Isolation and Properties of a New Species of Ribonucleic Acid Synthesized in Sporulating Cells of *Saccharomyces cerevisiae*

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A new species of ribonucleic acid (RNA) was detected in sporulating culture of *Saccharomyces cerevisiae*. This RNA was isolated by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis and partially characterized. It has a sedimentation coefficient of approximately 20*S* and a nucleotide composition distinct from other known RNA species in yeast, and it hybridizes with nuclear but not with mitochondrial deoxyribonucleic acid.

Previous papers from our laboratory have described the genetic (3, 5) and physiological (4, 13) control of sporulation in *Saccharomyces cerevisiae*. During the course of sporulation, a new species of ribonucleic acid (RNA) was detected which had a unique mobility on polyacrylamide gels, was preferentially synthesized during sporulation, and was produced only in cells capable of sporulating or entering the early stages of meiosis when placed under conditions of sporulation (7). This paper describes the isolation and characterization of this RNA species and demonstrates that it is distinct from the other known classes of RNA in yeast.

MATERIALS AND METHODS

Organism. *S. cerevisiae* strain Y-290 was used in the experiments.

Media and growth condition. Media and cultural conditions for vegetative growth and sporulation were those described previously (7).

Extraction, isolation, and purification of 20S RNA. After 12 hr of incubation in KAc medium, cells were harvested, washed, and disrupted after freezing in a French press (7). To prevent RNA degradation, sodium dodecyl sulfate and water-saturated phenol were added immediately after thawing of the homogenate. RNA was extracted three times with the phenol, precipitated by addition of 2 volumes of cold alcohol, and stored at −20 C. RNA was dissolved in a small volume of 0.1 M sodium acetate buffer (*p*H 5.0) containing 0.1 M NaCl. The clear supernatant liquid after centrifugation was layered on a 10 to 20% sucrose gradient solution made in the same buffer and centrifuged at 52,000 × *g* for 18 hr with an SW25.1 or SW25.2 rotor in a Beckman-Spinco LS 65B ultracentrifuge. Fractions 12 to 17 in

Fig. 1 (21*S* region) were collected and pooled. RNA was precipitated with 2.5 volumes of cold 95% ethanol and stored at −20 C for at least 16 hr. 20*S* RNA was further purified by a second sucrose gradient centrifugation.

The partially purified 20*S* RNA from the second sucrose density gradient centrifugation was further purified by preparative polyacrylamide gel electrophoresis (10 by 100 mm tubes) as described in the previous paper (7). A small amount of toluidine blue O was added to follow RNA separation during electrophoresis. This dye does not affect the migration rate of RNA (10). The 20*S* RNA regions of several gels were sliced out with a razor blade and pooled. RNA was recovered from the gels electrophoretically (J. N. Hansen, *unpublished data*). The recovered RNA was precipitated by addition of three volumes of cold 95% ethanol and stored at −20 C. Since this preparation contained small amounts of 18*S* ribosomal RNA (rRNA) and other ultraviolet-absorbing materials derived from polyacrylamide gels (Fig. 2), a final purification was carried out by sucrose density gradient centrifugation. Fractions 13 to 17 (Fig. 2) were pooled, and the purified RNA was precipitated with ethanol.

Determination of nucleotide composition of RNA. Purified 20*S* RNA was dissolved in 0.05 M NH₄ acetate buffer (*p*H 5.5) and digested with ribonuclease T₁ and T₂ (0.025 units/ml and 25 μg/ml, respectively) for 3 hr at 37 C. The digest was thoroughly lyophilized to remove NH₄ acetate and then dissolved in small amounts of 0.05 M phosphate buffer (*p*H 3.5). Nucleotides were separated at 70 C by a linear gradient of 0.1 and 0.3 M potassium phosphate buffer (*p*H 3.5) in a Picker nucleotide analyzer (model LSC 1000; Varian Aerograph, Walnut Creek, Calif.). The relative amount of each nucleotide was determined by measuring the area under each peak. In the case of ³²P-labeled 20*S* RNA, the eluate was fractionated directly into scintillation vials,

and the nucleotide composition was determined from the distribution of ^{32}P radioactivity.

DNA-RNA hybridization and CsCl density gradient centrifugation. Deoxyribonucleic acid (DNA) was extracted and purified from whole cells (Y-290) at early stationary phase of growth (14) and denatured by heating in 0.1 N NaOH at 100 C for 5 min. Denatured DNA was incubated with ^{32}P-labeled 20S RNA in 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 7.4) containing 50% formamide and 0.33 M KCl at 37 C for 4 hr. The nucleic acids were precipitated by the addition of three volumes of cold 95% ethanol, stored at -20 C for 18 hr, and dissolved in 1 ml of 2× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The solution was then incubated with 25 µg of ribonuclease 1 per ml and 0.25 units of T_1 per ml at room temperature for 1 hr in 2 × SSC. After precipitation with ethanol, the DNA and DNA-RNA hybrid were dissolved in 2.5 ml of 1 × SSC. A 3.25-g amount of solid CsCl was added and, after reading the refractive index, 1.5 ml of paraffin oil was overlayered on the solution. Centrifugation was carried out at 25 C for 50 hr at 86,000 × g in a Beckman Spinco SW50 rotor.

Chemicals. ^{32}P-phosphate (carrier-free) was purchased from New England Nuclear Corp., Boston, Mass.; CO_2-stabilized formamide was from Fisher; and ribonuclease-free sucrose was from Schwarz Bio-Research Inc., Orangeburg, N.Y. Cesium chloride (optical grade powder), from the Harshaw Chemical Co., Solon, Ohio, was used without further purification. Other chemicals used were reagent grade.

RESULTS

To characterize the classes of RNA synthesized during sporulation, *S. cerevisiae* Y-290 was transferred at early stationary phase to KAc medium containing 0.1 mCi of ^{32}P phosphate per 0.1 mg per ml and aerated for 12 hr at 30 C. Bulk RNA was extracted from this culture and fractionated by sucrose gradient centrifugation (Fig. 1). The major RNA classes (18S and 26S) are present after 12 hr of sporulation. The optical density profile of bulk RNA in the sucrose gradient is very similar to that found in actively growing cells (for comparison see profile of T_0 cells in Fig. 3). All major classes of RNA are labeled during the 12-hr sporulation. Inspection of Fig. 1 reveals that the radioactivity profile does not strictly parallel total RNA. The radioactivity profile in the larger 26S rRNA region is asymmetric and contains a shoulder which corresponds to the new 20S RNA species previously reported to be preferentially synthesized during the early stages of sporulation (7).

To purify this RNA, fractions shown in Fig. 1 containing the highest specific activity between 26S and 18S RNA (fractions 12 to 17) were pooled, precipitated with ethanol, and subjected to further purification by sucrose gradient centrifugation and polyacrylamide gel electrophoresis (Table 1). At each stage of purification, analyt-
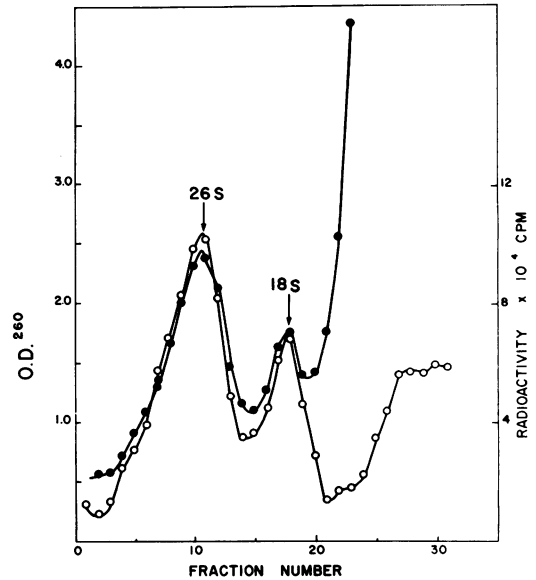

FIG. 1. *Profile of RNA extracted from sporulating cells in sucrose density gradient centrifugation. Cells (Y-290) were grown in YEP medium to the early stationary phase of growth, transferred to KAc medium containing ^{32}P-phosphate (1 mCi/mM), and incubated for 12 hr at 30 C with vigorous shaking. RNA was carefully isolated, layered onto a 10 to 20% linear sucrose gradient, and centrifuged at 52,000 × g for 18 hr at 5 C. After centrifugation, 30-drop fractions were collected and analyzed for radioactivity (●) and optical density at 260 mm (O), respectively.*

TABLE 1. *Isolation and purification of 20S RNA[a]*

| Step | Total | | Specific activity[b] | 20S RNA[c] (%) |
	OD_{260}	^{32}P[b] (10⁴ counts/ min)		
I. Crude RNA preparation	174.5	451.9	25.9	2.5
II. Sucrose gradient	46.8	115.1	24.6	7.4
III. Sucrose gradient	17.6	59.1	37.9	22
IV. Gel electrophoresis[d]		2.2		95
V. Sucrose	0.23	1.5	64.4	100

[a] Cells of Y-290 were incubated in KAc medium containing ^{32}P-phosphate (0.1 mCi per 0.1 mg per ml) for 12 hr at 30 C. RNA was extracted as described in Materials and Methods.

[b] Expressed as 10³ counts per minute at optical density at 260 nm (OD_{260}).

[c] The percentage of RNA present in each preparation as 20S RNA was determined by polyacrylamide gel electrophoresis (7).

[d] This preparation contains low-molecular-weight OD_{260} absorbing materials as shown in Fig. 2.

ical polyacrylamide gels were run to determine purity of each RNA sample (7). The first two sucrose density gradients each increased the purification of 20S RNA threefold. Further purification was done by polyacrylamide gel electrophoresis. Efficiency of purification by preparative

electrophoresis was remarkably high, but the yield in this step was very low, primarily owing to the poor RNA extraction from the gels. Since the RNA extracted from gels contained significant optical density (at 260 nm) absorbing materials which fail to sediment in sucrose gradient centrifugation and small amounts of 18S rRNA, a final sucrose gradient centrifugation was added to the purification procedure (Fig. 2). The main peak fractions (13 to 17) were pooled, and the RNA was precipitated by addition of three volumes of cold 95% ethanol. The purified 20S RNA preparation (bottom row, Table 1) was the RNA employed in subsequent experiments on the properties of 20S RNA. Purified 20S RNA was homogeneous by two criteria. First, in toluidine blue O-stained polyacrylamide gel electropherograms, the RNA preparation was electrophoretically pure. In a second test, the labeled 20S RNA was mixed with bulk RNA from T_0 cells and centrifuged in a sucrose density gradient (Fig. 3). The ^{32}P radioactivity sediments in the sucrose gradient as a single, sharp peak and is clearly displaced from the other major RNA species present in T_0 cells. The relative sedimentation coefficient of labeled RNA, on the basis of 26S and 18S rRNA, was approximately 22S.

Base composition of 20S RNA. Purified ^{32}P-labeled 20S RNA (Table 1) and 26S and 18S cytoplasmic rRNA were hydrolyzed by ribonuclease and analyzed for their base composition. The results (Table 2) reveal significant differences between these RNA species. The guanine plus cytosine (GC) composition of 20S RNA is 54.7% compared to 50.3 and 45.6% for 26S rRNA and 18S rRNA, respectively. In addition, the pyrimidine/purine ratio of 20S RNA (1.30) is significantly higher than in cytoplasmic rRNA (0.92 to 1.01). As reported elsewhere (H. Morimoto and H. O. Halvorson, Proc. Nat. Acad. Sci. U.S.A., *in press*), mitochondrial rRNA contains approximately equal proportions of purine and pyrimidine bases but has a considerably lower GC ratio (30.2 to 33.3%). These findings, and the results from the DNA-RNA hybridization experiment detailed below, eliminate the possibility that 20S RNA and the closely sedimenting large RNA species from mitochondrial ribosomes are identical. Only transfer RNA has a GC content similar to that of 20S RNA; however, sharp dissimilarities in their base composition and size indicate that these are distinct species of RNA.

The data in Table 2 clearly indicate the uniqueness of 20S RNA. Although this RNA sediments in sucrose gradients and migrates on polyacrylamide gels as a homogeneous species, it is still possible that it is composed of a heterogeneous
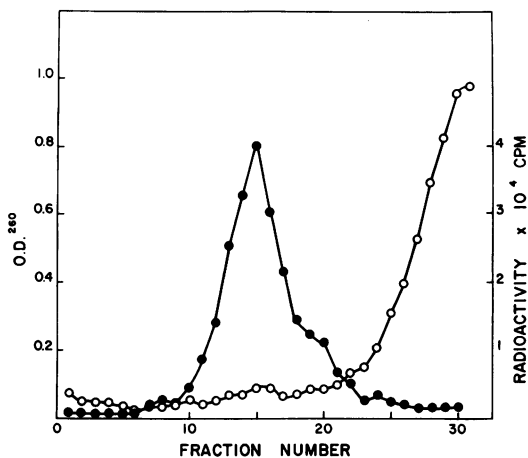

FIG. 2. *RNA profile in the final step of the purification by sucrose gradient centrifugation. The 20S RNA fraction from polyacrylamide gel electrophoresis was dissolved in 1 ml of 0.1 M Na-acetate buffer (pH 5.0). Centrifugation and fractionation were carried out as described in the legend for Fig. 1. Optical density at 260 nm (O); ^{32}P radioactivity (●).*
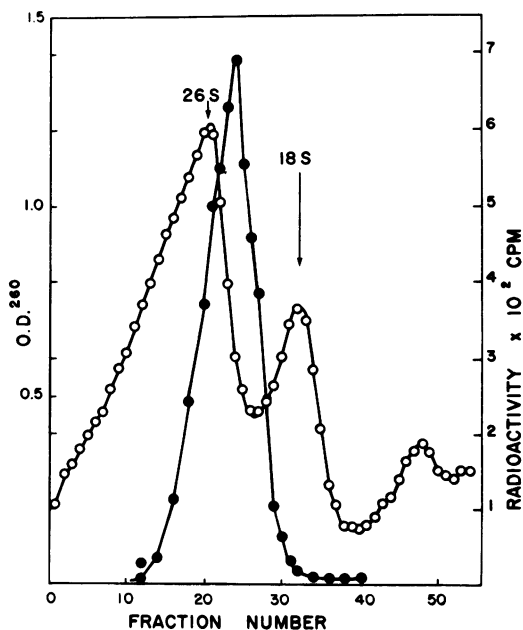

FIG. 3. *Sucrose density gradient centrifugation of purified ^{32}P-20S RNA in the presence of unlabeled bulk RNA from T_0 cells. Mixture of purified ^{32}P-20S RNA (5,000 counts per min per 3.5 μg) and total RNA from T_0 cells (1.5 mg) was layered on top of a 10 to 20% linear sucrose gradient and centrifuged as in Fig. 1. Fractions (20 drops) were collected. Optical density at 260 nm (O); radioactivity (●).*

TABLE 2. *Base composition of RNA species of yeast*[a]

RNA species	Molar per cent ratio[b]					
	CMP	UMP (dTMP)	AMP	GMP	GC	Reference
20S RNA	32.1	24.4	20.9	22.6	54.7	
26S cytoplasmic rRNA	23.8	23.3	26.4	26.5	50.3	
18S cytoplasmic rRNA	22.6	28.2	26.2	23.0	45.6	
24S mitochondrial rRNA	16.1	34.0	32.6	17.2	33.3	10
16S mitochondrial rRNA	12.9	36.4	33.3	17.4	30.3	10
Transfer RNA	24.2	26.5	18.8	30.5	54.7	14
5-Min pulse-labeled RNA	20.1	25.2	32.6	21.9	42.0	6
Nuclear DNA	17.2	33.7	31.5	17.6	34.8	6
Mitochondrial DNA	10.0	37.5	41.3	11.3	21.3	17

[a] Sporulating cells were labeled with ^{32}P as described in Materials and Methods. Purified cytoplasmic ribosomal subunits were isolated as described elsewhere (14). See Table 1 for purification of 20S RNA. RNA samples were dissolved in 0.05 M NH_4 acetate buffer (pH 5.5) containing 10^{-3} M ethylenediaminetetraacetic acid (EDTA) and then dialyzed against 0.05 M NH_4 acetate buffer for 5 hr to remove Mg^{2+} ions and EDTA. The RNA samples were reprecipitated with ethanol, dissolved with NH_4 acetate buffer, and digested with T_1 and T_2 ribonuclease for 3 hr at 37 C. The digest was lyophilized overnight to remove NH_4 acetate and dissolved in 0.1 M phosphate buffer, pH 3.5. The relative amount of each base was determined either by measuring the area under each nucleotide peak eluted at 70 C by a linear gradient of 0.1 M and 0.3 M potassium phosphate (pH 3.5) in a Picker nucleotide analyzer or by measuring the radioactivity in each peak in a Packard Tricarb scintillation counter. The values represent the average of duplicate determinations by both methods of analysis.

[b] Abbreviations: CMP, cytidine monophosphate; UMP, uridine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; GC, guanosine plus cytosine.

mixture of molecules with similar charge and size.

Nuclear origin of 20S RNA. Since sporulation is associated with the development of the system for acetate metabolism (4) and the synthesis of mitochondrial rRNA (7), it is of interest to determine whether 20S RNA is of nuclear or mitochondrial origin. To explore this question, ^{32}P-labeled purified 20S RNA was hybridized under conditions of DNA excess (DNA/RNA = 2.3 × 10^3) with denatured DNA extracted from whole cells at the early stationary phases of growth. After ribonuclease treatment, 87% of the input radioactivity was still associated with the DNA-RNA hybrids as analyzed by CsCl density gradient centrifugation (Fig. 4). Nuclear DNA bands as a sharp, single homogenous peak indicative of a large molecular weight population. Mitochondrial DNA appears as a broad light shoulder on the nuclear DNA band. Under the hybridization conditions employed (high DNA/RNA ratio with the majority of the ^{32}P-RNA hybridizing to DNA), it is unlikely that sufficient RNA was hybridized to shift the density from single-stranded DNA to hybrid. Thus, the distribution of ^{32}P radioactivity strongly suggests that nuclear but not mitochondrial DNA contains regions complementary to 20S RNA.

DISCUSSION

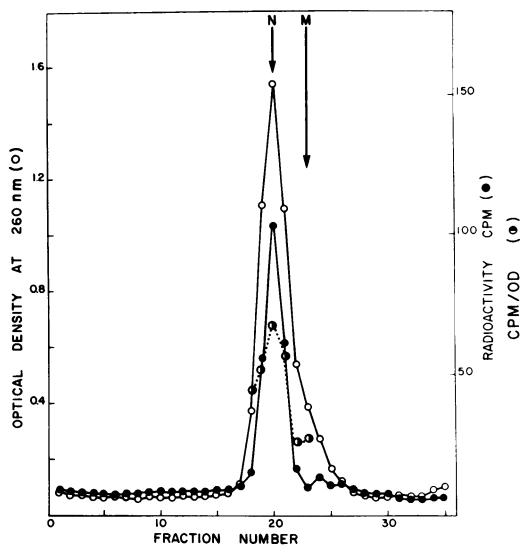The results presented here and elsewhere (7) clearly demonstrate that several species of high-

FIG. 4. *DNA-RNA hybrids in CsCl density gradient centrifugation. Denatured DNA (230 μg) from T_0 cells (Y-290) was hybridized with 0.1 μg of purified ^{32}P-20S RNA (4,000 counts per min per μg) in the presence of 50% formamide at 37 C for 4 hr, treated with ribonuclease, and centrifuged in CsCl (final density; 1.70 g/ cm^3) at 86,000 × g for 50 hr at 25 C. Ten-drop fractions were collected and, after addition of 0.5 ml of distilled water, were assayed for optical density at 260 nm (O) and for radioactivity (●), and the ratio of radioactivity/optical density at 260 nm was calculated (◑).*

molecular-weight RNA are preferentially synthesized during sporulation. These species include the two mitochondrial rRNA species (7) and, as shown here, a third RNA species which is separable from cytoplasmic rRNA both by its sedimentation rate in sucrose gradients and by its mobility in polyacrylamide gel electrophoresis. The size of this latter RNA species is as yet uncertain. Bishop et al. (1) and Loening (9) reported that the *S* value and molecular weight of RNA can be determined by the relative electrophoretic migration rate in polyacrylamide gels. The *S* value of this RNA as previously measured by polyacrylamide gel electrophoresis (7) is 20*S* and, as reported here, by sucrose density gradient centrifugation is 22*S*. No attempt was made to resolve this discrepancy, and the designation of 20*S* obtained from our standardized electrophoretic system will be used for convenience. On the basis of electrophoretic mobility, the molecular weight of 20*S* RNA is approximately 0.85×10^6 daltons. The molecular weights of cytoplasmic 18*S* and 26*S* rRNA species of yeast are 0.72×10^6 and 1.3×10^6 daltons, respectively (9).

One possible origin for 20*S* RNA is that it represents one of the high-molecular-weight precursors of rRNA. Darnell (2) recently reviewed the evidence that in eucaryotic cells the precursor to rRNA is transcribed as a high-molecular-weight species which is subsequently cleaved in several stages to 1.7×10^6- and 0.7×10^6-daltons rRNA species with the loss of some oligonucleotide components. After methylation, these are incorporated into ribosomal particles. The 38*S* RNA species which have characteristics suggesting an rRNA precursor have been reported in *S. carlsbergensis* (11) and in *Schizosaccharomyces pombe* (8, 15). Taber and Vincent (16) found that the 38*S* RNA contains sequences homologous to both rRNA species. This RNA is further methylated and the methyl groups are chased into transfer RNA. Molecules with intermediate molecular weights between the large RNA precursor and mature rRNA have been detected in *S. carlsbergensis* (quoted in 17). In *S. cerevisiae*, Udem and Warner (*personal communication*) observed a 35*S* precursor RNA which is first converted to 27*S* RNA and then to either 25*S* rRNA or to 20*S* RNA, the immediate precursor of 17*S* rRNA.

The base composition of 20*S* RNA (Table 2) makes unlikely the possibility that it is a precursor of 18*S* rRNA. The 20*S* RNA contains 54.7% GC and a pyrimidine/purine ratio of 1.32. 18*S* rRNA, on the other hand, has a GC content of 45.6% and a lower (1.05) pyrimidine/purine ratio. The 20*S* RNA is richer in cytosine and

lower in guanosine than 18*S* RNA. In the absence of knowledge of the heterogeneity of 20*S* RNA, one cannot exclude the possibility that it contains subspecies which are precursors of 18*S* rRNA. During meiosis, both 20*S* and mitochondrial rRNA are synthesized in preference to cytoplasmic rRNA. The possibility that 20*S* RNA is an intermediate in mitochondrial rRNA synthesis is also unlikely, based on its composition (Table 2) and the finding here that it contains hybridizable sequences homologous to nuclear but not mitochondrial DNA.

Experiments are now in progress to further define the role of 20*S* RNA in the meiotic process.

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