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Genetics

Isolation of genes expressed preferentially during sporulation in the yeast *Saccharomyces cerevisiae*

(microbial development/cDNA probe/differential plaque hybridization/meiosis and ascosporogenesis)

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ABSTRACT **A library of *Saccharomyces cerevisiae* DNA in the vector λ Charon 28 was probed for sequences complementary to cDNA made from poly(A)$^+$ RNA isolated from the well-sporulating yeast strain AP1 a/α. The RNA was isolated from cells that had been incubated 7, 9, 11, and 13 hr in sporulation medium. DNA complementary to poly(A)$^+$ RNA from α/α (nonsporulating) AP1 was used as a control, and 46 bacteriophage that gave a stronger response with a/α cDNA than with α/α cDNA were obtained in a screening of three yeast genomes worth of DNA. Two of the bacteriophage appeared to contain a/α-specific genes, in that they hybridized to cDNA from vegetative a/α RNA. The rest appeared to correspond to a/α genes expressed preferentially during sporulation. Restriction endonuclease analysis of four of the cloned sequences revealed a single major region of transcription in each; these regions ranged in size from 2.5 to 4.0 kilobases. RNA blot analysis showed that, in three of the four cases, transcripts of two different sizes were homologous to the cloned sequence. In all four cases, the homologous transcripts appeared at about 7 hr and were decreasing in amount by 13 hr. These results provide evidence for transcriptional control of genes expressed during sporulation and for at least one group of genes that is turned on at about the time of meiosis I in sporulation.**

The ascomycete *Saccharomyces cerevisiae* has a relatively complex life cycle that includes a morphologically defined cell cycle, mating and zygote formation, and meiosis and ascosporogenesis. The combined techniques of genetics and molecular biology have been used to elucidate several aspects of this life cycle and have led to a number of advances in our understanding of the cell cycle (1) and the control of mating type (2–6). The study of meiosis and sporulation (7), however, has heretofore lagged, partly because of the difficulty of isolating and characterizing mutations in genes expressed only in diploids (8).

Meiosis and ascosporogenesis is a complex process involving macromolecular synthesis (9, 10), recombination (11), and turnover of preexisting cellular components (12). It occurs in cells expressing both a and α mating type alleles on a shift to medium lacking ammonia and glucose but containing a respirable carbon source. Cells expressing only one *MAT* allele (haploid a or α, diploid α/α or a/a cells) do not undergo the process; such cells fail to undergo premeiotic DNA synthesis (13, 14) or any of the subsequent events that have been monitored. Meiosis and sporulation are therefore under the control of the mating type locus, although nutritional signals must also play a role in the initiation of the process.

The product of the developmental program, an ascus containing (usually) four haploid spores, is morphologically distinct from vegetative cells. In a typical (well-sporulating) diploid, 60–80% of the cells achieve this differentiated state in a relatively synchronous process that lasts 16–24 hr under the conditions we use (15). Physiological studies have shown that premeiotic S phase occupies the period from 3 to 7 hr (in the culture as a whole, the S period of individual cells appears to last about 1 hr), recombination occurs over roughly the same period, beginning and ending about half an hour later than S phase, and meiosis I takes about 120 min, from 8 to 10 hr (9, 12). Meiosis II follows immediately and is completed by 12 hr. Immature asci are detectable at 10 hr in some cells, and ≈80% of the final level of asci is found in the culture by 16 hr, with the remainder forming over the next 8 hr.

Several attempts to identify specific gene products involved in this process have failed, despite the existence of numerous mutations affecting gene products needed in sporulation but not for vegetative growth (8). One- and two-dimensional gel analyses of proteins synthesized during a pulse label of sporulating cells have identified few (16) or no (12, 17, 18) proteins specific to sporulation. This may be because the abundance of these gene products is simply too low to be detected on gels.

Recently, workers in this laboratory have identified an enzyme, sporulation amyloglucosidase (SAG), that occurs only in a/α cells in sporulation medium and whose appearance is dependent on the progression of the cells well into the pachytene stage of meiotic prophase (19, 20). It seemed worthwhile to ask whether a group of genes was expressed during the time that this enzyme activity appears, how large this hypothetical group might be, and whether these genes share common functional (e.g., duration of transcription) and structural properties.

We have therefore screened a *Saccharomyces cerevisiae*–λ Charon 28 (21) library for bacteriophage containing sequences specifically expressed from 7 to 13 hr in sporulation medium. Although we have not yet identified the *SAG* gene, we have found 46 hybrid phage that contain sequences more highly expressed in sporulating MATa/MATα cells than in otherwise isogeneic MATα/MATα cells. The characterization of six of these sequences is reported here.

MATERIALS AND METHODS

Radioisotopes and Enzymes. [^{32}P]dCTP (>400 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Restriction enzymes and oligo(dT) primer were from Bethesda Research Laboratories and were used as recommended by the supplier. DNA polymerase and avian myeloblastosis virus reverse transcriptase were from Boehringer Mannheim and Life Sciences, respectively. Nitrocellulose sheets and filter discs were from Schleicher

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Abbreviations: SAG, sporulation amyloglucosidase; kb, kilobase pairs.

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& Schuell. Formamide for hybridization (Aldrich) was deionized with Amberlite (Aldrich) before use. *Drosophila* cytoplasmic RNA was a gift from Jerry Dodgson.

Growth Conditions and Preparation of RNA. The *Escherichia coli* strain used for growth of the bacteriophage was KH802, a derivative of K-12. *S. cerevisiae* strains used were AP1 **a**/α and its isogeneic asporogenous derivative AP1 α/α (12). The cells were ordinarily grown and sporulated as described (15) in acetate pregrowth medium. Vegetative or sporulating yeast cells were harvested by centrifugation, washed in ice-cold sterile distilled water, and broken by mixing in a Vortex with glass beads (18) or in a Bronwill homogenizer (22). Poly(A)$^+$ RNA was prepared as described (22).

Library Construction. A *S. cerevisiae* library was constructed in λ Charon 28. DNA from *S. cerevisiae* strain AH22 was extracted by the method of Hereford *et al.* (23) and digested with *Mbo* I to yield fragments of ≈15 kilobase pairs (kb). Fifteen- to 20-kb fragments were isolated by sucrose gradient centrifugation, and these were ligated to Charon 28 arms prepared by digestion with *Bam*HI (24). The ligated DNA was packaged *in vitro* (25), plated, and amplified. Minipreparations of λ DNA were used for all experiments, essentially as in Maniatis *et al.* (24).

Differential Plaque Hybridization. The method used was essentially that of St. John and Davis (26). Aliquots of the library were plated onto *E. coli* KH802 on NZCYM (24) and grown to yield ≈300 plaques per plate. Isolated plaques were picked using sterile toothpicks into an ordered array on freshly poured lawns of *E. coli* KH802 (≈50 μl of an overnight culture) and allowed to grow for 8–12 hr. This amplification was necessary to allow detection of the hybridization signals from a majority of the plaques; only 10–20% of the plaques yielded signals when filters were made directly from the original plaque plates whereas >90% of the clones gave detectable signals with both probes when patch plates were used (26). Duplicate nitrocellulose filter replicas were prepared from the plates essentially by the method of Benton and Davis (27). Hybridization was carried out as in Engel and Dodgson (28).

Preparation of Hybridization Probes. ^{32}P-Labeled cDNA was prepared from poly(A)$^+$ RNA by the method of St. John and Davis (26), except that 40 mM sodium pyrophosphate was included in the reaction mixture. The reaction was started by the addition of reverse transcriptase, allowed to proceed at 40°C for 60–90 min, and terminated by the addition of base. Hydrolysis of RNA and separation of the cDNA from the unincorporated nucleotides was done as in St. John and Davis (26). Probes were used at a concentration of 1 to 2 ng/ml. Nick-translation of cloned DNA was accomplished by the method of Rigby *et al.* (29).

Agarose Gel Electrophoresis and Nitrocellulose Filter Hybridization. Agarose (Bethesda Research Laboratories) gels were run submerged in 40 mM Tris·HCl/20 mM NaOAc/1 mM EDTA, pH 7.5, at 60–100 mA and stained with ethidium bromide (1 μg/ml). The gels were 0.4%, 0.7%, or 1.2% agarose, depending on the experiment.

DNA from agarose gels was transferred to nitrocellulose by the method of Southern (30) and hybridized to cDNA prepared as described by St. John and Davis (26). For RNA blots, ≈5 μg of poly(A)$^+$ RNA per lane was denatured with glyoxal (31) and loaded onto 1.5-ml agarose gels (30) and electrophoresed. Then, the RNA was transferred to nitrocellulose by the method of Thomas (32) and hybridized to nick-translated DNA as described above. Dot blots were prepared by spotting 5-μl aliquots of the λ phage stocks onto nitrocellulose filters. The DNA was denatured and neutralized. The filters were baked as described for differential plaque hybridization and then hybridized to cDNA probes prepared as described above.

RESULTS

Differential Plaque Hybridization as a Screen for Sporulation-Specific Genes. Differential plaque hybridization, a modification of the plaque filter hybridization method of Benton and Davis (27), allows the isolation of genes whose RNAs are present at increased concentration in one population of cells compared with another. This method involves the preparation of two [^{32}P]cDNA probes, one against each of the RNA preparations to be compared, and hybridization of these probes to duplicate nitrocellulose filter replicas of a collection of λ clones on a Petri plate. Clones that hybridize strongly to one probe but not to the other contain genes that are expressed at different levels in the two populations. This method has been used successfully in yeast to isolate galactose-inducible genes (26) and genes expressed at high levels in low-phosphate medium (33).

We have used differential plaque hybridization to screen a λ Charon 28–*S. cerevisiae* library for sequences expressed preferentially during sporulation. A [^{32}P]cDNA probe was prepared against RNA isolated from MAT**a**/MATα cells after 6–13 hr of incubation in sporulation medium (Fig. 1) and, as a comparison, a similar probe was made from an otherwise virtually isogeneic MATα/MATα strain that is incapable of sporulation. The RNA preparation from the sporulating MAT**a**/MATα cells should contain sequences transcribed specifically during sporulation that the MATα/MATα cells would lack. These might include those coding for products required for premeiotic DNA

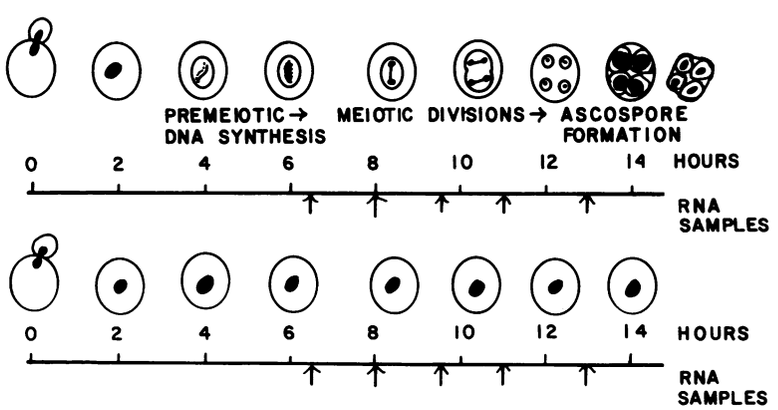

FIG. 1. Preparation of hybridization probes. One-liter cultures of the sporulation-proficient strain AP1 **a**/α (*Upper*) and the asporogenous AP1 α/α strain (*Lower*) were grown to 2 × 10^7 cells/ml and shifted to sporulation medium. The cells were allowed to progress into sporulation and 500-ml samples were taken at the indicated times for the preparation of RNA. The samples from each culture were pooled for the preparation of poly(A)$^+$ RNA, and [α-^{32}P]cDNA probes were prepared against these RNAs and used for differential plaque hybridization.

synthesis, recombination, the two meiotic divisions, and spore formation, as well as RNAs that are stored in the spore in preparation for dormancy and germination. The RNA sequences common to both probes would include those homologous to housekeeping genes or to genes induced by starvation. That these latter are the predominant sequences is suggested by the fact that two-dimensional gel electrophoresis of the proteins made in sporulation medium by sporulating-proficient and sporulation-deficient cells gives similar or identical patterns (16–18).

Isolation of Sporulation-Specific Genes. Aliquots of the library were plated in top agar onto *E. coli* KH802, individual clones were picked and amplified by patching into an ordered array onto freshly poured lawns of KH802 and duplicate nitrocellulose filters were prepared and hybridized. Clones that gave stronger signals with the MATa/MATα probe were picked and retested. The second test was necessary to purify the phage and also served to eliminate false positives, which represented >50% of the clones chosen on the first test, whether the same cDNA preparation was used for both the first and the second test or the probes were different. A sample autoradiograph from such an experiment is shown in Fig. 2. Plaques 1 and 2 gave stronger signals with the a/α probe than with the corresponding α/α probe on the original plate. On retesting, however, only clone 2 isolates hybridized preferentially with the a/α probe. Such clones were retained and phage stocks were prepared.

We screened ≈3,700 plaques and retained a total of 46 clones that appeared to contain differentially expressed genes. These clones varied at least 10-fold in the intensities of the hybridization signals obtained as well as in the intensity differences between the a/α and α/α probes. The dot blots (Fig. 3 *A* and *B*) show that some clones (e.g., nos. 1, 11, 16, 34, and 42) gave extremely intense signals with the a/α cDNA but weak signals with the corresponding α/α probe while others (e.g., nos. 2, 4, and 7) showed only slight qualitative differences. The 46 clones ought to represent some 15 genes, assuming that each is represented only once per haploid genome and that no highly expressed genes that are not sporulation specific are located nearby.

It is possible that the differentially expressed genes isolated in this screen were actually MATa/MATα specific, rather than sporulation specific, because the only criterion for selection was that the clones hybridize more strongly to the probe made from MATa/MATα cells in sporulation medium than to the corresponding MATα/MATα probe. That this is not the case is shown in Fig. 3 *C* and *D*. [^{32}P]cDNA probes were prepared to poly(A)$^+$ RNA from vegetatively growing MATa/MATα and MATα/MATα cells and the signals obtained when the 46 clones were hybridized to the two probes were compared. Most clones hybridized equally well to the two vegetative probes and more weakly to both than to the cDNA from sporulating cells. Clones 7 and 69 were exceptions to this since they hybridized strongly to both MATa/MATα probes and weakly to both MATα/MATα probes. These two clones, therefore, contain a/α rather than sporulation-specific genes. Clone 16 was surprising, since it hybridized more strongly to the MATα/MATα probe prepared from vegetative cells than to a comparable probe from a/α cells. Subsequent analysis showed that the apparent α/α-specific sequence was located ≈5 kb from the sporulation-specific sequence within the same clone (see below).

Restriction Maps and Time of Appearance of the Sporulation-Specific Transcripts. We wanted to determine whether each clone contained a single sporulation-specific gene or several and that time during sporulation at which the corresponding transcripts appeared. Six clones, nos. 1, 11, 16, 32, 34, and 42 were chosen for this analysis, because they gave particularly clear differential signals. Restriction maps were constructed and the locations of the differentially transcribed portion in each was determined by Southern blotting (30) using [^{32}P]cDNA from sporulating MATa/MATα cells as the probe. As shown in Fig.

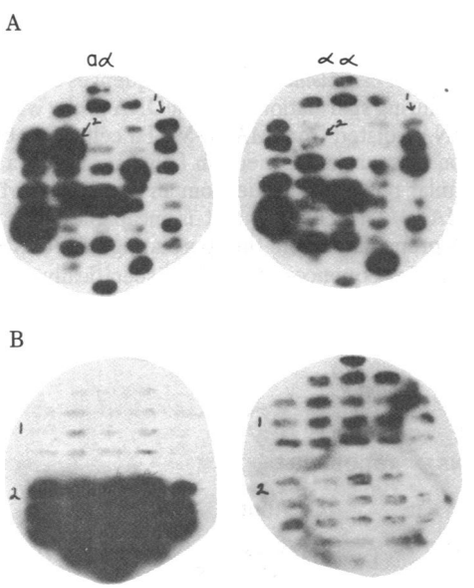

FIG. 2. Differential plaque filter hybridization as a screen for sporulation-specific genes. Aliquots of the λ library were patched onto *E. coli* KH802, and duplicate nitrocellulose filter replicas were prepared and hybridized to [^{32}P]cDNA probes prepared against RNA from sporulating MATa/MATα cells and nonsporulating MATα/MATα cells. The hybridized blots were washed and exposed to x-ray film and the signals obtained with the two probes were compared. Clones 1* and 2* (these numbers refer to the order of testing and are not related to clone numbers referred to in the text) appeared to contain sporulation-induced genes on the first test (*A*) but only clone 2 appeared positive on the second test (*B*).

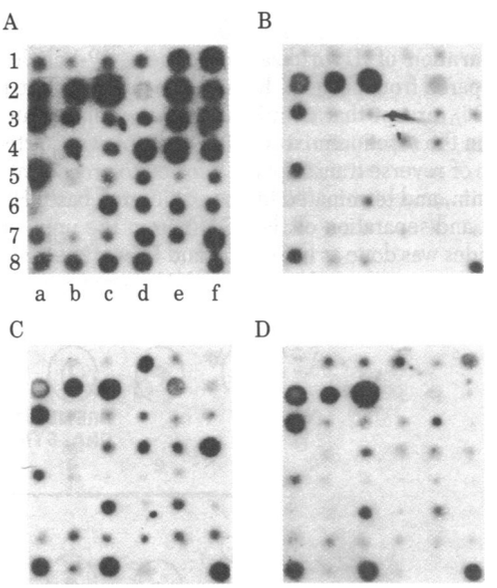

FIG. 3. Dot blot analysis of the 46 clones. Dot blots were prepared from isolated phage that appeared to contain sporulation-inducible genes and hybridized to [^{32}P]cDNA probes prepared against poly(A)$^+$ RNA from MATa/MATα cells undergoing sporulation (*A*), MATα/MATα cells incubated in sporulation medium (*B*), MATa/MATα cells growing vegetatively (*C*), and MATα/MATα growing vegetatively (*D*). The clones referred to in the text are 1, position 1a; 2, 1b; 4, 1c; 7, 1d; 11, 1e; 16, 1f; 32, 2c; 34, 2f; 42, 3f; and 69, 4f. Equal amounts of YEP13 DNA were included as controls (position 8f).

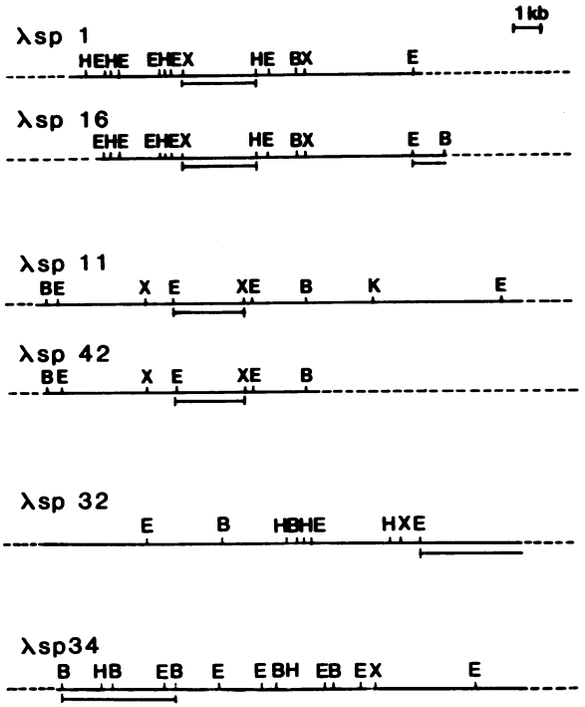

FIG. 4. Restriction enzyme analysis of selected clones. Single and double restriction enzyme digests of the purified λ DNAs were analyzed on 1.2% and 0.4% horizontal agarose gels. Size standards were restriction enzyme-digested λ and YEP13 DNAs. Enzymes used were E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; X, *Xba* I; K, *Kpn* I. The dotted lines represent the λ Charon 28 arms and the heavy lines represent yeast DNA. The thick lines shown below the maps represent the approximate locations of the sporulation-specific sequences within each phage, as determined by Southern blotting of the gels followed by hybridization to the ^{32}P-labeled MATa/MATα and MATα/MATα probes used to isolate the clones originally. Clones 11 and 42 contained *Hin*dIII sites but these are not shown on the maps.

4, clones 11 and 42 apparently contain the same sporulation-specific gene, as do clones 1 and 16. Clones 32 and 34 differ from each other as well as from the other clones. The maps of the two pairs of clones (nos. 1 and 16; nos. 11 and 42) are similar to each other, although not identical. In each case, the sporulation-specific gene could be located on a single restriction fragment <3 kb long, as shown by the bars in Fig. 4. The small size suggests that each clone contains only one, or possibly two, closely linked sporulation-specific genes.

The restriction fragments flanking those containing the sporulation-specific gene also hybridized to the **a**/α probe but at a greatly diminished intensity. It was not possible to determine whether this was the result of low levels of transcription from a flanking gene or whether the transcribed portion of the sporulation-specific gene extended past the fragments shown. In all cases except clone 34, the indicated fragment also hybridized to the α/α probe to some extent. This may reflect a low level of transcription of the "sporulation gene" by MATα/MATα cells or the presence of an adjacent transcription unit that is expressed in common between MATa/MATα and MATα/MATα cells.

We have used RNA blot analysis (34, 32) to determine the time of appearance of the transcripts corresponding to these genes and their sizes. AP1 **a**/α cells growing vegetatively were shifted to sporulation medium and aliquots were removed from the culture at intervals during sporulation for the preparation of poly(A)$^+$ RNA. This RNA was denatured with glyoxal, electrophoresed on 1.5% agarose gels (31), and blotted to nitrocellulose. The blots were hybridized to λ clone DNA that had been labeled *in vitro* with [^{32}P]dCTP by nick-translation (28).

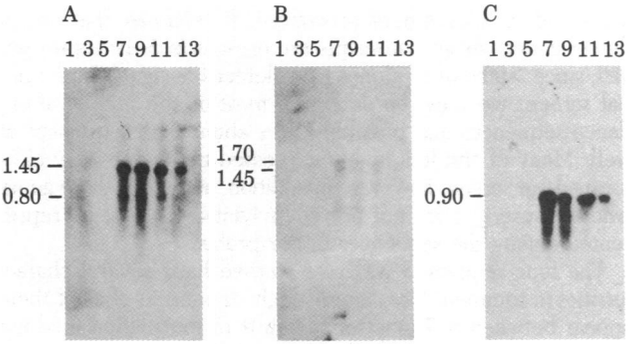

FIG. 5. RNA blot analysis of clones 16 (*A*), 34 (*B*), and 42 (*C*). Poly(A)$^+$ RNA was prepared from MATa/MATα cells incubated in sporulation medium for 1, 3, 5, 7, 9, 11, and 13 hr. Five-microgram samples of these RNAs were denatured with glyoxal, electrophoresed on 1.5% agarose gels, blotted to nitrocellulose, and hybridized to cloned DNAs labeled *in vitro* with [α-^{32}P]dCTP by nick-translation. Autoradiography was done at −70°C for 3–8 days with one intensifier screen. Numbers on the left of the gels indicate sizes (kb) of the developmentally regulated transcripts.

As shown in Fig. 5, clones 16 and 34 each hybridized to two developmentally regulated transcripts rather than one, and clone 32 also hybridized to two distinct transcripts (data not shown). Clone 42 hybridized to one major transcript, but other faint bands were also present. We do not know whether the multiple bands represent transcription of the same gene from different promoters, processing of the larger transcript, or hybridization of the cloned DNA to messages from two closely linked developmentally regulated genes. It is also possible that the second transcripts are from different but related genes elsewhere in the genome. None of these transcripts was detected in blots made to RNA from α/α cells, although such transcripts may have escaped detection because they were present at low levels.

DISCUSSION

Although a considerable amount is known about the physiology of the developmental pathway leading to recombination, meiosis, and ascosporogenesis in *S. cerevisiae* and extensive genetic analysis has been done (8), molecular approaches have been somewhat unproductive. The genetics has provided part of the explanation for this lack of progress in that it has been calculated that a relatively small number of genes are sporulation specific—i.e., required for the process but otherwise dispensable (8). If the proteins that are the products of these genes are present in low abundance, the failure to see specific proteins on two-dimensional gels is easily explained. We sought to identify the genes involved in sporulation directly, by differential plaque hybridization, with the hope that this method would be sensitive enough to detect sporulation-specific sequences. Although there is no doubt that our screen would fail to detect certain classes of genes, it has found a number of sequences that are preferentially expressed in **a**/α cells in sporulation medium.

There are two major groups of genes that will have escaped our screen. One of these is the class that comprises genes located close to a gene actively transcribed in α/α cells in sporulation medium. On the average, 15% of our clones gave a signal with α/α cDNA so strong that we would not have been able to detect an **a**/α difference. Thus, we may have missed 5–10 clones. The more important class contains genes whose transcripts are present in very low abundance or unstable to iso-

lation and cDNA synthetic procedure. We estimate that we can detect genes whose transcripts are present in 5–10 copies per cell; since >90% of all clones gave detectable signals in the initial screen, we must be detecting most of the middle abundance sequences and possibly lower abundance sequences as well. Many of the less highly expressed transcripts may have escaped our screen, however. In addition, of course, those genes whose transcripts are not polyadenylylated will not be represented among the sequences in our probe.

The four sequences we have studied have several characteristics in common. Most interestingly, transcripts of all of them appear between 5–7 hr after the shift to sporulation medium and decrease in abundance by 11–13 hr. The fact that our probe was prepared from mRNA isolated from cells at various stages of sporulation, the earliest of which was at 7 hr, may account for this coincidence. On the other hand, it may be that a significant number of sporulation-specific genes actually begin to be transcribed at this time, and our group is a representative sample. The fact that the *SAG* gene product appears at about this time, as shown by methods independent of the hybridization screen used here, is interesting in this context.

A second significant characteristic of three of these sequences is the presence of two coordinately controlled transcripts. A number of possible explanations exist for these transcripts. One is that we are seeing either processing events or a failure to terminate transcription part of the time. Another possibility is that there are two promoters for each gene, both of which are developmentally regulated. Finally, it is possible that each clone contains two clustered genes, both of which are sporulation specific. The last possibility might be examined by R-looping experiments. Whatever the explanation, it seems likely that the phenomenon may have some developmental significance; while dual transcripts from a single gene are not unknown in yeast (35, 36), they are rare and it seems improbable that three separate genes would exhibit this behavior by chance.

The role of these differentially expressed genes in the sporulation process is not known. It is possible that they represent genes whose products are required for the meiotic divisions or spore formation; they may also represent transcripts that are stored in the spore in preparation for germination. It is known that the spore contains a large number of transcripts (22) and that these include histone RNAs as well as those for glycolytic enzyme (D. Kaback, personal communication). Some of the clones we have identified may correspond to these RNAs.

The success of this screening method in identifying developmentally controlled genes whose transcripts are present in relatively low abundance is somewhat surprising. Although analogous methods have been used to identify conidiation-specific genes in *Aspergillus* (37), in that system the probe was enriched by "cascade hybridization" for specific sequences (38). By amplifying the "receptor" DNA on the filter by simple patching, we have been able to bypass the enrichment step. We expect that analysis of the structure and transcriptional control of genes described here may give important insights into sporulation in particular and eukaryotic development in general.

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