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Nature and Timing of Some Sporulation-Specific Protein Changes in *Saccharomyces cerevisiae*

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During meiosis and spore formulation in *Saccharomyces cerevisiae*, changes that occur in **a**/α diploids, but not in isogenic nonsporulating **a**/**a** diploids, have been detected in cellular polypeptides. These were found by the technique of prelabeling growing cells with $^{35}SO_4^{2-}$ and suspending them in sulfur-free sporulation medium. Under the conditions used, about 400 polypeptides were detected by two-dimensional gel electrophoresis, and 45 were altered during sporulation; of these, 21 changes were specific to **a**/α strains. These alterations were mainly due to the appearance of new polypeptides or to marked increases in the concentrations of a few polypeptides produced during vegetative growth. They could have been due either to modifications of existing polypeptides present in growing cells or to de novo synthesis of new gene products. They occurred at characteristic times during sporulation; whereas the majority of changes took place early (within the first 6 h in sporulation conditions), there were several changes characterizing the later stages of sporulation. Ten of the $^{35}SO_4^{2-}$-labeled polypeptides were also labeled with ^{32}P in the presence of [^{32}P]orthophosphate; of these, three were previously found to be sporulation specific. One of these was phosphorylated at all stages of sporulation and was labeled when [^{32}P]orthophosphate was added either during growth of the culture or 1 h after transfer to sporulation medium. Another was labeled in the same way by adding ^{32}P at either time, so that by 7 h in sporulation medium it was phosphorylated, but was dephosphorylated by 24 h. The third sporulation-specific peptide was labeled in extracts prepared at 7 h in sporulation medium (but not at 24 h) when [^{32}P]-orthophosphate was added during presporulation growth, but not when [^{32}P]-orthophosphate was added 1 h after transfer of the culture to sporulation medium. This polypeptide appeared early during sporulation; it is probably phosphorylated as it appears and is dephosphorylated at some time between 7 h and 24 h of sporulation.

Yeast sporulation has attracted attention both as a simple eucaryotic differentiation process and as a system in which to study meiosis and meiotic recombination (4, 8, 11, 23). From genetic data it has been estimated that about 50 loci code for indispensable sporulation functions (9), yet few biochemical changes that are specific to sporulating cells have been found (3, 7, 15, 25). Therefore, little is known about the role of gene expression in the process, its timing, or its regulation in molecular terms, and further progress depends on the identification of more sporulation-specific events. Several attempts to distinguish specific protein changes by pulse-labeling sporulating cultures with amino acids and subsequently examining their polypeptide complement by one-dimensional (12) or two-dimensional (18, 22) polyacrylamide gel electrophoresis have met with little success. Changes were detected in these studies, but they also occurred in nonsporulating cells placed under sporulation conditions. Some of these results may have been due to differential uptake of added amino acids by different subpopulations of cells in sporulating cultures; the bulk of labeled amino acids may be taken up by vegetative cells or those at an early stage in sporulation (6). To avoid this problem we have sought to identify sporulation-specific changes in polypeptides by labeling the cellular proteins continuously throughout presporulation growth in the presence of $^{35}SO_4^{2-}$ and then by using two-dimensional gel electrophoresis to analyze the labeled polypeptides of cells transferred to sulfur-free sporulation media. This technique has the advantage that it enables the detection of changes arising from modifications to existing labeled proteins as well

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as from de novo synthesis (the label in this case being incorporated via protein turnover); it does not, however, allow direct distinction between these two possibilities.

In preliminary experiments sporulation-specific changes were detected by using this approach (26). The present report has extended this to show that these changes are sequential, each occurring at a characteristic time during sporulation. Moreover, since some of the changes detected by this method may be due to modifications of preexisting polypeptides, we have tested for phosphorylation as one of the types of post-translational alteration possible.

MATERIALS AND METHODS

Strains and cultivation. *Saccharomyces cerevisiae* diploid strain JW-1, *MATa/MATα ARG4-17/arg4-17 his5-2/his5-2 LEU2-1/leu2-1 lys1-1/lys1-1 URA3-1/ura3-1 spd1-1/spd1-1 TRP1-1/trp1-1*, which sporulates well, was constructed from haploids derived from a number of dissections using strain D609-28C (Cold Spring Harbor), X2315-14C (Berkeley Yeast Genetic Stock Center) and ID16D (5) as parents. The nonsporulating strain JW-2, homozygous for *MATa* but otherwise isogenic with JW-1, was derived from JW-1 by ultraviolet-induced gene conversion.

Strains were grown in a defined low-sulfate presporulation medium (18) containing 2% (wt/vol) galactose as the carbon source and with supplements added as previously described (26), except that Na_2SO_4 was added at a concentration of 10 mg/liter, and Na_2HPO_4 was reduced to 10 mg/liter.

Sporulation was induced by the procedure of Fast (10) by harvesting exponential cultures at a density of 10^7 cells per ml and transferring them to sporulation medium (2% [wt/vol] potassium acetate containing 20 mg of L-histidine per ml and 40 mg of L-lysine per ml) at a density of 5×10^7 cells per ml.

Labeling of polypeptides. The cellular polypeptides were labeled continuously throughout the presporulation growth phase in the presence of 10 μCi of $^{35}SO_4^{2-}$ (Radiochemical Centre, Amersham) per ml, and no sulfur-containing compounds were added to the sporulation medium. Where indicated, [^{32}P]orthophosphate was added, either at 40 μCi/ml during growth in presporulation medium (in the presence or absence of $^{35}SO_4^{2-}$) or at 200 μCi/ml 1 h after transfer to sporulation medium.

Extraction and analysis of polypeptides. Samples containing 10^8 cells were taken, and the cells were pelleted and washed with buffer A [10 mM tris(hydroxymethyl)aminomethane-hydrochloride, (pH 7.4), 5 mM $MgCl_2$] at 0°C. Cells were suspended in 400 μl of buffer A containing pancreatic ribonuclease and 2 mM phenylmethylsulfonyl fluoride at 0°C, and an equal volume of acid-washed glass beads (40 mesh) was added. Cells were broken by two 2-min vibrations in a Vibromix with cooling on ice throughout; 30 μl of buffer A containing deoxyribonuclease was then added, and the mixture was left for 10 min at 0°C. Samples were then freeze-dried for storage until required for analysis by two-dimensional gel electrophoresis.

Labeled polypeptides were separated by two-dimensional polyacrylamide gel electrophoresis, in the first dimension by isoelectric focusing (pH 3 to 10) and in the second dimension in a 5 to 15% (wt/vol) gradient of acrylamide in the presence of sodium dodecyl sulfate by the method of O'Farrell (19). For autofluorography the resulting slab gels were dehydrated and impregnated with 2,5-diphenyloxazole (1, 14) before being dried onto filter paper (Whatman 3MM) and exposed to X-ray film (Kodak XH-1 or XRP-) as described previously (26).

Discrimination of ^{35}S- from ^{32}P-labeled polypeptides. To match on autofluorograms the pattern of ^{32}P-labeled polypeptides with that obtained from ^{35}S labeling, autofluorograms of gels containing both isotopes were prepared immediately after drying of the gel by exposing to X-ray film at −80°C for 7 days. After a lapse of 3 months (corresponding to the half-life of ^{35}S), during which time the ^{32}P activity was reduced 60-fold, the gels were reexposed for 14 days. In this way polypeptides labeled only with ^{35}S showed as spots of about equal intensity in the pairs of autofluorograms of the same gel. Any ^{32}P-containing spots were much reduced (or disappeared almost completely if no ^{35}S was present) in the second exposure. Results were checked by cross-reference to autofluorograms obtained from cells labeled only with ^{32}P.

pH and molecular weight estimation. The pH gradient in the first dimension was estimated by cutting six duplicate isoelectrically focused tube gels into 5-mm slices. Each slice was immersed in 2 ml of degassed distilled water for 10 min, and the pH was measured. Plots of pH versus slice number enabled the pH scale to be estimated for Fig. 2.

Molecular weights of polypeptides seen in the second dimension were estimated by comparison with standard proteins, including bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, and lysozyme, separated on a number of the gels. In these instances the gels were stained with Coomassie brilliant blue R (2.5%, wt/vol), destained, and then subjected to autofluorography as described above.

RESULTS

Under the conditions used in these experiments strain JW-1 began premeiotic deoxyribonucleic acid replication 3 h after resuspension in sporulation medium and commitment to meiotic segregation (determined by the appearance of *leu2* auxotrophs) after 4 h; asci could first be detected after 12 h (Fig. 1). The majority (about 75%) were four spored, whereas most of the remainder were three spored. During growth in presporulation medium before transferring the cells to sporulation conditions, 40% of the available $^{35}SO_4^{2-}$ was taken up into trichloroacetic acid-precipitable material, and labeling of the cells reached a steady state (data not shown). Disruption of the cells by mixing with glass beads in a Vibromix resulted in greater than 80% breakage of cells, asci, and spores. The extracts finally obtained from each sample represented approximately 50% recovery of the trichloro-

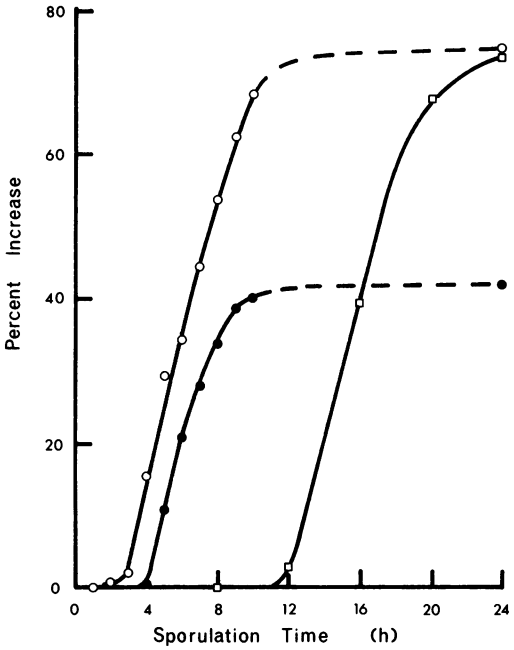

FIG. 1. *Timing of some sporulation events in strain JW-1 as described in the text. Symbols:* ○, *deoxyribonucleic acid per milliliter of culture;* ●, *commitment to meiotic segregation, plotted as twice the percentage of leu2 segregants observed;* □, *percentage of asci.*

acetic acid-precipitable label; the remainder was found in the insoluble debris and glass beads after centrifugation (3,000 × *g*, 1 min).

Sporulation-specific protein changes. A composite diagram (Fig. 2) of the readily detectable polypeptides from *S. cerevisiae* was constructed by comparing the autofluorograms obtained by two-dimensional polyacrylamide gel electrophoresis of ^{35}S-labeled extracts prepared at 2-h intervals throughout sporulation. Each extract was analyzed at least in duplicate, and each gel was exposed for autofluorography for several different times.

Of about 400 polypeptides that could be detected, 45 were found to have altered in cells during 24 h in sporulation medium (Table 1), confirming the results of a preliminary study (26). The important result is that 21 of the changes (Table 1) were due to the appearances of polypeptides only in sporulating **a**/α JW-1 and were therefore sporulation specific. The remaining 17 appearances were less interesting in that they were common to both sporulating (JW-1) and nonsporulating (JW-2) cells.

Only 14 of the sporulation-specific changes were due to the appearance of polypeptides not detectable in vegetative cells; the remaining 7 changes were the result of significant increases in the amounts of polypeptides that were already present at lower concentration in vegetative cells (Fig. 3).

Of the remaining seven polypeptides that changed (Table 1), one (no. 42 in Fig. 2) disappeared completely, and two more (no. 6 and 25) were markedly reduced in concentration only in nonsporulating JW-2 under sporulation conditions. Similarly, one polypeptide (no. 15) disappeared and two (no. 8 and 9) decreased in amount in both strains. Furthermore, one other (no. 41) appeared in both strains, but was much more evident in sporulating JW-1.

The above changes occurred in polypeptides from a wide spectrum of molecular weights, in the range from 20,000 to over 150,000 with most from 40,000 to 90,000. Appearances of proteins of high molecular weight would be unlikely to be due to proteolytic changes occurring during extraction. All possible precautions were taken to avoid artifacts of this nature in accordance with the practice of others (21). Moreover, a mixed cell extraction procedure was used to eliminate the possibility that changes were due to proteolysis during extraction. Unlabeled sporulating cells were mixed with labeled vegetative cells, and extracts were made in the usual way. No significant difference between the labeled polypeptide patterns of the mixture and of the 0-h extracts was found.

Timing of protein changes. By examining fluorograms of extracts prepared at successive 2-h intervals after transfer of cells to sporulation medium, individual polypeptides were seen to alter at characteristic times. Figure 3 illustrates the changes that occurred in polypeptides numbered 34 to 37 in Table 1 (with molecular weights about 45,000; pI about 6.0). The majority of changes detected, both sporulation-specific changes and those common to both cell types, occurred within the first 6 h in sporulation medium (Table 1)—at least 6 h before the first signs of ascus formation. Of the 21 sporulation-specific changes, 10 were detectable after only 2 h in sporulation medium, and 8 of the rest had occurred within a further 4 h. There was no apparent difference in timing between the appearance of new polypeptides and those that underwent an increase in concentration.

Most of the new polypeptides that appeared in both sporulating and nonsporulating strains did so within 2 h of transfer to sporulation medium, but the majority of changes involving an increase in a polypeptide already present in vegetative cells took place later, between 4 and 8 h, and most of the other types of alteration (no. 6, 8, 9, 15, 25, and 42) occurred even later.

Phosphorylation of polypeptides during sporulation. Taking the above results in conjunction with the pulse-labeling studies reported by others (12, 13, 20, 24), it seems likely that some of the changes reported here were the result of modifications of polypeptides present in the vegetative cell before sporulation was initiated. Since phosphorylation of proteins can be readily detected by using [^{32}P]orthophosphate as the label, its possible involvement in sporulation was assessed. Extracts were prepared from sporulating cells that were labeled in four ways: (i) cells were labeled throughout growth with both $^{35}SO_4^{2-}$ and [^{32}P]orthophosphate and then transferred to label-free sporulation medium; (ii) $^{35}SO_4^{2-}$-labeled vegetative cells were suspended in sporulation medium to which [^{32}P]orthophosphate was added after 1 h; (iii) cells were labeled during growth with [^{32}P]orthophosphate only before transfer to label-free sporulation medium; and (iv) unlabeled vegetative cells were transferred to sporulation medium to which [^{32}P]orthophosphate was added after 1 h. These protocols were chosen to distinguish the following: proteins that are phosphorylated at all times, proteins that are phosphorylated only under sporulation conditions, and proteins that are dephosphorylated either between 1 and 7 h or between 7 and 24 h of sporulation. In protocols ii and iv of the above the [^{32}P]orthophosphate was added 1 h after transfer of the cells to sporulation medium to allow a significant proportion of the population to begin sporulation. It should be noted, however, that **a**/α diploids transferred to sporulation conditions only take up phosphate after a lag of 2.5 h. This may affect the ability to detect some very early modifications involving phosphate, although for one sporulation-specific change discussed below (polypeptide no. 20) an early phosphorylation may have been found.

Samples from cultures labeled by protocols i, ii, and iii were taken at 0, 7, and 24 h after transfer to sporulation medium; samples from cultures labeled by protocol iv were taken at 7 and 24 h after transfer. By comparing the autofluorograms obtained before and after decay of the phosphorus isotope it was found that 10 of the polypeptides previously detected by ^{35}S labeling contained the ^{32}P label at the times indicated in Table 2. Five of these (C through G in Fig. 1 and Table 2) were found by ^{35}S labeling to be present in vegetative as well as sporulating cells; one, F, was phosphorylated at all times. C, D, E, and G, however, were not phosphorylated in vegetative cultures (0 h), but were phosphorylated during sporulation (Table 2). Two polypeptides (no. 5 and 30) that were previously detected by ^{35}S labeling to appear in both **a**/**a** and **a**/α diploids after transfer to sporulation medium were also phosphorylated whenever present.

Three sporulation-specific polypeptides (no. 7, 20, and 41) were phosphorylated; no. 7 was phosphorylated at both 7 and 24 h, and it may therefore have been phosphorylated from the time of its appearance as a new polypeptide. Polypeptide no. 41 was labeled with ^{32}P at 7 h (but not at 24 h) by all methods of labeling used (i through iv), whereas no. 20 was of interest since it was labeled at 7 h (but not 24 h) only when ^{32}P was present in the presporulation medium (protocols ii and iv). From the timing data, polypeptide no. 20 appeared within 2 h of transfer of cells to sporulation medium; thus, it is likely that this polypeptide is phosphorylated as it appears and is dephosphorylated by 24 h.

Finally, two previously undetected spots presumed to be polypeptides (they were not detectable by ^{35}S labeling) were also phosphorylated within 7 h of transfer of cells to sporulation medium. These are shown clearly in Fig. 4 (spots A and B), which illustrates the detection of polypeptides labeled in extracts prepared after 7 h in sporulation medium to which [^{32}P]orthophosphate was added at 1 h (Fig. 4a) and their location by the double-labeling technique described above (Fig. 4b and c). The extent of ^{32}P label in spots A and B decreased during the later stages of sporulation such that by 24 h, B could no longer be detected and A could barely be detected.

For a number of the above changes, the appearance of a polypeptide may have been due to the phosphorylation of a preexisting protein. However, for two at least (no. 20 and 41), this explanation does not hold since these polypeptides appeared in about the same position on gels after apparent dephosphorylation. In these cases phosphorylation did not alter the pI of the polypeptides sufficiently for them to be detected as marked changes in the broad range of pH gradient used during isoelectric focusing.

DISCUSSION

These results have confirmed and extended those of the initial study (26) and have shown that changes in proteins that are specific to the process of sporulation in yeast can be detected by using continuous presporulation labeling techniques, and that there appears to be definite control over the timing of these changes.

Many of the changes, both sporulation-specific changes and those common to both sporulating and nonsporulating **a**/**a** diploids (proba-

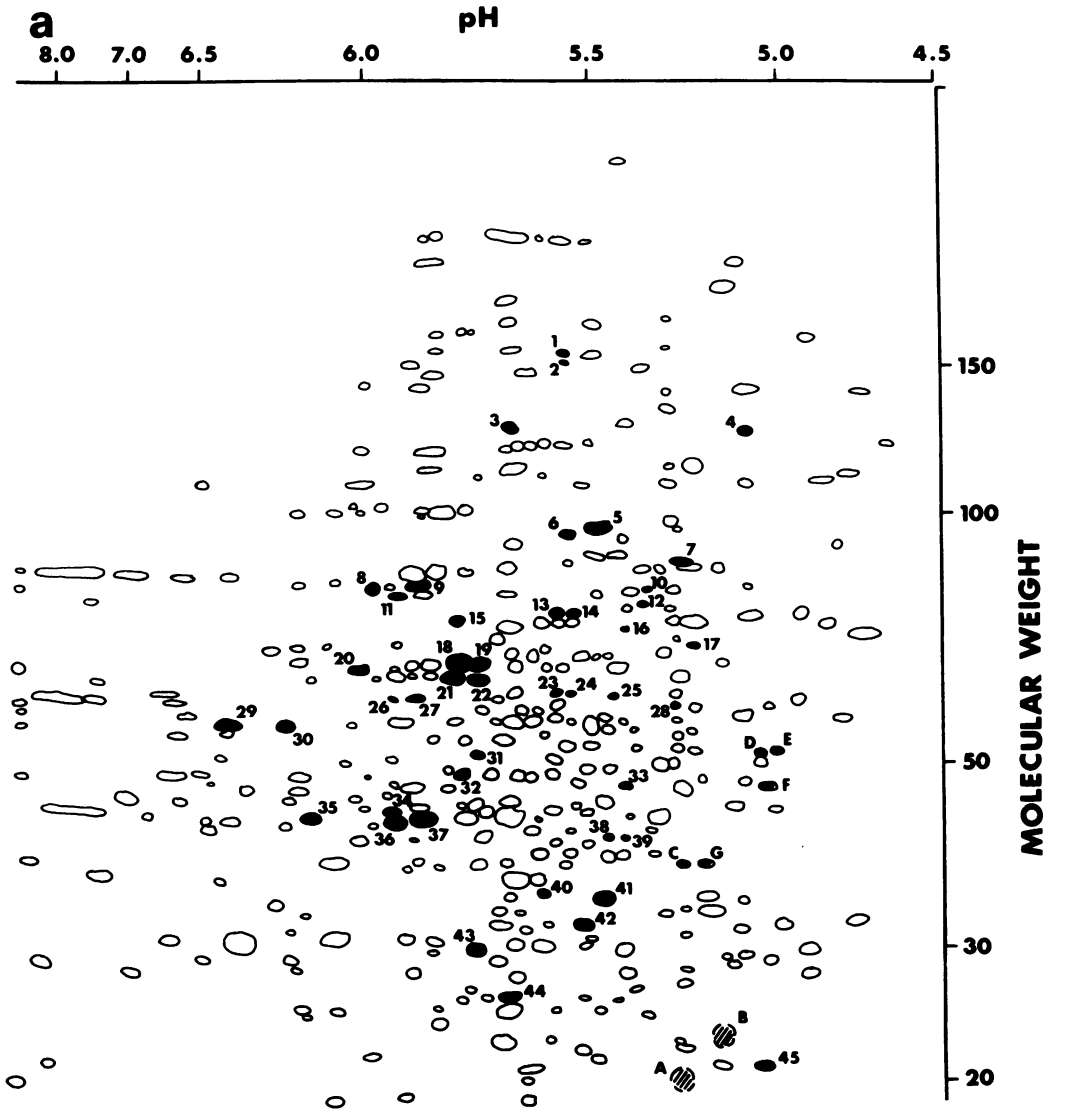

FIG. 2. *a, Composite diagram of the more prominent polypeptides detected by autofluorography of ^{35}S-labeled yeast proteins. The polypeptides were separated by two-dimensional gel electrophoresis as described in the text. Polypeptides undergoing changes during sporulation are indicated as blacked-in spots and numbered for reference to Tables 1 and 2. A and B indicate the two components detected by [^{32}P]orthophosphate labeling only. Molecular weights are given $\times 10^{-3}$. b, Autofluorogram obtained on an extract of cells from a 24-h sporulation culture that had been prelabeled with $^{35}SO_4^{2-}$ as described in the text. This was used as the basis for Fig. 2a.*

bly resulting from the change in media), were noticeable within the first 6 h after transfer to the sporulation medium. Mature asci did not appear under the same conditions until 12 to 14 h after resuspension, and by comparison with the time course of sporulation events obtained by us and others these early sporulation-specific changes were taking place before cells became committed to meiosis and corresponding to the periods when deoxyribonucleic acid synthesis and recombination were occurring (23). They also coincide with the times at which the rates of synthesis of ribonucleic acid and protein are maximal (16). Despite this preponderance of early changes, there are nonetheless a number of later ones that are important since they define the sequential nature of the changes and provide useful markers of the later stages of sporulation.

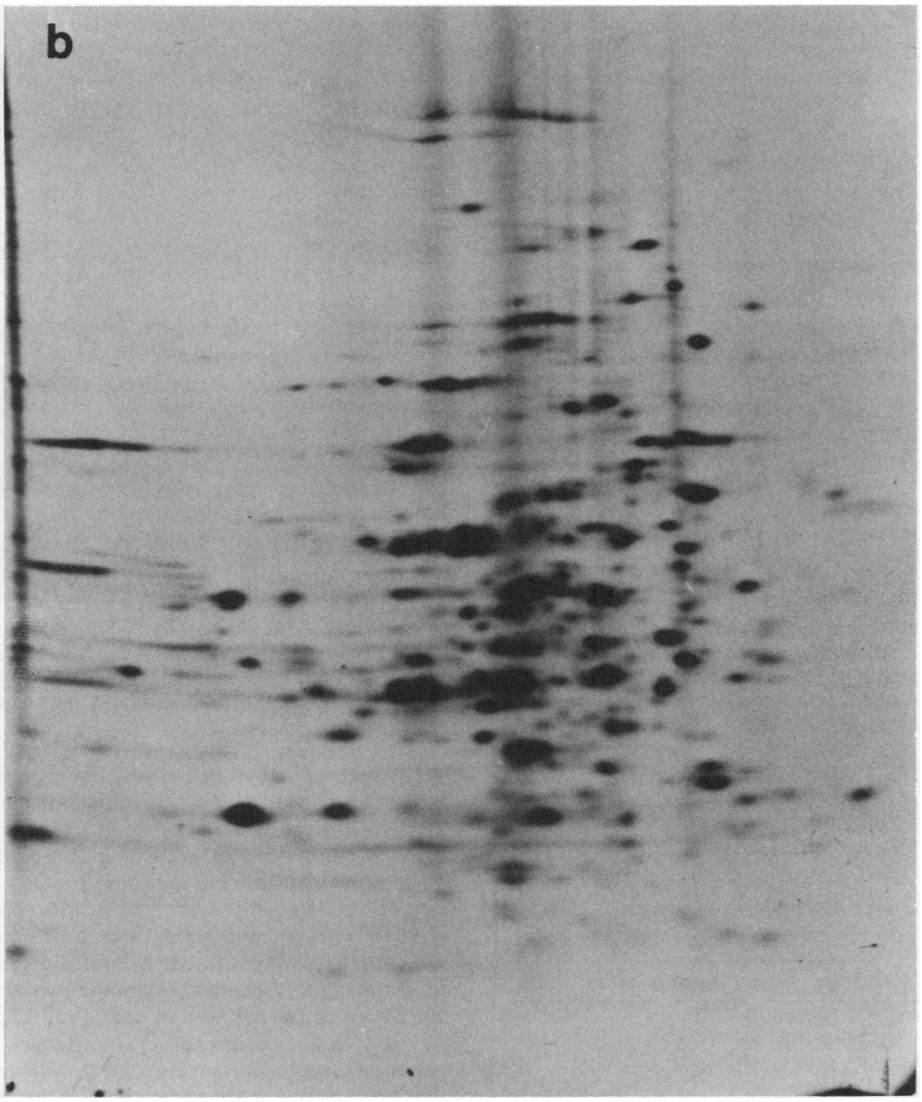

FIG. 2. *b*.

TABLE 1. *Nature and timing of significant changes in polypeptides during sporulation*

Nature of change	Identity of polypeptide[a] at time of first appearance[b] (h):				
	2	4	6	8	16 to 24
Sporulation specific					
New	7, 17, 20, 26, 27, 35	11, 16, 23, 38	24, 39		1, 2
Concentration increase	4, 13, 14, 37	45		10, 28	
Common					
New	29, 40, 43, 44	12	33		
Concentration increase	5, 18, 19	3, 31, 32	21, 22, 30, 34, 36		
Other alterations		41	8, 15	9, 42	6, 25

[a] Polypeptides are numbered according to the scheme given in Fig. 1.
[b] Unless otherwise stated in the text, the change persists for the remainder of the 24-h period.

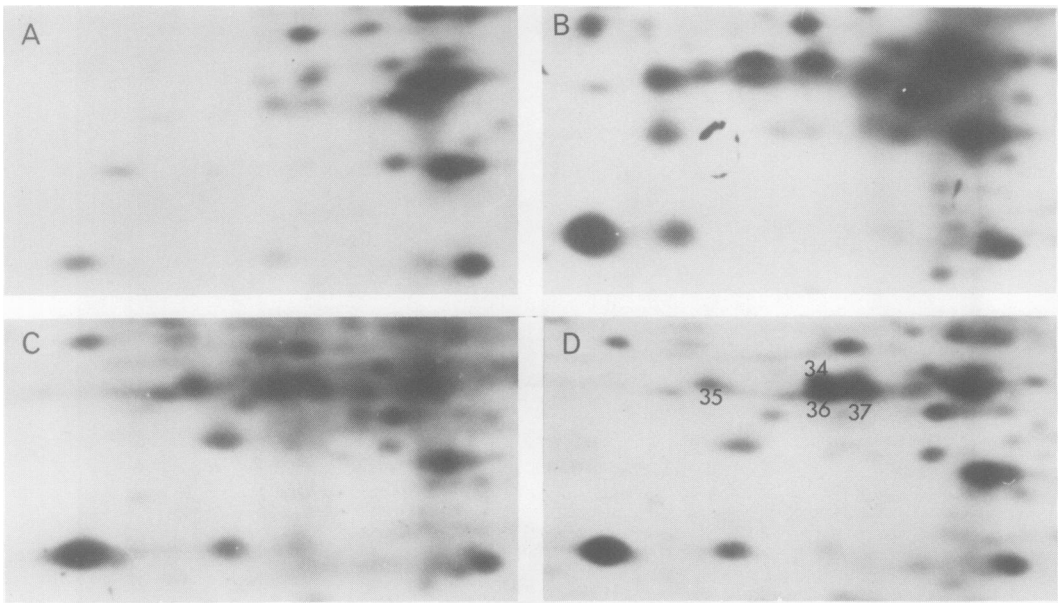

FIG. 3. *Timing of changes in some polypeptides during sporulation. Autofluorograms of the separated ^{35}S-labeled polypeptides were obtained on cultures that had been prelabeled with $^{35}SO_4^{2-}$ before resuspension in sulfate-free sporulation medium. Samples were taken at (A) 0 h, (B) 6 h, (C) 8 h, and (D) 24 h after resuspension. Only the small region of each autofluorogram including polypeptides no. 34 to 37 and those with molecular weight about 45,000 and pI around 6 is shown.*

TABLE 2. *Phosphorylation of cellular polypeptides during yeast sporulation*

Polypeptide[a]	Phosphorylation of polypeptides				
	[^{32}P]orthophosphate added only in presporulation medium			[^{32}P]orthophosphate added after 1 h in sporulation medium	
	0 h[b]	7 h	24 h	7 h	24 h
5	−	+	+	−	−
7	−	+	+/−	+	+
20	−	+	−	−	−
30	−	−	−	+	−
41	−	+	−	+	−
A	−	+	+/−	+	+
B	−	+	−	+	−
C	−	+	+	+	+
D	−	+	+	+	+
E	−	+	+	+	+
F	+	+	+	+	+
G	−	+	+	+	+

[a] Polypeptides that were phosphorylated were correlated with those numbered or lettered in Fig. 2 by using the ^{35}S, ^{32}P double-labeling technique described in the text and illustrated in Fig. 4.

[b] Extracts were prepared at the times indicated at the head of each column.

At least one of these changes may be concerned with a spore surface protein (2) which may be the antigenic determinant reacting with antibody prepared against whole spores (22).

Recently, biochemical studies of yeast sporulation have tried to find specific proteins that are synthesized uniquely during sporulation to provide the means of analyzing the control of gene expression during a simple process of eucaryotic cell development. The results of several studies using pulse-labeling with L-[^{35}S]methionine and subsequent polyacrylamide gel electrophoretic separation of the labeled proteins (12, 20, 24) were remarkable in that they appeared to show that whereas there are changes in the patterns of proteins synthesized in cells in sporulating conditions, the same changes occurred in sporulating (**a**/α diploid) and nonsporulating (**a**/**a**, α/α diploid; **a**, α haploid) cells.

It is premature to suggest from these results that there are no detectable changes in gene expression that are uniquely concerned with the process of sporulation in yeast, and that all of the changes reported here are due to protein modification rather than to de novo synthesis. Pulse-labeling has been done using [^{35}S]methionine and therefore would not have detected changes in proteins containing few methionine residues. Moreover, sporulating cells raise permeability barriers (17), and nonsporulating cells present in sporulating yeast populations can take up some amino acids preferentially (6). Recently, however, a repetition of the pulse-labeling and polyacrylamide gel electrophoresis

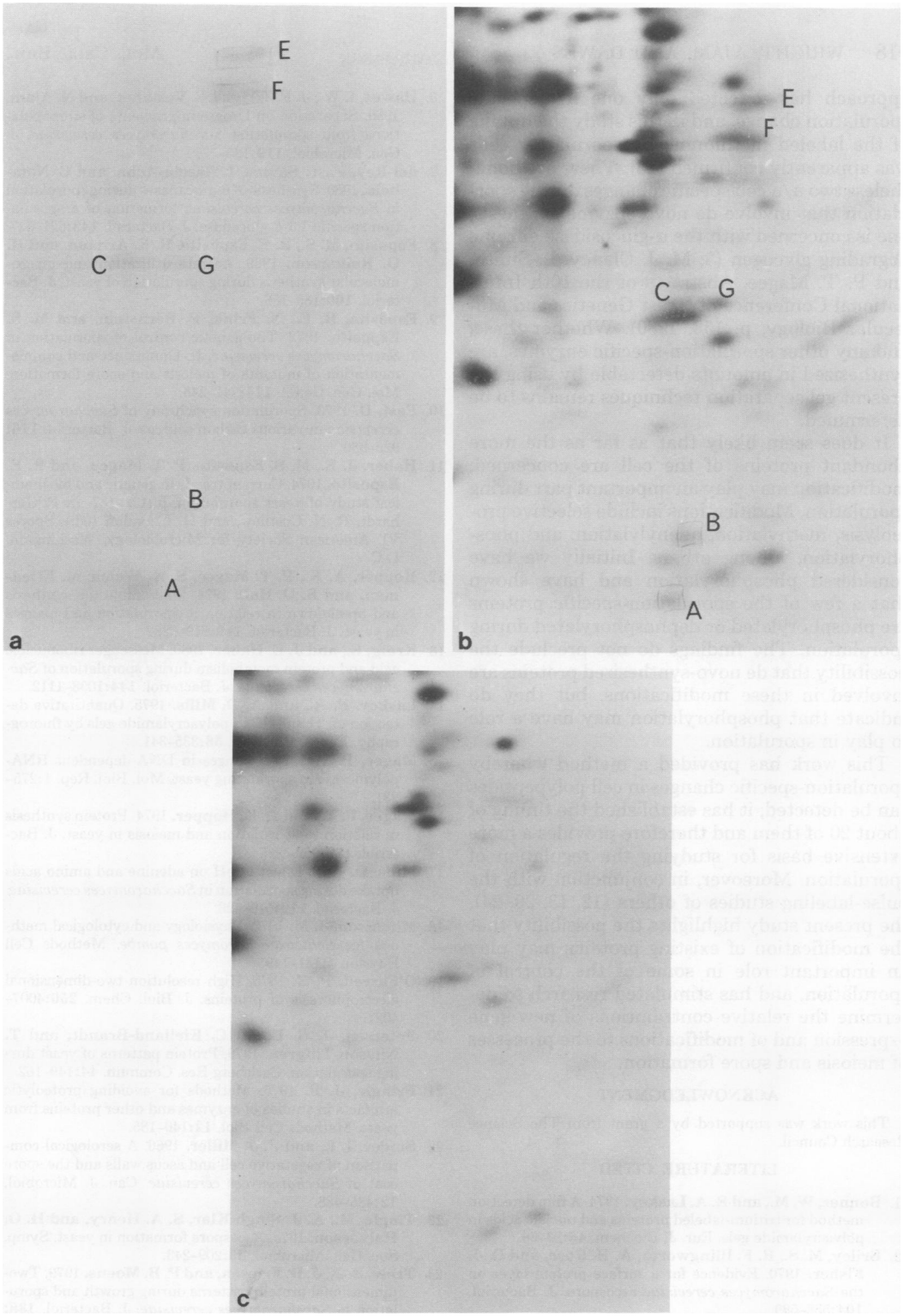

FIG. 4. *Polypeptides phosphorylated during sporulation. Autofluorograms obtained from 7-h sporulation cultures to which [^{32}P]orthophosphate had been added 1 h after resuspension in sporulation medium. a, ^{32}P labeling only. b, Cultures were prelabeled with $^{35}SO_4^{2-}$ before resuspension in sporulation medium, and [^{32}P]orthophosphate was added after 1 h. This autofluorogram was prepared as soon as possible after two-dimensional electrophoresis. c, Same gel as used to obtain b, but stored for 3 months before reexposure for twice the period. Under these conditions the ^{32}P label has decayed almost completely, but similar ^{35}S exposures were obtained. Spots A and B can be seen clearly, together with a number of other ^{32}P-labeled polypeptides. The region shown in these autofluorograms corresponds to the lower right section of Fig. 2 (molecular weights, 15,000 to 50,000; pI, from 4.5 to 5.5).*

approach has detected only one a/α-specific sporulation change, and in this study the uptake of the labeled methionine by sporulating cells was apparently confirmed (13). There are nonetheless two a/α cell specific changes during sporulation that involve de novo enzyme synthesis; one is concerned with the α-glucosidase enzyme degrading glycogen (3; M. J. Clancy, L. Smith, and P. T. Magee, Abstracts of the 10th International Conference of Yeast Genetics and Molecular Biology, p. 153, 1980). Whether these, and any other sporulation-specific enzymes, are synthesized in amounts detectable by using the present gel separation techniques remains to be determined.

It does seem likely that as far as the more abundant proteins of the cell are concerned, modification may play an important part during sporulation. Modifications include selective proteolysis, methylation, adenylylation, and phosphorylation, among others. Initially we have considered phosphorylation and have shown that a few of the sporulation-specific proteins are phosphorylated or dephosphorylated during sporulation. The findings do not preclude the possibility that de novo-synthesized proteins are involved in these modifications, but they do indicate that phosphorylation may have a role to play in sporulation.

This work has provided a method whereby sporulation-specific changes in cell polypeptides can be detected; it has established the timing of about 20 of them and therefore provides a more extensive basis for studying the regulation of sporulation. Moreover, in conjunction with the pulse-labeling studies of others (12, 13, 20, 24), the present study highlights the possibility that the modification of existing proteins may play an important role in some of the control of sporulation, and has stimulated research to determine the relative contributions of new gene expression and of modifications to the processes of meiosis and spore formation.

ACKNOWLEDGMENT

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