Protein Patterns during Sporulation in Fission Yeast

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Various strains (wild-type and mutants) of *Schizosaccharomyces pombe* were subjected to sporulation conditions, and analysed with regard to changes in their protein patterns. Trisbuffer-soluble proteins (TS) and DNA-binding proteins (DB) were denatured and separated by polyacrylamide electrophoresis. After endogenous nitrogen depletion (the apparent trigger of conjugation and sporulation) the following alterations were observed: larger TS-proteins were proteolytically degraded; the pattern of DB-proteins underwent several selective changes (reduction, enforcement, or new appearance of individual bands) ; an endonucleolytic DNase activity appeared. Most of these changes were expressed before conjugation, and did not appear in sterile strains. They could, however, be provoked by an abrupt shift to nitrogen-free medium, even in sterile strains. Inhibition studies with cycloheximide revealed at least two phases of protein synthesis specifically needed for sporulation: a very sensitive phase before conjugation, and a more resistant phase lasting until 0.5 h before spore formation. Medium exchange between heterothallic cultures (h^+ and h^-) effected certain sporulation-specific alterations of the DB-protein pattern, indicative of diffusible mating-type factors in the culture fluid.

The sporulation cycle of the fission yeast *Schizosaccharomyces pombe* consists of several successive phases: sexual agglutination, conjugation, meiosis and spore formation. From this sequence, meiosis is of the most general interest because of its fundamental importance to sexual reproduction in all eukaryotes. It is still not possible yet to describe the meiotic processes in molecular terms precisely. An essential requisite in approaching this goal is a correlation of biochemical data with structural and ultrastructural and, above all, genetic observations.

In *S. pombe* the isolation of mutants blocked at various steps of the sporulation cycle has turned out to be relatively simple [1–4]. Hence we tried to analyse these mutants by biochemical criteria. As a first step in the search for meiosis-specific proteins we compared the protein patterns of meiotic and non-meiotic cells by polyacrylamide gel electrophoresis.

Meiosis is, however, closely coupled to the cessation of growth and to sporulation in *S. pombe*, so that these processes can hardly be investigated independently. Cells can only enter the sporulation cycle after the culture medium has been depleted, especially from nitrogen sources [5]. Under this condition, cellular growth is impeded, and vegetative cell division phases out after the normal temporal order of DNA replication and cell division has been interrupted [6].

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We therefore analysed protein patterns on two different levels: (i) The spectrum of all the trisbuffer-soluble proteins (TS-proteins) was supposed to reveal bulk changes in response to starvation, whereas (ii) more subtle differences expected to be meiosis-specific might be enriched in a fraction with DNA-binding properties (DB-proteins), recoverable by affinity chromatography. As a first indication of enzymic differences we observed a potent endonucleolitic DNase activity in cells from sexually induced cultures.

In order to correlate the observed changes more closely to specific stages, we followed various culture parameters in different strains and mutants. We also report on medium exchange experiments between heterothallic cultures indicative of diffusible interactions in the early induction phase.

Materials and Methods

Strains and media. All the mutants used are derived from homothallic strains (h^{90}) of *Schizosaccharomyces pombe*, and have been described previously [1–3]. Six different groups of strains were investigated: (i) h^{90}, h^+, h^- – the normal mating types of the original wild-type strain; (ii) mutants still able to self-conjugate, yet blocked before the first meiotic division – *mei1-B102*, *mei3-B71*, *mei4-B2*; (iii) mutants which can only conjugate with h^+ and h^{90}: *map1-A80* (no self-agglutination), *map2-A207* (self-agglutinable) ; (iv) mutants which can only conjugate with h^- and h^{90}: *mam1-A50* (no self-agglutination), *mam2-A84* (self-agglutinable) ;

(v) a mutant blocked during cell fusion: *fus1-B20*; and (vi) sterile strains (*ste*) which do not sexually react with any standard mating type: *A17, A28, A76, A90, A104.*

Culture media were used as previously described [5, 6]. The synthetic sporulation medium (SSL) was modified to contain 250 mg/l aspartic acid (instead of 200 mg).

Growth and sporulation conditions. Stationary cells from a YEL (yeast extract liquid) preculture were inoculated into SSL at a titer of 8×10^5 to 2×10^6 cells/ml. The SSL cultures were shaken at 30 °C in Erlenmeyer flasks. In order to induce sporulation, the cells were either allowed to grow further in the same medium [5] or, at a titer of 1×10^7 cells/ml, they were transferred into SSL medium without a nitrogen source [6].

Buffer solutions. "Crude-extract buffer": 20 mM Tris HCl (pH 7.5), 1 mM β-mercaptoethanol, 1 mM Na_3EDTA, 50 mM NaCl; "DNase buffer": crude-extract buffer plus 10 mM $MgCl_2$, 2 mM $CaCl_2$.

Protein and DNA determination. Protein concentrations were determined by a modified Lowry procedure [7]. DNA was fluorometrically measured using 3,5-diaminobenzoic acid [8].

Protein labelling. Proteins of log-phase cells were labelled for three cell generations during vegetative growth with 0.035 – 0.1 μCi/ml [U-^{14}C]protein hydrolysate (Amersham-Buchler, Braunschweig). Proteins formed during sporulation phase were labelled with 0.02 – 0.07 μCi/ml from the beginning of agglutination to the onset of spore formation.

Endonuclease test. Double-stranded covalently closed circular λdv DNA was isolated by standard procedures [9]. The endonuclease reaction was done as follows. TS-proteins (2 mg/ml in crude-extract buffer) 0.04 ml, and λdv DNA (0.7 mg/ml) 0.06 ml were added to DNase buffer 0.3 ml. In certain tests 0.2 mM ATP was added. After 30 min at 20 °C the reaction was stopped with 0.4 ml chloroform/isopropanol (96 : 4), and the DNA was precipitated with ethanol. After separation on acrylamide agarose gels and staining of the DNA with ethidium bromide [10] the gels were photographed under ultraviolet illumination.

Isolation of trisbuffer-soluble (TS-) proteins. Cells of 40 ml samples were harvested by centrifugation, washed three times with ice-cold water, frozen in liquid nitrogen, and stored at – 20 °C (up to 2 d) before processing. A "Microdismembrator" (B. Braun, Melsungen) was used to break the cells. The vibration chamber, the agitating tungsten carbide ball, and the cell pellets were cooled with liquid nitrogen, before the assembled containment was agitated for 1 min. The debris was taken up in 1.5 ml crude-extract buffer (cooled in ice). After protein dissolution, the homogenate was centrifuged for 30 min at $10\,000 \times g$. The supernatant contained the TS-proteins.

Isolation of DNA-binding (DB-) proteins. Both DNA acrylamide and DNA cellulose columns were used, containing native and denatured calf thymus DNA in a 1 : 1 ratio. The DNA acrylamide columns [11], 1.1 cm in diameter and 10 ml packed volume, were operated from bottom to top at a flow rate of 6 ml/h. DNA cellulose columns [12] are workable at higher DNA concentrations (3 mg *vs* 0.1 mg DNA per 1 ml packed volume) as compared to DNA acrylamide. With both types of columns the DB-proteins were isolated according to Alberts and Herrick [13]. Cells from a 400 ml culture were processed as described for the TS-proteins.

The cell debris was resuspended in 12 ml of a buffer appropriate for the elimination of endogenous DNA, which was either precipitated with 10% polyethyleneglycol (PEG, mol wt 6000) in crude-extract buffer containing 2 M NaCl, or digested with 0.1 mg/ml pankreatic DNase I (3.1.4.5) for 1 h at 20 °C in DNase buffer. Subsequent steps were carried out at 4 °C. The PEG- or DNase-treated extracts were centrifuged (15 min at $10\,000 \times g$), dialysed against crude-extract buffer (24 h, 3 changes), and centrifuged again (1 h at $150\,000 \times g$, or 2.25 h at $100\,000 \times g$). The supernatants were adjusted to 2 mg/ml in protein and to 10% glycerol, and 11.5 ml were loaded on to a DNA cellulose column. The resulting protein : DNA ratio of 2.5 : 1 falls into the lower linear part of the saturation curve for these columns, where the recovery of DB-proteins is still independent of the protein load. After loading, the columns were washed with 10 vols crude-extract buffer, containing 10% glycerol. The DB-proteins (2 – 3% of the proteins loaded) were eluted with 2 M NaCl crude-extract buffer, collected, dialysed against water, and lyophilized in 10 ng protein portions.

SDS gel electrophoresis. Proteins were prepared and separated in a neutral SDS buffer system [14] by means of slab gels [15]. The slabs were 1 mm thick, and consisted of 8.5 cm separating gel, 1 cm

spacer gel, 1.5 cm sample slots. Linear gel gradients (as used for the separation of proteins less than 32 000 Daltons) were set up using a conventional gradient mixer. Marker proteins were used for calibration [16]. Gels were stained with Coomassie blue and destained with 7% acetic acid [17]. For autoradiography, the slabs were dried under vacuum.

Evaluation of protein patterns. The destained gels were illuminated from behind, and photographed. Absorption profiles were obtained directly from the destained gels, using a Joyce Loebl "Chromoscan MK II". Photographs, absorption curves and original gels were used in combination to identify the protein bands in the different patterns. The scan curves to be matched were superimposed on an illumination box, and were evaluated by comparison with the gels and photographs. Profile differences were only recognized after they had been reconfirmed in the original gels.

Results

Cell division and sporulation. Liquid cultures of *S. pombe* can be induced to sporulate (after conjugation) by a shift of growing cells from nitrogen-containing to nitrogen-free synthetic medium [6], or by extended incubation in the nitrogen-containing medium (SSL) beyond the point of nitrogen depletion [5]. In shift-induced sporulation, cell division ceases at the beginning of agglutination, whereas heterothallic cells (unable to sporulate without partner cells) can start an additional division cycle [6].

Similar results, observed without shift, are shown in Fig. 1. In the sporulating h^{90} culture cell division ceases within 3 h after agglutination. In the h^- culture the rate of cell division decreased transiently, but division was later on resumed until the final h^{90} titer was exceeded by 60%. All the self-agglutinable strains investigated (*mei4*, *mei3*, *mei1*, *fus1*, *mam2*, *map2*), as well as an h^+/h^- mixture, coincided with h^{90} as far as cell division is concerned, whereas all sterile or heterothallic strains unable to self-agglutinate (h^+, *mam1*, *map1*, *ste-A17*, *ste-A104*) followed the h^- growth curve.

Protein degradation before sporulation. As a first approach to analyse protein metabolism in sporulating and non-sporulating *S. pombe* cultures, patterns of trisbuffer-soluble (TS-) proteins were developed by SDS polyacrylamide gel electrophoresis. In Fig. 2 these patterns are shown for h^{90} cells, which had been harvested at various times before or during the sporulation cycle (in SSL without shift). Although distinctive pattern transformations did not accompany the different stages of sporulation, progressive disappearance of larger proteins was indicated by these gels. In order to obtain a quantitative comparison of different strains, the entire protein patterns were scanned and integrated by planimetry. For each pattern the fraction of proteins larger than 18 000 Daltons relative to its total protein content was plotted against the sampling time. The value obtained at a titer of 1×10^7 cells/ml was set to 100%. In Fig. 3 (a, b and c) these data are compared for three cultures: h^{90} (sporulating), h^- (heterothallic), and *ste-A17* (sterile). Other agglutinable cultures (*mei4*, *mei3*,

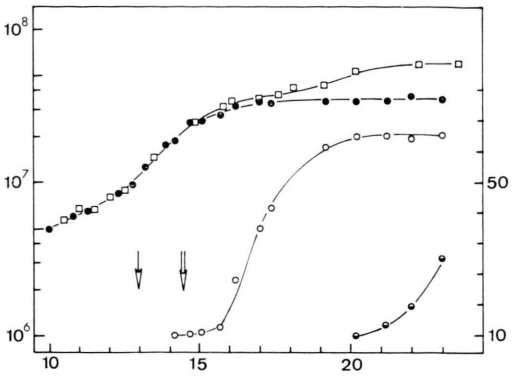

Fig. 1. *Abscissa:* time after inoculation (hours); *ordinate:* (left) cell number, h^- (□), h^{90} (●); (right) % zygotes (○), % asci (◒). Culture parameters after nitrogen depletion. Both cultures (h^- and h^{90}) were inoculated with 1×10^6 cells/ml in SSL medium, and incubated without shift. The arrows indicate the times of nitrogen depletion according to ref. [5], and of visible agglutination (double arrow).

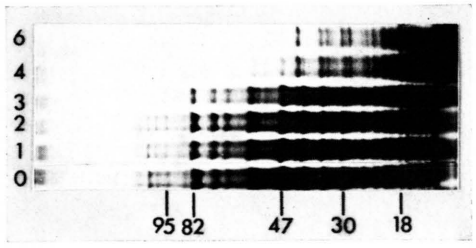

Fig. 2. *Abscissa:* molecular weight of proteins in 10^3 daltons; *ordinate:* time (hours) after a titer of 1×10^7 cells/ml had been reached. Trisbuffer-soluble proteins of h^{90} before and during sporulation. The gel consisted of a 10% to 20% linear gradient.

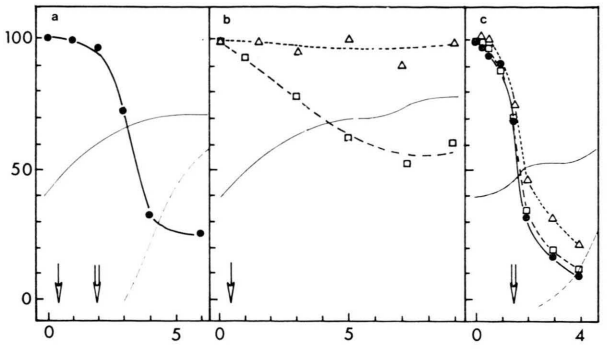

Fig. 3. *Abscissa:* time (hours) after a titer of 1×10^7 cells/ml had been reached; *ordinate:* relative fraction of proteins larger than 18,000 daltons. Protein degradation of different strains after endogenous nitrogen depletion (a, b), and after shift into nitrogen-free medium (c). Strains: h^{90} (●), h^- (□), *ste-A17* (△). Lightly drawn curves and arrows indicate the culture parameters of Fig. 1.

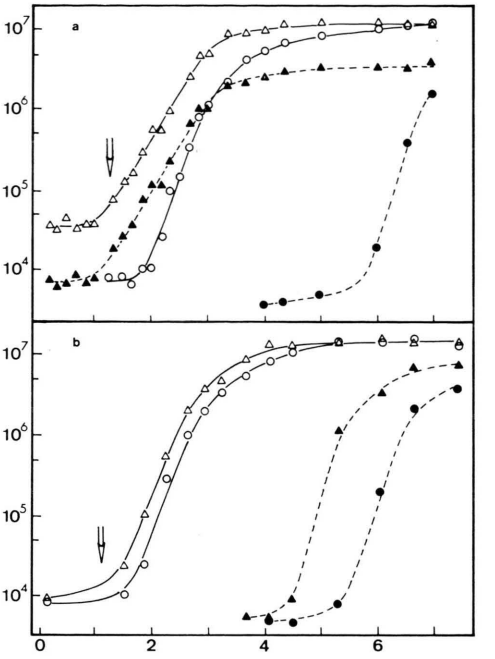

Fig 4. *Abscissa:* time (hours) after mixing of the heterothallic cultures (h^+ and h^-); *ordinate:* number of zygotes or asci (per ml). Inhibition of conjugation and sporulation by cycloheximide at 10 μg/ml (a), or 100 μg/ml (b). Triangles indicate zygotes (△) and asci (▲) of treated cultures. Circles indicate zygotes (○) and asci (●) of untreated controls.

mei1, *fus1*, *mam2*, *map2*, and h^+/h^- mixture) matched the h^{90} culture, other heterothallic non-agglutinable strains (h^+, *mam1*, *map1*) matched the h^- culture, and another sterile strain (*ste-A104*) matched the *ste-A17* culture.

Apparently, under these conditions, protein degradation proceeded the more the further the respective cells could advance towards sexual reproduction. These differences, however, vanished when cells of the same strains as before were shifted to nitrogen-free medium (Fig. 3 c, where h^{90}, h^- and *ste-A17* were again selected as representatives).

Inhibition of protein synthesis. To inquire for how long cells of a sporulatable culture depended on newly synthesized proteins to accomplish sporulation, cycloheximide (10 or 100 μg/ml) was added at various times. For these experiments h^+ and h^- cells had been mixed at a 1 : 1 ratio from parallel cultures (in SSL) at 3.5×10^7 cells/ml. Samples were removed at 10 min intervals and incubated with cycloheximide until 12 h after the time of h^+/h^- mixing, when all samples were chilled on ice, so that cells, zygotes and asci could be counted at a convenient time later on. Cycloheximide at 10 μg/ml was able to inhibit both conjugation and sporulation only when it was added before agglutination had started (Fig. 4 a). At a higher concentration (100 μg/ml), however, it blocked sporulation until 1 h before the first spores would have appeared (Fig. 4 b). When cells were shifted to nitrogen-free medium, cycloheximide (100 μg/ml) also prevented protein degradation (*cf.* Fig. 3 c).

Endonuclease activity. No sporulation-specific enzyme activities have as yet been described for *S. pombe*. On the other hand, endonucleases are implied in most theories of genetic recombination for the initiation of cross-overs. It was of interest, therefore, to look for endonucleolytic activities during the sporulation cycle. Hence, different strains were tested using double-stranded covalently closed circular λdv DNA as a substrate. With the exception of log-phase h^{90}, all the strains shown in Fig. 5 were harvested 6 h after the titer of 1×10^7 cells/ml had been passed (in SSL, see Fig. 1). Crude extracts of h^{90} in log-phase or of the sterile strains did not contain endonuclease activities, either with or without the addition of ATP – ATP-dependent nucleases have been discerned in bacterial recombination (*cf.* Radding [18]). Extracts of all other strains were able to degrade the circular DNA. These strains, therefore, contained at least one active endonuclease during stationary or sporulation phase.

Fig. 5. Endonuclease activity of different strains against λdv DNA circles. The strong band at the top corresponds with dimer DNA circles. The lower band consists of monomeric DNA molecules. Trails: h^{90} spo-phase (a), h^{90} log-phase (b), b+ATP (c), DNA control+ATP (d), *ste-A17*+ATP (e), a+ATP (f), DNA control (g), *ste-A17* (h), *mei3* (i), *fus1* (j), *mei1* (k), *mei4* (l), h^+ (m), h^- (n), *map1* (o), *map2* (p), *ste-A104* (q).

DNA-binding (DB-) proteins. After the patterns of the TS-proteins had not revealed marked differential changes related to sporulation, a selected group of proteins, recoverable by DNA affinity chromatography [13], was studied. This fraction should be enriched for structural or enzymatic chromatin proteins specific for meiosis. In Fig. 6,

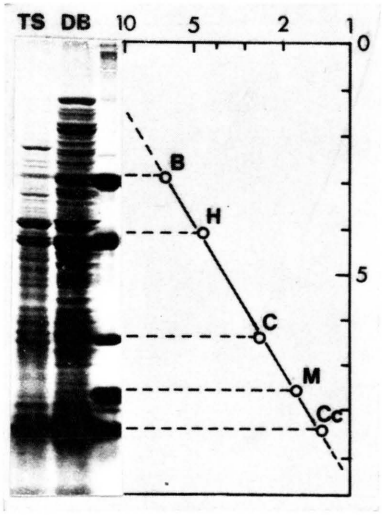

Fig. 6. *Abscissa:* molecular weight of marker proteins in 10^4 daltons; *ordinate:* distance (cm) from the top of the gel. Calibration of protein size. The gel consisted of a 10% to 20% linear gradient. Trails: trisbuffer-soluble (TS) and DNA-binding proteins (DB) of h^{90} log-phase cells, and marker proteins: bovine serum albumin (B), chicken serum albumin (H), chymotrypsin (C), myoglobin (M), cytochrome-c (Cc).

TS-proteins and DB-proteins from DNase-treated crude extracts of h^{90} log-phase cells were separated and matched with standard proteins for calibration. Very few bands are common to both patterns. Hence, most DB-proteins were isolated selectively.

Fig. 7 shows the DB-protein patterns of h^{90} log-phase, h^{90} at the beginning of ascus formation ("spo-phase", 5 h after agglutination) and a heterothallic self-agglutinable strain *mam2*, also harvested 5 h after agglutination. Comparison of both the h^{90} patterns reveals that, unlike the before mentioned TS-proteins, the DB-proteins underwent distinctive changes preliminary to sporulation. These differences appeared at an early stage of the developmental sequence, since all changes were expressed by mutant strains that are blocked between agglutination and sporulation. Mutant *mam2* is included in Fig. 7 as the earliest-blocked representative of this group (*map2*, *fus1*, *mei1*, *mei3*, *mei4* gave identical results). Degradation of larger proteins was much more selective among DB-proteins than in TS-proteins. Disappearing bands were mostly found in the upper region. Some bands were not reduced or even amplified, and several new bands appeared. When proteins (synthesized after agglutination had started) were labelled by incorporation of radioactive amino acids, the patterns of DB-proteins developed by autoradiography coincided with the stained patterns (Fig. 9, data only shown for shift experiments). This was supportive evidence that the proteins of the modified pattern were synthesized as such during the induction phase of mating.

The DB-protein patterns of sterile strains or non-agglutinating heterothallic ones showed only minor changes to the log-phase pattern. As shown in Fig. 8, the pattern produced by the sterile strain *ste-A17* fairly resembled that of h^{90} log-phase, whereas h^- (similar to h^+, *mam1*, *map1*) developed a pattern somewhat intermediate to the h^{90} spo-phase profile.

Yet, similar to the results regarding proteolytic activity, the differences between agglutinating strains, non-agglutinating heterothallic strains, and sterile strains were much smaller in shift experiments to nitrogen-free SSL medium. In Fig. 9, instead of h^-, the protein pattern of a 1 : 1 mixture ($h^+ + h^-$) between the crude extracts of both heterothallic strains is shown. This mixture was used as a control to check whether factors of both extracts

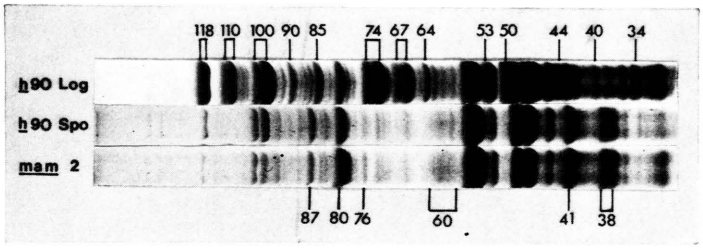

Fig. 7. DB-proteins from log-phase and spo-phase cultures in SSL without shift (10% gel). Numbers (mol wt in 10^3 daltons) indicate bands that are typical for log-phase (above), or spo-phase (below).

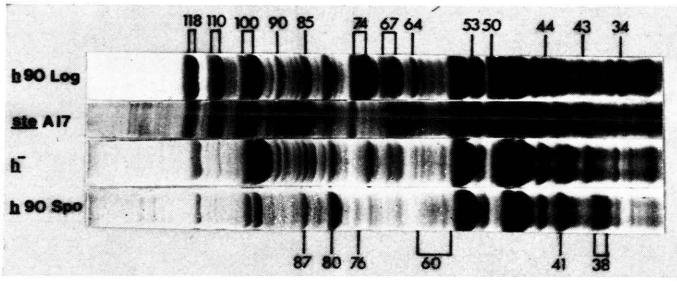

Fig. 8. Intermediate patterns (*ste-A17* and h^-) of DB-proteins from SSL cultures without shift (10% gel; numbers as in Fig. 7).

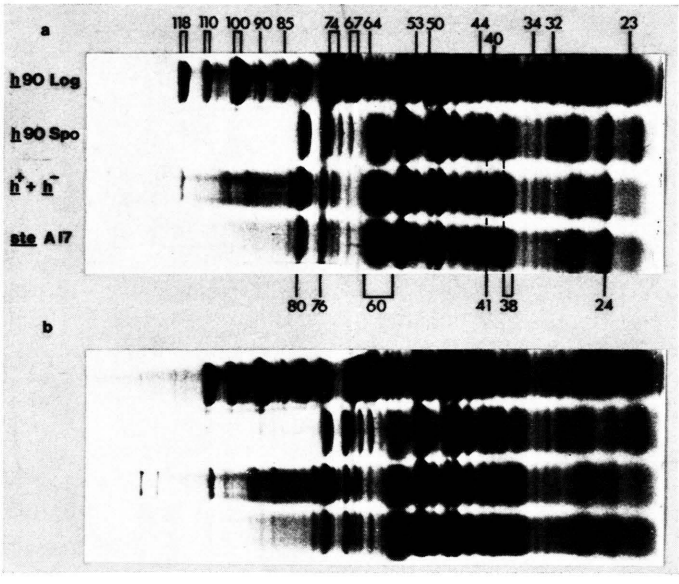

Fig. 9. Development of the same gel by staining (a) and autoradiography (b) DB-proteins were extracted after shift into nitrogen-free medium. The gel consisted of an 8% to 18% linear gradient (numbers as in Fig. 7).

could cooperatively produce the full spo-phase pattern during the isolation procedure of the DB-proteins. As shown in Fig. 9, the patterns of $h^+ + h^-$ and *ste-A17* were almost identical, and still different from the h^{90} pattern. Certain log-phase bands were differently reduced (74, 53, 50, 40), and a few spo-phase bands were present only in h^{90} (41 and double band 38).

Induction of the spo-phase pattern. It is still debatable whether all the observed changes in proteolytic activity and in protein patterns are specific to the sporulation cycle, or whether most of them occur merely as results of medium exhaustion and transition into stationary phase, as might be suggested by the shift results. Further evidence that the changes were not entirely unspecific was obtained from medium exchange experiments between heterothallic cultures (Fig. 10). Cultures (in SSL) of h^+ and h^- were grown in parallel to a titer of 3.5×10^7 cells/ml. The cells were then pelleted by centrifugation, the supernatants were exchanged, and the cells were incubated for 2 h in the medium

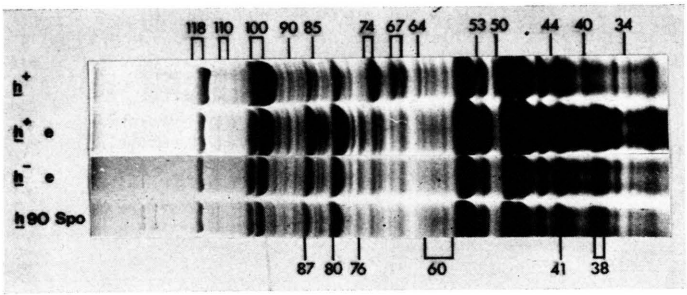

Fig. 10. DB-proteins after medium exchange (e) between heterothallic cultures (h^+ and h^-) (10% gel).

of the other mating type. The DB-proteins isolated from both these cultures revealed a pattern identical to that of h^{90} spo-phase. Also the breakdown of TS-proteins effected by this medium exchange was almost the same as in h^{90}. Similar experiments with sterile strains, however, did not produce significant changes.

Discussion

The comparison of vegetative and conjugating cells in *S. pombe* has shown that the start of the sexual cycle in these cells is accompanied by various biochemical changes. The results obtained from incubation in sporulation medium without a shift (relying on the endogenous depletion of consumable nitrogen) indicated that the early cessation of cell division, the degradation of soluble proteins (the larger ones in particular), the altered pattern of DNA-binding proteins, and the newly appearing endonuclease activity were specific for the sexual cycle in this yeast, since sterile mutants did not undergo these alterations. Upon an artificial shift to nitrogen-free medium, however, the differences between sterile and conjugating strains were reduced to but a few bands in the patterns of DNA-binding proteins (Fig. 9). Hence, most of the observed changes were specific only in a relative sense (see below). Their rather loose coupling with sporulation, therefore, gave but circumstantial answers to the central questions: Which changes of cellular metabolism are, in fact, specific for sporulation; and why do the cells respond differently to the slightly altered experimental procedure?

The expression of the biochemical changes observed under sporulation conditions varied with two parameters primarily: the genetic outfit of the cells (mating-type alleles and other relevant mutations), and the mode of nitrogen depletion. When cells exhausted the nitrogen source slowly by their own consumption, the various strains responded differently. They could be grouped in correspondence to three quantitative levels up to which they progressively approached the fully altered pattern of sporulation-proficient wild-type strains. These groups contained the sterile mutants, the non-agglutinative heterothallic strains, and the sexually agglutinating ones.

The abrupt interruption of their nitrogen supply, however, incited all the cells to the strongest response, irrespective of their sexual capacity. One explanation could be that it is the perturbation brought about by the experimental shift that releases the constraints altogether which normally keep control at different threshold levels. Another interpretation might assume inhibitory compounds accumulating in the culture fluid during vegetative growth. An indication that such compounds are likely to exist and even might be mating-type specific comes from certain medium exchange experiments (K. Friedmann, unpublished data). When the culture media of heterothallic strains were exchanged some time before the cells were mixed to allow conjugation, then zygote formation started about 20 min earlier as compared to the controls (mixed without previous exchange). Mating-type specific factors might interact with the postulated inhibitory compounds, thereby producing the normally observed step-wise appearance of the sporulation-specific pattern. The shift experiment, on the other hand, would dilute out these compounds and release all cells from the inhibitory control. Mating-type specific factors with similar properties have already been demonstrated in budding yeast [19]. – Yet another observation in favour of anti-sporulation factors produced during vegetative growth is the following dilution effect (E. Limpert and R. Egel, unpublished data). If diploid log-phase cells are transferred to SSL medium at too low a titer

(less than 10^4 cells/ml), division rate is severely retarded, and precocious sporulation is induced in a considerable fraction of the culture.

All the changes observed by our methods (including the newly appearing endonuclease) took place well before conjugation. Hence our series of mutants has not, as yet, defined additional steps in biochemical terms. Nevertheless, protein synthesis is clearly needed beyond the fusion of cells, as demonstrated by the cycloheximide experiments. Yet, the nature of the proteins then synthesized remains to be evaluated. The chances are that most of these might be structural or membrane-bound proteins which, therefore, have been missed by our extraction procedure.

The observations reported here are most appropriately summarized by the assumption of two periods of protein synthesis essential to sporulation. The first round is brought about by nitrogen starvation and is associated by a major breakdown of preexisting proteins. The second phase only starts after agglutination and leads to products that require more sophisticated techniques for their detection. Comparable results, at least with regard to proteolytic activities under sporulation conditions, have also been reported for *Saccharomyces* [20].

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[1] C. Bresch, G. Müller, and R. Egel, Molec. Gen. Genet. **102,** 301 (1968).
[2] R. Egel, Molec. Gen. Genet. **121,** 277 (1973).
[3] R. Egel, Molec. Gen. Genet. **122,** 339 (1973).
[4] J. H. Meade and H. Gutz, Genetics **83,** 259 (1976).
[5] R. Egel, Planta **98,** 89 (1971).
[6] R. Egel and M. Egel-Mitani, Exptl. Cell Res. **88,** 127 (1974).
[7] A. J. Shatkin, Fundamental Techniques in Virology (K. Habel and N. P. Salzman, eds.), p. 231, Acad. Press, New York 1969.
[8] J. M. Kissane and E. Robins, J. Biol. Chem. **233,** 184 (1958).
[9] G. Hobom and D. S. Hogness, J. Mol. Biol. **88,** 65 (1974).
[10] U. Petterson, C. Mulder, H. Delius, and P. A. Sharp, Proc. Nat. Acad. Sci. U.S. **70,** 200 (1973).
[11] L. F. Cavallieri and E. Caroll, Proc. Nat. Acad. Sci. U.S. **67,** 807 (1970).
[12] R. M. Litman, J. Biol. Chem. **243,** 6222 (1968).
[13] B. Alberts and G. Herrick, Methods in Enzymology, **vol. 21** (L. Grosman and K. Moldave, eds.), p. 198, Acad. Press, New York 1971.
[14] U. K. Laemmli, Nature **227,** 680 (1970).
[15] F. W. Studier, J. Mol. Biol. **79,** 237 (1973).
[16] A. L. Shapiro, E. Vinuela, and J. V. Maizel, Biochem. Biophys. Res. Commun. **28,** 815 (1967).
[17] H. R. Maurer and F. A. Dati, Analyt. Biochem. **46,** 19 (1972).
[18] C. M. Radding, Annu. Rev. Genet. **7,** 87 (1973).
[19] L. E. Wilkinson and J. R. Pringle, Exptl. Cell. Res. **89,** 175 (1974).
[20] A. K. Hopper, P. T. Magee, S. K. Welch, M. Friedman, and B. D. Hall, J. Bacteriol. **119,** 619 (1974).