26 (see below and Table 1).]

(*v*) The four remaining newly obtained clones could not be accounted for by probes made to the existing purified species. To directly investigate the products of these clones

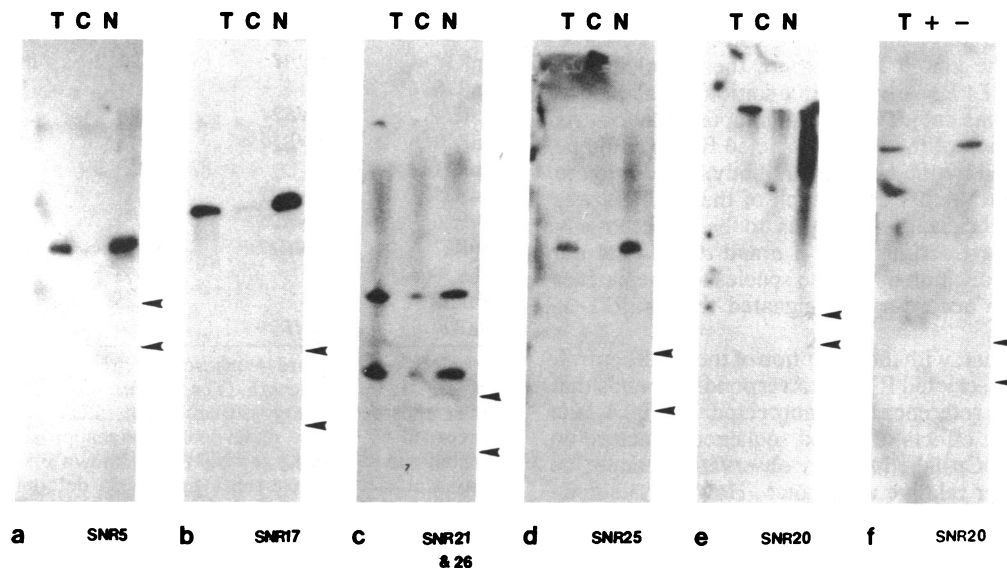

FIG. 3. RNA blot hybridization analysis, using *Bam*HI clones. (*a–e*) Nick-translated clones were used to probe blots of total RNA (T) or RNA from nuclear (N) and cytoplasmic (C) fractions. (*f*) Total RNA (T) was fractionated over oligo(dT)-cellulose columns to generate poly(A)$^+$ (+) and poly(A)$^-$ (−) RNAs. Arrowheads indicate positions of 5S and 5.8S rRNAs. Estimated sizes are given in Table 1.

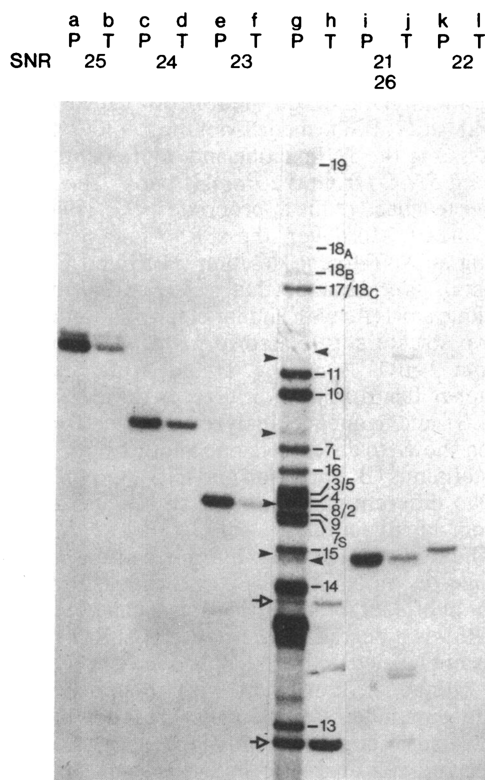

FIG. 4. Hybrid-selection of yeast RNA with *Bam*HI clones. RNAs were hybrid-selected as described in *Materials and Methods*, and 25% of the eluate was labeled with [5'-^{32}P]pCp (50 μCi; 1 Ci = 37 GBq) and subjected to immunoprecipitation. The immunoprecipitates (P) were analyzed in a 6% acrylamide/7 M urea sequencing gel; 1% of the starting material (T) is shown for comparison. The reduced precipitability of snR26 relative to snR21 is not understood. The reference lanes (g and h) were obtained by immunoprecipitation of total low molecular weight RNA. Filled arrowheads indicate alignment of hybrid-selected species against the total immunoprecipitate; open arrowheads mark 5S and 5.8S RNAs, low amounts of which contaminate the hybrid-selected species prior to immunoprecipitation.

and to confirm the snR21 result just discussed, we performed the following experiment.

Hybrid Selection Analyses. Total low molecular weight RNA was prepared and used for hybrid selection by these *Bam*HI clones. The eluted RNAs were then labeled with [5'-^{32}P]pCp, using T4 RNA ligase. In addition, an aliquot of the labeled RNA was subjected to immunoprecipitation. As shown in Fig. 4, each of the hybrid-selected RNAs could be immunoprecipitated by the anti-cap antibody. In addition to possessing this snRNA hallmark, each of these RNAs was shown by blot hybridization analysis to be preferentially enriched in nuclear fractions (Fig. 3 *c* and *d* and data not shown). We thus conclude that these species are themselves snRNAs and have accordingly designated them snR21–26 (see Table 1).

It is interesting that, with the exception of the snR8 control, each of the hybrid-selected RNAs corresponds to bands that are very faint in the reference immunoprecipitate (Fig. 4, lane g). Due to possible effects of 3' end sequence/structure on the efficiency of pCp labeling, this observation cannot be used *per se* to infer relative abundance. However, as comparison of Fig. 2, lane a, with Fig. 4, lane g, shows, the patterns of *in vivo*- and *in vitro*-labeled RNAs generally correspond well with one another in the size range of snR14 to snR17. It is particularly obvious that snR24, -25, and -26 comprise minor components in both the *in vivo* and *in vitro* profiles; higher-resolution gels allow alignment of snR21–23. Although direct quantitation of these species is not possible by this approach, we estimate, based on the relative intensities visible in Fig. 2 (lane a) and a suitable overexposure of Fig. 4 (lane g), that snR21–26 are on the order of 1/5th to 1/20th as abundant as the major class.

DISCUSSION

Our description of the snRNA family in *Saccharomyces cerevisiae* is summarized in Table 1. The results presented here, in conjunction with earlier studies (15, 16), have led to a number of conclusions unanticipated by work in metazoans. There the picture is dominated by a small group (U1–U6) of highly abundant snRNAs [200,000–1,000,000 molecules per cell (23)]. In contrast, our current estimates place the number of unique yeast snRNAs at two dozen, with their abundance spanning a range from 10 to 500 molecules per haploid cell. Moreover, we think it likely that these are minimum estimates; for example, it seems certain that there are more minor species than those for which we now have clones. Finally, we have shown that at least one yeast snRNA (snR19) is ≈600 nt long and another (snR20) exceeds 1000; in comparison, U3, the largest capped snRNA yet described in metazoans, is 216 nt (24).

Our demonstration that yeast has an unexpectedly large number of snRNA species, some of which are relatively minor, has important ramifications for our conceptual framework for snRNA function. In considering these, the first question that arises is whether or not this situation is likely to be unique to yeast. As pointed out previously (15), it is

Table 1. Summary of *Saccharomyces cerevisiae* snRNAs

Species	Clone	Length, nt	Class	Ref(s).
snR13		≈125	Major	
snR14	*SNR14*	≈163	Major	*
snR21	*SNR21*	≈165	Minor	
snR15		≈168	(Var.)	
snR22	*SNR22*	≈170	Minor	
snR7$_s$	*SNR7*	178	Major	
snR7$_l$		213	Major	†
snR2		≈185	Major	
snR9	*SNR9*	188	Major	15‡
snR8	*SNR8*	189	Major	15‡
snR23	*SNR23*	≈190	Minor	
snR4	*SNR4*	≈192	Major	15, 16
snR5	*SNR5*	≈193	Major	‡
snR3	*SNR3*	194	Major	15, 17
snR16		≈204	(Var.)	
snR24	*SNR24*	≈220	Minor	
snR10	*SNR10*	245	Major	15, 18
snR11		≈259	Major	
snR25	*SNR25*	≈265	Minor	
snR26	*SNR26*	≈270	Minor	
snR17	*SNR17*	≈311	Major	
snR18(a–c)		≈350	Major	
snR19		≈625	Major	
snR20	*SNR20*	>1000	Major	

snRNA species are numbered arbitrarily and are presented in order of increasing length. (The absence of snR1 and snR6 is due to their apparent identity with snR7 and snR17, respectively.) When preceded by ≈, the chain lengths are approximations based on mobility in denaturing gels relative to known size markers; in other cases, nucleotide sequences have been determined as described [Ref(s).]. "Class" refers to the relative abundance of the snRNAs, as described in the text. (Var.) indicates species whose yields are variable in different preparations.

*P. Siliciano and C.G., unpublished data.

†B. Patterson and C.G., unpublished data.

‡R. Parker, L. Simmons, E. Shuster, P. Siliciano, and C.G., unpublished data.

possible that yeast snRNAs might carry out more specialized and/or diversified functions than their metazoan analogues; indeed, the identification of minor classes would be particularly compatible with this hypothesis. Moreover, the sequences obtained to date show only modest (17) or no (ref. 18; unpublished data) compelling homology at the primary structure level to sequences reported from metazoans, consistent with the possibility that mammalian counterparts to these particular yeast RNAs do not exist. As discussed recently (18), it seems less likely that the yeast species are functional analogues to mammalian RNAs which are too far diverged to be structurally recognizable. The alternative explanation for the lack of obvious primary sequence homology is that the metazoan counterparts have not been identified.

In fact, several recent experimental findings favor the prediction that the snRNA profile of higher eukaryotes will turn out to be more complex than has been assumed. Foremost among these is the discovery of a hitherto unknown snRNA in sea urchin; U7 is present in only 1/30th the abundance of U1 and has the apparently specific function of generating the mature 3′ ends of histone mRNAs (25–27). In contrast to the predominant view of snRNAs as mediating general classes of processing reactions, we must now entertain the intriguing possibility that U7 is only the first of many snRNAs that will be found to perform a function that can be considered "target-gene specific." *A priori*, one would expect such snRNAs to be present in low abundance relative to the "general reaction" types, accounting for the prior failure to notice them against the background of snRNAs numbering in the hundreds of thousands of molecules. Indeed, Busch and colleagues (28) have identified three snRNAs in rodents (U8–U10) that are 1/10th as abundant as the most minor species previously identified, U6. Thus, one plausible way to reconcile the otherwise discrepant pictures of snRNA complexity between higher and lower eukaryotes centers on the fact that we can readily discern the existence of yeast species present at several molecules per cell, whereas detecting snRNAs of such a low abundance in HeLa cells requires the search for RNAs 4–5 orders of magnitude less abundant than the predominant species.

The direct resolution of the question of whether higher eukaryotes share a similar diversity of snRNAs will ultimately require comparative structural and functional analyses. In any event, it appears that at least two dozen unique snRNAs contribute to the makeup of a unicellular eukaryote; these findings suggest that we should be prepared to reconsider our previous prejudices as to the number and kinds of different roles played by snRNAs.

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