DEPENDENCY RELATIONS BETWEEN EVENTS IN MITOSIS IN \textit{SCHIZOSACCHAROMYCES POMBE}

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SUMMARY

The dependency relationships between various events in mitosis in the fission yeast \textit{Schizosaccharomyces pombe} have been investigated using a combination of approaches. The events concerned are those controlled by specific genes, a step or steps in mitosis sensitive to benomyl, and protein synthesis.

The anti-microtubule agent benomyl was shown to inhibit mitosis specifically, while not significantly affecting the accumulation of RNA or protein. DNA synthesis was not directly affected, though accumulation ceased after mitosis was inhibited. The inhibition of mitosis was readily reversible and was followed by a synchronous cell division.

Reciprocal shift experiments were carried out using benomyl in conjunction with temperature-sensitive mutants defective in mitosis. Consideration of these experiments and the transition points of the mutants allowed the mutant and the steps controlled by the respective genes to be ordered functionally. Group (i) consisted of \textit{cdc6-23}, which showed an early transition point: the benomyl-sensitive step was dependent on completion of the \textit{cdc6} step.

Group (ii) consisted of \textit{cdc2-33} and \textit{cdc27-K3}, which had transition points close to the time of mitosis itself. The benomyl-sensitive step was dependent on the \textit{cdc2} and \textit{cdc27} steps, but the relationship between these gene-controlled steps was uncertain. Group (ii) processes may be dependent on those in group (i), or they may act independently on separate pathways. Group (iii) mutants consist of \textit{cdc1-7}, \textit{cdc13-117} and \textit{cdc25-22}. These have transition points close to mitosis, and act interdependently with benomyl. It is likely, though not directly proven, that all group (iii) processes are dependent on those in group (ii).

The strikingly different terminal phenotype of \textit{cdc117} compared with other mutants of mitosis allowed further analysis of the group (iii) mutants according to the terminal phenotypes of double mutants of \textit{cdc13-117} with other mutants. Double mutants with \textit{cdc1-7}, \textit{cdc2-33}, \textit{cdc25-22} and \textit{cdc27-K3} all showed a terminal phenotype unlike \textit{cdc13-117}, and similar to the other single mutant parent. It is therefore likely that the \textit{cdc13} step is dependent on \textit{cdc1}, 2, 25 and 27; that is, later in mitosis, consistent with its unusual terminal phenotype.

Two mutants defective in stages of the cell cycle other than mitosis were subjected to reciprocal shifts with benomyl. The process controlled by \textit{cdc15-140}, an event in septum formation, was dependent on the benomyl-sensitive step. The other mutant, \textit{cdc10-129}, with a defect very early in the cycle, has been proposed to arrest at a stage analogous to 'start' in budding yeast. The completion of the \textit{cdc10} function was shown to be dependent on the occurrence of mitosis in the previous cycle.

Cells arrested early in mitosis by \textit{cdc2-33} were able to undergo mitosis when returned to the permissive temperature in the presence of cycloheximide. This supports previous observations that protein synthesis is not required for mitosis once the process has started. Extrapolation of this result to the normal cycle suggests that sequential switching on of \textit{cdc} genes is not the basis of the dependency relations determined in this study, and that periodic synthesis of proteins coded by \textit{cdc} genes may not be an important part of the mechanism that controls the sequence of events in mitosis.
INTRODUCTION

The cell division cycle is composed of an ordered series of events. The constancy of the sequence has led investigators to pose two questions: what mechanism maintains the relative order of events, and what determines their absolute timing in the cell cycle? The events under consideration are: firstly, the major periodic processes of DNA replication, mitosis and cell division; and secondly, the component processes that make up the major ones. The isolation of conditional mutants defective in cell-cycle progress (Hartwell, 1978; Simchen, 1978; Nurse, Thuriaux & Nasmyth, 1976; Nasmyth & Nurse, 1981) has allowed the component events to be analysed in some detail. One approach that has proved useful is to examine the dependency relationships among the various events. Establishing that a particular event is dependent on completion of another is important in itself, and may provide clues as to the molecular bases of the events. Such an approach is particularly valuable in the case of processes controlled by the products of cell-cycle-specific genes, since progress in identifying such gene products biochemically has been rather limited (Morris, 1980; Nasmyth, 1977; Bisson & Thorner, 1977; Dickinson, 1981).

In the fission yeast Schizosaccharomyces pombe, a number of temperature-sensitive mutants defective in cell-cycle progress (cdc mutants) have been isolated and characterized (Nurse et al. 1976; Fantes, 1979; Roy & Fantes, 1981). These studies have revealed that, as in other systems, mitosis and DNA replication are mutually interdependent and must alternate, while later stages of the cycle, septum formation and cell separation, are dependent on completion of at least some steps in mitosis. Specifically, mutants defective in mitosis are usually (though not invariably; see below) unable to septate or undergo cell division.

The events leading up to and including mitosis in S. pombe are of particular interest, since entry into mitosis is a major control point in the cell cycle. Initiation of mitosis is closely correlated with the attainment of a critical cell size. This size can be altered physiologically (Fantes & Nurse, 1977) or by mutations at either the wee1 or the cdc2 locus (Nurse, 1975; Thuriaux, Nurse & Carter, 1978; Fantes, 1981). Mutations in the cdc2 gene are of two types: one class are typical temperature-sensitive lethals with a defect in early mitosis, while the other class are phenotypically wee or small size mutants (Nurse & Thuriaux, 1980; Fantes, 1981). This and other evidence led Nurse & Thuriaux (1980) to conclude that the cdc2*-controlled step occurring just before mitosis is in fact a rate-limiting step regulating entry into mitosis.

Apart from cdc2, several other genes required for events leading up to or in mitosis have been identified (Nurse et al. 1976; Nasmyth & Nurse, 1981). The work presented here describes some properties of mutants defective in mitosis, namely cdc1, cdc2, cdc6, cdc13, cdc25 and cdc27.

First, the final time of action in the cell cycle of each gene product was determined by estimating the transition point (Nurse et al. 1976). Similar experiments were carried out using the microtubule inhibitor benomyl.

Second, reciprocal shift experiments were carried out using pairwise combinations of cell-cycle blocks, in the way described by Jarvik & Botstein (1973) for bacteriophage
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Table 1. Mutant strains of *S. pombe*

<table>
<thead>
<tr>
<th>Mutant allele</th>
<th>Defective cell cycle stage</th>
<th>Terminal phenotype</th>
<th>Transition point</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdc1-7</td>
<td>Mitosis</td>
<td>Uninucleate cells with 2C DNA content</td>
<td>0.63</td>
</tr>
<tr>
<td>cdc2-33</td>
<td>Mitosis</td>
<td>Uninucleate cells with 2C DNA content</td>
<td>0.75</td>
</tr>
<tr>
<td>cdc6-23</td>
<td>Mitosis</td>
<td>Uninucleate cells with 2C DNA content</td>
<td>0.25</td>
</tr>
<tr>
<td>cdc13-117</td>
<td>Mitosis</td>
<td>Uninucleate cells with 2C DNA content; partial and aberrant septa present.</td>
<td>0.72</td>
</tr>
<tr>
<td>cdc25-22</td>
<td>Mitosis</td>
<td>Uninucleate cells with 2C DNA content</td>
<td>0.64</td>
</tr>
<tr>
<td>cdc27-K3</td>
<td>Mitosis</td>
<td>Uninucleate cells with 2C DNA content</td>
<td>0.66</td>
</tr>
<tr>
<td>cdc10-129</td>
<td>Initiation of DNA replication</td>
<td>Uninucleate cells with 1C DNA content</td>
<td>-0.23</td>
</tr>
<tr>
<td>cdc15-140</td>
<td>Septum formation</td>
<td>Multinucleate cells</td>
<td>0.74</td>
</tr>
</tbody>
</table>

assembly, and used by Hartwell and colleagues to investigate the early stages of the *Saccharomyces* cell cycle (reviewed by Hartwell, 1978; and discussed in detail by Pringle, 1978).

Third, it is sometimes possible to determine the dependency relation between two gene-controlled events, provided the single mutants have distinguishable terminal phenotypes. Comparison of the double mutant’s terminal phenotype with those of the single mutant parents can at least eliminate certain possible dependency relationships. For this study, use was made of the multiseptate phenotype of cdc13-117, which differs from that of the other mutants used.

Fourth, the role of protein synthesis in mitosis in *S. pombe* was examined.

**MATERIALS AND METHODS**

**Organisms**

The wild-type strains 972 h- and 975 h+ of *Schizosaccharomyces pombe* (Gutz, Heslot, Leupold & Loprieno, 1974) and mutants derived from them were used throughout. The mutant strains used were all conditionally defective in the ability to complete the cell division cycle at 35-36 °C, while growth and division at 25 °C were essentially normal. Mutant cdc25-22 has been described by Fantes (1979), cdc27-K3 by Nasmyth & Nurse (1981), and other mutants by Nurse et al. (1976). Relevant characteristics of the mutants are shown in Table 1.

**Media and growth conditions**

Cultures were grown in EMM 2 medium (Mitchison, 1970) as modified by Nurse (1975), at 25 or 36 °C in water baths, with shaking. Shifts between temperatures were performed by transferring flasks from one bath to another, with an equilibration time of 2-3 min under the conditions used.

Benomyl (methyl-1-(butylcarbamoyl)-benzimidazol-2-ylcarbamate), a gift from DuPoint Nemours & Co., was added to medium as a solution in ethanol (5 mg/ml) to a final concentration.
of 20 μg/ml (69 μM). The medium was then autoclaved. Shifts between benomyl-containing and benomyl-free medium were carried out by filtering cells onto a membrane filter (Oxoid; 0.45 μm pore size) and washing and resuspending them in fresh medium.

Cycloheximide (Sigma) was added from a stock solution (5 mg/ml) in water to a final concentration of 100 μg/ml. This concentration was found by Polanshek (1977) to inhibit the rate of incorporation of radioactive leucine by more than 90% within 2 min of addition, and by more than 95% for the following 90 min.

Selection synchronized cultures

The selection method used was the elutriator rotor method described by Creanor & Mitchison (1979).

Estimation of cell number and macromolecular content; visualization of nuclei

Cell number was determined using a Coulter particle counter as described by Mitchison (1970). Protein, DNA and RNA were estimated according to Nurse & Thuriaux (1977). Cells were stained to visualize nuclei as described by Nurse et al. (1976).

Transition points for temperature-sensitive mutants were determined from the residual cell division after shifting an asynchronous culture from permissive to restrictive temperature (Nurse et al. 1976). Similarly, the transition point for benomyl was estimated from the residual division after transfer of asynchronous cells to benomyl medium.

RESULTS

Transition points of cdc mutants

Asynchronous cultures of the mutants used were shifted from 25 to 36 °C, and the fractional increase in cell number at 36 °C determined. Transition points were calculated from these data (Table 1). Of the six mutants defective in mitosis, five showed transition points within about 0.1 cell cycle of mitosis itself, which occurs at 0.75. The other strain, cdc6-22, showed a transition point of 0.25 (Table 1). The strain cdc15-124, defective in septum formation, showed a transition point of 0.74, shortly before septation. Strain cdc10-129, defective in the initiation of DNA replication, showed a transition point of −0.23, or 0.77 in the previous cell cycle, very shortly after the previous mitosis. The values obtained for transition points were similar to those reported previously (Nurse et al. 1976; Nasmyth & Nurse, 1981). The clustering of the transition points of all but one of the mitotic mutants around a value of 0.7 means that the relative order of function of the respective gene products cannot be deduced from information about timing alone. Rather, the type of dependency analysis mentioned in the Introduction must be applied.

Effect of benomyl on S. pombe

Cells of the wild-type 972 growing asynchronously at 25 °C were transferred from minimal medium to the same medium containing benomyl at 20 μg ml⁻¹ (Fig. 1). The fraction of binucleate cells fell from about 5% to zero within the first half hour after shift, and remained low for several hours. This suggested a specific inhibition of mitosis, and consistent with this, cell number reached a plateau about 1.5 h after the shift, which was maintained for 3 h thereafter. Some 28% of the cells divided between the time of shift and the plateau, giving a transition point of 0.64 for the
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Fig. 1. Effect of benomyl on growth and division of strain 972. Cells growing in minimal medium at 25 °C were transferred to medium containing benomyl (20 μg/ml) at time zero. Ordinate: logarithmic scale for cell no./ml (× 10^4) (○); DNA content/ml (× 50) (●); RNA content/ml (◇); protein content/ml (× 0.2) (□). In bracket: % binucleate cells (■).

action of benomyl, close to the timing of mitosis. The inhibition of cell division was a consequence of the block to mitosis, since those cells that had passed mitosis at the time of transfer to benomyl were able to complete the events of septation and cell separation. Benomyl-treated cells arrested as uninucleate cells without septa. The cells had a higher macromolecular content after the treatment, since RNA and protein accumulation continued through the division block (Fig. 1). This increase was reflected in an increase in cell length (data not shown).

DNA synthesis was inhibited by the addition of benomyl (Fig. 1). This was probably a pleiotropic effect due to the dependency of DNA replication on prior mitosis (Nurse et al. 1976), rather than a direct inhibition of DNA replication by benomyl, for two reasons. (1) The DNA content per nucleus of cells treated with benomyl for 4–5 h was about 28 fg, close to the 2C value of 33.8 fg (Nurse et al. 1976), indicating accumulation in G1 phase rather than G0. (2) In a separate experi-
After 3 1/4 h in the presence of benomyl, cell number started to increase gradually. This was observed in several experiments, though the rate of escape from the division block varied considerably. Increasing the benomyl concentration to 30 μg/ml made no difference, while further increases resulted in the appearance of abnormal, swollen or branched cells. The concentration of 20 μg/ml was therefore used in subsequent experiments. None of the mutant strains used was significantly more or less sensitive to benomyl when grown at 25 °C than the wild-type.

**Reversibility of benomyl arrest**

After 3 1/4 h in the presence of benomyl, cells of strain 972 were washed free of the inhibitor, and transferred to fresh medium at 25 and 35 °C (Fig. 2). In the 25 °C culture, cell number remained nearly constant for 1 h, after which there was a synchronous burst of division. The fraction of cells dividing was about 75 %, showing a high degree of recovery from the mitotic block. In the culture shifted to benomyl-free medium at 35 °C, the pattern of division was similar, though the initial plateau was not as pronounced, and a second synchronous round of division was observed.
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Table 2. Experimental schedule for reciprocal shifts

<table>
<thead>
<tr>
<th>Procedure (1)</th>
<th>1st incubation</th>
<th>2nd incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth of culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. 25 °C, -benomyl</td>
<td>B. 36 °C, -benomyl (3 h)</td>
<td>C. 25 °C, -benomyl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. 25 °C, + benomyl</td>
</tr>
</tbody>
</table>

| Procedure (2) | | |
|---------------|----------------|
| A. 25 °C, -benomyl | E. 25 °C, + benomyl (3½ h) |
| | F. 25 °C, - benomyl |
| | G. 36 °C, -benomyl |

These experiments established that benomyl inhibition of mitosis was sufficiently reversible to be used in reciprocal-shift experiments with cdc mutants.

Reciprocal shifts

Each mutant defective in mitosis and cdc15-140, defective in septum formation, was subjected to two experimental procedures, outlined in Table 2. In the first, cells growing at 25 °C (culture A) were shifted to 36 °C (culture B) and held at this temperature for 3 h. This ensured that essentially all cells were arrested at the cdc block, since the generation time of wild-type cells under these conditions is 2½ h.

After 3 h, a portion of culture B was shifted to 25 °C (culture C) and a second portion to medium containing benomyl at 25 °C (culture D). Culture C was monitored as a control for recovery of the cdc mutant from the division block. Only if a round of division occurred soon after transfer to 25 °C would the observations on culture D be meaningful. This was so in all cases except cdc5-140 (see text, below). The occurrence of a division round in culture D would indicate that the cells had passed the benomyl-sensitive step while arrested at the cdc block. Absence of division showed failure to complete the benomyl-sensitive step.

The second experimental procedure was the converse of the first: cells were transferred from culture A to medium containing benomyl at 25 °C (culture E). After 3½ h of incubation, portions of culture B were transferred to benomyl-free medium at 25 °C (culture F) and 36 °C (culture G). The division patterns of cultures F and G were followed: F acted as a control for recovery from the benomyl block, and in each case a synchronous round of division was observed similar to that in the wild-type (Fig. 2). The behaviour of culture G showed whether cells had completed the step controlled by the particular cdc gene during the period of benomyl treatment. In this second experiment cells were incubated in the presence of benomyl for only 3½ h, compared with the generation time at 25 °C of around 4½ h. This was done because after 3½ h incubation with benomyl cells started to escape from the division block, as mentioned above. Applying benomyl for less than a full cycle time meant that not all cells had arrested at the first (benomyl) block by the time of the second shift. Therefore some cells did not have the opportunity to complete the cdc gene-controlled event during the benomyl incubation, whether or not they were able to do so, since their progress through the cycle was insufficient to reach the step controlled by the cdc gene. These cells would therefore not divide when transferred to culture G.
at 35 °C. The overall effect was to reduce the number of cells able to divide in culture G, even if the majority of cells were able to do so. Nevertheless, it was in practice possible to distinguish between the occurrence of a partial round of division, and no division at all.

Mutant strains carrying the various cdc defects were subjected to the two experimental procedures described above. For convenience the mutants are considered here in four groups.

Mutant cdc6:23 comprises the first group. This mutant and all other known mutant alleles (Nasmyth & Nurse, 1981) have transition points substantially before mitosis, at 0.25 as determined in this study (Table 1). This is substantially earlier than the benomyl transition point. Consistent with this, the reciprocal shifts indicated that the
benomyl-sensitive step was dependent on completion of the \textit{cdc6} step. Essentially no division was observed in procedure (1) in Table 2, while the majority (74\%) of cells were able to divide after the second procedure (Fig. 3).

The second group of mutants consisted of \textit{cdc2-33} and \textit{cdc27-K3}. A typical experiment with \textit{cdc2-33} is shown in Fig. 4, and very similar results (not shown) were obtained with \textit{cdc27-K3}. Fig. 4 shows that little division occurred in culture D (benomyl present) while a synchronous division was observed in culture C (control, without benomyl). This shows that either the benomyl-sensitive step was dependent on completion of the \textit{cdc2} step (and the \textit{cdc27} step) or that each \textit{cdc} gene-controlled step was interdependent with the benomyl-sensitive step. The results of procedure (2) exclude the latter possibility, since a substantial fraction of cells divided in culture G at 36 °C. It therefore seemed probable that the benomyl-sensitive step was dependent on the \textit{cdc2} and \textit{cdc27} steps. One point of concern was the low fraction (40\%) of cells dividing in culture G compared with 70\% in the control culture F. A possible reason for this was that not all cells were blocked at the benomyl-sensitive step during the first incubation in procedure (2), as described above. To test this possibility, a
Fig. 5. Effect of presynchronization on reciprocal shift with strain _cdc2-33_. Cells of strain _cdc2-33_ were grown at 25 °C, shifted to 36 °C for 3 h, then shifted to 25 °C in the presence of benomyl for 3 h. At time zero, portions were shifted to benomyl-free medium at 25 °C (○) and 36 °C (●).

A similar experiment was performed, with the difference that _cdc2-33_ cells were presynchronized by incubating at 36 °C for 3 h before applying procedure (2). The following period of incubation with benomyl was reduced to 3 h since the degree of asynchrony in the cells was reduced in the starting population. Fig. 5 shows that after presynchronization and transfer to 25 °C in the presence of benomyl, cells transferred to benomyl-free medium at 25 °C (equivalent to culture F) underwent a rapid round of division between 0 and 1.5 h after transfer. Following this partially synchronous division there was a slowing in rate and then a second rapid rise. The two periods of rapid increase represent successive divisions that overlap slightly, giving rise to a period of slow increase around 2.5 h. Taking the start of the first synchronous division as zero time, and the end as the middle of the overlap period, about 70% of the cells were estimated to participate in the first division.

In the culture finally shifted to 36 °C, cell number rose and reached a plateau corresponding to a fractional increase in cell number of 67% (Fig. 5). Thus all cells able to divide at 25 °C are also able to do so at 36 °C. This experiment shows that presynchronization of _cdc2-33_ cells increased the fraction able to divide after the second shift in procedure (2) to 67% (Fig. 5) compared with only 40% without presynchronization (Fig. 4). Thus initial asynchrony of the population and incomplete arrest by benomyl is the likely reason for the incomplete division in experiments such as those shown in Fig. 4. Other experiments where cells were presynchronized
by selection gave results similar to those of Fig. 5 (data not shown), again confirming this idea. All these observations point to the conclusion that the benomyl-sensitive step in the cell cycle is dependent on completion of the steps controlled by *cdcz* and *cdcs*.

The third group of mutants consists of *cdci*, *cdcs*, and *cdcz*. Cells of these strains showed essentially no division after either of the reciprocal shift procedures in Table 2. Data for a typical experiment with *cdcz* are shown in Fig. 6. Recovery from the first division blocks (cultures C and F) was rapid and essentially complete while there was virtually no division in the corresponding experimental cultures D and G. This suggests that the steps controlled by *cdci*, 13 and 25 are each functionally interdependent with the step sensitive to benomyl.

One mutant strain defective in septation, but not in nuclear division, makes up the fourth group. Imposition of the benomyl block followed by release and shift to 36 °C (i.e. procedure (2)) resulted in no cell division (Fig. 7). This is consistent with the
previous observation that septation is dependent on mitosis (Nurse et al. 1976). In the opposite shift, where the cdc block was applied before the benomyl block, the results were less clear. The difficulty here was the poor recovery of cells previously arrested by cdc15:140 at 25 °C (Fig. 7); only 20–25% of the cells divided within 3 h of transfer. Poor recovery of mutants defective in septation after return to the permissive temperature has been observed with other mutations (W. Haddow, personal communication). However, despite the low fraction of cells dividing at 25 °C in the absence of benomyl, a similar number were able to divide in the presence of benomyl (Fig. 7). It therefore seems reasonable to conclude that the cdc15 step is dependent on the benomyl-sensitive step, consistent with the dependency of septation on mitosis. The conclusion also confirms the observation on the wild-type (Fig. 1) that benomyl has no direct effect on processes later in the cell cycle than mitosis.

**Dependency of ‘start’ on mitosis**

The mutant cdc10:129 is defective in an early event of the cell cycle during G₁, and shows a very early transition point under the conditions used here (Nurse et al. 1976). Recently it has been proposed that the process controlled by the cdc10 gene is analogous to the early event of the S. cerevisiae cell cycle termed ‘start’ (Nurse &
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Fig. 8. Dependency of the cdc10 step on prior mitosis. Cells of strain cdc10-129 growing at 25 °C were shifted to benomyl medium and incubated at 25 °C (○). At the times indicated, portions were transferred to benomyl-free medium at 25 °C (△) and 36 °C (▲).

Bissett, 1981). The transition point of −0.23 (i.e. 0.77 in the previous cycle) suggested that the product of the cdc10 gene completed its function almost simultaneously with the previous mitosis at 0.75. To investigate whether cells were able to execute the cdc10 function independently of the completion of mitosis, the following experiment was performed. Cells of cdc10-129 growing at 25 °C were shifted to benomyl medium for 3 1/2 h, by which time cell division had ceased, and most cells were arrested at the benomyl-sensitive step in mitosis. The point at issue was whether these cells had passed the cdc10 step in the subsequent cycle while arrested. Release of the benomyl block while imposing the cdc10 block at 36 °C should lead to at least one round of division whether or not the cells had passed the cdc10 block. This is because no cdc10 function is required between mitosis (strictly, the benomyl-sensitive step) and the cell division subsequent to the mitosis previously blocked by benomyl. The inability to pass the cdc10 step would be reflected in a single round of division. On the other hand, if cells had passed the cdc10 step during the 25 °C incubation in benomyl, they would have been able to undergo a second division. The results (Fig. 8) show that in the
culture shifted to 36 °C, a single round of division occurred, followed by a slow increase in cell number probably due to slight leakiness of the cdc10-129 mutation at 36 °C. During the course of the experiment a control culture shifted to benomyl-free medium at 25 °C showed one complete round of division followed by a short plateau and part of a second division. The ability of the 25 °C culture to increase in number at least threefold while the 36 °C culture only doubled shows that only one division could occur at the restrictive temperature for cdc10-129. This in turn suggests strongly that the cdc10 step cannot be completed until mitosis in the previous cycle has taken place, so that 'start' is dependent on the previous mitosis.

**Double mutant phenotypes**

As mentioned in the Introduction, some information about dependency relations between cell cycle events can be obtained by examining the terminal phenotypes of double mutants, that is, by applying two blocks simultaneously. This required that the terminal phenotypes of the single mutants be distinguishable. Of the mutants used
here, only cdc13:117 had a terminal phenotype readily distinguishable from that of other mutants. Therefore, double mutants of cdc13:117 with cdc1:7, cdc2:33, cdc25:22 and cdc27-K3 were constructed and cells examined after 5-6 h incubation at 36 °C. The single cdc13:117 mutant arrests with a partial septum, or with multiple septa near the centre of the cell (Nasmyth & Nurse, 1981). None of the other single mutants forms a septum under these conditions, and it was found that the double mutants did not form septa either. This suggests that the presence of a block caused by any of cdc1, cdc2, cdc25 and cdc27 prevents the cdc13 step, which otherwise leads to an unusual phenotype, from taking place. Alternative explanations are however possible in such situations, as discussed thoroughly by Pringle (1978).

Protein synthesis requirement for mitosis

The observation that protein synthesis is required up to a particular point in G1 phase, after which mitosis can occur without further protein synthesis, has been made in several systems (see Mitchison, 1971, pp. 208-234, for a review). Polanshek (1977) showed that blocking protein synthesis in S. pombe prevented the entry of cells into mitosis, unless cells were beyond 0-65 in the cell cycle at the time of block. This observation has been confirmed for the different strains and growth conditions used in this study. The existence of mutants defective in specific stages of the cell cycle allowed investigation of the role of protein synthesis in finer detail. As indicated by experiments presented here and elsewhere (Nurse & Thuriaux, 1980), the cdc2+ gene product acts early in mitosis and plays an important role in controlling the timing of mitosis. I carried out the following experiment to investigate the relationship between the protein synthesis requirement and the cdc2-controlled step. A growing culture of cdc2:33 cells was shifted from 25 to 36 °C and incubated for 4 h at the high temperature. Portions of the culture were shifted back to 25 °C either in the presence or absence of cycloheximide to inhibit protein synthesis. Cells incubated without cycloheximide underwent a synchronous division, shown by a peak in the fraction of septated cells and a rapid rise in cell number (Fig. 9). In the cycloheximide-treated
culture, septated cells appeared rather later than in the control culture, but finally 75% of the cells were septated. This fraction did not decline, but remained high for several hours. Only a very small rise in cell number occurred in the cycloheximide culture. These observations showed that septation, though not separation of daughter cells, had occurred in the presence of cycloheximide. Examination of cells stained with Giemsa at the end of the experiment showed that the septa formed were often aberrant, frequently consisting of double structures. Similar structures were previously reported by Polanshek (1977), under very similar conditions using a wild-type strain. However the septated cells almost always contained two nuclei, and unseptated binucleated cells were also present. In total, 84% of the cells contained two nuclei. This strongly suggested that cells prealigned at the cdc2-33 block did not need to synthesize any proteins in order to complete mitosis. However, protein synthesis was required for normal septation and cell separation.

DISCUSSION

I report in this paper the use of the microtubule agent benomyl as a specific inhibitor of mitosis in S. pombe, as reported for other fungi (Morris, 1980; Quinlan, Pogson & Gull, 1980). Among a group of benomyl-resistant mutants of Aspergillus nidulans, defective in the structural gene for β-tubulin, one has been identified as showing altered mitotic behaviour (Oakley & Morris, 1981). Similar mutants of S. pombe recently isolated show simultaneous benomyl resistance and a cold-sensititive defect in mitosis (Roy & Fantes, 1981), and it will be interesting to see whether another group of mutants resistant to various benzimidazole derivatives shows any alteration in mitosis (Yamamoto, 1980).

The specificity of action of benomyl has allowed the agent to be used in reciprocal-shift experiments in conjunction with temperature-sensitive cdc mutants. All but two of the mutants investigated in this study have a conditional defect in mitosis; the others, defective in an early G1 event and septation, are considered below. Of the mitosis mutants available in our collection, cdc5 and cdc28 could not be investigated since all available mutant alleles were somewhat leaky and did not arrest completely at 36 °C. Strains carrying representative temperature-sensitive alleles of cdc1, 2, 6, 13, 25 and 27 were subjected to the reciprocal-shift procedures shown in Table 2. The detailed results are described above; major conclusions are outlined below and in Fig. 10. The step controlled by cdc6 is completed substantially earlier in the cell cycle than mitosis, and the benomyl-sensitive step is functionally dependent on its completion. The steps controlled by cdc2 and cdc27 are completed shortly before mitosis, and their completion is essential before the benomyl-sensitive step can occur. Thus cdc6, cdc2 and cdc27 all sequence before benomyl in Fig. 10. However, it is not possible to draw firm conclusions about interrelationships between the functions controlled by these genes. The early transition point of cdc6-23 and all mutant alleles tested (Nasmyth & Nurse, 1981) means that the step controlled by cdc6 is unlikely to be dependent on the cdc2 step or the cdc27 step. The earlier transition point of cdc6 compared with cdc2 and cdc27 also makes it unlikely that the cdc6 step is inter-
dependent with either of the other gene-controlled steps. This leaves the possibilities that cdc6 and cdc2 are independent of one another or that cdc2 is dependent on cdc6. The same arguments apply to the relation between cdc6 and cdc27. It is likely that cdc2 controls an early step in one pathway leading to mitosis, because of its involvement in the mechanism determining the time of mitosis (Nurse & Thuriaux, 1980). The cdc6 step might occur earlier on the same pathway as cdc2 or on a parallel pathway. The cdc27 step may well be dependent on the rate-limiting cdc2 step, but the evidence here is insufficient to lead to definite conclusions.

In strains cdc1, 13 and 25, the absence of division after both reciprocal shifts with benomyl suggests that each gene product acts interdependently with the benomyl-sensitive process. However, criticisms of drawing unambiguous conclusions from such situations have been made by several authors, notably Pringle (1978), Hereford & Hartwell (1974) and Jarvik & Botstein (1973). Even if the relationship between cdc1, 13 and 25 is one of interdependence with the benomyl step, no conclusion can be drawn about the dependency relations between the three cdc genes. However, the position of cdc13 in the developmental pathway could be determined from the terminal phenotypes of double mutants of cdc13-117 with other cdc mutants. In each of the double mutants examined, the terminal phenotype resembled that of the single mutant parent other than cdc13-117, in that no multiple septa were observed. In contrast, cdc13-117 itself exhibited multiple septa. This argues that the cdc13 step is dependent on the steps controlled by each of cdc1, 2, 25 and 27, since the presence of a mutation in any of these genes suppresses the abnormal terminal phenotype of cdc13-117 (Fig. 10). This is consistent with the nature of the abnormal phenotype, which is presumably beyond the point in the cell cycle at which septum formation is initiated, i.e. later than the arrest points of other mutants in mitosis.

The overall dependency relations between the benomyl-sensitive step and cdc gene-controlled steps in mitosis are shown in Fig. 10. On the basis of reciprocal-shift experiments and transition points the mitosis mutants can be grouped into three classes: cdc6, with an early transition point makes up class (i); cdc2 and cdc27, with transition points close to mitosis and sequencing before benomyl are in class (ii); and cdc1, 13 and 25 with similar transition points but sequencing interdependently with benomyl form class (iii); the benomyl-sensitive step itself can be considered to be in class (iii). Possible relations between classes (i) and (ii) are discussed above. It is probable that all class (iii) functions are dependent on those in class (ii), though this has only been directly demonstrated for benomyl among the class (iii) blocks. It is not possible to draw conclusions about the relationships between cdc mutations in the same class on the basis of reciprocal-shift experiments. For instance cdc1 and cdc25 are both interdependent with benomyl action, but this does not necessarily imply that cdc1 and 25 are interdependent with one another. If, as seems likely, benomyl acts on microtubules to prevent assembly into a spindle, it is conceivable that the cdc1 and cdc25 gene products might interact with different parts of the tubulin molecule. The temporal order of the interactions might not be important, in which case the cdc1 and cdc25 functions would be independent of one another. This is one hypothetical instance; other possibilities can doubtless be imagined in which the relationships
between $cdci$ and $cdc25$ are different. Without further information about the molecular roles of the gene products no firm conclusions can be drawn.

It is of interest to compare the results described here with ultrastructural observations on mitosis in wild-type (McCully & Robinow, 1971) and $cdc$ mutant (King & Hyams, 1982) strains of $S. pombe$. In a detailed study of a wild-type strain, McCully & Robinow (1971) described the following events: duplication of the spindle pole body (SPB) or kinetochore equivalent; local expansion of the nuclear envelope initiating separation of the two SPBs; simultaneous appearance of a microtubule bundle joining the SPBs; elongation of the nucleus coincident with spindle extension; and finally, separation of the chromatin and nucleolar material into daughter nuclei. Strains defective at the $cdci$, 2, 6, 25 or 27 locus have been examined after incubation at the restrictive temperature, using electron microscopy, by King & Hyams (1982). In $cdc2-32$ and $cdc6-23$, nuclei were spherical or rectangular with an unreplicated SBP (except for 20% of $cdc2-33$ cells that showed a replicating SPB, for reasons discussed by King & Hyams). Such nuclear morphologies are similar to those in late interphase in the wild-type reported by McCully & Robinow (1971), and are consistent with $cdc2$ and $cdc6$ acting early in mitosis, in agreement with the present study. Cells of $cdc27-K3$ had a terminal phenotype similar to $cdc2-33$ and $cdc6-23$, again consistent with a block point in early mitosis. Strain $cdci-7$ arrested with a replicating SPB, and some cells showed arrest at a later stage with a dumbbell-shaped nucleus, each half of which contained chromatin, nucleolar material and an SPB. Whatever the reason for the presence of two terminal phenotypes, both are clearly 'later' than the phenotypes of $cdc2$, 6 and 27, consistent with the functional classification of this study (Fig. 10).

The situation for $cdc2$ alleles was complex, with three different mutant alleles showing different terminal phenotypes. However, in one mutant ($cdc25-43$) the nucleus was very elongated, and contained separated SPBs and microtubule bundles (King & Hyams, 1982), again consistent with a block late in mitosis (cf. Fig. 10). Thus there is good general agreement between the two methods of analysis about classification of mutants into those affecting early as opposed to late blocks.

The observation that protein synthesis is required for progress toward mitosis, but is not necessary for the event itself, has been made for several systems (Mitchison, 1971, pp. 208–234), including $S. pombe$ (Polanshek, 1977; unpublished results of the author). The existence of cell cycle mutants has allowed more accurate positioning of the protein synthesis transition point in relation to the initiation of mitosis. Cells arrested at the $cdc2$ block are able to undergo mitosis when returned to the permissive temperature in the presence of cycloheximide at a concentration that effectively inhibits protein synthesis (Fig. 9). Mitosis is delayed in these cells as compared with the control culture shifted to 25 °C without cycloheximide, suggesting that de novo synthesis of the $cdc2-33$ gene product may play a role in the normal recovery process when protein synthesis is permitted. The experiment implies that cells were able to traverse the cell cycle from a stage early in mitosis, immediately following the $cdc2$ stage, to complete the process. Since the $cdc2^+$ gene is concerned with the initiation of mitosis, it may be that under the particular conditions used, all the steps in mitosis subsequent to initiation can take place in the absence of protein synthesis. Extra-
Dependency in yeast mitosis

Interpolation of this result to the normal cell cycle is consistent with the suggestion of Polanshek (1977) that at the time mitosis is initiated all the proteins required for the process are present. In particular the products of genes such as cdc11+, cdc13+ and cdc25+ may be present in sufficient quantity for at least one cycle of mitosis. This has profound implications for the physiological and molecular bases of some of the dependency relations shown in Fig. 10. One possibility proposed by Hartwell (1976) is that sequential switching on of cdc genes forms the basis of some dependency relations. This seems unlikely in the present case since there may well be sufficient product of each of three cdc genes present at the cdc2 stage, and the functions controlled by these genes are dependent on completion of cdc2+ function. It is more likely that the sequential occurrence of events is controlled by processes at post-transcriptional levels. This is consistent with the failure to demonstrate periodic gene expression during the Saccharomyces cell cycle for some 120 proteins (Elliott & McLaughlin, 1979). Another relevant study showed that the amounts of several cdc gene products present in each cell were sufficient to allow several cycles to occur (Byers & Sowder, 1980). Such experiments, together with the present study, suggest strongly that a large part of the control of the cell division cycle must operate at the level of cytoplasmic interactions, rather than involving a complex cascade of gene switching. This does not exclude the possibility that switching on some genes at particular times may be important in overall cycle control, though direct evidence for this is at present lacking.

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REFERENCES


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