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# PREMEIOTIC DNA SYNTHESIS IN FISSION YEAST

R. EGEL and MICHIKO EGEL-MITANI

*Institutes for Biology (III and II), University of Freiburg i Br., D-7800 Freiburg, BRD*

## SUMMARY

Sporulating and various non-sporulating strains of *S. pombe*, especially several mutants deficient in conjugation or meiosis, were compared with respect to DNA synthesis under sporulation conditions. Meiosis and sporulation were induced by a transfer to nitrogen-free medium. As synchronized mitotic division was observed in all the strains as a first response to the shift, reducing the DNA amount per cell from the replicated state in G2 to the unreplicated state in the G1 phase of the cell cycle. Cells of the heterothallic wild-type strains ($h^+/h^+$ or $h^-/h^-$) accumulated in G1 with respect to DNA synthesis when they were incubated separately. In a mixed culture of these strains a period of enhanced DNA synthesis was observed after the start of zygote formation. This period of synthesis was absent in mutant *fus*1, where only prezygotes accumulated. Hence we conclude that in zygotic meiosis the premeiotic DNA synthesis is confined to zygotes after conjugation has been completed. In the diploid sporulating wild-type strain ($h^+/h^-$), capable of azygotic meiosis without prior conjugation, premeiotic DNA synthesis occurred between 2½ and 5 h after the shift to the sporulation medium. There was no significant premeiotic DNA synthesis observed in diploid cells of the meiosis-deficient mutants *mei*1 or *mei*3, whereas premeiotic DNA synthesis proceeded normally in mutant *mei*4, which is blocked at a stage after commitment to meiosis in opposition to both the other mutants.

The vegetative cell cycle of the fission yeast *Schizosaccharomyces pombe* has been investigated in considerable detail [1, 2]. *S. pombe* is also a well established test organism for genetic analysis [3, 4]. Relatively little, however, is known as yet about the regulation of the meiotic cycle in this yeast. The sequence of sexual reproduction is the outstanding event of differentiation in the life cycle of this unicellular fungus. Usually this sequence consists of the ordered succession of sexual agglutination, conjugation, nuclear fusion, meiosis, spore formation, and ascus lysis. This series can, however, be disconnected in diploid strains (e.g. $h^+/h^-$), in which meiosis and sporulation can proceed without prior conjugation ('azygotic meiosis').

The controlling factor of prime importance for the initiation of conjugation and zygotic meiosis as well as of azygotic meiosis is the mating type locus. This genetic region, consisting of at least two genes, is known to specify the three phenotypically expressed mating types $h^+$, $h^-$ and $h^{90}$. Cells of the homothallic mating type $h^{90}$ are self-fertile, showing sporulation in a clonal culture, whereas the heterothallic strains $h^+$ or $h^-$ can only sporulate in a mixed culture.

A genetic approach to a further analysis of the meiotic events in *S. pombe* has led to the isolation of mutants that are blocked at certain steps along the sporulation cycle, especially during conjugation and meiosis [5]. A detailed comparison of mutant and wild-type strains should elucidate which functions are normally governed by the affected genes.

Conditions for studying conjugation and

meiosis in liquid culture have been described [6], where nitrogen starvation was shown to be a critical prerequisite. Conjugation can be blocked by *fus*1 mutations. In this case only prezygotes accumulate, which can fully revert to vegetative cells when they are transferred to fresh growth medium. The genes necessary for the first meiotic division have been assigned to early vs late functions, since mutants of three genes (*mei*1, *mei*2, *mei*3; see also the following remark) are blocked before commitment to meiosis, whereas mutant *mei*4 is only blocked after commitment [7]. The present paper discusses the occurrence of premeiotic DNA synthesis in wild-type and mutant strains.

*Remark*

We suggest that the previously used symbol *meI* [5, 7] be replaced by the symbol *mei* to specify genes involved in the first meiotic division. In addition, we suggest the symbol *mes* to specify genes involved in the second meiotic division, hitherto called *meII* [5]. These alterations will conform with the three-letter code now generally adopted in microbial genetics.

## MATERIALS AND METHODS

*Strains and media.* The mutants defective in conjugation or in meiosis were described previously [5, 7]. They were derived from $h^{90}$ strains of *Schizosaccharomyces pombe* (Lindner). Mutant *fus*1 (B20) fails to complete conjugation, since the separating walls are not dissolved. Mutant *mei*1 (B102) carries a mutation at the mating type locus. This mutant, and mutant *mei*3 (B71) do not reach a stage of commitment to meiosis. Mutant *mei*4 (B2) is still blocked before the first meiotic division, but cells of this mutant become committed to the meiotic pathway.

Diploid strains heterozygous for mating type ($h^+/h^-$) were constructed by the use of selective markers (complementing *ade*6 alleles). Diploid strains homozygous for mating type ($h^+/h^+$ or $h^-/h^-$) were obtained as segregants from tetraploid meiosis [8].

Media were described previously [7]. Only the synthetic sporulation medium (SSL) was modified to a nitrogen-enriched version (SSL + N) and a nitrogen-free formula (SSL − N). SSL − N was composed as follows: (per liter) glucose 10 g, $KH_2PO_4 \cdot 2H_2O$ 250 mg, $MgSO_4 \cdot 7H_2O$ 500 mg, $CaCl_2 \cdot 2H_2O$ 100 mg, $H_3BO_3$ 500 $\mu$g, $CuSO_4 \cdot 5H_2O$ 40 $\mu$g, KI 100 $\mu$g, $FeCl_3 \cdot 6H_2O$ 200 $\mu$g, $MnSO_4 \cdot 2H_2O$ 400 $\mu$g, $H_2MoO_4 \cdot 2H_2O$ 160 $\mu$g, $ZnSO_4 \cdot 7H_2O$ 400 $\mu$g, biotin 10 $\mu$g (in 1 ml 50% ethanol), calcium pantothenate 1 mg, nicotinic acid 10 mg, *m*-inositol 10 mg. (Adenine was not included in this version, even when adenine requiring strains were used.) SSL + N contained (per liter) ammonium acetate 2 g, aspartic acid 200 mg, and adenine 75 mg in addition to the ingredients of SSL − N.

*Sporulation conditions.* The strains were maintained in nutrient-rich liquid culture (YEL = yeast extract liquid) in order to suppress the induction of azygotic meiosis. The precultures were diluted into SSL + N to a cell density of $1–3 \times 10^5$ cells/ml, and grown overnight. After having reached a cell density of $5–8 \times 10^6$ cells/ml, cells were harvested by centrifugation, washed once in SSL − N, resuspended in SSL − N at the original volume, and incubated at 30°C on a rotary shaker (ca 200 rpm).

*Cell counts.* The frequencies of cells, zygotes and spores were calculated from hemocytometer counts. Agglutinated samples were dispersed by brief sonication. Zygotes were counted as two cell equivalents.

*Radioactive label in RNA and DNA.* Cells were labelled with uracil-6-H3 (21 000 mCi/mmol, Amersham-Buchler, Braunschweig) for 5 generations in SSL + N (1–2 $\mu$Ci/ml). Cells were shifted to sporulation conditions, i.e. to SSL − N, and no further label was added. Aliquots of 0.5 or 1 ml were removed for nucleic acid determination. For RNA measurement, a sample was precipitated by an equal volume of cold 10% trichloroacetic acid (TCA). The precipitate was collected on membrane filters (SM 11306, Sartorius, Göttingen), and washed with 10 ml of 5% TCA. The filters were dissolved in 5 ml methoxyethanol, and methoxyethanol-toluene based scintillation fluid was added [9]. Vials were agitated for 10 min on a rotary shaker, and counted in a Beckman scintillation system. DNA was estimated after alkaline hydrolysis of RNA. Samples were incubated overnight at room temperature in 1 N NaOH, chilled in ice, and, in the presence of carrier DNA (100 $\mu$g/sample), precipitated by cold TCA (10% final concentration). After standing in ice for 15 min, precipitates were collected by filtration, and prepared for scintillation counting as before. DNA was estimated as the average count of 10 parallel samples.

In later experiments (figs 3, 6) only three parallel samples were counted for each point, but in these cases the sampling error, which caused problems especially in heavily agglutinated cultures, was reduced by the following schedule: At the time of the shift to the sporulation medium each culture was divided into 2.5 ml aliquots in 70 ml test tubes, which were then incubated on a reciprocating shaker. At each sampling time the entire contents of a test tube was dispersed by ultrasonic treatment before samples were distributed for DNA determination (3 times 0.5 ml into 0.5 ml 2 N NaOH) or for cell counting (0.5 ml into 4.5 ml 1% formaldehyde as a preservative).

In these experiments the filters containing the acid precipitates were dried in open vials at 60°C for 3–5 h before they were counted in a toluene-based scintillation fluid without methoxyethanol.

## RESULTS

### *Sporulation after medium exchange*

Conjugation, meiosis and sporulation in *S. pombe* can be observed in a low-nitrogen synthetic medium (SSL) after the nitrogen source has been consumed [6]. This condition was accentuated in shift-down experiments, when cells where harvested from nitrogen-enriched medium during late log phase, and resuspended in nitrogen-free medium. A fairly synchronous mitotic division was usually observed as a first response to the shift. This division was reflected by a pronounced peak in cell plate percentage (the fraction of septated cells, which can serve as a measure of synchronization) and a subsequent stepwise increase in cell number (fig. 1). In the case of haploid cells ($h^{90}$) the first zygotes were formed after 2 h, and the first developing spores appeared after 6 h. In sporulating diploid strains ($h^+/h^-$) there was no need for conjugation, and sporulation started about 3 h earlier than in haploid strains. The initial round of mitotic cell division did, however, take place (fig. 2).

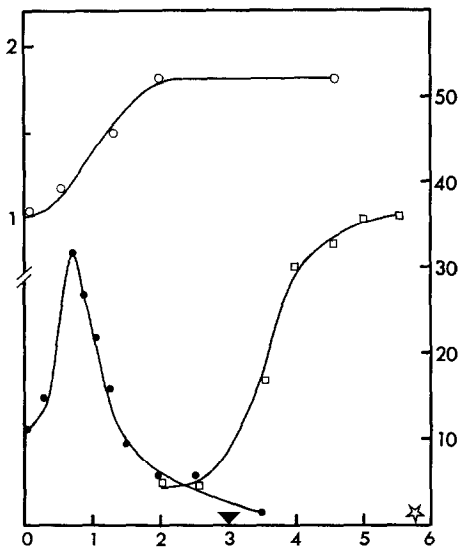


*Fig. 1. Abscissa:* time after transfer to SSL − N (hours); *ordinate: (left)* cell number (○), initial value $8 \times 10^6$ cells/ml; *(right)* % cell plates (●), % zygotes (□).

Cellular changes after N-starvation in a haploid strain. Wild-type cells of a haploid strain ($h^{90}$) were shifted from SSL + N to SSL − N medium. Cell density, cell plate percentage, and zygote percentage were determined. The beginning of agglutination (▼), and the first appearance of visible spores (☆) are also indicated in the diagram.

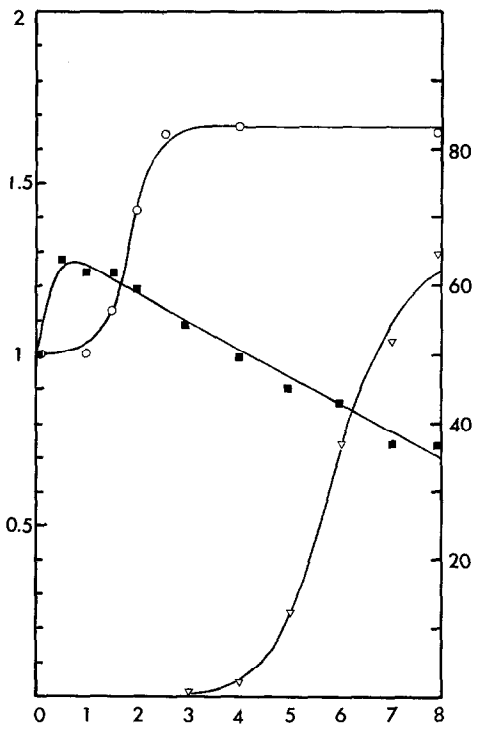


*Fig. 2. Abscissa:* time (hours); *ordinate: (left)* cell no. (○), $^3$H cpm in RNA (■), initial value $5.6 \times 10^5$ cpm; *(right)* % cells containing developing spores (▽). Cellular changes, and RNA degradation in a diploid strain. Wild-type cells of a diploid strain ($h^+/h^-$) were shifted to sporulation conditions, after being labelled for 5 generations with uracil-6-H3. The numbers of cells, and of developing asci, and the radioactive counts in nucleic acids (predominantly RNA) were determined as described in Materials and Methods.

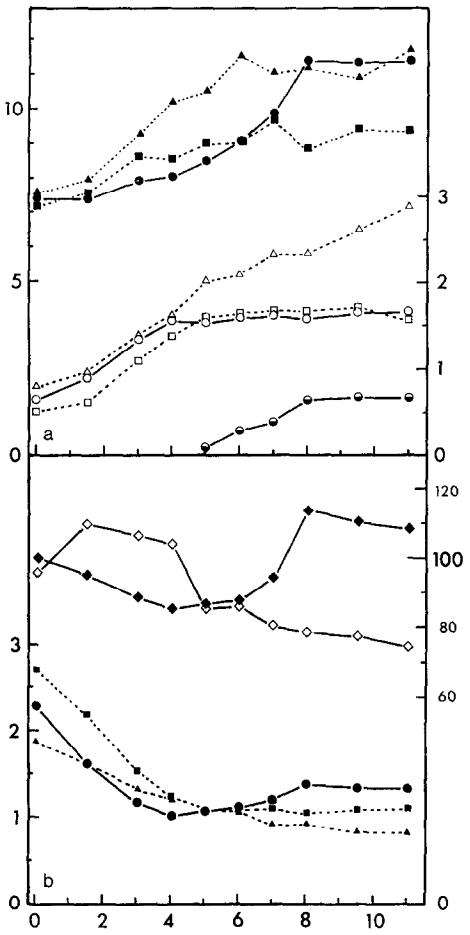


*Fig. 3. Abscissa:* time (hours); *ordinate:* (*a, left*) $10^2$ cpm ($^3$H DNA)/0.5 ml; (*a, right*) $10^7$ cells/ml; (*b, left*) $10^{-4}$ cpm/cell; (*b, right*) %.

Changes in cell number and DNA content in zygotic meiosis. Heterothallic strains ($h^+/h^+$, $h^-/h^-$) were shifted to SSL − N, and incubated separately or as a 1:1 mixture. (*a*) DNA content and cell number were determined according to Materials and Methods. DNA content: ▲, $h^-/h^-$, ■, $h^+/h^+$; ●, $h^+/h^+ \times h^-/h^-$; cell number: △, $h^-/h^-$; □, $h^+/h^+$; ○, $h^+/h^+ \times h^-/h^-$ total cells, ◒, ditto conjugated cells. (*b*) For the DNA content (◆), and for the cell number (◇) the ratios of these data in the mixed culture over the average in the separately incubated cultures are plotted as % of the average values, i.e. $100 \times (h^+/h^+ \times h^-/h^-) : 1/2 \times ((h^+/h^+) + (h^-/h^-))$. The DNA amounts per cell are also shown, using the same symbols as in (*a*) for DNA content.

## *Label in RNA*

When cells were prelabelled with radioactive uracil for five generations before the shift, their nucleic acids were considered to be uniformly labelled. At that time, ca 50% of the input radioactivity was recovered as acid-precipitable material (predominantly RNA; only 2% of the incorporated radioactivity was found to be alkali-resistant as DNA). After the shift to SSL − N without additional label, the cells had to rely on their pool of nucleotides and on turnover of pre-existing macromolecules for any new synthesis of nucleic acids. RNA was considerably degraded under these conditions (fig. 2). All the strains tested, the mutants included, showed this degradation of RNA of 40–60%. The initial increase immediately after the shift to SSL − N medium was also consistently observed. It may either be caused by a transient disturbance of the cells owing to the manipulations during the shift, or represent a depletion of the nucleotide pool before the degradation starts.

The degradation of RNA, at least after the first 30 min, should provide sufficient precursors for the DNA synthesis discussed in the following section, especially since the total amout of DNA is only ca 2% of the normal RNA content. Furthermore, this newly fed pool of nucleotides should have the same specific radioactivity as the pre-existing nucleic acids. Hence, any increase in radioactivity in DNA should be proportional to the amount of newly synthesized DNA.

## *Label in DNA*

*Zygotic meiosis.* Diploid strains homozygous for one of the heterothallic mating types ($h^+/h^+$ or $h^-/h^-$) are unable to sporulate when they are separately incubated. (Diploid strains were used in order to conform with the following experiments on azygotic meiosis.) When these strains were shifted to SSL − N, cell numbers increased more than two-fold. (For unknown reasons, the $h^+/h^+$ strain

of the experiment depicted in fig. 3 did not parallel the $h^-/h^-$ strains as closely as in other experiments, but this discrepancy will not affect the main conclusions to be drawn from this experiment. One reason may have been a higher proportion of $h^{90}$ cells, which spontaneously arise in every $h^+$ culture.) DNA synthesis took place in both these strains, but it did not keep pace with cell division, and gradually the DNA amount per cell declined to half its initial value (fig. 3*b*).

The third culture of this experiment was made up by mixing the above-mentioned strains (1:1 by volume) at the time of the shift. In this culture 40% of the cells had formed zygotes by the end of the experiment. Any deviation of this culture from the average of the separately incubated strains is relevant to the events associated with the sporulation cycle. The respective ratios of the cell numbers, and of the DNA contents are plotted in the upper part of fig. 3*b*. Initially, there was a small elevation in cell number, but then this parameter fell below the average of the control strains. Cell division seems to have been stopped before the appearance of the first zygotes. A different pattern emerged from the comparison of the DNA contents. Here, the DNA content remained below average until conjugation had started. Only thereafter did the relative DNA content rise again, and finally exceed the average. This pattern indicates a temporary repression of DNA synthesis until the first zygotes have been formed. The following rise probably represents the round of premeiotic DNA synthesis. Simultaneously the DNA amount per cell increased as well. The absolute increase seems to be small; however, 60% of the cells were still unconjugated.

The conclusion that premeiotic DNA synthesis occurred only after conjugation was strengthened by the experiments with mutant *fus*1. (This mutant had to be used as a haploid strain, since azygotic meiosis in diploid strains is not blocked by this mutation.) When this mutant was shifted to SSL − N, only prezygotes accumulated. The DNA amount per cell dropped to about half its value in vegetative cells, and a later increase was never observed (fig. 4).

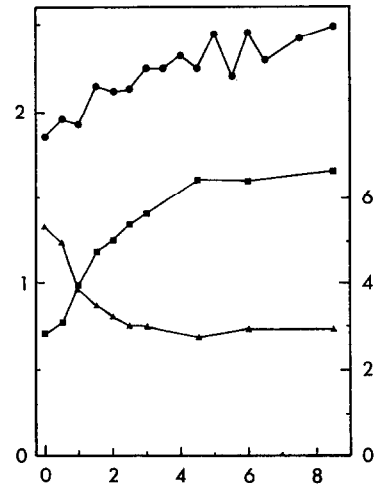


*Fig. 4. Abscissa:* time (hours); *ordinate: (left)* $10^3$ cpm ($^3$H DNA)/0.5 ml, or $10^7$ cells/ml; *(right)* $10^{-4}$ cpm/cell.

Changes in cell number and DNA content in the conjugation-arrested mutant *fus*1. ●, DNA content; ■, cell number; ▲, DNA/cell.

*Azygotic meiosis.* In the heterozygous diploid strain ($h^+/h^-$) the premeiotic DNA synthesis occurred in the period 2.5–5 h after the shift (fig. 5) between the last cell division and the first appearance of spores. Both the synchrony of cell division, and the efficiency of sporulation, were lower in this experiment than in the one shown in fig. 2, and the actual time needed for this DNA synthesis in a single cell might have been considerably shorter. The premeiotic DNA synthesis is also reflected by the rise in the DNA amount per cell. (Note that a developing ascus is counted as one cell, not the four developing spores.)

When the sporulating wild-type strain was

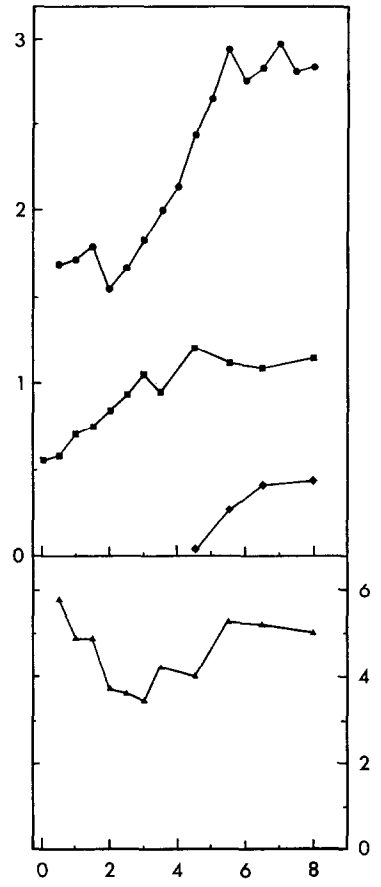


*Fig. 5. Abscissa:* time (hours); *ordinate: (left)* $10^3$ cpm ($^3$H DNA)/0.5 ml, or $10^7$ cells/ml; *(right)* $10^{-4}$ cpm/cell.

Changes in cell number and DNA content in azygotic meiosis. A culture of the heterozygous wild type strain ($h^+/h^-$) was shifted to SSL − N, and analysed. ●, DNA content; ■, cell No.; ◆, No. of developing asci; ▲, DNA/cell.

compared with three non-sporulating, meiosis-deficient mutants (fig. 6), the commitment to meiosis studied previously [7], and the ability to undergo premeiotic DNA synthesis appeared to be correlated. DNA synthesis comparable to wild-type was only observed in the mutant *mei*4, which is blocked after commitment to meiosis. The mutants carrying earlier blocks (*mei*1 or *mei*3) did not undergo this round of DNA synthesis. During this experiment five cultures of each strain were shifted at 1.5 h intervals, so that the initial cell numbers varied between $3 \times 10^6$ and $1.5 \times 10^7$ cells/ml. As the general trends were similar in the parallel cultures of each set, the differences between the fours sets have to be assigned to the different mutations rather than to deviating culture conditions at the time of the shift. (The meiosis-deficient mutants were homozygous for the respective mutation, and in the case of *mei*3 and *mei*4 heterozygous for mating type $h^+/h^-$; *mei*1 is a mutation of the mating type locus itself, allelic to $h^+$ and $h^-$.)

## DISCUSSION

Cells of *S. pombe* are known to lack a measurable G1 phase during the vegetative cycle

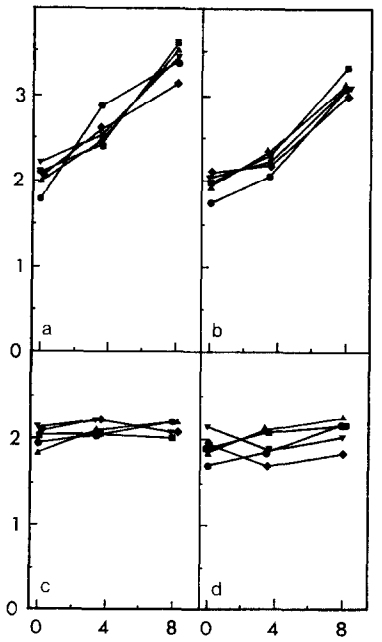


*Fig. 6. Abscissa:* time (hours); *ordinate:* $10^3$ cpm ($^3$H DNA)/0.5 ml.

Changes in DNA content in meiosis-deficient mutants. Diploid strains of wild-type ($h^+/h^-$) (*a*), or mutants *mei*4 (*b*), *mei*1 (*c*), *mei*3 (*d*) were analysed with respect to their DNA content. Of each strain five cultures were shifted at 1:5 h intervals, the initial cell density varying from $3 \times 10^6$ to $1.5 \times 10^7$ cells/ml, each set in the order (● ▲ ■ ▼ ◆).

[2]. In other words, cells at the time of division have passed through S phase, so that they already contain the doubled amount of DNA necessary for the next division. This is the basis on which the decrease of the DNA content per cell induced by the shift has to be discussed.

The present data indicate that such G2 cells cannot be induced to undergo meiosis directly. The first response to the shift to sporulation conditions is another vegetative cell division, no matter whether haploid or diploid cells are subjected to the shift. Since DNA synthesis is absent, or at least relatively low during this period, cells emerging from this division are in G1 phase of the cell cycle. In this respect, therefore, nitrogen starvation has similar consequences to phosphate starvation, which has also been shown to stop growth at the G1 phase level [2]. The pronounced synchrony of this division is brought about by a shortening of the normal cell cycle. Cells divide at an earlier time than they would have done if they had remained under growth conditions. This conclusion can be drawn from the uniform rise of the cell plate percentage after the shift (fig. 1).

Zygotes are only formed by G1 phase cells, since cells of one of the heterothallic mating types ($h^+/h^+$ or $h^-/h^-$), and even prezygotes of the mutant *fus*1 arrested during conjugation accumulate at this stage. A comparable condition has also been described for *S. cerevisiae*, in which case, however, conjugation proceeds during log phase, and synchronization at G1 phase is accomplished by specific factors excreted into the medium. These factors arrest cells of the opposite mating type at G1 phase until the completed zygotes resume growth and division [10, 11].

In *S. pombe* conjugation is confined to a stage where cells accumulate at G1 phase by other means (N-starvation), and analogous factors might not be required. Yet apparently, even in this yeast vegetative DNA replication is halted in prezygotes until the formation of zygotes has been accomplished. The data of fig. 3, especially the deviations induced in the mixed culture with respect to the control cultures incubated separately, provide further evidence for mating-type-specific intercellular interactions before the start of conjugation. Previous attempts [6, 8] have not revealed any cell-free factors inducing conjugation or azygotic meiosis in *S. pombe*, but the mating-type-specific repression of DNA synthesis and of cell division may be a proper test system to look for such factors again.

The present paper also provides further evidence for a genetic control of premeiotic DNA synthesis separate from that of vegetative replication. Analogous to what has been found in *S. cerevisiae* [12, 13], in *S. pombe* the mating type locus (*mei*1) regulates the onset of premeiotic DNA synthesis, and a second gene (*mei* 3) is likewise involved in this process. The separate control of vegetative vs premeiotic replication is also pronounced in *S. cerevisiae* by a dominant mutation affecting premeiotic DNA synthesis exclusively [14], and by three recessive ones [15].

It is still not clear whether vegetative and premeiotic replication are also different by physical criteria. Such differences might gain importance for a better understanding of the events leading to meiotic recombination. The work described can serve as a basis for studying the process of premeiotic DNA synthesis in greater detail.

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