Cell Division: a Separable Cellular Sub-cycle in the Fission Yeast
Schizosaccharomyces pombe

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Carbon-limited growth of Schizosaccharomyces pombe in chemostats under certain conditions of dissolved oxygen concentration and temperature gave rise to multiseptate and branched hyphal cells. On the basis of these observations it is suggested that fission can be uncoupled from growth, nuclear processes and septation.

INTRODUCTION

The concept of the cell cycle emerged from the laboratories of Hughes (1952), Pelc (Howard & Pelc, 1953), and Mazia (1956) about a quarter of a century ago. Based upon observations that all elements of the cell cycle were not always tightly coupled, Mitchison (1971) depicted the cell cycle as two parallel sub-cycles usually locked together. Thus nuclear events and cell division were considered to be tightly coupled in a DD (DNA-division) sub-cycle, whereas loosely coupled growth processes were considered to make up the parallel G (growth) sub-cycle. Subsequently, uncoupling has been discussed in general by Hartwell (1974) and specifically with respect to the DD sub-cycle of the fission yeast by Nurse et al. (1976).

By growing the fission yeast Schizosaccharomyces pombe in glucose-limited chemostat cultures in which the imposed growth rate was held constant while temperature and aeration rate were varied, we have obtained multiseptate and branching states which were readily reversible by further changes. These results are difficult to explain in terms of the two sub-cycle model of Mitchison (1971), but could be explained by uncoupling of the DD sub-cycle.

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METHODS

Organism and culture conditions. Cultures of the haploid strain of Schizosaccharomyces pombe (NCYC 132, ATCC 26192) were maintained in 10 ml amounts of malt extract broth (MEB, Oxoid; 2% w/v) in 1 oz bottles at 32 °C. A strain, S2, which had been twice recloned was used in all experiments.

Glucose-limited experimental cultures were grown in chemostats at growth rates imposed by the rate of addition of defined medium (EMM2; 0.2% w/v, glucose; Mitchison, 1970). Chemostats (culture volume about 500 ml), described previously (Johnson et al., 1978), were custom-made (Pegasus Industrial Specialists, Agincourt, Ontario, Canada). Cultures were agitated and aerated by a Vibro Mixer (Chemopac, Hoboken, N.J., U.S.A.) with a hollow stainless steel shaft through which sterile air was passed (Martin, 1980). Temperature was controlled by the circulation of water through the fermenter jackets. Culture pH was maintained by means of a pH control system (pH 40, New Brunswick Scientific Co.) through the addition of sterile 1 M-KOH. Dissolved oxygen concentration, [DO], was measured with a DO analyser (DO40, New Brunswick Scientific Co.).

Although our conclusions are based upon many similar experiments, we describe in detail only portions of a single chemostat run in which the dilution rate (D) was 0.069 h⁻¹ (mass doubling time, t₀, 10 h), and the

Abbreviations: BI, branching index; DI, division index; MSI, multiseptation index; [DO], dissolved oxygen concentration.

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controlled experimental variables were the pH, the [DO] of the medium, and the temperature of growth. The method for inoculating and operating chemostats was as follows: defined medium in chemostats was inoculated with late-exponential or stationary phase cultures of \textit{S. pombe} in EMM2 or MEB (10 ml in 1 oz bottles) at 32 °C. Cultures were allowed to grow to stationary phase before dilution with EMM2 (0.2% glucose) was started; collection of experimental data was begun only after a minimum of five volume changes. In the experiment described, the chemostat culture had grown for 43 d during which time qualitative data were collected. On day 43, conditions were set at $D = 0.06$ h$^{-1}$, temp. = 32 °C, pH = 5.5 and [DO] = 35%. After 72 h, collection of quantitative data related to changes in environmental conditions was begun.

**Observations of cell division.** The division index (DI, the proportion of septate cells), the multiseptation index (MSI, the percentage of 'cells' with more than one visible septum) and the branching index (BI, the percentage of 'cells' having branch points) were all determined by phase-contrast microscopy.

Cells were classified into seven easily scored stages of division (plus non-dividing cells) by fluorescence microscopy of Calcofluor-stained living cells (Johnson et al., 1974, 1977). Samples of 2250 cells were counted. The following stages were scored (see Fig. 1): those having (a) no apparent septum; (b) only a vestigially apparent primary septum; (c) a primary septum less than one-half closed; (d) a primary septum more than one-half closed; (e) a completely closed primary septum; (f) a primary septum less than one-half eroded; (g) a primary septum more than one-half eroded; and (h) only a vestigially remaining primary septum. Since different culture conditions affected different patterns according to this classification, the terms 'early' and 'late' become somewhat subjective. We use 'early' to refer to a cell found in the septum elaboration stages of division (Fig. 1 b–d), and 'late' to refer to the septum fission stages of division (Fig. 1 f–h), regardless of the proportion of division time required to attain the particular stage. ('Fission' becomes restricted in definition by this convention to only the septum erosion stages, and is no longer synonymous with the entire division process.) Two other points should be obvious: each class established by light microscopy might readily be sub-classified by electron microscopy; and the stage showing no sign of primary septum elaboration (Fig. 1a) might be blocked at initiation of primary septum elaboration (and hence could be classed as a division step) or might be blocked at any one of a number of pre-initiation steps which are crucial, but nevertheless not division steps. In sum, these criteria seem different from and have been found more convenient than those applied by Nurse et al. (1976).

**RESULTS AND DISCUSSION**

**Preliminary observations**

A glucose-limited chemostat culture of \textit{S. pombe} NCYC 132 S$_{2,2}$ was maintained at constant doubling time ($t_d = 10$ h) over a period of 71 d. During the first 43 d, qualitative observations showed that the DI, the MSI and the BI of the culture could be varied reversibly by changing the temperature, pH or [DO]. As a consequence of these observations, the culture was kept going and was examined critically for quantitative changes in the DI, MSI and BI.
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Fig. 2. Photofluoromicrographs of S. pombe cells stained with Calcofluor White M2R New. The brightly stained regions are septa. (a) A uniseptate during fission; (b) multiseptates with branches. Original magnification: (a) 1000 x; (b) 430 x.

The normal phenotype

At day 43, the chemostat was adjusted to 32 °C, pH 5-5 and [DO] = 35%. After 72 h under these conditions the chemostat-grown cells had the sausage-like morphology usually seen in batch culture (Fig. 2a). The growth habit was also like that seen in batch culture: cells extended at one end and divided by cross-wise septation and fission at approximately the mid-point. When extension resumed at the beginning of the cell cycles of the resultant progeny, it usually occurred at the older end, i.e. following Mitchison's rule of initiation (Mitchison, 1957). These cells could not be seen to differ from cells grown as batch cultures in MEB medium in spite of the fivefold longer generation time (10 h) imposed by the dilution rate of the chemostat.

Modulated division index

On day 46, the pH of the chemostat culture of S. pombe was adjusted to 3.75. After growing for 18 h at 32 °C and [DO] about 20%, the culture had a DI of about 10% (Fig. 3), a DI considered normal for asynchronous cultures in EMM2 (Mitchison, 1970). When the temperature of the chemostat was dropped to 20 °C there was a significant decrease in the DI (Fig. 3, arrow 1), coincident with a considerable increase in mean cell length (data not shown), and the OD_{660} dropped from 0.48 to about 0.27 over about 1.5 d. A steady state was not attained. After 1.5 d, about three mass doubling times, these longer cells began to divide, and the DI then increased to over 40% before experimental conditions were changed. The length of the cells was no longer remarkable, but an asynchronous culture with a DI almost as high as the highest previously published for a synchronous culture of S. pombe (Miyata & Miyata, 1978) was noteworthy.

After 3.5 d the rate of aeration was raised to increase the [DO] to about 58% from about 30% (Fig. 3, arrow 2). The OD_{660} remained at about 0.26 for a further 18 h, then reached a plateau at
Fig. 3. Changes of the division index (DI, proportion of septate cells, □) and of the branching index (BI, proportion of cells with branches, ×) in a chemostat culture of *S. pombe* in response to altered culture conditions. The pH was held constant at 3.75. At 1, the temperature was reduced from 32 °C to 20 °C. At 2, the dissolved oxygen concentration, [DO], was increased from 30% to 58%. At 3, the [DO] was decreased from 55% to 28%. The multiseptation index (MSI), if plotted, would be superimposed upon or fall slightly lower than the BI.

about 0.36, where it remained for two weeks. The acid product titration remained constant through the entire time. For about 2 d after the aeration rate was increased, the DI dropped as rapidly as it had risen; the rapid fall was followed by a long, slow pseudohysteretic decline in DI from about 24% to about 15% two weeks later. At this time, the [DO] was decreased again, this time to about 28% (Fig. 3, arrow 3). This change initiated a slow rise in DI that continued over about 6 d until experimentation ceased. In summary, the combination of low temperature (20 °C) and low [DO] (about 30%) produced a very high DI in these asynchronous cultures, but raising only the [DO] was enough to effect a decline in DI, rapidly for 2 d, then slowly for about 12 d.

The fact that the DI changed from 10% to over 40% in a continuously asynchronous chemostat culture indicates that the proportion of the cell cycle time spent in recognizable division by the typical cell must have been increasing. It would appear, therefore, that the modulated DI is secondary to a modulated cell cycle.

The initial decline of the DI in response to the lowered temperature suggests that the cells which had already initiated their septa could proceed reasonably quickly to complete division. The rates of activity of relevant enzymes were presumably decreased by the lowered temperature, but the slow-down did not seem to be onerous. On the other hand, cells which had not passed a critical point for elaboration of their septa before the temperature was dropped simply did not start to divide – initiation was markedly delayed and consequently the DI fell.

**Multiseptation and branching**

In a glucose-limited chemostat culture at 20 °C, pH 3.75, poising the [DO] at 30% resulted in the appearance of multiseptate and branched hyphal cells (Fig. 2b), as well as in the increases in DI and in mean cell length noted above. The hyphal cells began to appear about 1 d after the DI began to rise (BI, Fig. 3, arrow 1) but when the [DO] was increased from 30% to 60%, their numbers and the DI declined rapidly (Fig. 3, arrow 2).

Multiseptation did not bear a simple relationship to the DI (Fig. 4a), nor did branching (Fig.4b). At first glance, the MSI and BI seemed to show a smooth, curvilinear relationship to the DI. But an examination of the same data plotted in a chronological way (Fig. 3) shows that all the low BI values and most of the high BI values occurred while the aeration rate was low, [DO] about 30%; the intermediate BI values were mostly found during the pseudo-hysteretic period (between days 8 and 17, Fig. 3). The MSI and BI values remained at or near 0 until the DI exceeded 20%. Thus multiseptation and branching were associated with a high DI, but the relationships were not entirely simple.
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Raising the aeration rate resulted in a prompt fall in the DI, BI (Fig. 3) and MSI. The fact that these three values all fell more or less in synchrony suggests that multiseptation and branching are dependent upon continuous replenishment of the supply of slow divisions in the population of cells. If newly-extended divisions are important to the maintenance of the DI, MSI, and BI, then all divisions are proceeding to completion, no matter how slowly. Multiseptation and branching seem closely related to the primary lesion, the extended presence of the septum; but are secondary phenomena. The fact that they co-vary (Fig. 4c) suggests that they are equally secondary to the same defect.

A multiseptate cell first arises when a cell initiates a second growth sub-cycle before the processes terminating the first cycle have been completed. Extension at the old end follows Mitchison's rule of initiation just as though division had been completed, hence the uniseptate overlong cell grows at both ends. Eventually one or both 'halves' might complete their second extension sub-cycles and initiate new septa to become bi- or triseptate cells. A branched cell results when the overlong multiseptate cell initiates yet a third cycle of extension at the 'older' ends, i.e. those formed by the first septation; ends which are not yet fully formed because the division process is incomplete but which are, nevertheless, the old ends. However, because extension initiated at these incomplete ends is in a sideways mode, branching occurs. Finally, altering growth conditions either by simply increasing the [DO] to about 60% at pH 3.75 and 20 °C (Fig. 3), or, in other experiments, by changing the temperature to 32 °C and the pH to 5.5 at [DO] about 20% (data not shown), allowed most of these multiseptate cells to finish their septation–fission processes and return to the yeast state. These results suggest that nuclear processes were proceeding apace, and were in these experiments coupled more tightly with extension (G, sub-cycle) than with division. We explain these observations in terms of the possible existence of three potentially separable sub-cycles (G, growth; D, division; and N,
Fig. 5. Diagrammatic representation of the cell cycle of *S. pombe* separated into bi-sub-cycles (a) or tri-sub-cycles (b, c, d). (a) The bi-sub-cycle proposed by Mitchison (1971), consists of a G (growth) sub-cycle and a DD (DNA-division) sub-cycle. (b) This is a tri-sub-cycle having: G sub-cycles; D (division) sub-cycles showing the minimal condition for bi- or trisepate cells – F$_S$(1) continuing concurrently with G$_{E(2)}$ and overlapping at least briefly with F$_S$(1), and N (nuclear) sub-cycles. (c) This is a tri-sub-cycle having: G sub-cycles; D sub-cycles showing the minimal condition for branching – F$_S$(1) continuing concurrently with G$_{E(2)}$, overlapping with F$_S$(1), and overlapping at least briefly with G$_{E(2)}$, and N sub-cycles. (d) Showing the normal condition as a tri-sub-cycle is (d), with G, D, and N sub-cycles. The blanks represent metabolism (mostly of unknown nature) related to subsequent steps. I$_E$: initiation of extension; I$_O$: initiation of DNA synthesis; I$_M$: initiation of mitosis; F$_S$: formation of septum and fission; G$_E$: growth by extension; P$_M$: passage through mitosis; G1, S$_1$ first gap; S$_0$, DNA synthesis; G2, S$_2$ second gap; subscript numerals in brackets denote the pertinent cycle.
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Table 1. Distribution of division stages of S. pombe as influenced by culture conditions

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Stage*</th>
<th>No. counted</th>
<th>No. as percentage of Σb−h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch, exponential phase†</td>
<td>a</td>
<td>1756</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>44</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>136</td>
<td>27.5</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>131</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>112</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>35</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>g</td>
<td>14</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>h</td>
<td>22</td>
<td>4.5</td>
</tr>
<tr>
<td>Σb−h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemostat‡</td>
<td>a</td>
<td>1581</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>17</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>28</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>146</td>
<td>21.8</td>
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<tr>
<td></td>
<td>e</td>
<td>115</td>
<td>17.2</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>218</td>
<td>32.6</td>
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<tr>
<td></td>
<td>g</td>
<td>55</td>
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<td></td>
<td>h</td>
<td>90</td>
<td>13.5</td>
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<tr>
<td>Σb−h</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* See Fig. 1 and Methods.
† In 10 ml MEB in closed 1 oz bottle; 32 °C.
‡ Glucose-limited growth; pH = 5.5; [DO] = 55-60%; 20 °C; see Methods.

nuclear; as shown by Nurse et al., 1976) running in parallel in the cell cycle of the fission yeast rather than two (G, growth; and DD, DNA-division) as proposed by Mitchison (1971). The N sub-cycle includes all the non-divisional events remaining when the DD sub-cycle is split. The three separated sub-cycles are illustrated diagrammatically in Fig. 5 to show the minimal uncoupling necessary to generate multiseptate cells (Fig. 5b) and branching cells (Fig. 5c). We conclude that multiseptation is evidence for a D sub-cycle lasting longer than one ordinary cell cycle, and that branching is evidence for a D sub-cycle lasting longer than two. Both multiseptation and branching can thus be predicted from knowledge of the generalized growth habit of the organism, once uncoupling of the sub-cycles is accepted.

Differential distribution of division stages

Samples from an exponential phase MEB batch culture and from the chemostat at 20 °C, pH 3.75, [DO] 55-60% (day 16, Fig. 3), were analysed for their distributions of division stages (Table 1). The two cultures were markedly different. In the MEB culture, about 63% of the dividing cells were in the early septum-elaboration stages (Fig. 1b–d), slightly over 20% were in the middle, complete primary septum, stage (Fig. 1e), while only about 15% were in the late septum-fission stages (Fig. 1f–h) of cell division. Thus, dividing fission yeast cells grown in MEB spent about four times as long in the septum elaboration stages as they did in the fission stages. In contrast about 54% of the dividing cells in the chemostat culture were in the late stages, slightly less than a fifth in the middle, and about 29% were in the early stage (Table 1). Thus the chemostat-grown cells spent about half as long in the septum elaboration stages as they did in the fission stages. The extent of the differences leads us to suggest that it was the fission step that was retarded and which led ultimately to the hyphal state (Fig. 2b).

Concluding remarks

No single cellular character reported above was unique to chemostat cultures. Branched cells in still batch cultures in MEB are found if the incubation temperature is 18 °C (Mitchison, 1970) and were illustrated in the first treatise on S. pombe (Lindner, 1893). Nevertheless, the chemostat approach had certain advantages, chief among these being the capacity to reverse the kinetics of
multiseptation and branching merely by changing chemostat conditions, and the ability to impose a constant culture doubling time, so that one immediately thinks of changes within the cell cycle when faced with secondary changes such as changes of morphology, or altered DI.

The culture temperature and [DO] were each varied independently while the doubling time was held constant, and each gave a different insight. Lowering the temperature resulted in a markedly altered cellular morphology, and thus chemostat studies of cell division in known conditional (temperature-sensitive) mutants could be very instructive. The effect of changing the [DO] emphasizes the delicate balance between normal morphogenesis and conditions of oxygenation which become more limiting to some portions of some cell sub-cycles than to others. Septum fission in *S. pombe* appears to be more sensitive to low [DO] than septum elaboration, with a resulting tendency towards the hyphal form. The sensitivity of morphogenesis to oxygen has been observed previously for species of *Mucor* (Rogers et al., 1974; Sypherd et al., 1978). With the latter organism, however, the mycelial form occurs at high rather than low [DO].

The fact that G and N sub-cycles of a new cycle can be initiated before septum fission in an earlier D sub-cycle is completed suggests that coupling among sub-cycles does not depend upon monitoring of the fission process. The cell might couple by monitoring completion of some earlier D sub-cycle process, such as the completion of the primary or secondary septum. However, none of this implies that fission is trivial and not monitored in other senses: we have noted elsewhere (Johnson et al., 1977) the very close regulation of the lytic enzymes required for fission and the catastrophic consequences to the cell of impaired regulation of those lytic enzymes.

The argument of Mitchison (1971) for splitting the cell cycle into two sub-cycles was that one sub-cycle (G) could continue while the other was blocked. We here discuss the splitting of the DD sub-cycle into a D and an N on a similar conceptual ground: the initiation of a new cycle or cycles even though a portion of an earlier cycle was uncoupled and blocked. It is evident that uncoupling can and does occur in other yeasts in many culture conditions and not solely in fission yeasts under chemostat conditions. For examples, when brewing yeasts exhibit ‘chain formation’, they too have their D sub-cycle uncoupled from N and G events. Brown & Hough (1965) found ‘pseudohyphae’ produced by *Saccharomyces* in chemostats with low growth rates when glycerol was the carbon source. Thompson & Wheals (1981) have described unstable ‘filaments’ of *Saccharomyces* produced in chemostats with high growth rates imposed. These they believe to be different from chain formation, but the filaments clearly are a consequence of comparable uncoupling. When ordinary batch cultures of fission yeasts reach stationary phase, the cells become shorter (Johnson, 1968; Mitchison, 1970) and stop in G1, suggesting repressed G and N sub-cycles uncoupled from the D sub-cycle which has gone one more turn. These observations led to a primitive model of modified cell cycles (Johnson, 1968).

Resolution of the cell cycle into three parallel sub-cycles by culturing conditions allows further insight into regulatory cross-connections between and among events comprising these sub-cycles. Characterization of the uncompleted septation–fission process should indicate which early D sub-cycle functions must be completed before the next G sub-cycle is initiated, and also which later D functions can be slowed without hindering progression of either the G or the N sub-cycles.

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