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Methionine-Dependent Synthesis of Ribosomal Ribonucleic Acid During Sporulation and Vegetative Growth of *Saccharomyces cerevisiae*

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Methionine limitation during growth and sporulation of a methionine-requiring diploid of *Saccharomyces cerevisiae* causes two significant changes in the normal synthesis of ribonucleic acid (RNA). First, whereas 18*S* ribosomal RNA is produced, there is no significant accumulation of either 26*S* ribosomal RNA or 5.8*S* RNA. The effect of methionine on the accumulation of these RNA species occurs after the formation of a common 35*S* precursor molecule which is still observed in the absence of methionine. During sporulation, diploid strains of *S. cerevisiae* produce a stable, virtually unmethylated 20*S* RNA which has previously been shown to be largely homologous to methylated 18*S* ribosomal RNA. The appearance of this species is not affected by the presence or absence of methionine from sporulation medium. However, when exponentially growing vegetative cells are starved for methionine, unmethylated 20*S* RNA is found. The 20*S* RNA, which had previously been observed only in cells undergoing sporulation, accumulates at the same time as a methylated 18*S* RNA. These effects on ribosomal RNA synthesis are specific for methionine limitation, and are not observed if protein synthesis is inhibited by cycloheximide or if cells are starved for a carbon source or for another amino acid. The phenomena are not marker specific as analogous results have been obtained for both a methionine-requiring diploid homozygous for *met13* and a diploid homozygous for *met2*. The results demonstrate that methylation of ribosomal RNA or other methionine-dependent events plays a critical role in the recognition and processing of ribosomal precursor RNA to the final mature species.

The conditions that enable diploid cells of *Saccharomyces cerevisiae* to sporulate are considerably different from those that support vegetative growth (6, 7, 18, 28). Diploid cells that have been preadapted to growth on an oxidative carbon source will sporulate when placed in a medium such as potassium acetate in the absence of a nitrogen source (3, 4, 6). Sporulation occurs under pseudo-starvation conditions so that continued biosynthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein depends on the depletion of preexisting pools of nucleotides and amino acids within the cell, and on greatly increased turnover of nucleic acids and protein (3, 5). Compared to vegetative growth doubling times of 2 to 3 h, sporulation is a slow process in which premeiotic DNA synthesis is not completed until approximately 10 h, and the appearance of mature ascospores requires about 20 h (3, 5).

Only diploid cells heterozygous for the mating type alleles (*a*/α) are capable of completing sporulation (22). Premeiotic DNA synthesis occurs in *a*/α cells, but not in diploids of *a*/*a* or α/α genotype (23). However, there is very little difference between sporulating and nonsporulating diploid strains in terms of the synthesis of lipids (9), carbohydrates (12), or protein and total RNA (10). Few sporulation-specific biochemical events have been identified.

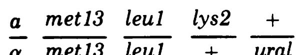
One sporulation-specific event that has received attention is the appearance of 20*S* RNA (11, 26). This species has been found only in *a*/α diploids and not in haploid or diploid strains placed in sporulation medium (11). The new species appears during the period that 26*S* and 18*S* ribosomal RNA (rRNA) are synthesized, and accumulates as a stable species. 20*S* RNA was shown by hybridization to be approximately 70% homologous to 18*S* ribosomal RNA; however, unlike rRNA in yeast, this 20*S* RNA is nearly unmethylated (26). This methyl-deficient, sporulation-specific 20*S* RNA is thus

quite different from a transient, methylated 20S precursor to 18S RNA found in vegetative cells (30).

Of the possible changes in the environment and physiology of sporulating cells that might cause the appearance of unmethylated 20S RNA, a restriction in the degree of methylation of some of the ribosomal precursor RNA seemed to us most likely. We therefore constructed methionine-requiring a/α diploid strains to determine whether the level of accumulation of 20S RNA is dependent on the availability of a methyl donor. We also examined the possibility that methionine limitation of vegetative cells would also result in the appearance of unmethylated 20S RNA. Our results show that methionine-dependent events play a central role not only in the appearance of unmethylated 20S RNA in vegetative cells, but also in the maintenance of an equimolar accumulation of 26S and 18S ribosomal RNA. We have interpreted these observations in terms of a model for rRNA processing in which the probability of complete methylation of precursor RNA determines the ratio of 26S, 20S, and 18S RNA.

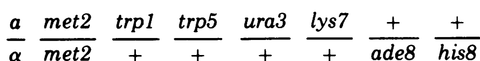
MATERIALS AND METHODS

Strains. Two diploid strains of *S. cerevisiae* were used in this study. Strain J1, methionine and leucine requiring, has the following genotype:



$$\frac{a}{\alpha}\ \frac{met13}{met13}\ \frac{leu1}{leu1}\ \frac{lys2}{+}\ \frac{+}{ura1}$$

Strain J2, methionine requiring, has the following genotype:



$$\frac{a}{\alpha}\ \frac{met2}{met2}\ \frac{trp1}{+}\ \frac{trp5}{+}\ \frac{ura3}{+}\ \frac{lys7}{+}\ \frac{+}{ade8}\ \frac{+}{his8}$$

Parent haploid strains were from the Yeast Stock Center at Berkeley, Calif.

Cultivation. Strains were usually grown at 30 C in liquid YEPD medium (1% yeast extract, 2% peptone [Difco], and 2% dextrose) or minimal medium (0.6% yeast nitrogen base without amino acids [Difco], 2% dextrose). Minimal medium was supplemented where indicated with 0.33 mM L-leucine and 0.13 mM L-methionine. In some experiments cells were grown vegetatively in acetate growth medium initially developed by Roth and Halvorson (23) and modified by Küenzi et al. (14). This medium (AC) contains 1% potassium acetate, 0.6% yeast nitrogen base without amino acids, 0.5% yeast extract, 0.5% peptone, and 1.02% potassium biphthlate, pH 5.5.

Sporulation. Cells were grown either to stationary phase in YEPD medium (approximately 2.2×10^8 cells/ml) or exponentially in AC medium to 5×10^7 cells/ml. The cells were harvested, washed twice in distilled water, and resuspended at a density of approximately 2×10^7 cells/ml in 1% potassium acetate, pH 7, containing leucine or methionine as indicated. For sporulation, cells were aerated at 30 C on a gyratory shaker in Erlenmeyer flasks with a volume capacity 10 times larger than the volume of liquid sporulation medium used. Sporulation was monitored by counting the percent of asci among cells in the population.

Isolation of RNA. Cells were harvested by centrifugation, washed in water, and suspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), 0.1 m NaCl, 0.003 M sodium ethylenediaminetetraacetate (EDTA), 20% glycerin, and 1 M sorbitol, and then broken in a French press by the method described by Bhargava and Halvorson (1). The lysate was mixed with an equal volume of distilled, water-saturated phenol preserved with 0.1% 8-hydroxyquinoline. The emulsion was shaken in a wrist-action shaker at 4 C for 15 min, and the phases were separated by centrifugation for 10 min in a Sorvall HB4 swinging-bucket rotor at 8,000 × *g*. The aqueous layer was retained and extracted with phenol twice more. The final aqueous layer was extracted four times with an equal volume of anhydrous ether to remove phenol. The aqueous phase was then mixed with 2 volumes of cold ethanol and RNA-precipitated for 1 h at −20 C. The precipitate was collected by centrifugation at 8,000 × *g* for 10 min, resuspended in TES buffer (0.01 M Tris [pH 7.4], 0.1 M NaCl, and 0.003 M EDTA), and reprecipitated by ethanol to remove some carbohydrate. The second precipitate was collected by centrifugation and dried in a desiccator under vacuum at room temperature. The dried RNA was resuspended in TES buffer.

Polyacrylamide gel electrophoresis. Polyacrylamide disk gels were prepared by the method of Loening (15). In general, large rRNA species were resolved by the electrophoresis of RNA on 3% polyacrylamide gels, 0.9 by 10 cm, run for 12 h at 8 mA per gel. Examination of smaller-molecular-weight RNA was carried out on 4% polyacrylamide gels run for 3 h at 10 mA per gel.

Frozen gels were sliced into 1-mm sections on a Joyce-Loebel gel slicer. For gels which contained ^{32}P and ^{14}C, the slices were dried on filter paper strips and counted in a Beckman LS 250 liquid scintillation counter using PPO-POPOP [4.0 g of 2,5-diphenoloxazole per liter and 0.5 g of 1,4-bis-2-(5-phenyloxazolyl)benzene per liter dissolved in toluene].

Gels containing tritium were analyzed by placing each slice in a disposable 3-dram (about 10 ml) glass vial to which was added 1 ml of a solution containing 58.5% Protosol (New England Nuclear), 39% toluene, and 2.5% water (J. Warner, personal communication). The vials were capped and gently agitated at room temperature for at least 6 h. The treated gel slices were then counted in a liquid scintillation counter after the addition of 8.5 ml of PPO-POPOP.

Polyacrylamide gels were also analyzed by densitometry. Gels were stained with a 0.01% toluidine blue in 1% acetic acid for 8 h and destained by soaking in 1% acetic acid for 20 h with several changes of 1% acetic acid. The gels were scanned in a Joyce-Loebel double-beam recording microdensitometer.

Preparation of ribosomal subunits. Cells were grown in YEPD medium to stationary phase and resuspended in sporulation medium with or without

methionine for 12 h. Cells (25 ml) were harvested, washed, and resuspended in 5 ml of buffer containing 0.1 m NaCl, 0.01 M Tris (pH 7.4), and 0.05 mM $MgCl_2$. After breaking the cells at 4 C in the French pressure cell at 6000 lb/in^2, 0.5% sodium deoxycholate and 0.5% Brij-58 were added. Cellular debris was removed by centrifugation at 12,000 × *g* for 10 min, and 0.5 ml of the supernatant was layered on a 16-ml, 10 to 47% sucrose gradient containing the buffer specified above. The ribosomal subunits were separated by centrifugation at 25,000 rpm in an SW27.1 rotor for 6 h.

Measurement of protein synthesis. The incorporation of radioactive amino acids into protein was determined by the method of Rodenberg et al. (21). Generally, samples were incubated with [^{3}H]lysine, quenched with an equal volume of cold 10% trichloroacetic acid, and then boiled for 30 min. The samples were then filtered on membrane filters (Millipore Corp.), and washed with cold 5% trichloroacetic acid containing 25 μg of L-lysine per ml and then 2 ml of cold 95% ethanol. The dried filters were counted in a liquid scintillation counter with PPO-POPOP.

Radioisotopes. [8-^{14}C]adenine (6.53 mCi/mmol), [2-^{3}H]adenine (8.06 Ci/mmol), L-[*methyl*-^{3}H]methionine (6.3 Ci/mmol), L-[4,5-^{3}H(N)]lysine (55 Ci/mmol), and carrier-free [^{32}P]phosphoric acid were obtained from New England Nuclear.

Materials. Acrylamide, bis-acrylamide, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Eastman Kodak; L-methionine, L-leucine, 8-hydroxyquinoline, and S-adenosyl methionine were obtained from Sigma Chemical Co.; potassium acetate and dextrose were from Fisher Scientific Co.; and potassium biphthalate was from Baker.

RESULTS

The diploid strain J1, which requires both methionine and leucine, was used to study the effect of methionine limitation on the synthesis of rRNA during sporulation. Sporulation of strain J1 depended on the addition of methionine to the medium, as shown in Table 1. The pattern of rRNA synthesis was also changed by the availability of methionine (Fig. 1). In the presence of methionine, the gel electrophoresis pattern of RNA which accumulates during sporulation is similar to that observed with other diploid strains (11, 26). 26*S* RNA and 18*S* RNA were made in the same equimolar proportion as was found in vegetative cells; a significant amount of 20*S* RNA also appeared. On the other hand, when methionine was absent from sporulation medium, the accumulation of rRNA became asymmetric (Fig. 1B). Newly synthesized 18*S* RNA and 20*S* RNA accumulated during incubation in sporulation medium, but virtually no new 26*S* RNA was found.

TABLE 1. *Effect of amino acid limitation on sporulation of J1*[a]

Addition to sporulation medium	Sporulation (%)
Methionine and leucine	58
Methionine without leucine	47
Leucine without methionine	0
None	0

[a] J1 was grown to stationary phase and resuspended in 1% KAc, ph 7, which was supplemented with 0.15 mM methionine and/or 0.3 mM leucine. Sporulation was measured after 48 h.

The effect of methionine limitation during sporulation appeared to be primarily on the de novo synthesis of 26*S* RNA. The 26*S* RNA labeled with ^{14}C during vegetative growth was not selectively degraded, and was still found in an equimolar ratio with ^{14}C-labeled 18*S* RNA. Methionine limitation had very little effect on the total amount of 18*S* RNA synthesized in the presence or absence of methionine, as seen by a comparison of the amount of ^{32}P-labeled 18*S* RNA synthesized during sporulation relative to 18*S* labeled with ^{14}C during the vegetative phase (Fig. 1). It should be emphasized that the amount of new rRNA synthesized during sporulation represented approximately 50% of the total rRNA found at the end of sporulation. This was clearly illustrated by densitometer tracings of stained polyacrylamide gels of the RNA isolated after incubation in the presence or absence of methionine (Fig. 2). The lack of new 26*S* RNA synthesis in the absence of methionine was reflected in a markedly reduced 26*S* RNA peak relative to 18*S* RNA (Fig. 2B). The pattern was identical to that which would be obtained by adding the ^{14}C and ^{32}P patterns in Fig. 1. Thus, methionine limitation during sporulation of strain J1 resulted in a lack of accumulation of 26*S* RNA without significant alteration of the synthesis of 18*S* rRNA.

Appearance of 18S RNA in ribonucleoprotein particles. The 18*S* RNA that was produced during sporulation in the absence of 26*S* RNA accumulation sedimented as a particle that is quite similar to a normal ribosomal subunit. This is shown clearly in the sedimentation of 60*S* and 40*S* ribosomal subunits in sucrose gradient (Fig. 3). Cells that were prelabeled during vegetative growth with [^{14}C]adenine were incubated in sporulation medium containing ^{32}P, in the presence and absence of methionine. After 12 h the cells were harvested and the ribosomal subunits were prepared as described in Materials and Methods. When methionine was present during sporulation, newly synthesized RNA appeared in both the 60*S* and 40*S* ribosomal subunits (Fig. 3A). However, when cells were incubated in the

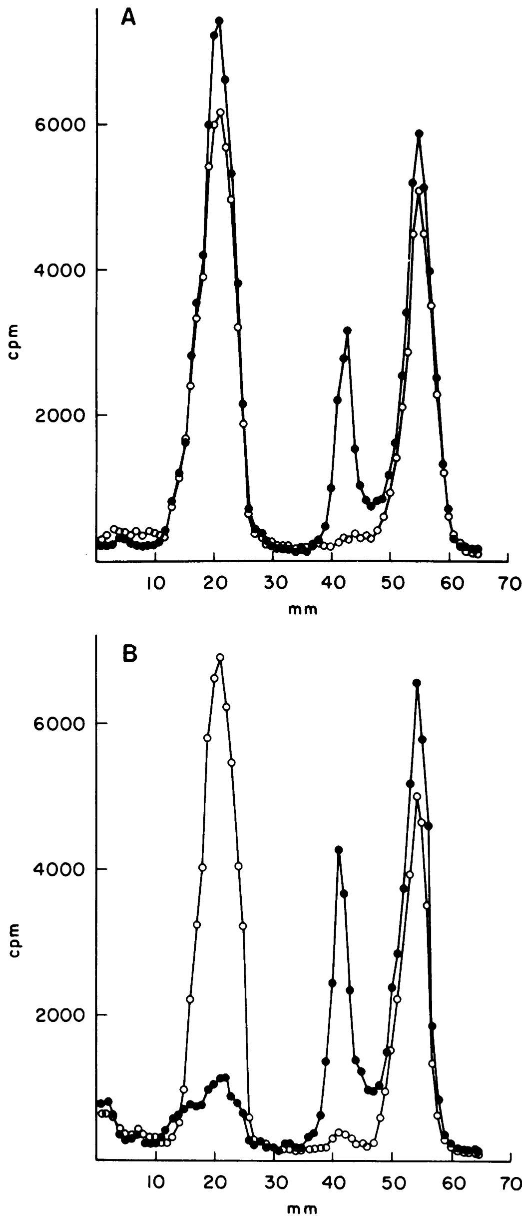


FIG. 1. *The effect of added methionine on RNA synthesis during sporulation of strain J1. Cells were grown to stationary phase in the presence of 0.2 μCi of [^{14}C]adenine per ml, washed, and resuspended in 1% potassium acetate (pH 7), containing 0.33 mM L-leucine and 0.13 mM L-methionine (A) or no additional methionine (B). RNA synthesized during sporulation was labeled with 0.2 μCi of [^{32}P]phosphoric acid per ml, added at T_0. Cells were harvested after 16 h (T_{16}), and RNA was extracted and run on polyacrylamide gels. (A) RNA synthesis during sporulation in the presence of L-methionine. RNA labeled during vegetative growth is found in 26S and 18S RNA (○), whereas RNA synthesized during sporulation (●) is found in 26S, 20S, and 18S RNA. (B) RNA synthesis during sporulation in the absence of added methionine. Although both ^{14}C-labeled 26S and 18S RNA from vegetative growth are found (○), virtually no ^{32}P-labeled 26S RNA accumulates during sporulation (●).*

absence of methionine, newly synthesized RNA was found incorporated only into the 40*S* RNA region (Fig. 3B). This experiment indicated that 18*S* RNA (and possibly 20*S* RNA) made in the absence of 26*S* RNA was nevertheless incorporated into a particle similar, if not identical to, the 40*S* ribosomal subunit.

Concentration range of methionine dependence of sporulation and RNA synthesis. When small amounts of methionine were added to sporulation medium, both the percent of sporulation and the amount of 26*S* RNA which accumulated relative to 18*S* production increased. Sporulation and 26*S* RNA exhibited very similar methionine concentration dependence (Fig. 4). For example, if 0.05 mM methionine was added to the medium, the percent of sporulation was approximately 80% of that observed when higher concentrations of methionine were added, and the amount of 26*S* RNA that accumulated was about 85% of maximum (Fig. 5).

Methionine dependence for sporulation was also observed in another methionine-requiring diploid, J2 (homozygous for *met2*). Methionine dependence during sporulation of both strains J1 and J2 was also observed when cells were sporulated after exponential vegetative growth in acetate medium.

Effect of methionine concentration on 20S RNA. The 20*S* RNA synthesized during sporulation was found even in the presence of high concentrations of methionine; however, one could observe a change in the proportions of 20*S* RNA and 18*S* RNA over a range of methionine concentration. In the absence of added methionine, strain J1 synthesized 20*S* and 18*S* RNA in a ratio of 0.51 (Fig. 1B). The presence of 200 mM methionine in sporulation medium resulted in a ratio of 0.31 (Fig. 1A), whereas at an intermediate methionine concentration (Fig. 5) the ratio was 0.38. Under sporulating conditions, the amount of 20*S* RNA synthesized by a methionine-requiring diploid could be reduced by the addition of methionine, but not abolished.

Methionine dependence of 5.8S RNA synthesis. Udem and Warner (30) demonstrated that 5.8*S* RNA found in the yeast ribosome is closely associated with 26*S* RNA and is derived from the same 27*S* ribosomal RNA precursor

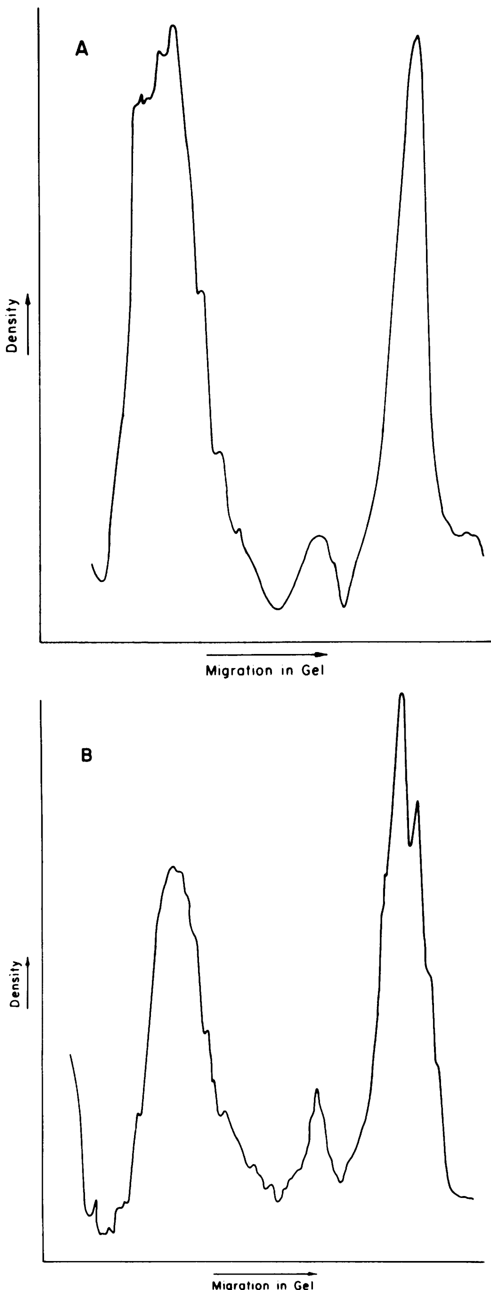


FIG. 2. *Total rRNA present after sporulation for 16 h in the presence (A) or absence (B) of L-methionine. Samples of RNA prepared for the experiment shown in Fig. 1 were also analyzed by staining polyacrylamide gels with toluidine blue and measuring total RNA as described in Materials and Methods. (A) The ratio of 26S to 18S RNA after strain J1 was sporulated in the presence of methionine is approximately 1.85. (B) The ratio of 26S to 18S after sporulation in the absence of methionine is approximately 0.95.*

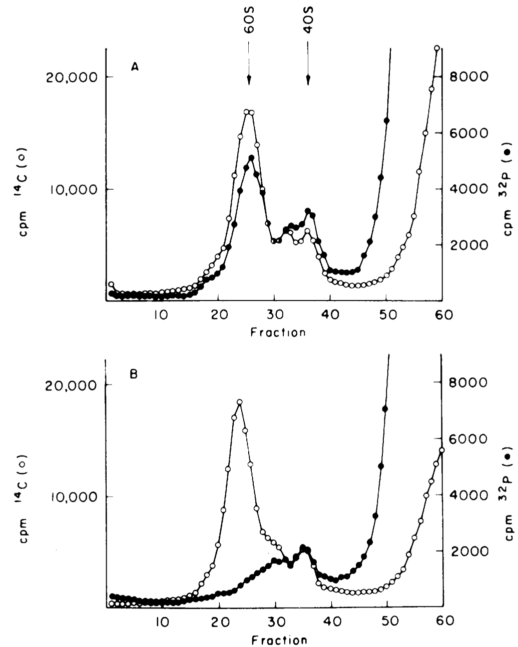


FIG. 3. *Separation of ribonucleoprotein particles formed after sporulation in the presence and absence of methionine. Cells were prelabeled during vegetative growth with 0.2 μCi of [^{14}C]adenine per ml and labeled during sporulation with 0.4 μCi of [^{32}P]phosphoric acid per ml. After 12 h, cells were harvested and ribosomal subunits were prepared as described in Materials and Methods. (A) Both 60S and 40S particles containing ^{32}P-labeled RNA are found after sporulation of strain J1 in the presence of methionine. (B) Only 40S particles labeled with ^{32}P are found after incubation in the absence of methionine.*

molecule. It was, therefore, of interest to determine whether the accumulation of 5.8*S* RNA during sporulation was also methionine dependent. Labeled RNA was extracted from cells sporulating in the presence or absence of methionine and separated by gel electrophoresis. Figure 6 shows the region of the polyacrylamide gels containing 5.8*S*, 5*S*, and 4*S* RNA. 5.8*S* RNA accumulated only when methionine was added to the acetate sporulation medium. The diminished synthesis of 5*S* RNA in strain J1 was also observed in other strains undergoing sporulation. There was no significant effect of the concentration of methionine on the accumulation of 4*S* RNA. The absence of newly synthesized 5.8*S* RNA without the addition of methionine indicated that the accumulation of 26*S* RNA and 5.8*S* RNA was coordinately regulated.

Kinetics of methionine-dependent RNA synthesis. Measurements of the total accumulation of rRNA after 16 h of sporulation did not demonstrate whether the lack of accumulation

of 26S RNA resulted from alterations of normal transcription or from changes during the processing of precursors of rRNA. Labeling for shorter periods during sporulation indicated that the lack of accumulation of 26S RNA was most likely due to a post-transcriptional event.

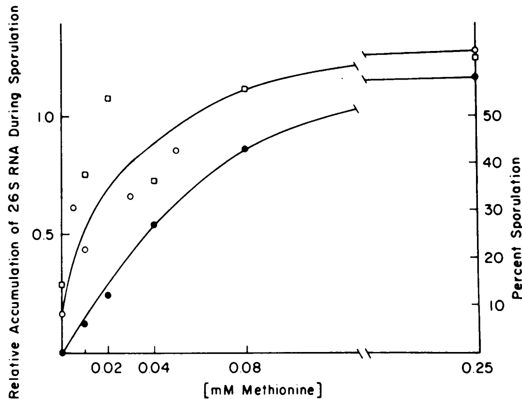


FIG. 4. *Effect of increasing L-methionine concentration on sporulation (●) and relative 26S RNA accumulation (O, □). For each measurement, cells were labeled with [^{14}C]adenine during vegetative growth and with ^{32}P during sporulation (cf. Fig. 1) in the presence of the indicated additional L-methionine. After 16 h, cells were harvested and the RNA was extracted and run on polyacrylamide gels. The accumulation of ^{32}P-labeled 26S RNA was then normalized by dividing by the ratio of ^{14}C-labeled 26S to 18S RNA. The percent of sporulation was determined after 48 h.*

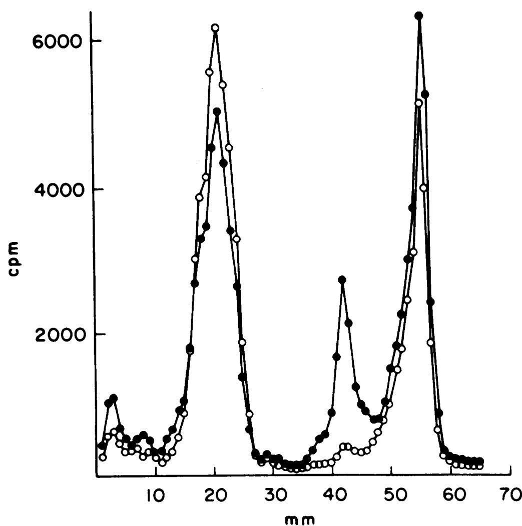


FIG. 5. *Accumulation of 26S RNA during sporulation in the presence of 50 × 10^{-6} M L-methionine. Cells labeled vegetatively with [^{14}C]adenine (O) and during sporulation with [^{32}P]H_3PO_4 (●) were analyzed as described in Fig. 1.*

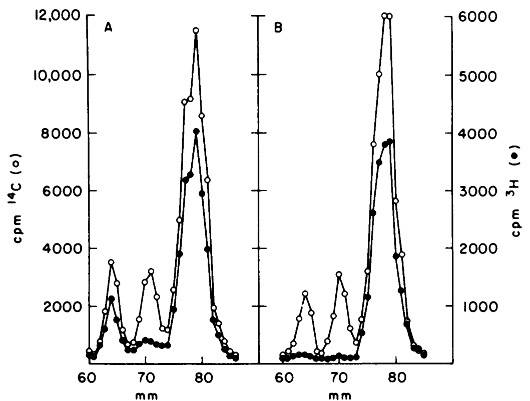


FIG. 6. *Effect of methionine deprivation on the synthesis of 5.8S, 5S, and 4S RNA during sporulation of strain J1. Strain J1 was prelabeled with 2 μCi of [^{14}C]adenine per ml (O) and labeled during sporulation with 20 μCi of [^{3}H]adenine per ml (●) for 17 h. (A) In the presence of 0.15 mM L-methionine, 5.8S and 4S RNA both accumulate. (B) In the absence of added methionine, virtually no 5.8S RNA is synthesized.*

Because rRNA synthesis is much slower during sporulation than during vegetative growth (26), labeling of RNA for a 2-h interval yields a pattern on gel electrophoresis in which the 35S initial ribosomal precursor, 27S and 20S secondary precursors, and the mature 26S and 18S species are normally all seen. After 6.5 h in sporulation medium, strain J1 was labeled with [^{3}H]adenine for 2 h and the RNA was extracted for gel electrophoresis (Fig. 7). RNA synthesized in the presence or absence of methionine contained a distinct 35S rRNA precursor. As compared to RNA synthesized in the presence of methionine, the 27S precursor in the methionine-limited RNA extract was distinctly diminished, and virtually no 26S RNA was seen. The rate of degradation of 26S RNA or its 27S precursor must have been rapid ($t_{1/2}$ < 1 h) to yield no 26S RNA in the 2-h labeling interval.

The presence of a 35S RNA component even when 26S RNA was not found suggested that the effects of methionine limitation during sporulation were exerted after the transcription of a complete 35S precursor. A clearer demonstration could be accomplished by following the fate of labeled 35S precursor RNA in the absence of additional RNA synthesis. After incubation of J1 without methionine for 6.5 h, cells were labeled for 40 min with [^{3}H]adenine and then allowed to continue RNA processing, after the addition of a large excess of unlabeled adenine to prevent further accumulation of labeled RNA. After 60 min (Fig. 8), tritium radioactivity was found primarily in the 35S precursor

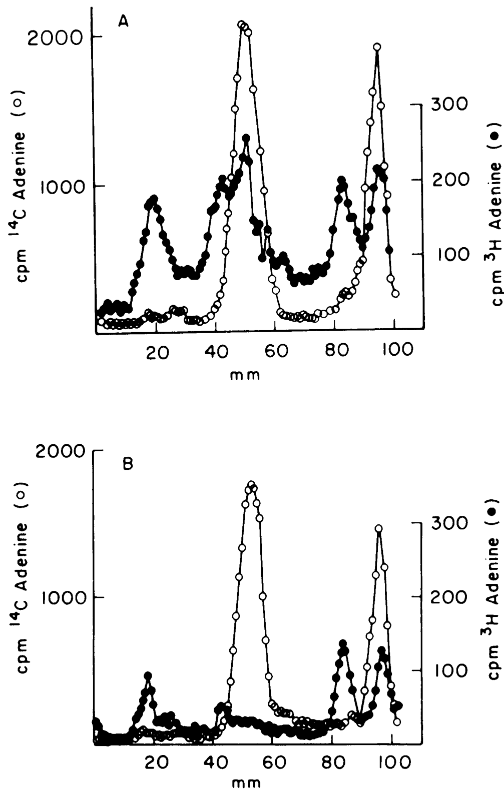


FIG. 7. *Accumulation of labeled RNA for 2 h during sporulation in the presence (A) or absence (B) of methionine. Stationary phase strain J1 labeled with 0.25 μCi of [^{14}C]adenine per ml was washed and resuspended in 1% potassium acetate plus leucine, with or without methionine. After incubation for 6.5 h, the cells were labeled with 13.3 μCi of [^{3}H]adenine per ml for 2 h, after which RNA was extracted and separated by polyacrylamide gel electrophoresis.*

region of the gel although some counts were found in 27S and 20S precursor regions. By 120 min, the 35S component was greatly reduced and a significant accumulation of 18S and 20S RNA was seen. No 26S RNA was found, however, and the amount of 27S precursor was also small. By 240 min, the distribution of label was similar to that found after long periods of labeling. The shoulder to the left of the 20S RNA peak in the 240-min profile may have resulted from an unstable breakdown product of some of the larger precursor forms, as it was not seen in longer labeling experiments. This pulse-chase experiment again suggested that normal 35S precursor RNA is transcribed, but that subsequent processing—either to the 27S precursor or to the final 26S form—is altered. The processing of the 35S transcript to 20S and 18S RNA was apparently not methionine-dependent.

Relation of methionine limitation to protein synthesis. The effects of methionine limitation during sporulation did not simply reflect a cessation of protein synthesis. The addition of 200 μg of cycloheximide per ml after 6 h in sporulation medium containing methionine completely prevents the formation of ascospores and also leads to a cessation of rRNA production (26). Cells labeled with ^{32}P after 6 h in sporulation medium (T_6) were harvested at T_{12}

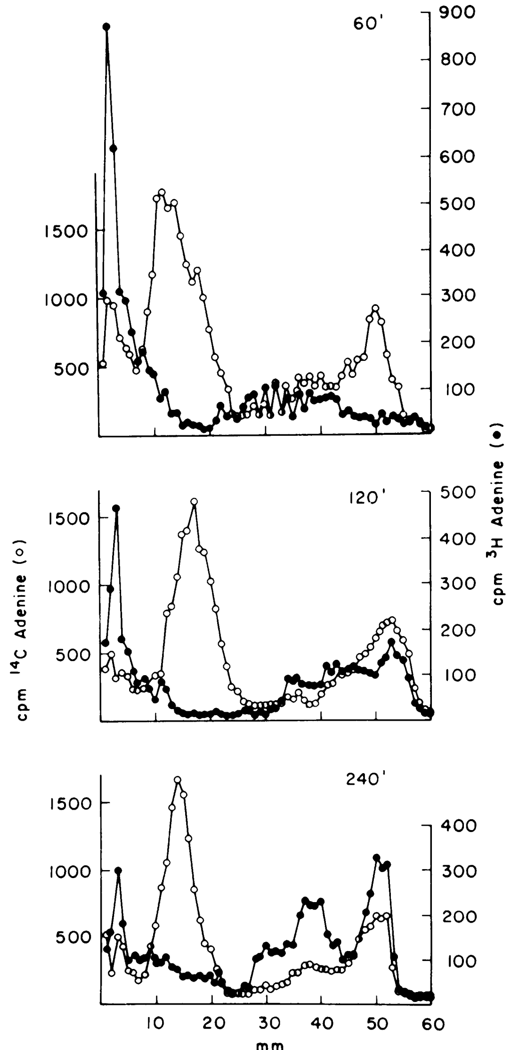


FIG. 8. *Pulse-chase labeling of RNA synthesized at $T_{6.5}$ during the sporulation of strain J1 in the absence of L-methionine. Cells were labeled with ^{14}C during vegetative growth, resuspended in 1% potassium acetate (pH 7), containing 0.3 mM leucine and labeled with [^{3}H]adenine, as described in Fig. 6. The accumulation of ^{3}H-labeled RNA (●) was monitored at 60, 120, and 240 min from the time of [^{3}H]adenine addition.*

and the RNA was extracted and characterized by polyacrylamide gel electrophoresis (Fig. 9). When protein synthesis was thus inhibited, neither 26*S* nor 18*S* RNA accumulated to a significant degree. A recognizable 20*S* peak was found, however. Inhibition of protein synthesis did not lead to the accumulation of 18*S* RNA in the absence of 26*S* RNA, as observed when methionine was limiting.

When protein synthesis was measured in sporulating strain J1 deprived of methionine, one found that the rate of incorporation of [^{3}H]lysine into trichloroacetic acid-insoluble material was between 70 and 80% of that observed in cells given methionine (Table 2). Thus, unless the synthesis of certain proteins essential for rRNA processing was especially methionine dependent, it seems that the primary effect of methionine limitation was not on the synthesis of proteins.

Methionine dependence of rRNA synthesis in vegetative cells. The accumulation of 18*S* rRNA without concomitant accumulation of newly synthesized 26*S* RNA was not restricted to cells undergoing sporulation in the absence of methionine. Very similar results were observed when J1 cells growing vegetatively were starved for methionine. In the experiment illustrated in Fig. 10, cells of strain J1 were grown exponentially in minimal medium supplemented with both methionine and leucine. Cells were centrifuged, washed twice in minimal medium, and resuspended in medium from which either methionine or leucine was absent. Under deprivation of either methionine or leucine, growth, as measured by an increase in optical density, was terminated by 45 to 50 min. RNA synthesis in this starving state was measured by adding [^{3}H]adenine at this point. Incorporation of adenine into RNA was allowed to continue for 3 h, at which time the cells were harvested and broken, and the RNA was extracted. The RNA synthesized in minimal medium supplemented with both amino acids was indistinguishable from the normal steady-state pattern (Fig. 10A). Cells supplemented with methionine but without leucine showed a drastically reduced synthesis of both stable rRNA species, but the ratio of 26*S* and 18*S* RNA remained approximately equimolar (Fig. 10C). On the other hand, cells supplemented with leucine but without methionine showed the same asymmetric production of rRNA that was observed during sporulation (Fig. 10B). In this case the ratio between ^{3}H-labeled 26*S* and 18*S* RNA was 0.53 versus 2.05 for [^{14}C]RNA labeled prior to starvation. The total amount of RNA synthesized during methionine starvation was also considerably less than that observed for cells supplemented with both methionine and leucine. Under methionine starvation during vegetative growth there was also an accumulation of a 20*S* RNA.

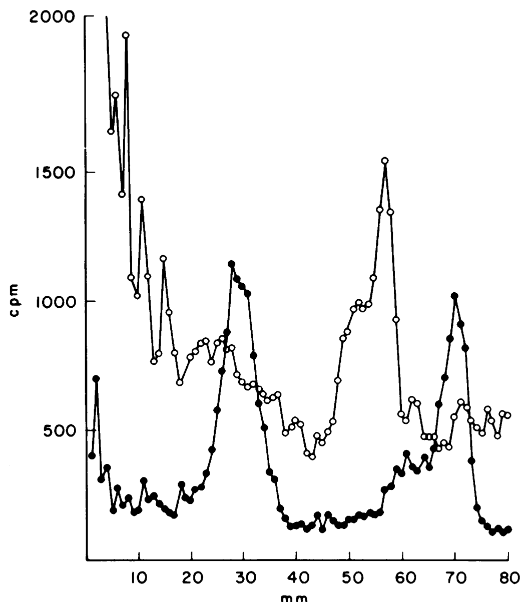


FIG. 9. *Effect of cycloheximide on accumulation of RNA during sporulation. Strain J1 labeled with 0.2 μCi of [^{14}C]adenine per ml was grown to stationary phase in YEPD medium and placed in sporulation medium in the presence of both 0.3 mM leucine and 0.15 mM methionine. At T_6, 1 μCi of [^{32}P]H_3PO_4 per ml and 200 μg of cycloheximide per ml were added and the cells were incubated until T_{12}. Only 20S RNA is found as stable ^{32}P-labeled species (O), whereas both 26S and 18S ^{14}C-labeled RNA (●) are found. No sporulation was found in cells incubated for 38 h.*

TABLE 2. *Effect of methionine deprivation on protein synthesis during sporulation of J1*[a]

Sporulation medium	Counts/min incorporated in 30 min			
	T_4	T_6	T_7	T_8
With methionine	1,197	1,002	625	394
Without methionine	1,403	882	570	281

[a] J1, grown to stationary phase in YEPD, was washed and placed in sporulation medium containing leucine but with or without methionine (see Materials and Methods). At intervals, duplicate 0.5-ml volumes were removed, mixed with 1.25 μCi of [^{3}H]lysine, and incubated at 30 C for 30 min. Samples were quenched with an equal volume of 10% trichloroacetic acid and assayed for incorporation into trichloroacetic acid-insoluble protein. Sporulation of J1 with methionine was 47% at T_{36} and 0% without methionine.

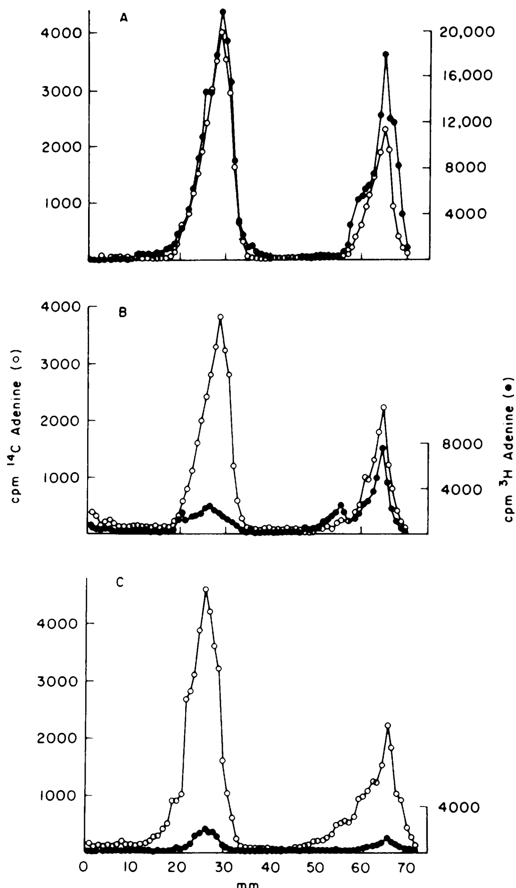


FIG. 10. *Accumulation of rRNA during amino acid starvation of vegetative cells. Strain J1 was grown exponentially in minimal medium containing leucine and methionine and 0.5 μCi of [^{14}C]adenine per ml. Cells were then washed in minimal medium and incubated in the absence of methionine (B) and leucine (C). After 45 min, 2 μCi of [^{3}H]adenine per ml was added to each culture, cells were harvested, and RNA was extracted after 3 more h. (A) Synthesis of RNA in the presence of both leucine and methionine. (B) Accumulation of ^{3}H-labeled RNA (●) in the absence of methionine. (C) Accumulation of ^{3}H-labeled RNA (●) in the absence of leucine.*

When the methionine-requiring diploid strain J2, homozygous for *met2*, was examined under similar conditions of vegetative starvation for methionine, exactly analogous results were obtained.

Lack of methylation of 20S RNA from methionine-limited vegetative cells. The appearance of a stable 20*S* RNA in vegetative cells raised an important question: is this 20*S* form fully methylated as is the normally short-lived vegetative precursor of 18*S* RNA (30), or is it unmethylated similar to that observed during sporulation? The degree of methylation of 20*S* RNA that accumulated during methionine starvation of vegetative strain J1 was examined by labeling with [*methyl*-^{3}H]methionine. The specific activity of the tritiated methionine was high enough so that the final concentration of methionine was low enough (0.2×10^{-3} mM) not to perturb the asymmetric accumulation of rRNA (cf. Fig. 3).

Vegetative J1 cells were starved for either methionine or leucine, as described above, and then labeled with [*methyl*-^{3}H]methionine and [^{14}C]adenine. After 3 h, the RNA was extracted and separated on polyacrylamide gels (Fig. 11). Cells starved for leucine showed an insignificant production of RNA and no incorporation of methyl groups into any RNA. On the other hand, cells starved for methionine (Fig. 11B) showed an accumulation of methyl label in the 18*S* fraction but not in the 20*S* component that was observed by ^{14}C incorporation. It appeared that the 20*S* RNA that could be produced under these vegetative starvation conditions was in fact similar to the 20*S* RNA observed during sporulation, in that neither was extensively methylated (Fig. 11B). This 20*S* species is found in vegetative cells only with methionine limitation of growth, and not when growth is inhibited by leucine starvation or by the addition of cycloheximide (30).

DISCUSSION

rRNA synthesis in *S. cerevisiae* is extremely sensitive to the availability of methionine. We have noted two different changes in RNA synthesis when methionine-requiring diploids of yeast are deprived of methionine. First, the normal equimolar accumulation of rRNA species becomes asymmetric, with only 18*S* RNA being produced. Second, methyl-deficient 20*S* RNA accumulates as a stable form.

Control of balanced rRNA synthesis by methionine. During normal vegetative growth or sporulation of *S. cerevisiae*, equimolar amounts of 26*S*, 18*S*, and 5.8*S* RNA are synthesized. Cells inhibited by cycloheximide or starved for a carbon source or the amino acid leucine have a greatly reduced production of rRNA, but an equimolar ratio is maintained (26, 30). The idea that the synthesis of rRNA and ribosomal subunits is tightly regulated has been given more support by studies of nine different mutants of yeast temperature sensitive for rRNA production (8) in which neither one ribosomal subunit nor one rRNA species accumulated in the absence of the other at the restrictive temperature (32).

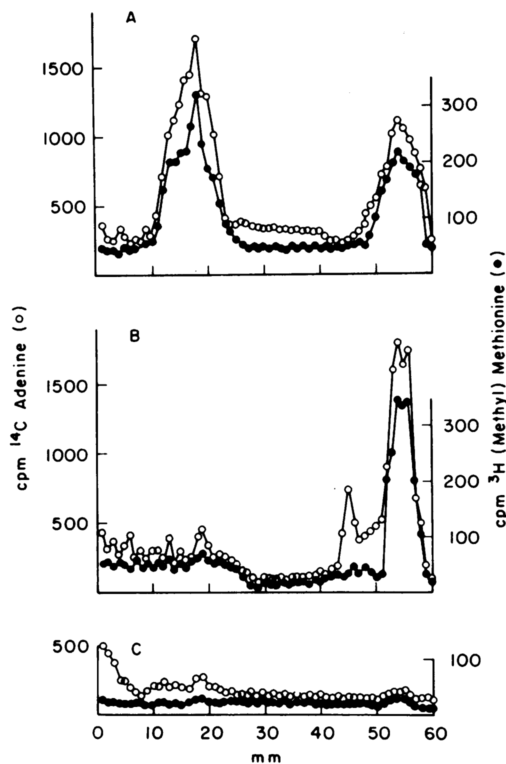


FIG. 11. *Methylation and synthesis of RNA during amino acid starvation of vegetative strain J1. Unlabeled cells were grown exponentially in minimal medium plus leucine and methionine, and resuspended in the absence of methionine (B) or leucine (C). After 45 min, both 1 μCi of [¹⁴C]adenine (O) and [methyl-³H]methionine (●) were added. For cultures A and C containing methionine, 10 μCi of [methyl-³H]methionine per ml was added; for B, without methionine, 1 μCi of methionine per ml was added. After 3 more h, RNA was extracted and analyzed by polyacrylamide gel electrophoresis. (A) After 3 h, only 26S and 18S RNA are present and methylated when cells are grown in the presence of leucine and methionine. (B) In the absence of methionine, both 20S and 18S RNA accumulate, while only 18S RNA is methylated. (C) In the absence of leucine, virtually no RNA synthesis or methylation is found.*

In contrast, when methionine-requiring strains are restricted for methionine, the synthesis of rRNA becomes markedly asymmetric—18*S* RNA is found without a concomitant formation of 26*S* or 5.8*S* RNA. The 35*S* precursor RNA common to all three rRNA species is synthesized even in the absence of methionine, so that the lack of accumulation of 26*S* and 5.8*S* RNA apparently occurs by a restriction in one or more processing steps leading to the 27*S* precursor or to the final 26*S* and 5.8*S* cleavage products. Because general protein synthesis inhibitors or other modes of inhibition do not yield an asymmetric accumulation of rRNA, we assume that the essential methionine-dependent process involves the methylation of rRNA.

Our results with *S. cerevisiae* differ from those reported for *S. carlsbergensis* (19), in which only the large (45*S*) precursor rRNA was found after methionine starvation. The difference most likely rests on the fact that Retel and Planta (19) examined yeast protoplasts starved for 3 h for methionine and then labeled for only 5 min with [*methyl*-³H]methionine.

The effects of methionine limitation on rRNA synthesis seen in yeast are also different from those reported in HeLa cells (31). In HeLa cells, methionine limitation leads to a reduction in both 18*S* and 28*S* RNA synthesis. Nevertheless, the results of methionine deprivation in HeLa cells are distinctly different from those observed when cells are inhibited by cycloheximide (33), puromycin (25), or starvation for valine (17). The clear distinction between methionine-dependent effects and those of other inhibitors led Vaughan et al. (31) to propose that methylation of ribosomal precursor RNA plays a crucial role in the accumulation of the mature ribosomal species. Although the details of rRNA processing in HeLa cells (16) and yeast are different, in both cases essential methionine-dependent steps can be inferred.

Recently a temperature-sensitive mutant in a hamster cell tissue culture line was described (29). At the restrictive temperature, these cells produce 18*S* RNA without 28*S* RNA, and the 32*S* precursor of 28*S* RNA is degraded. Whether this phenotype is methionine dependent is not known.

Synthesis of methyl-deficient 20S RNA. Stable, methyl-deficient 20*S* RNA can be found in vegetative cells starved for methionine. This observation clearly demonstrates that the formation of such 20*S* RNA is not a sporulation-specific event, as previously believed (7, 11, 26). There are, however, differences in the accumulation of this species in vegetative and sporulating cells. An understanding of these differences may permit a more complete description of the relation of 20*S* RNA synthesis to the accumulation of methylated rRNA.

During sporulation, unmethylated 20*S* RNA appears in all *a*/α diploid strains that have been examined (11, 26). In wild-type strains or in strain J1 when methionine is added to sporulation medium, 20*S* RNA is found along with the equimolar accumulation of both 26*S* and 18*S* rRNA. When strain J1 is sporulated in the absence of methionine, rRNA synthesis does

become asymmetric and the ratio of 20S to 18S RNA increases. Nevertheless, under sporulating conditions, 20S RNA synthesis does not require that balanced rRNA synthesis be affected.

In vegetative cells, methyl-deficient 20S RNA has only been seen when methionine-requiring cells are starved for methionine, and rRNA appears in the absence of 26S RNA. The methyl-deficient 20S RNA is stable, in contrast to the transient, methylated 20S precursor of 18S RNA that can also be observed in vegetative cells (30). The exact relationship between these two 20S forms, one methylated, the other virtually unmethylated, is not certain, but preliminary experiments that we have performed indicate that the two 20S species co-migrate on polyacrylamide gels. Whether there are still differences in length and which, if any, nucleotide sequences are always methylated are not yet known.

The independence of 20S RNA accumulation from altered rRNA accumulation in sporulating cells may reflect the significant physiological differences between sporulating and vegetative cells. Cells normally grow at pH 6 or below, whereas the pH of sporulation medium rapidly rises to 8.5 or 9 (4, 6). Cells do not grow vegetatively at pH 8.5, whereas sporulation medium buffered at pH 6 will not support sporulation (6, 18). The differences in rRNA and 20S RNA production may reflect pH-dependent steps in sporulating cells.

It is also possible that the observed pattern of RNA synthesis is made up of several distinct patterns from different cells. Sporulating cells are heterogeneous and not all cells in the population sporulate (6, 7), so that it remains possible that some cells produce only 26S and 18S RNA while others yield only 20S RNA (cf. Fig. 9). By fractionating cells into those that contain spores and those that do not, by density equilibrium banding in a centrifuge, we have found 20S RNA in both fractions (Haber, unpublished results). Finding 20S RNA in vegetative cells starving for methionine makes more certain that both methyl-deficient 20S and methylated 18S RNA accumulate in each individual cell.

Role of methylation in rRNA processing. Yeast rRNA is extensively methylated. In *S. carlbergensis*, 43 sites are methylated in 26S RNA and 26 sites are methylated in 18S RNA (13). The number of different methylating enzymes is not known, but there are likely to be. several, as several different nucleotide base methylations have been found, as well as 2-O-methylation of specific ribose sites (20). Methylation takes place primarily or exclusively at the 35S precursor stage, and all of the methylation is restricted to portions of the molecular which become the 27S and 20S precursors of the mature, methylated 26S and 18S forms (30).

Under conditions of methionine limitation of growth or sporulation, one finds the accumulation of methylated 18S RNA in the absence of 26S and 5.8S RNA. In addition, methyl-deficient 20S RNA which is homologous to 18S RNA also accumulates. We suggest that the 27S and 20S regions of the common ribosomal precursor RNA are processed essentially independently. Only precursor regions which are completely methylated will accumulate as stable RNA, whereas partially methylated precursor molecules will be transported to the cytoplasm and degraded. The results represented in this paper further suggest that, under conditions of methionine deprivation, methylation of at least some sites on the 27S precursor region is less likely than methylation of the 20S portion. Similarly, these may be one or more "critical sites" on the 20S region which must be methylated prior to any other sites in this region. Any 20S molecule in which no methylation of these initial sites has occurred might remain in the nucleus, protected from ribonuclease degradation, even while methylated 18S RNA accumulated in the cytoplasm. The ratio of 26S:20S:18S RNA will thus depend on the probabilities of methylating different nucleotide sequences in the common precursor molecule.

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