

# Relationship Between Sporulation-Specific 20S Ribonucleic Acid and Ribosomal Ribonucleic Acid Processing in *Saccharomyces cerevisiae*

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The nature and properties of the 20S ribonucleic acid which accumulates only during the sporulation of *Saccharomyces cerevisiae* were examined. The 20S ribonucleic acid (RNA) has a base composition considerably different from ribosomal RNA species and is virtually unmethylated. The 20S RNA did, however, exhibit approximately 70% homology with 18S RNA by RNA-deoxyribonucleic acid filter hybridization competitions. The 20S RNA showed a hybridization saturation plateau level 30 to 40% higher than 18S, consistent with measurements of the size difference in polyacrylamide gels. Pulse-chase experiments in the presence and absence of cycloheximide indicate that the 20S RNA has a presumptive relationship to the 20S ribosomal RNA precursor normally observed only in short pulse-labeling in vegetative cells.

A number of major biochemical and structural changes accompany sporulation of *Saccharomyces cerevisiae*. One of the most striking events is the appearance of a sporulation-specific 20S ribonucleic acid (RNA). Kadowaki and Halvorson (6, 7) showed that this RNA is not produced by haploid cells even when placed in sporulation medium, nor by diploid cells (capable of sporulation) during the vegetative state. The 20S RNA was found to accumulate to a level of approximately 4% of the total cellular RNA by the end of sporulation. The role of this RNA was not identified.

Several observations have been published which add to the confusion about the identity of 20S RNA in yeast. Udem and Warner (17) observed that, during the processing of ribosomal RNA in vegetative cells, the 20 and 27S precursor RNA species accumulate prior to the formation of the two mature ribosomal RNA species. The 20S RNA which appears during vegetative growth has only a transient existence and does not remain as a stable species. On the other hand, Van Den Bos and Planta (18) observed a 20S RNA during the vegetative maturation of ribosomal RNA in *S. carlsbergensis* and reported that a one-dimensional polyacrylamide gel electrophoresis of a ribonuclease of a T<sub>1</sub> digest of this 20S RNA was not at all similar to the mature 18S RNA in this

species. Thus, in *S. cerevisiae*, vegetative RNA processing precedes the obligate 20S RNA precursor, whereas in *S. carlsbergensis* 20S RNA, which is transiently labeled during vegetative growth, may not be a precursor of the final 18S RNA.

It therefore seemed necessary to further characterize the sporulation-specific 20S RNA which appeared as a stable species in *S. cerevisiae*. Additional chemical characterization included reexamination of the base composition of this RNA species and a determination of the degree of methylation of 20S RNA. In addition, the homology between 20S RNA and 18S RNA was examined by RNA-deoxyribonucleic acid (DNA) hybridizations and by hybridization competition experiments. Finally, the kinetics of RNA labeling during sporulation was examined to give some indication of the precursor product relationship between 26, 18, and 20S RNA.

## MATERIALS AND METHODS

**Organism and cultivation.** *S. cerevisiae* D649, a diploid strain, was used in these experiments. The conditions for cultivation in yeast extract-peptone (YEP) medium and sporulation in potassium acetate medium were previously described (5).

**Isolation of RNA.** The cells were broken in the French press as described by Bhargava and Halvorson (1) except that the homogenizing buffer con-

tained 0.005 M tris(hydroxymethyl)aminomethane (Tris; pH 7.8), 0.05 M NaCl, 0.0015 M ethylenediaminetetraacetic acid (EDTA), 1 M sorbitol, and 20% glycerin. Crude RNA was prepared by shaking the cell lysate with an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline as a preservative (12). The suspension was shaken, and the phases were separated by centrifugation. The aqueous phase was removed and extracted with phenol. The RNA was precipitated by the addition of two volumes of ethanol and collected by centrifugation. The precipitate was suspended in TES buffer (0.01 M Tris [pH 7.8], 0.1 M NaCl, 0.003 M EDTA) and extracted five times with an equal volume of ether to remove the phenol. Ethanol was added to the aqueous phase to a final concentration of 67%, and the precipitated RNA was collected by centrifugation. The crude RNA was further purified by polyacrylamide gel electrophoresis.

**Polyacrylamide gel electrophoresis.** Gels (3%; 9 mm diameter) were prepared by the method of Loening (9) and run at 10 mA per gel for 12 hr for both analytical and preparative procedures. The gels were sliced into 1-mm sections on a Joyce-Lobel gel slicer. For analytical runs, the slices were dried onto paper strips and counted by using toluene-based scintillation cocktail containing 4 g of 2,5-bis-(2-[5-tert-butylbenzoxazolyl])-biophene per liter in a Beckman LS250 liquid scintillation spectrometer. Where  $^3\text{H}$  was used, slices were dissolved with 0.5 ml of  $\text{H}_2\text{O}_2$  at 80 C for 8 hr. The dissolved slices were then counted in 10 ml of Bray's solution (3). In preparative procedures, the gel slices were placed in scintillation vials containing 1 ml of a GE buffer (0.005 M EDTA, pH 7.2, 0.1 M NaCl) and counted directly using Cerenkov emissions. Peak slices were pooled, and an additional 1 ml of GE buffer was added. The slices were allowed to elute overnight in the cold with gentle agitation. The  $^{32}\text{P}$  counts (85–90%) were eluted in this fashion. Ethanol was then added to a final concentration of 55%. The resulting precipitate was then collected on columns of CF11 cellulose (Whatman) and eluted with 0.005 M EDTA, pH 7.2, containing 13% ethanol. The eluted material was lyophilized, suspended in distilled water, and further purified by gel filtration on G10 Sephadex to remove solubilized acrylamide.

**Labeling procedures.** Cells were grown in YEP medium containing either 0.1  $\mu\text{Ci}$  of  $^{14}\text{C}$ -adenine or 2  $\mu\text{Ci}$  of  $^3\text{H}$ -adenine per ml;  $^{32}\text{P}$  was then added to the sporulation medium at 0.5  $\mu\text{Ci}/\text{ml}$ .

For preparative and short-term labeling,  $^{32}\text{P}$  was added to the sporulation medium at the level of 0.5 mCi per ml.

**$^3\text{H}$ -DNA preparation.** Three 1-liter cultures of YEP medium containing 1 mCi of  $^3\text{H}$ -adenine (23.4 Ci/mole, New England Nuclear) per ml were inoculated using a late logarithmic-phase preculture. Cells were grown for 20 hr and harvested by centrifugation at 4 C. The cells were washed once with cold, distilled water, once with cold buffer (0.15 M NaCl, 0.1 M EDTA, 2% sodium dodecyl sulfate [SDS]), and suspended in the same buffer to a concentration of approximately 1 g of cells per ml of buffer. Cells were placed in an Eaton pressure cell and frozen by

cooling in a dry ice-ethanol bath for 5 min. Cells passed through the cell under pressure of 4,000 psi. Solid NaCl was added to the extract to a final concentration of 1 M. Pronase (Calbiochem), preheated for 5 min at 80 C, was added to a final concentration of 1 mg/ml. This extract was incubated for 2 hr in a boiled dialysis bag at 37 C against SSC (0.15 M NaCl, 0.15 M sodium citrate, pH 7.0). This extract was deproteinized by shaking with an equal volume of chloroform-isoamylalcohol (24:1) for 30 min on a wrist shaker. The mixture was centrifuged, the upper layer was removed, and the DNA was precipitated by the addition of two volumes of ice-cold ethanol. The precipitate was collected by centrifugation to avoid any preferential loss of ribosomal RNA (rRNA). The crude precipitate was dissolved in  $0.1 \times \text{SSC}$  and made up to  $1 \times \text{SSC}$  with  $10 \times \text{SSC}$ . Ribonuclease was added to a final concentration of 5  $\mu\text{g}$  of pancreatic ribonuclease (Calbiochem) per ml and 10 units of  $\text{T}_1$ -ribonuclease (Calbiochem) per ml. The ribonuclease mixture was boiled for 5 min to remove any deoxyribonuclease activity.  $\alpha$ -Amylase (10  $\mu\text{g}/\text{ml}$ ; Calbiochem) was added to remove carbohydrate contamination. The crude DNA was incubated for 4 hr in a boiled dialysis bag against SSC. The Pronase step was repeated, and the DNA was ethanol-precipitated and suspended in  $0.1 \times \text{SSC}$ . Residual protein contamination was removed by treating the DNA with chloroform-isoamylalcohol until no protein was detectable and the DNA was precipitated by ethanol.

**Fixation of DNA to nitrocellulose filters and estimation of specific activity.** The method of fixation of DNA to nitrocellulose filters was that of Schweitzer et al. (15); 5  $\mu\text{g}$  of denatured  $^3\text{H}$ -DNA was fixed to each 27-mm filter (Schleicher and Schuell, Bac-T-Flex, type B-6). For hybridization, each 27-mm filter paper was cut into three circles of 13 mm diameter. The specific activity of each sample was obtained by spotting 10  $\mu\text{g}$  of sample on a 12-mm Schleicher and Schuell type B-6 filter. Filters were dried and dissolved in ethylacetate. Samples were counted in a Beckman LS-250 liquid scintillation counter in the presence of 10 ml of toluene POP-POPOP (2,5-diphenyloxazole; 1,4 bis-[2-(5-phenyloxazolyl)]-benzene). Each 13-mm filter contained approximately 1  $\mu\text{g}$  of DNA (6,100 counts per min per  $\mu\text{g}$ ).

**RNA-DNA hybridization procedures.** The filter hybridization method of Gillespie and Spiegelman (8) was used with several modifications. Hybridizations were carried out in f30SS buffer containing 0.3 M NaCl, 0.3 M sodium citrate, 30% (v/v) stabilized SDS as described by Bonner et al. (2) and Spiegelman (Ph.D thesis, Univ. of Wisconsin, 1972). Two 13-mm DNA filters and a blank filter were incubated with RNA in paraffin-oil-sealed vials containing 0.4 ml of solution. The RNA used in hybridizations was partially degraded to an average size of  $10^2$  nucleotides by heating as an aqueous solution in a boiling-water bath for 5 min before preparing the hybridization buffers. All hybridizations were carried out at 37 C.

Unlabeled 18S RNA from vegetative cells was

prepared by phenol extractions from cells disrupted by the French press. Vegetative RNA was then separated by centrifugation in a 5 to 20% sucrose gradient in a buffer containing 0.1 M NaCl, 0.01 M Tris (pH 7.4), and 0.001 M MgCl<sub>2</sub>. The 18S RNA peak fractions were pooled, precipitated by ethanol, and suspended in water for hybridizations.

The concentrations of all RNA species were determined spectrophotometrically. A slight correction for acrylamide contamination in the RNA species obtained by preparative gel was also made. Acrylamide absorbs ultraviolet light (UV) approximately 3.5 times more at 250 nm than at 260 nm, whereas pure RNA has a spectrum in which the absorbance at 250 nm is approximately 0.85 that of 260 nm. The presence of low levels of acrylamide does not appear to interfere with filter hybridizations.

Hybridizations were carried out for 20 hr, after which the filters were washed, treated for 1 hr with ribonuclease (8), washed, and dried. Excess salt was removed by washing with 1 ml of distilled water (15), and the dried filters were then dissolved in 1 ml of ethyl acetate for counting in PPO-POPOP. The amount of RNA per microgram of DNA hybridized was determined by counting <sup>3</sup>H and <sup>32</sup>P activity of the filters.

**Determination of RNA base ratios.** RNA was hydrolyzed in 0.2 N NaOH at 37 C for 18 hr. The hydrolysate was then spotted on Whatman 3MM chromatography paper and saturated with a buffer containing 0.5% pyridine, 5% acetic acid, pH 3.5 (14). The individual bases were then separated by a Savant electrophoresis system at 5,000 V for 2 to 3 hr. The radioactive areas which were localized by autoradiography on the electropherogram were then excised and counted to determine the relative amount of radioactivity in each base.

**Materials.** Materials were obtained from the following: 3 MM paper and CF11 cellulose from Whatman Paper Co.; <sup>3</sup>H-adenine (6.1 Ci/mM), [<sup>8-<sup>14</sup>C</sup>]adenine (6.6 mCi/mMole), L-[methyl-<sup>3</sup>H]methionine (147 mCi/mMole), and carrier-free <sup>32</sup>P-phosphoric acid from New England Nuclear Corp.; pancreatic ribonuclease from Worthington; T<sub>1</sub> ribonuclease from Calbiochem; 8-hydroxyquinoline from Sigma; and acrylamide and bisacrylamide from Eastman Kodak.

## RESULTS

**Further chemical characterizations of 20S RNA.** Kadowaki and Halvorson (6, 7) have described the kinetics of appearance of 20S RNA during sporulation. To explore whether the 20S is derived from preexisting 26S RNA, the experiment shown in Fig. 1 was conducted. In this experiment, vegetative cells were prelabeled with <sup>3</sup>H-adenine, washed, and transferred to sporulation medium containing <sup>32</sup>P as described above. After 18 hr in sporulation medium, RNA was extracted and separated on polyacrylamide gels, and the varying fractions

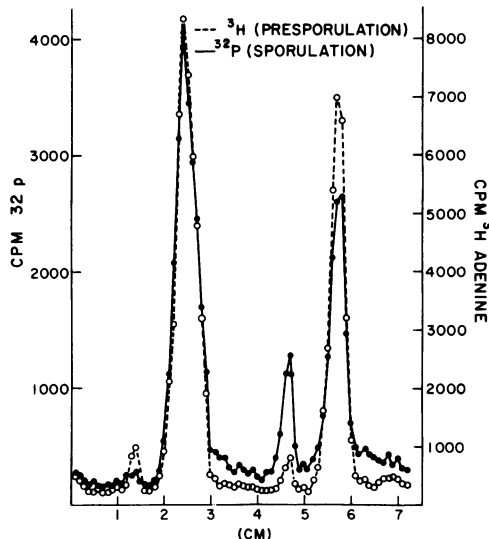


FIG. 1. Appearance of 20S RNA during sporulation. Cells were grown to stationary phase in YEP-dextrose medium containing 5  $\mu$ Ci of <sup>3</sup>H-adenine per ml. After washing, the cells were suspended in sporulation medium containing 5  $\mu$ Ci of <sup>32</sup>P-orthophosphate per ml. Cells were harvested after 17 hr, and the RNA was extracted and separated by polyacrylamide gel electrophoresis as described in *Materials and Methods*. The vegetative label, <sup>3</sup>H-adenine (---), is accumulated almost exclusively in the two ribosomal RNA peaks. During sporulation as followed with <sup>32</sup>P (—), a third peak, 20S RNA, is also found.

were sliced and measured for <sup>3</sup>H and <sup>32</sup>P. As can be seen, pre-labeled RNA is almost exclusively in 26 and 18S fractions with a ratio of 1.7:1. In <sup>32</sup>P labeling, a 20S component is clearly evident, and the ratio of 26S to 18S has been increased to 2:1. Also note that a small fraction of <sup>3</sup>H counts are included in the 20S peak. As has been previously shown (5), RNA turnover is evident during sporulation. Undoubtedly this reflects recycling of pre-labeled RNA to the adenine pool and reincorporation into RNA during sporulation, since no 20S stable RNA peak is observed when RNA is prepared from vegetative cells.

If the 20S RNA component represents a late processing precursor for ribosomal RNA, one would anticipate that this species would be methylated. To test this possibility, cells were grown to stationary phase in YEP, washed, and transferred to sporulation medium containing 5  $\mu$ Ci of L-[methyl-<sup>3</sup>H]methionine and 0.1  $\mu$ Ci of <sup>32</sup>P per ml. RNA was extracted after 18 hr and fractionated as described in Fig. 1. It is evident from Fig. 2 that both 26 and 18S are

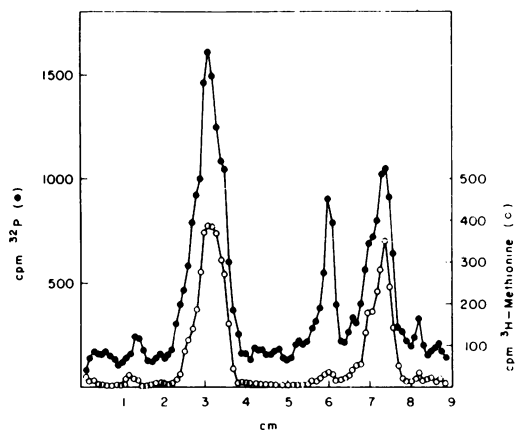


FIG. 2. Methylation of 20S RNA in sporulating *S. cerevisiae*. Stationary-phase cells were transferred to sporulation medium containing 5  $\mu$ Ci of L-[methyl- $^3$ H]methionine per ml and 0.1  $\mu$ Ci of  $^{32}$ P-orthophosphate per ml. RNA was extracted after 18 hr and separated by polyacrylamide gel electrophoresis. Gels were cut into 1-mm slices, dissolved in hydrogen peroxide, and counted in a liquid scintillation counter.

substantially methylated as expected. However, 20S RNA contains little, if any, label from methionine and therefore is not significantly methylated. It is of interest that, in vegetative cells, methylation appears to occur at the 35S precursor level (17). Thus, in the case of the vegetative cell, when the 20S RNA precursor of 18S RNA appears it is already methylated.

In characterizing the nature of the 20S RNA, the RNA base compositions were reexamined. RNA was prepared as described above. After the removal of acrylamide, the separated 26, 20, and 18S were subjected to alkaline hydrolysis, and the bases were separated by paper electrophoresis. The individual bases were identified, and the spots were excised and counted for  $^{32}$ P. The data in Table 1 confirm a higher guanine plus cytosine (GC) content in the 20S RNA. 26S RNA and 18S RNA have similar, but not identical, base compositions; 20S RNA has a significantly elevated C content and a significantly reduced adenosine (A) content. The G content of 20S RNA is also higher than that of 18S RNA. Thus, if 20S RNA is a precursor of 18S, the additional (nonconserved) RNA sequence would be enriched in C and to a lesser extent G.

**Evidence that 20S RNA is a precursor of 18S ribosomal RNA.** To test the possibility that 20S RNA is a precursor of 18S ribosomal RNA, RNA-DNA hybridization and hybridization competition experiments were carried out.

20S RNA and 18S RNA labeled with  $^{32}$ P were isolated from sporulated cells as described above. As seen in Fig. 3, essentially similar saturation plateau results (ca. 0.7% of the DNA) were observed when labeled 18S and 20S RNA from sporulating cells were hybridized with denatured nuclear DNA from the *S. cerevisiae* strain D649. The saturation plateau

TABLE 1. Comparison of base compositions of 20S RNA with ribosomal RNA species<sup>a</sup>

RNA species	C (%)	A (%)	G (%)	U (%)	%GC
26S					
1	20.3	26.8	27.9	25.0	
2	19.5	26.5	28.5	25.5	
3	19.6	26.6	28.4	25.3	
Avg	19.8	26.6	28.4	25.3	48.2
20S					
1	28.6	18.3	29.4	23.7	
2	28.6	18.5	29.1	23.8	
3	27.5	18.7	29.7	23.1	
4	28.6	18.6	29.1	23.7	
Avg	28.6	18.6	29.1	23.7	57.7
18S					
1	19.5	27.0	25.4	28.1	
2	19.8	26.5	25.6	28.2	
3	19.4	25.7	25.5	29.3	
4	19.6	26.4	25.9	28.1	
Avg	19.6	26.4	25.6	28.4	45.2

<sup>a</sup> RNA labeled with  $^{32}$ P-orthophosphate was isolated from sporulating cells according to the procedures given in Materials and Methods. The RNA species were purified by polyacrylamide gel electrophoresis, and the base compositions were then determined after alkaline hydrolysis and separation of the mononucleotides by paper electrophoresis as described above. Abbreviations: C, cytosine; A, adenosine; G, guanine; U, uridine; %GC, percent guanine plus cytosine.

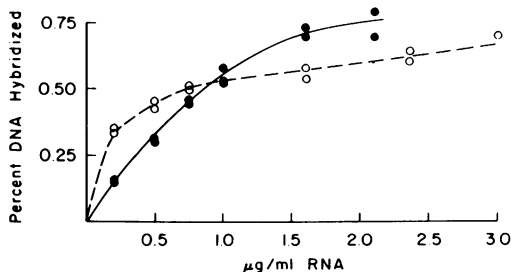


FIG. 3. RNA-DNA filter hybridizations of 18 and 20S RNA. RNA labeled with  $^{32}$ P was isolated and fractionated from sporulated cells as described above. The percent DNA hybridized was measured for both 18S (---) and 20S (—) RNA.

is somewhat greater than that of the 18S ribosomal RNA although the concentration dependence of the two saturation curves is somewhat different. Since prior heat treatment was employed to denature and to degrade partially the RNA prior to hybridization, it is possible that 20S RNA is more stable to the heat treatment by virtue of possible greater secondary structure. In this case, the kinetics of the two hybridizations may be slightly different. A second problem arises in the measurement of the rather small quantities of 20S RNA available from the preparative gels. Trace concentrations of acrylamide will absorb in the UV from 250 to 280 nm and lead to a slight overestimation of the RNA concentration. This slight overestimation would be greater for 20S RNA which was isolated in lower amounts.

To demonstrate the homology between 18 and 20S RNA, the competition of 20S RNA hybridization by unlabeled 18S RNA isolated from vegetative cells was carried out (Fig. 4). In the control experiments, labeled 18S RNA from sporulated cells is competed completely by 18S RNA from vegetative cells. However, in the parallel competition experiment employing labeled 20S RNA from sporulating cells and unlabeled 18S RNA from vegetative cells, a portion of the 20S RNA was not competed by the 18S RNA. From a double reciprocal plot of the competition data of Fig. 4, one can estimate that 30% of the 20S RNA is nonhomologous with the 18S RNA. Thus, the findings from the hybridization competition experiments are consistent with the saturation plateaus in showing a larger size of the 20S RNA. The remaining 70% is clearly homologous to 18S ribosomal RNA by hybridization criteria.

**Kinetics of ribosomal RNA synthesis during sporulation.** To understand the accumulation of 20S RNA during sporulation, it is necessary to follow the kinetics of ribosomal RNA synthesis during this same period. To follow the processing of precursors into mature ribosomal RNA, cultures were sporulated for 6 hr and then labeled for 10 min with  $^{32}\text{P}$ . Excess unlabeled inorganic phosphate in 0.5% potassium acetate, pH 9, was added after 10 min to a final concentration of 0.5 M. Samples were removed at intervals and harvested, and DNA was isolated as described above. Figure 5 shows the distribution of counts from polyacrylamide gels at various times during the chase experiments. It is of interest that during sporulation the chase is not completely effective. Even after the addition of excess inorganic phosphate, the total amount of  $^{32}\text{P}$  continues to accumulate, although at a rate of 0.10 that

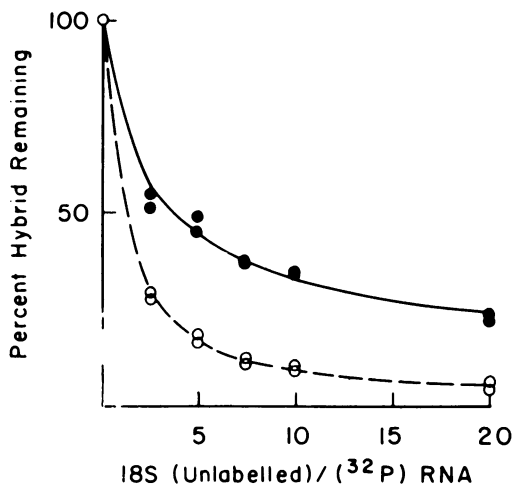


FIG. 4. Competition hybridizations of labeled 18 and 20S RNA with unlabeled 18S RNA. Labeled RNA (0.5  $\mu\text{g}/\text{ml}$ ) was hybridized to DNA filters in the presence increasing amounts of unlabeled 18S RNA. The competition of 18S versus 18S (---) extrapolates to zero, whereas the 20S competition curve (—) extrapolates to approximately 30%.

prior to the chase. One possibility is that during the initial labeling period polyphosphate reserves accumulate, as is well known in vegetative cells and resting cells (11, 16), and during the chase this polyphosphate reserve may be preferentially utilized for RNA synthesis. The time course of ribosomal RNA processing in sporulating cells is remarkably slow. In the vegetative cell, the 27 and 20S ribosomal precursor RNA appear within 5 min of the addition of the label, and 26 and 18S stable RNA species appear by 10 min (4). In contrast in the sporulating cell, the 27S and 20S precursors do not appear in significant quantities until after 1 hr. At this time, no significant amount of 26 or 18S ribosomal RNA is observed. After 120 min, both the precursors and the mature species are evident. It is not until 240 min that the same pattern observed in long-term labeling experiments is found. At this time, no 27S precursor is detectable; however, a significant amount of 20S RNA still remains. Although the lack of a complete chase may lengthen the total time of processing before the final stable RNA pattern is observed, it is clear that at least for the first hour, ribosomal RNA processing is unaffected by a residual accumulation of RNA. Furthermore, the processing of ribosomal RNA in the sporulating cell is approximately 10 times slower than that found in vegetative spheroplasts (18).

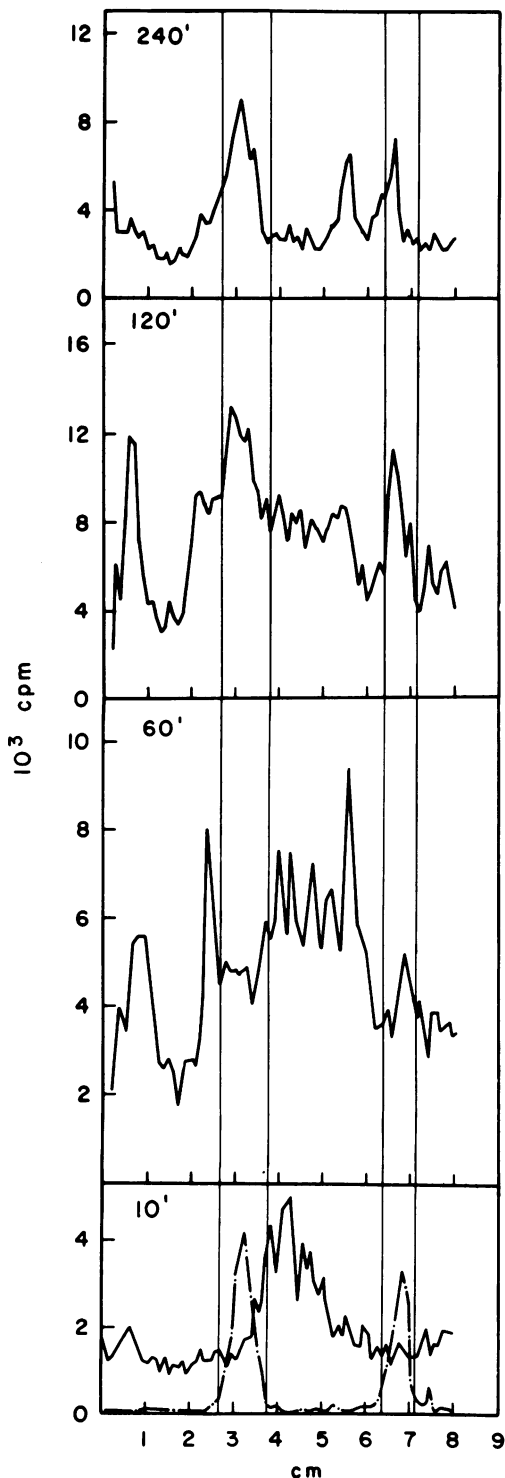


FIG. 5. Accumulation of  $^{32}\text{P}$  into ribosomal RNA in sporulating cultures of *S. cerevisiae*. Stationary-phase cells in YEP containing 0.1 mCi of  $^{14}\text{C}$ -ade-

Because ribosomal RNA processing is known to be modulated by the synthesis of ribosomal protein (17), it was also of interest to see whether or not the addition of cycloheximide significantly altered the pattern of accumulation of precursor and stable species during sporulation. The effect of the addition of 250  $\mu\text{g}$  of cycloheximide per ml is shown in Fig. 6. The processing of RNA during the chase experiments was identical to that previously observed in the absence of cycloheximide for the first hour (Fig. 5). At 120 min, however, the cycloheximide treated samples did not show any accumulation of 26 or 18S RNA, even though both precursor peaks accumulated to a very significant extent. After 240 min, the pattern still does not reveal the presence of any mature ribosomal RNA species. In addition, the 27S peak appears to have been substantially lost, whereas the 20S peak remains. It is also interesting to note that the addition of cycloheximide apparently increases the total incorporation of  $^{32}\text{P}$  into RNA during this experiment.

## DISCUSSION

One of the most intriguing features of the sporulation of *S. cerevisiae* has been the accumulation of a unique 20S RNA species which accumulates to 4 to 5% of the total RNA. In the present paper, evidence is provided that the majority of this 20S RNA fraction contains nucleotide sequences homologous to that of the mature 18S rRNA. In this respect, the 20S RNA from sporulation may be closely related to the 20S RNA reported by Udem and Warner (17) as an intermediate in the processing of 18S rRNA in yeast. If 20S were a precursor of 18S rRNA, one would expect that (i) it would hybridize with nuclear cistrons from RNA and, (ii) that this hybridization would be substantially reduced when competed by mature 18S rRNA. From the studies of Schweizer et al. (15) and Rytel and Planta (13), there are approximately 140 cistrons for the 18S rRNA in the haploid genome of yeast (saturation plateau of 0.8% of the genome). As shown in Fig. 3, the saturation plateau of the

nine per ml were transferred into sporulation medium. After 6 hr,  $^{32}\text{PO}_4$  was added at 0.5 mCi/ml for 15 min, followed by an addition of excess inorganic phosphate. The RNA was extracted at the times indicated and analyzed as indicated in Materials and Methods. The vertical bar centered at 3.2 cm indicates the position of 26S RNA, and the bar at 6.8 cm is the peak position of 18S RNA, as determined from the vegetative steady-state label (---).

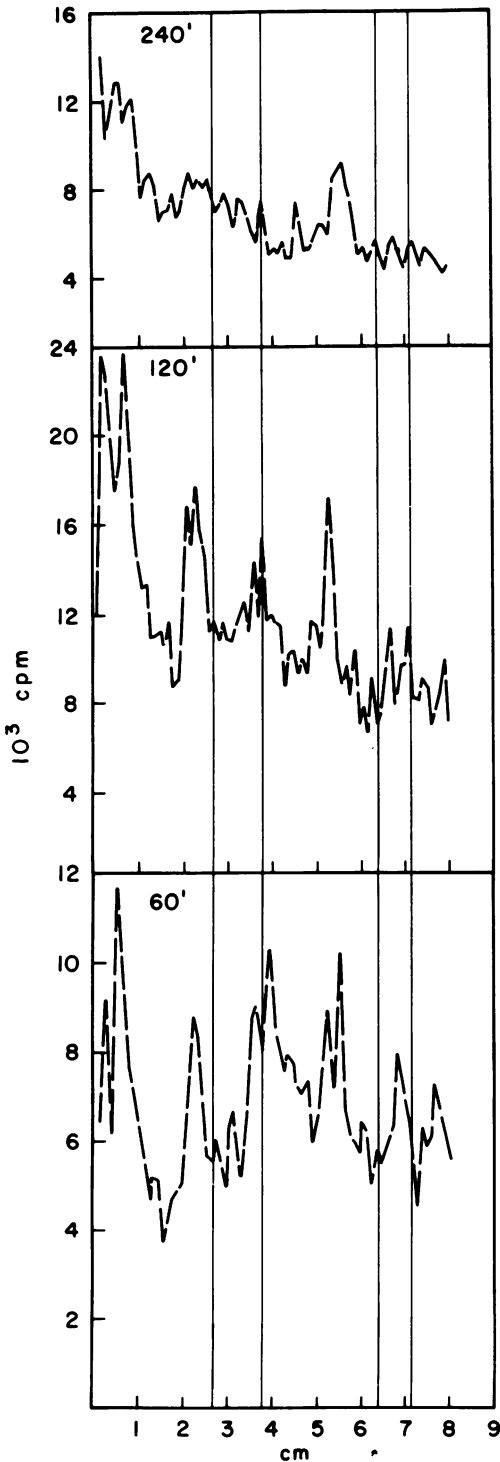


FIG. 6. Accumulation of  $^{32}\text{P}$  into ribosomal RNA in sporulating cultures of *S. cerevisiae* inhibited by cycloheximide. The conditions for this experiment

20S RNA with nuclear DNA is slightly greater than that of 18S rRNA. A precise measurement of the number of nuclear cistrons for 20S rRNA is difficult because of the lack of the exact molecular weight of the 20S rRNA and the limited supply available for careful quantitative measurements of the hybridization plateaus. If both 20S and 18S RNA are essentially single species of RNA, then the fact that they both hybridize to similar plateau levels indicates that there are approximately the same number of cistrons (140 per haploid genome) for each of these species. If the 20S species contain the entire 18S nucleotide sequence as well as a nonconserved region, the hybridization plateau for 20S should be greater than that for 18S. From inverse plots of data in Fig. 3 (not shown), 20S RNA hybridizes approximately 40% more than the 18S rRNA, which may reflect the presence of a nonhomologous RNA component.

From the competition experiments, one can estimate that 30% of the 20S RNA is not homologous with 18S. These findings, as well as those from the saturation experiments, indicate that 20S RNA is larger than 18S and that 60 to 70% of the two molecules are homologous.

From the kinetics of rRNA synthesis, it is evident that rRNA accumulation in the sporulating cell is quite slow as compared to that of the vegetative cell. In vegetative cells, RNA processing from the initial appearance of a 35S precursor to the formation of two stable rRNA species is complete in approximately 20 min (17). In the experiments presented here, the appearance to 35S RNA is observed after approximately 20 min; however, the formation of mature rRNA does not accumulate to a large extent until 60 min, and, in fact, precursor RNA species during the chase experiment are observed as late as 120 min in the chase experiment. The stable pattern of rRNA synthesis observed in a steady-state label cell during sporulation is not found until after 240 min of chase. During the chase experiments, the rRNA precursor peaks of 27S and 20S accumulate prior to the accumulation of the two mature rRNA species. During sporulation, the rate of protein synthesis is markedly reduced over the level found in the vegetative cell.

were identical to those of Fig. 5 except that 250  $\mu\text{g}$  of cycloheximide per ml was added simultaneously with the addition of the excess inorganic phosphate. The vertical lines indicate the position of 26 to 18S ribosomal RNA synthesized during the presporulation growth.

Udem and Warner (17) reported that the processing of vegetative rRNA to mature species is dependent upon protein synthesis. In the presence of cycloheximide, the ribosomal RNA precursors accumulated, but the appearance of the mature 26 and 18S species was not observed. Subsequent incubation of vegetative cells in the presence of cycloheximide also led eventually to the breakdown of both the 27 and 20S precursors with no accumulation into the stable species. Sporulated cells treated with cycloheximide exhibit very similar behavior. In the presence of cycloheximide, no accumulation of the mature 26S and 18S ribosomal RNA species was found. As shown in Fig. 6, however, the 20S RNA does not break down with subsequent incubation, whereas it appears that the 27S species is degraded over long time. From these results, we conclude that by polyacrylamide gel electrophoresis it is impossible to distinguish between the precursor 20S RNA which is accumulated by cycloheximide and the 20S RNA which remains stable during sporulation. By analogy with the results found in the vegetative cell, however, it would be expected that the precursor RNA which is accumulated in the presence of cycloheximide should be methylated, since, at least in the vegetative cell, all of the precursor RNA species are methylated prior to conversion to the mature species (17). Because of the difficulty of incorporating a significant amount of labeled methyl groups into RNA during a pulse experiment in sporulation, it was not possible to confirm directly this observation in the sporulating cell.

The interesting feature of the sporulation system is that submethylated 20S RNA accumulates in spite of the fact that 18S RNA as well as the larger species are methylated. It is possible that during sporulation at least a fraction of the ribosomal RNA cistrons are transcribed but not methylated. Vaughan et al. (19) have shown that in HeLa cells methionine starvation led to a deficiency in methylation and the accumulation of submethylated large precursor ribosomal RNA which was not processed to the final stable species. The appearance of methyl label into ribosomal RNA of the HeLa cells closely accompanies transcription (10, 17). Furthermore, methylation is restricted to the conserved regions (18 and 28S) of the ribosomal precursor. It is not unreasonable to propose that methylation may be required for final processing. For example, the processing of ribosomal RNA might require the attachment of some ribosomal proteins, as indicated by the accumulation of precursors

when protein synthesis is inhibited. The methylated sequences may be areas to which these proteins are bound.

Therefore, one could propose that methylation is limiting and that in some species only a fraction of the 35S ribosomal precursor molecules are methylated. The unmethylated 20S segments then would be restricted in further processing. Some molecules of 35S are non-methylated, and the 27S precursor portion is more readily degraded than the 20S due to differences in secondary structure. It is also possible that the appearance of both unmethylated 20S RNA and methylated 18S RNA may arise from two subpopulations of cells within the sporulation culture. At the present time, it is not possible to distinguish between these possibilities.

#### ACKNOWLEDGMENTS

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#### LITERATURE CITED

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