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Changing patterns of gene expression during sporulation in yeast

(*in vitro* translation/heat shock protein/nitrogen starvation/development)

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ABSTRACT **Analysis of RNAs isolated from the yeast *Saccharomyces cerevisiae* reveals a dramatic series of changes in protein coding sequences during sporulation. Shortly after transfer to sporulation medium, mRNAs for certain proteins are repressed while a broad array of mRNAs for other proteins is induced. Superimposed on this general increase in transcriptional activity is the very strong induction of a particular subset of heat shock mRNAs, the same subset that is induced during the normal course of oogenesis in *Drosophila*. At distinct times later in sporulation, two sets of abundant mRNAs are coordinately induced. Unlike the earlier changes in the message complement, these changes are unique to sporulating cells. As asci mature, one set of sporulation-specific RNAs is selectively degraded. The second set, as well as the broad array of mRNAs induced earlier in development, is retained in a highly stable and fully translatable form.**

In response to nutrient deprivation many bacilli and fungi undergo a series of complex genetic and morphological changes that lead to the formation of spores. Studies of sporulation have provided many important insights on the mechanisms of genetic regulation in prokaryotes. For instance, in *Bacillus subtilis* proteins unique to sporulation are synthesized in a tightly programmed sequence controlled by the elaboration of RNA polymerase subunits that redirect the specificity of the holoenzyme to different promoters (1, 2). The power of genetic analysis in the yeast *Saccharomyces cerevisiae* makes sporulation an attractive system for studying gene regulation in eukaryotes. The process is of further interest in these cells because it is concomitant with meiotic chromosome replication, recombination, and segregation.

Many studies have defined in detail the genetic, morphological, and biochemical landmarks of sporulation in *S. cerevisiae* (3). Unfortunately, attempts to elucidate the underlying changes in gene expression, particularly at the level of protein synthesis, have not met with comparable success (4–8). A major problem is the impermeability of sporulating yeast cells to radiolabeled precursors. In earlier studies, two approaches were taken to circumvent this problem. Wright and Dawes (7) relied on the reutilization of $^{35}SO_4$ introduced during presporulation growth to identify changes in protein synthesis arising during sporulation. Unfortunately, it was not possible to distinguish between changes due to *de novo* protein synthesis and those due to post-translational modification of preexisting proteins. Other studies (4–6, 8) used radioactive amino acids to pulse-label sporulating cells that had been permeabilized by a shift in pH. Although all of these studies identified changes in the protein complement of sporulating cells, similar changes were observed in asporogenous **a**/**a** or α/α cells cultured under the same conditions. Curiously, the enormous differences in cell structure between sporulating and nonsporulating cells could not be associated with the expression of new proteins.

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We have taken an alternative approach and isolated RNAs from yeast cultures at various times during the course of sporulation. The protein-coding capacities of these RNAs were determined by translation in cell-free lysates. To identify changes specific to the sporulation process, RNAs were also isolated from isogeneic **a**/**a** and α/α cells, which are incapable of sporulating. Our results demonstrate a dramatic series of changes in the message complement of sporulating cells. Two sets of abundant sporulation-specific messenger RNAs are induced in a strict temporal order. The first set is degraded as the second appears. The process culminates with the long-term storage of the second set of sporulation-specific RNAs together with a very broad array of coding species induced earlier in development. It should be noted that previous studies indicated that spores contained poly(A)$^+$ RNA associated with polysomes (9) but little was known about their protein-coding specificities.

MATERIALS AND METHODS

Yeast Strains. Several strains of *S. cerevisiae* were used. The diploid strain AP3 has the genotype

$$\frac{\mathbf{a}}{\alpha}\ \frac{ade1}{+}\ \frac{ade2}{ade2}\ \frac{gal1}{+}\ \frac{tyr1}{+}\ \frac{lys2}{+}\ \frac{his7}{+}\ \frac{ura1}{+}\ \frac{+}{ura3}\ \frac{+}{can1}\ \frac{+}{cyh2}\ \frac{+}{leu1}\ \frac{CSP^+}{CSP^+}.$$

The corresponding asporogenous cell types, **a**/**a** and α/α, are isogeneic to AP3 except at the *MAT* locus where they are homozygous. The diploid LM1 has the genotype

$$\frac{\mathbf{a}}{\alpha}\ \frac{ade1}{+}\ \frac{ade2}{ade2}\ \frac{+}{ade5}\ \frac{CAN^S}{can^R}\ \frac{gal\text{-}1\text{-}4}{+}\ \frac{his7\text{-}1}{his7\text{-}2}\ \frac{+}{leu1\text{-}12}\ \frac{lys2\text{-}2}{lys2\text{-}1}\ \frac{+}{met13d}\ \frac{+}{trp5d}\ \frac{tyr1\text{-}2}{tyr1\text{-}1}\ \frac{ura1}{+}\ \frac{+}{ura3\text{-}13}.$$

FD-1, the asporogenous **a**/**a** cell type that is isogeneic to LM-1, was created by culturing LM-1 in sporulation medium for 8 hr to induce recombination, returning the cells to growth, and then selecting for the ability to mate with α cells. This strain was unable to sporulate and when mated to an α/α tester strain produced asci with four spores of the expected genotypes.

Diploid SK-1, $\frac{\mathbf{a}}{\alpha}\ \frac{HO}{HO}$, diploid 186, $\frac{\mathbf{a}}{\alpha}\ \frac{+}{ade1}\ \frac{+}{his7\text{-}2}\ \frac{+}{leu1\text{-}12}\ \frac{+}{lys2\text{-}1}\ \frac{+}{met13d}\ \frac{trp1}{+}\ \frac{+}{trp5d}\ \frac{+}{tyr1\text{-}1}$, and diploid 1HD30, $\frac{\mathbf{a}}{\alpha}\ \frac{CAN^S}{can^R}$, were also examined.

Sporulation Conditions. Prior to sporulation, cells taken from fresh YEPD cultures were preadapted to respiratory metabolism for five to six generations in synthetic acetate medium (1% KOAc/0.67% yeast nitrogen base/5% phthalic acid, pH 5.5, supplemented with the appropriate auxotrophic requirements). At a density of 5×10^6/ml, cells were collected by centrifugation, washed once in sterile water, and resuspended in sporulation medium (1% KOAc, pH 7) (10). Sporulation cultures were maintained at 30°C with vigorous aeration in a New Brunswick rotary shaker.

Preparation of RNA. For each time point, $\approx 5 \times 10^8$ cells were collected by centrifugation and then suspended in 3 ml of ice-cold extraction buffer (100 mM Tris/100 mM LiCl/20 mM dithiothreitol, pH 7.5) (11). The cell suspension was added to a test tube (20 × 200 mm) containing 7 g of glass beads (0.5-mm diameter), 2.5 ml of phenol equilibrated to pH 7, 2.5 ml of chloroform, and 0.5 ml of 10% $NaDodSO_4$. This mixture was vigorously mixed in a Vortex for 4 min. The entire lysate, excluding the glass beads, was transferred to a 15-ml Corex tube. Calcium chloride was added to a final concentration of 0.01 M to precipitate the $NaDodSO_4$ and the lysate was sedimented at 16,000 × g. The aqueous phase was removed and ethanol and NaOAc were added to precipitate the RNA. The pellet was suspended in water and reprecipitated twice with ethanol. The nucleic acids were suspended in 3 M NaOAc and sedimented in an SW60 Ti rotor at 100,000 × g for 35 min to precipitate RNA. The pellet was washed with 70% ethanol, dried under vacuum, and resuspended in water. Concentrations were determined by spectrophotometric measurements at A_{260} and A_{280}. The RNA was examined by ethidium bromide fluorescence after electrophoresis through 1.4% agarose gels containing 10 mM methylmercury hydroxide (Alfa–Ventron, Danvers, MA).

***In Vitro* Translation of mRNA and Electrophoretic Analysis.** Equal amounts of RNA from each time point were added to a nuclease-cleared wheat germ lysate (Bethesda Research Laboratories) supplemented with creatine phosphate, creatine phosphokinase, 1.5 mM $Mg(OAc)_2$, 100 mM KOAc, all of the amino acids except leucine, and 20 μCi of [^{3}H]leucine at 120 Ci/mmol (1 Ci = 37 GBq; Amersham) in a total volume of 10 μl. The reactions were maintained at 20°C for 5 hr and then stopped by the addition of 20 μl of Laemmli sample buffer (2× concentrated) containing 5% $NaDodSO_4$/10% 2-mercaptoethanol/10% glycerol/bromphenol blue. Each translation reaction contained an equivalent concentration of yeast RNA previously determined to be below the level of saturation for the lysate. A 7.5-μl aliquot of each sample was separated on 10% $NaDodSO_4$/polyacrylamide gels (12). After electrophoresis, the gels were prepared for fluorography according to Laskey and Mills (13) and exposed to Kodak XAR-5 film for 2–5 days at −80°C.

RESULTS

Transfer to Sporulation Medium Initiates Complex Changes in the Message Population. A variety of strains of *S. cerevisiae* were used in this study. We will begin by describing results obtained with the diploid AP3, a strain that sporulates in a rapid and nearly synchronous manner and achieves high levels of ascus formation. Cells preadjusted to respiratory metabolism were collected in midlogarithmic growth and resuspended in nitrogen-deficient sporulation medium. Under these culture conditions, 90% of the cells completed sporulation within 24 hr.

At various intervals during sporulation, total cellular RNAs were extracted and their protein coding capacities were assessed in a cell-free wheat germ translation system. The translation products encoded by these RNAs are shown in Fig. 1. In this experiment, as in all others reported here, each translation reaction mixture contained an identical quantity of total yeast cellular RNA at a concentration that was well below saturation for the lysate. Thus, the changing intensities of individual protein bands during this time course reflect the changing abundance of their mRNAs.

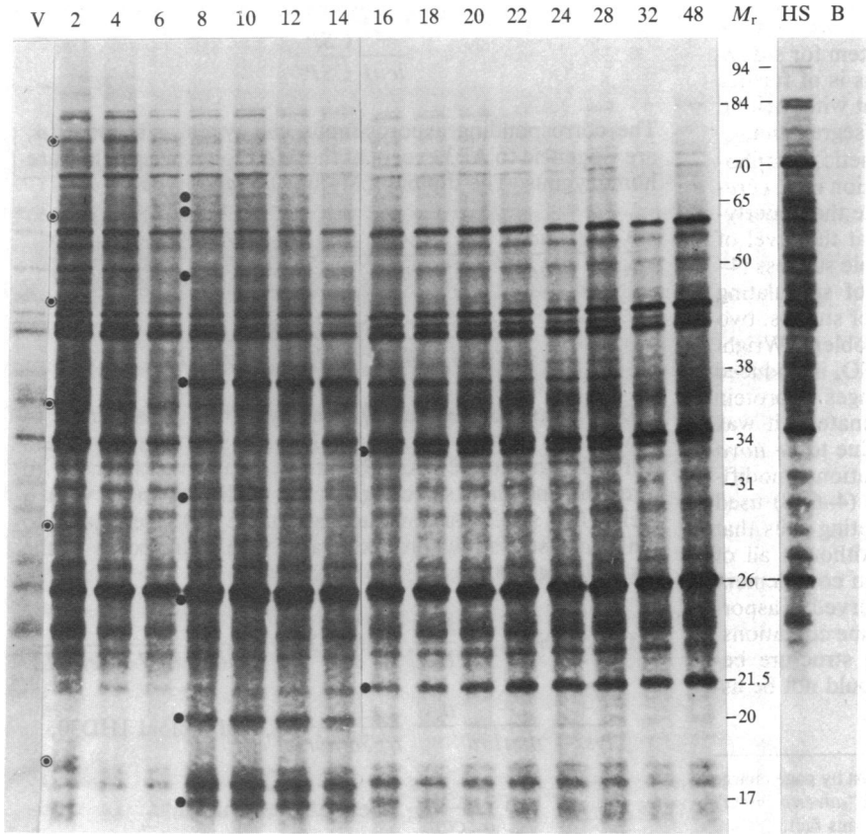

FIG. 1. Changing message composition of sporulating cells. RNAs were extracted from aliquots of a continuous culture of diploid AP3 (**a**/α) at various times after transfer to nitrogen-deficient medium. The RNAs were translated in a wheat germ lysate and the products were separated on $NaDodSO_4$/polyacrylamide gels. Lanes: V, vegetative translation products; 2–48, hr after transfer to nitrogen-deficient medium; HS, translation products from cells heat shocked at 39°C for 1 hr; B, translation reaction in the absence of exogenous RNAs (blank). M_r values are indicated × 10^{-3}. Dots (◉) to the right of lane V indicate polypeptides repressed during sporulation. Dots (●) to the right of lanes 6 and 14 hr indicate polypeptides induced during sporulation.

The initial response to the change in medium was a rapid increase in the concentration of RNAs encoding a broad range of polypeptides. RNAs isolated 2 hr after transfer to nitrogen-deficient medium directed twice as much incorporation into protein as RNAs from vegetative cells. Most, but certainly not all, of the induced species were already present during vegetative growth at low concentrations.

Previous studies of sporulating cells suggest that 50–70% of preexisting RNA is degraded during sporulation (4, 14). If this degradation occurred early in sporulation and primarily affected ribosomal RNA, it would provide a simple explanation for the apparent increase in message-coding species. Using our method of nucleic acid extraction, however, we have never observed a decrease in the concentration of ribosomal RNA during the early stages of sporulation. As shown in Fig. 2, a decrease in ribosomal RNA recovery is apparent only after ascus formation is complete. Since we obtain good recovery from isolated spores, this decrease is most likely due to the degradation of ribosomes left outside the spores in the ascus cytoplasm. Thus, the increase in translatable RNAs after 2 hr in sporulation medium is due to transcriptional activity.

Another change in the message population became apparent 4–6 hr after transfer to sporulation medium. Namely, a number of messages characteristic of vegetative growth were sharply diminished. A few examples detectable at the level of exposure shown in Fig. 1 are marked with dots.

Superimposed on the general increase in translatable RNAs, there was a dramatic induction of messenger RNA for a 26-kDa protein. This message was barely detectable in vegetative cells but within 6 hr of transfer to nitrogen-free medium it was the most intensely translated species in the *in vitro* reaction. Its protein product comigrated on NaDodSO$_4$/polyacrylamide gels with the 26-kDa yeast heat shock protein (hsp26). (An *in vitro* translation reaction with RNAs isolated from heat-shocked cells is displayed on the far right of Fig. 1.) Message for a polypeptide of 84 kDa, which comigrated with another of the major heat shock proteins, was also induced at this time. Note, however, that no induction was observed of message for a 70-kDa protein, the size of the other major heat shock species. The induction of hsp26 and hsp83 mRNAs as well as the absence of induction of hsp70 mRNA were confirmed by hybridization of electrophoretically separated RNAs to cloned probes for the heat shock genes.

Later in sporulation two new sets of mRNAs were coordinately induced. The first appeared after 6 hr and encoded polypeptides of 17, 20, 25, 31, 38, 50, 65, and 68.5 kDa. These RNAs were maximally induced between 8 and 14 hr and then began to disappear. (The 25-kDa polypeptide is partially obscured by the 26-kDa polypeptide on this gel but migrates as a distinct species on gels with higher acrylamide concentrations; the 65- and 68.5-kDa polypeptides are less abundant than others in the set and are difficult to see on this particular film.) After 16 hr, a second set of RNAs, encoding 21.5- and 34-kDa polypeptides, was induced. These two RNAs continued to accumulate late into sporulation.

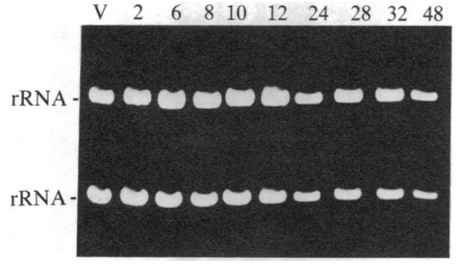

FIG. 2. Ethidium bromide fluorescence pattern of total nucleic acids from sporulating cells. Nucleic acids were extracted from aliquots of a culture of AP3 at various times after transfer to nitrogen-deficient medium. Prior to precipitation in 3 M sodium acetate, aliquots of total nucleic acids from each time point were electrophoresed through 1.4% agarose gels containing 5 mM methylmercuric hydroxide. Lanes: V, vegetative nucleic acids; 2–48, hr after transfer to nitrogen-deficient medium. Note the increase in a 20S RNA species late in sporulation as previously reported (15).

Some Changes in RNA Metabolism Are the Result of Nitrogen Starvation, Others Are Unique to Sporulating Cells. Since sporulation is induced by nitrogen starvation, it is necessary to distinguish changes that are unique to the developmental process of sporulation from those that are a consequence of altered growth conditions. Two nonsporulating diploid strains were grown in an identical manner and transferred to nitrogen-deficient sporulation medium. These strains are isogeneic with AP3 except at the mating-type locus where they are homozygous for **a** or α. The translation profiles of RNAs extracted at times that matched those for the sporulating diploid are shown in Fig. 3.

Initially, both the **a**/**a** and α/α strains responded to the change in medium in a manner similar to the **a**/α diploid. That is, many RNAs showed an enhanced abundance and several RNAs characteristic of vegetative growth were diminished over the first few hours (Fig. 3). Again, most prominent among the induced species were RNAs encoding polypeptides that comigrate with hsp26 and hsp84.

A dramatic difference between the sporulating diploid and its nonsporulating derivatives occurred after 8 hr in sporulation medium. Unlike the sporulating diploid, neither the **a**/**a** nor the α/α cells produced the coordinately regulated set of messages for the 17-, 20-, 25-, 31-, 38-, 50-, 65-, and 68.5-kDa polypeptides. Furthermore, no message coding for the 34-kDa polypeptide was observed. Although a 21.5-kDa-coding species was produced in these cells, it did not accumulate during late time points. If it is the same RNA that was observed in the sporulating diploid it is certainly not regulated in the same manner.

These experiments identify a series of changes in gene expression that are induced in AP3 cells by nitrogen starvation. Some of these changes appear specific to the sporula-

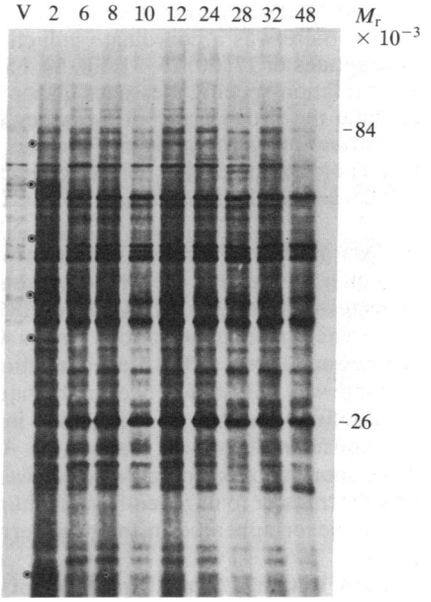

FIG. 3. Message composition of the nonsporulating diploid AP3 (α/α). RNAs were extracted from aliquots of a continuous culture at various times after transfer to nitrogen-deficient medium. Lanes: V, vegetative translation products; 2–48, hr after transfer to nitrogen-deficient medium. Dots (◉) indicate polypeptides repressed during sporulation.

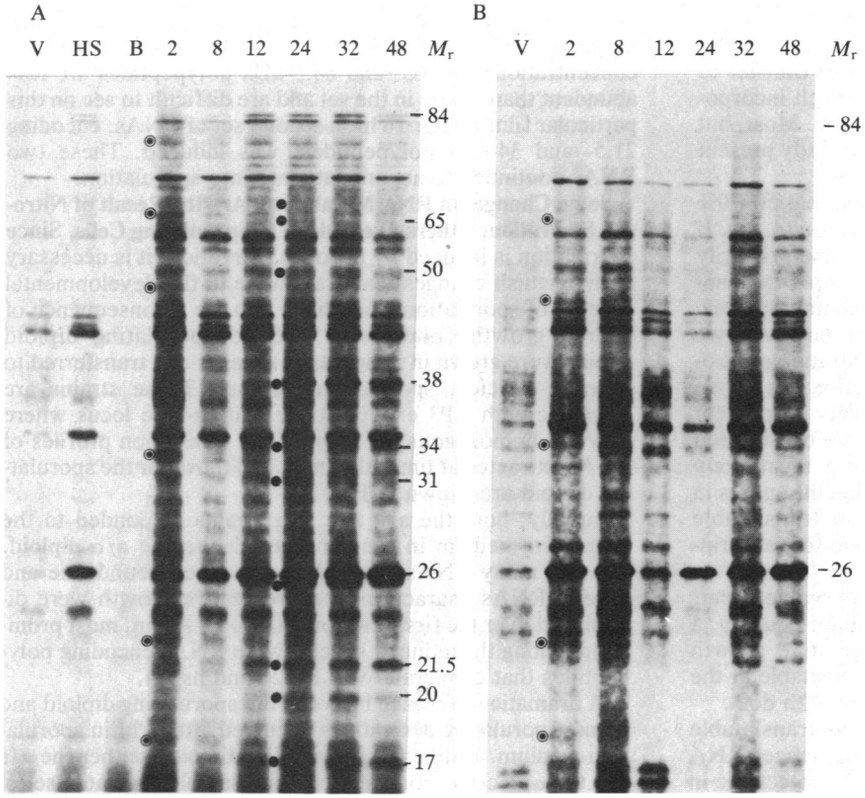

FIG. 4. (*A*) Changing message composition of strain LM-1 (**a**/α) during sporulation. RNAs were extracted and analyzed as indicated for Fig. 1. Lanes: V, vegetative translation products; HS, translation products from heat-shocked cells; B, translation reaction in the absence of exogenous RNA; 2–48, hr after transfer to nitrogen-deficient medium. (*B*) Message composition of the isogeneic nonsporulating diploid (**a**/**a**). M_r values are indicated $\times 10^{-3}$. Dots (◎) to the left of lane 2 hr (*A* and *B*) indicate polypeptides repressed during sporulation. Dots (●) to the left of lane 24 hr (*A*) indicate polypeptides induced during sporulation.

tion process since they do not occur in isogeneic strains that are unable to sporulate. To determine how characteristic these changes are, we extracted RNA from several **a**/α diploids undergoing sporulation and from an **a**/**a** diploid derived from one of them. In every strain, transfer to nitrogen-deficient medium was marked by the disappearance of certain vegetative mRNAs and an increase in the concentration of messages for a very broad array of proteins. At the same time there was a strong induction of RNAs encoding polypeptides of 26 and 84 kDa. Later in development and only in sporulating cells, there was a coordinate induction of messages for polypeptides of 17, 20, 25, 31, 38, 50, 65, and 68.5-kDa. These sporulation-specific messages disappeared from mature asci while the broad array of messages induced at the onset of sporulation were maintained. In each strain the second set of sporulation-specific RNAs coding for polypeptides of 21.5 and 34 kDa accumulated late in sporulation.

The translation profiles of RNAs extracted from the diploid LM-1 and an **a**/**a** isogeneic derivative are shown in Fig. 4. Other strains tested were SK-1, 1HD30, and 186. The only significant differences among the various strains were differences in the rates and levels of RNA accumulation that correlated in a simple way with differences in the rates and efficiency of sporulation. The rate of sporulation in LM-1, for example, was considerably slower than that in AP3. In this experiment the sporulation-specific messages were first observed 24 hr after transfer to nitrogen-free medium (Fig. 4*A*). These messages never appeared in the nonsporulating diploid.

Mature Spores Retain a Broad Spectrum of Messenger RNAs in a Fully Translatable State. The full spectrum of messengers induced earlier in development was retained throughout the course of sporulation. We wondered, therefore, whether these RNAs were packaged into spores, perhaps stored for future use. Asci from strain AP3 stored at room temperature for 2, 4, and 7 days or on plates at 4°C for 21 days were resuspended in buffer and processed by our usual RNA extraction method.

As shown in Fig. 5, mature asci retained the complete spectrum of messenger RNAs in a fully translatable form. No diminution of message was detectable after 3 weeks of storage. Experiments with fractionated asci indicated that most, if not all, of the message was stored within the spore itself. High levels of incorporation were obtained both from isolated spores and from whole asci but no translatable RNA was recovered from the debris of disrupted asci (data not shown). Translation of RNAs isolated from **a**/**a** cells also indicated an increase in message stability as a consequence of nitrogen starvation but not to the same extent as that found in spores.

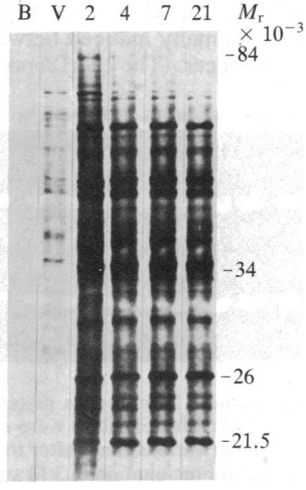

Fig. 5. Message composition of stored asci. RNAs were extracted from spores 2, 4, 7, and 21 days after sporulation and translated in wheat germ lysates. Lanes: B, translation products in the absence of exogenous message; V, vegetative translation products.

DISCUSSION

Our data demonstrate several dramatic changes in the pool of translatable RNA during the course of sporulation in *S. cerevisiae*. These changes fall into two categories: those that are associated with the starvation conditions required for sporulation and those that are concomitant with the development of asci. On transfer to nitrogen-free sporulation medium, both sporulating diploids and asporogenous diploids show a repression of certain RNAs characteristic of vegetative growth, an enhancement of many other RNAs and, shortly thereafter, the induction of RNAs for two of the major heat shock proteins. Hsp70 messenger RNA is not induced in these cells, in marked contrast with the response to heat shock and other stresses in which the induction of these three species is coupled. Thus, the production of hsp26 and hsp84 mRNAs in response to nitrogen starvation is not part of a stereotyped response to stress but part of a developmental adjustment to altered growth conditions.

In all of the $\mathbf{a}/\alpha$ diploids that we examined, these changes are followed by the coordinate induction of a set of messenger RNAs that encode polypeptides of 17, 20, 25, 31, 38, 50, 65, and 68.5 kDa. These RNAs are simultaneously induced no earlier than 6 hr into sporulation and disappear from the message population when spores have matured. Their induction is restricted to sporulating $\mathbf{a}/\alpha$ diploids. A second set of sporulation-specific messenger RNAs, coding for 21.5- and 34-kDa species, appears much later. The complex spectrum of messages induced earlier in development is packaged into spores and retained in a stable, highly translatable state.

The elaboration of sporulation-specific messages is tightly programmed. There is no trace of the first set of these messages at 6 hr; all are abundant at 8 hr. The second set is induced when the first set begins to disappear. The most likely explanation for the rapid accumulation of these RNAs is a change in transcription. The coordinate character of their appearance suggests that each member of the set is controlled by some common regulator.

Our method of analysis elucidates global changes in RNA metabolism during sporulation and defines sporulation-specific products, but it has a major limitation: we can detect only messenger RNAs that accumulate in substantial quantities. Our finding of 10 sporulation-specific protein products must therefore be considered an absolute minimum. Two recent studies using differential cDNA hybridizations have identified 14 or 15 sporulation-specific RNAs (16, 17). It may be that some of these transcripts do not have protein coding activity, or our methods may not detect them.

As this manuscript was being prepared for submission, Weir-Thompson and Dawes (18) published a related study using *in vitro* translation in reticulocyte extracts to analyze changes in translatable RNA during sporulation. In agreement with our findings, they observed a general increase in messenger RNA concentrations on transfer to nitrogen-deficient medium and a decrease in the abundance of particular vegetative mRNAs. However, only four sporulation-specific polypeptides were detected, leading to the conclusion that sporulation in *S. cerevisiae* does not involve the synthesis of abundant structural proteins. Based on the intensity of translation in wheat germ lysates, most of our sporulation-specific mRNAs appear to encode abundant proteins. An explanation for the discrepancy with our results lies in a major difference between the two *in vitro* translation systems. Reticulocyte lysates have high concentrations of signal recognition particle (SRP). This particle blocks the synthesis of proteins with signal sequences that are normally translated across cellular membranes. Typically, SRP inhibition can be released by supplementing reticulocyte lysates with microsomal membranes (19).

In experiments to be reported elsewhere, we have found that our most abundant sporulation-specific polypeptides are not produced in reticulocyte lysates in appreciable quantities unless those lysates are supplemented with microsomal membranes. This finding is of particular interest because electron microscopic analysis of developing asci has shown that spore walls are deposited within a double membrane that surrounds the immature spore (20, 21). It seems most reasonable then, that the messages that fail to translate in unsupplemented reticulocyte lysates code for structural proteins involved in spore wall assembly. The appearance of these messages at a time that closely coincides with spore wall formation (8 hr in AP3 and 24 hr in LM-1 cells) further supports this hypothesis (unpublished work).

At the end of sporulation this initial set of sporulation-specific messages completely disappears from mature asci. The second set of sporulation-specific messages, as well as the complex array of messages induced earlier in development, are retained in a highly translatable form and packaged into spores. During vegetative growth yeast messages have half-lives of 15–20 min (22). In spores no turnover of messages was detected in our experiments. Apparently, a specific mechanism of message stabilization is operating in sporulating cells. In this regard the induction of a particular subset of heat shock messenger RNAs is intriguing. A similar uncoupling of heat shock transcription occurs during the development of *Drosophila* oocytes. RNAs for hsp83, hsp28, and hsp26 are synthesized by nurse cells during normal development and are passed into the maturing oocyte. No synthesis of hsp70 is detectable in these tissues (23).

Both *Drosophila* oocytes and yeast spores are characterized by the transcription and storage of a diverse spectrum of messenger RNAs. The hypothesis that hsps might be involved in this process is strengthened by the fact that hsp26 in *Drosophila* has recently been shown to be a RNA-binding protein (24).

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