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Genetic Control of the Cell Division Cycle in the Fission Yeast *Schizosaccharomyces pombe*

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Summary. Twenty seven recessive temperature sensitive mutants have been isolated in *Schizosaccharomyces pombe* which are unable to complete the cell division cycle at the restrictive temperature. These mutants define 14 unlinked genes which are involved in DNA synthesis, nuclear division and cell plate formation. The products from most of these genes complete their function just before the cell cycle event in which they are involved. Physiological characterisation of the mutants has shown that DNA synthesis and nuclear division form a cycle of mutually dependent events which can operate in the absence of cell plate formation. Cell plate formation itself is usually dependent upon the completion of nuclear division.

Introduction

Temperature sensitive mutants blocked at various stages of the cell division cycle have been isolated in a number of organisms including *Escherichia coli* (Hirota, Ryter and Jacob, 1968; see review by Slater and Schaechter, 1974), *Saccharomyces cerevisiae* (Hartwell, 1974), *Chlamydomonas reinhardii* (Howell, 1974) and Chinese Hamster cells (Roscoe, Robinson and Carbonell, 1973). The most comprehensive investigation of such mutants in a eukaryotic organism has been undertaken by Hartwell and his associates using the budding yeast *S. cerevisiae*. They have established the dependency relationships between various events of the cell cycle (Hartwell, 1974), and have suggested that temporal programmes of gene activity leading to a cell cycle event may be initiated substantially before that event takes place (Culotti and Hartwell, 1971). Detailed studies of specific mutants also revealed the existence of new events of the cell cycle such as "start" (Hartwell, 1974) and hitherto unsuspected cell cycle controls such as a "timer" controlling bud emergence (Hartwell, 1971). The importance of these conclusions for an understanding of the cell cycle has led us to initiate a genetical investigation of the cell cycle and its controls in another simple eukaryote, the fission yeast *Schizosaccharomyces pombe*.

S. pombe has been used extensively both for research into the cell cycle (Mitchison, 1970) and for genetical studies (Gutz, Heslot, Leupold and Loprieno, 1974). Its cell cycle is basically similar to that of other eukaryotes and can be divided into G1, S, G2 and M periods (Mitchison and Creanor, 1971). The period of DNA synthesis (S) is very short and occurs at the beginning of the cell cycle simultaneously with cell separation. A long G2 period follows culminating in nuclear division (M) at 0.75 of a cell cycle. There is then a short G1 period ending with the S period of the next cell cycle. Cell division is marked by the formation of a cell plate (septum) across the middle of the cell followed by the separation of two equal sized daughter cells. This mode of cell division shows a greater similarity to that found in higher eukaryotes than does the budding mode of cell division found in *S. cerevisiae* and most other yeasts.

Previous work on the genetic control of the cell cycle in *S. pombe* has revealed a gene involved in the co-ordination between cell division and cell growth (Nurse, 1975). In the present report we describe the genetical and physiological characterisation of temperature sensitive mutants unable to undergo cell division because they are defective in some event leading up to DNA replication, nuclear division or cell plate formation.

Materials and Methods

1. Strains and Genetical Analysis

We have used the standard genetical procedures for *S. pombe* as described by Gutz et al. (1974). All the mutants derive from the

haploid wild type strain initially used by Leupold (1950) with mating type h^- (strain 972) or h^+ (strain 975). Mutants *lys* 1–131 and *ura* 5–294 were UV induced (Leupold, unpublished). The mating type allele *mei* 1–102 has been described by Egel (1973). The mutant strain *wee* 1–50 which divides at half the size of the wild type strain, was originally called *cdc* 9–50 (Nurse, 1975). It has been changed to *wee* 1–50 because of its fundamentally different properties from the rest of the cdc^- mutants; the name *wee* refers to the small size and Scottish origin of the mutant.

2. Media and Growth Conditions

The media malt extract agar (MEA) and yeast extract glucose agar (YEA) have been described in Gutz et al. (1974). Phloxin B (from Sigma) which stains dead cells was added to the YEA at 20 mg/l after the autoclaved medium had cooled below 60° C. The minimal medium used for characterisation of the cdc^- mutants was EMM2 (Mitchison, 1970), as modified by Nurse (1975).

Cultures were grown at 25° C and 35° C as described in Mitchison (1970). They were shifted between these temperatures by filtering cells on an Oxoid membrane filter and resuspending them in fresh medium at the new temperature. This procedure takes less than two minutes.

3. Temperature Sensitive Mutant Isolation

The wild type strain 972 h^- was mutagenised with nitrosoguanidine to 30% survival as described in Gutz et al. (1974), and plated at about 100 survivors per plate on YEA containing Phloxin B. After 4 days at 25° C, the colonies were replica plated and examined after overnight incubation at 25° C and 35° C. Those colonies which at 35° C showed poor growth or were stained with the Phloxin B (indicating a high proportion of dead cells), were isolated from the plate incubated at 25° C. These temperature sensitive mutants formed about 1% of the survivors.

4. Determination of Cell Number, DNA, RNA and Protein

Cell number was determined as described in Mitchison (1970). DNA and RNA was determined by the diphenylamine reaction and absorption at 260 nm respectively, as described in Bostock (1970). Protein was determined on the cell pellet left after the extraction step in 0.5 N perchloric acid at 70° C used in the DNA and RNA determination. The cell pellet was resuspended in water, an aliquot hydrolysed for 18 h in 1.0 N sodium hydroxide containing 2% sodium deoxycholate at 32° C, and protein determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

5. Nuclear and Cell Plate Staining

For nuclear staining, cells were filtered, smeared onto a slide, and heat fixed for 15 min at 80° C. The cells were rinsed in water, post fixed in freshly made Carnoy's fixative (ethanol 60 ml:chloroform 30 ml:acetic acid 10 ml) for 15 min, and rinsed in water again. They were then extracted for 30 min in 1.0 N hydrochloric acid at 60° C, rinsed in water, and stained in 0.05 M sodium phosphate buffer pH 6.8 containing 4% freshly added Giemsa stain (from Lamb) for 45–60 min. Nuclei were examined and photographed using a 100× planachromatic oil immersion objective on a Zeiss photomicroscope.

For cell plate staining, cells were centrifuged and resuspended in water containing 2 mg/ml of primulin (Streiblová and Beran, 1963). After staining for 5–10 min the cells were mounted in the primulin solution, and cell plates examined and photographed using a 100× oil immersion objective on a Nikon fluorescence microscope.

Results

Choice of Permissive and Restrictive Temperature for Mutant Isolation

Most previous studies of the cell cycle in *S. pombe* have been carried out at 32° C. In order to have a reasonable temperature range available for isolation of temperature sensitive mutants, 25° C and 35° C were chosen as the permissive and restrictive temperatures respectively. Below 25° C *S. pombe* grows inconveniently slowly, whilst above 35° C the cells become swollen and aberrantly shaped. The timing of the major cell cycle events of DNA synthesis, nuclear division and cell plate formation are similar at 25° C, 32° C and 35° C (Mitchison, 1970; Nurse, 1975 and unpublished). Generation times are different, being about 4 h at 25° C, and 2 h 25 min at 32° C and 35° C in minimal medium.

Isolation of Temperature Sensitive cdc^- Mutants

About 500 temperature sensitive mutants which formed colonies at 25° C but not at 35° C were isolated as described in Materials and Methods. Only a small proportion of these were expected to be defective in some function specific to the cell cycle at the restrictive temperature. Bonatti, Simili and Abbondandolo (1972) have suggested that these cdc^- mutants could be identified as producing abnormally elongated cells at restrictive temperature. Such elongated cells are produced when either DNA synthesis or nuclear division is prevented in *S. pombe* using inhibitors (Mitchison, 1974). Accordingly those mutants which formed elongated cells after 18 h incubation at 35° C were retained as presumptive cdc^- mutants.

These mutants were further screened microscopically and those which continued to divide after 5 h incubation at 35° C were discarded as too leaky to warrant further analysis. The remaining 28 cdc^- mutants stopped cell division within 5 h at 35° C, and continued to make RNA and protein at a rate similar to wild type (for examples of representative mutants see Figs. 4–7).

Genetic Analysis

a) Mendelian Segregation. Tetrad analyses of crosses between h^+ *lys* 1–131 and the various cdc^- mutants

showed that in all but one case they had the 2:2 segregation indicative of a mutation within a single gene. The one strain which failed to show 2:2 segregation, required two mutations to express fully the temperature sensitive phenotype and was not further investigated. One mutant, (strain 22) was sterile, leaving 26 mutants for further genetic characterisation.

b) Test of Allelism. The h^- and h^+ strains of each *cdc*$^-$ mutant were crossed pairwise at 25° C on MEA using the criss-cross technique of Leupold (Gutz et al., 1974). The crosses were streaked out on YEA containing Phloxin B and incubated at 35° C in order to detect wild type recombinants. In most crosses a high frequency of recombinants was observed indicating that the two crossed mutants mapped in unlinked genes. In the other crosses, no or little recombination was observed by this qualitative technique, indicating that the mutants belonged either to the same gene or to closely linked genes.

These two possibilities were tested using free spore analysis which enables a quantitative estimate of recombination frequency, as well as providing a test for complementation as discussed by Gutz et al., (1974). All but one pair of the closely linked *cdc*$^-$ mutants were found not to complement and thus defined single genes. Alleles within the same gene recombined at frequencies which were compatible with intragenic recombination (in *S. pombe* this is less than 500 recombinants per 10^6 spores), indicating that they resulted from different mutational events. One pair of closely linked *cdc*$^-$ mutants, *cdc* 4–8 and *cdc* 4–31, did complement, but exhibited a very low frequency of recombination (only 10 recombinants per 10^6 spores). The likeliest explanation is that this is a case of intragenic complementation.

Thus, the 26 *cdc*$^-$ mutants were allocated to 14 unlinked genes with no evidence for clustering. Chromosome mapping of these genes is in progress (Thuriaux and Kohli, unpublished) and has already shown that they are distributed on all of the three chromosomes known in *S. pombe*.

c) Recessivity. A representative allele of each *cdc* gene was tested for dominance by crossing a double mutant h^- *cdc*$^-$ *lys* 1–131 with a *mei* 1–102 *ura* 5–294 strain. The mating type allele *mei* 1–102 produces non sporulating diploids when crossed to an h^- strain (Egel, 1973). Diploids were selected by streaking onto minimal medium and incubating at 25° C. In each case the diploid was not temperature sensitive and was indistinguishable from a wild type diploid at 35° C. Thus the representative alleles of all the *cdc* genes were recessive.

Determination of Transition Points

An important characteristic of a *cdc*$^-$ mutant is its transition point on shift from the permissive to restrictive temperature. In studies of *cdc*$^-$ mutants of *S. cerevisiae* the transition point has also been called the execution point (Hartwell, 1974). All cells which are beyond it in the cell cycle can undergo one further cell division, whereas all cells before it cannot. As long as the transition point can be shown to be gene rather than allele specific, it should indicate the stage in the cell cycle when that gene product completes its function. If the transition point is determined for a single allele then it may only reflect the decay characteristics in activity of the particular thermosensitive product produced by that allele. Thus if the activity of the thermosensitive product takes a long time to decay at the restrictive temperature, then the gene product will continue to function and so cells will continue to divide. This will give a transition point which is earlier in the cell cycle than the time at which the gene product actually completes its function.

The transition point can be calculated from the cell number increase observed in a perfectly asynchronous exponentially growing culture after shift from the permissive to the restrictive temperature. The cell number increase reflects the proportion of cells in the culture which are beyond the transition point in the cell cycle, and can therefore divide at the restrictive temperature. Since the age distribution of cells throughout the cell cycle is skewed (Cook and James, 1964), the cell number increase on shift to the restrictive temperature has to be corrected using the equation which describes this age distribution (Howell, 1974). This type of analysis assumes that the age distribution of cells throughout the cell cycle is normal in the *cdc*$^-$ mutants when growing at the permissive temperature. This is likely to be the case with all the mutants described here except one, since they have generation times at 25° C which are very close to wild type. The one exception, sterile strain 22 which has a slow generation time and a late transition point (Table 1), is discussed more fully later.

The increases in cell number after shift to 35° C are given in Figs. 3–7 for the wild type strain h^- and several representative *cdc*$^-$ mutants. The transition points calculated from these and other similar experiments are given for all the *cdc*$^-$ mutants in Tables 1–3. In the wild type strain there is a short plateau of cell number between 50–80 min after shift to 35° C (Fig. 3). This pattern results from a temporary inhibition of nuclear division immediately after the shift (Nurse, 1975). A similar plateau was observed for several mutants which have early transi-

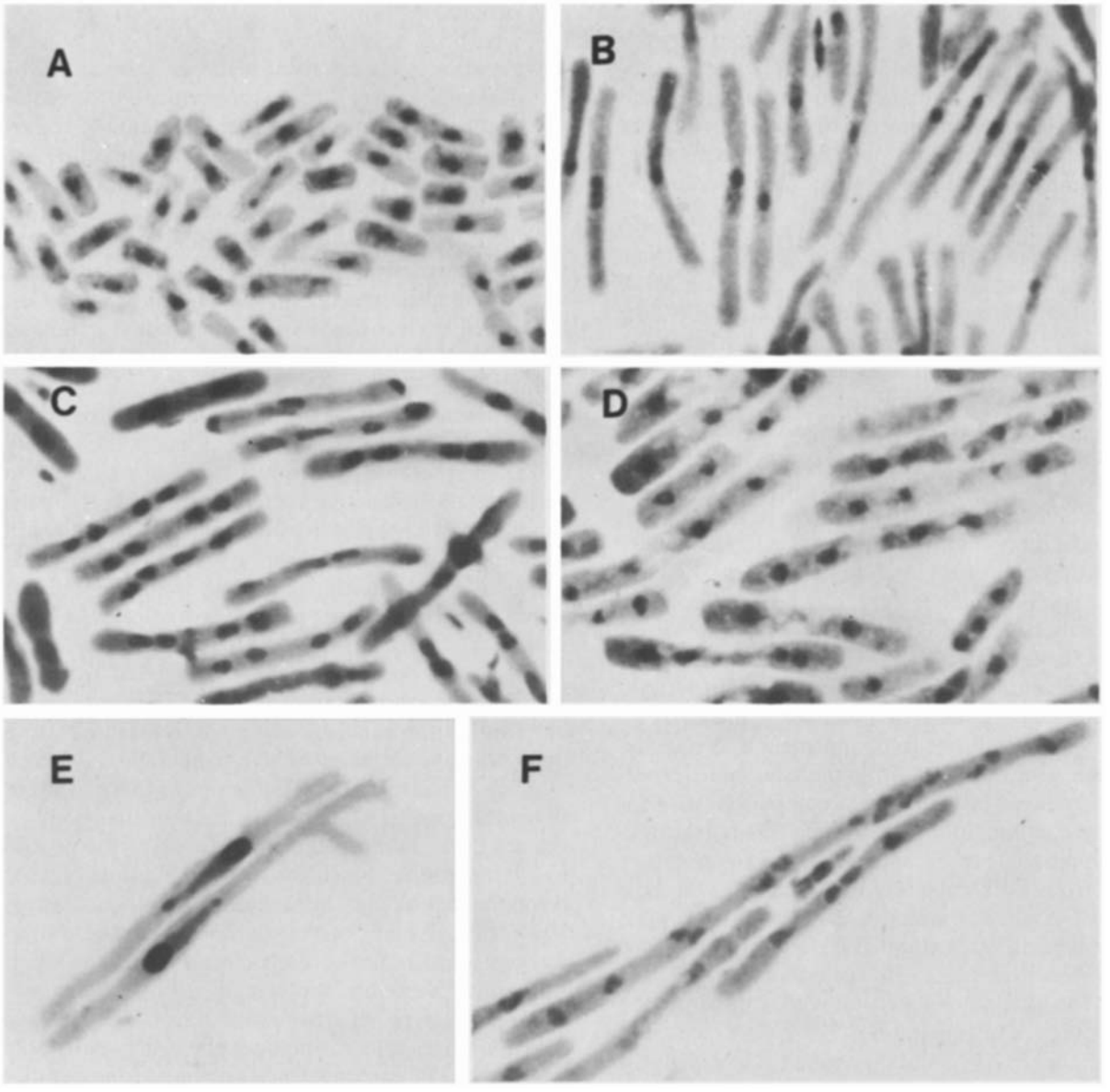

Fig. 1A–F. Nuclear staining of wild type strain and *cdc*$^-$ mutants. Cells stained with Giemsa after 5–9 h incubation at 35° C. All plates are at a final magnification of ×1,000. **A** 972 h$^-$ wild type strain after 5 h at 35° C. **B** *cdc* 2–33 h$^-$ nuclear division mutant after 5 h at 35° C. **C** *cdc* 7–24 h$^+$ early cell plate mutant after 5 h at 35° C. **D** *cdc* 3–6 h$^-$ late cell plate mutant after 9 h at 35° C. **E** Strain 22 nuclear division mutant after 5 h at 35° C. **F** *cdc* 7–24 h$^+$ early cell plate mutant after 9 h at 35° C

tion points (Figs. 4 and 6) and was ignored when calculating the transition point for these mutants.

Throughout this paper transition points are expressed as a fraction of a cell cycle with cell separation acting as 0.

Identification of Defective Cell Cycle Formations

Exponentially growing cultures at 25° C of the wild type strain 972 h$^-$ and the *cdc*$^-$ mutants were shifted to 35° C in liquid minimal medium for 7–8 h. Cells were examined microscopically after nuclear staining with Giemsa stain and cell plate staining with primulin, and the mutants divided in three cytological categories:

1. Elongated cells with a single nucleus (Fig. 1B). These were provisionally classified as DNA synthesis or nuclear division mutants. With one exception (*cdc* 13–117), none of these mutants formed cell plates (Fig. 2B).

2. Elongated cells with 4–8 nuclei per cell and no cell plates (Fig. 1C). These were classified as cell plate mutants blocked early in the process since no

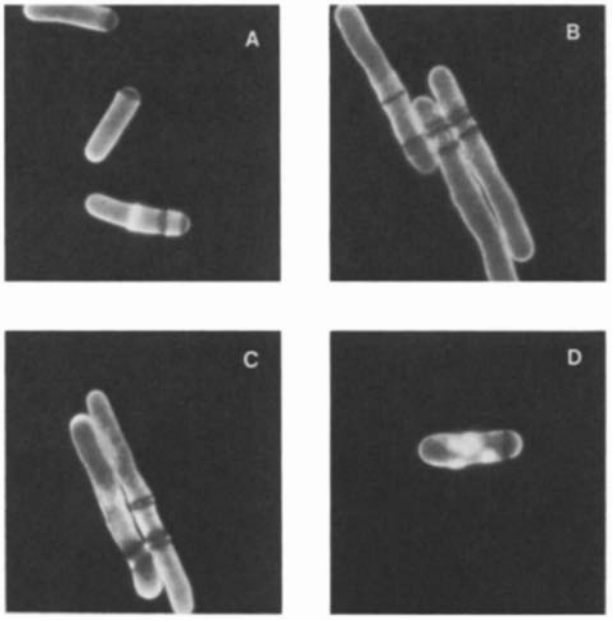

Fig. 2A–D. Cell plate staining of wild type strain and *cdc*⁻ mutants. Cells stained with primulin and examined by fluorescence microscopy after 5 h incubation at 35° C. All plates are at a final magnification of ×1,000. The cell plates and cell plate material show up as brilliant white lines and patches. The dark lines are birth scars. **A** 972 h⁻ wild type strain. **B** *cdc* 2–33 h⁻ nuclear division mutant. **C** *cdc* 7–24 h⁺ early cell plate mutant. **D** *cdc* 3–6 h⁻ late cell plate mutant

cell plate material was accumulated at 35° C (Fig. 2C). On prolonged incubation at 35° C these mutants may accumulate up to 16 nuclei per cell (Fig. 1F).

3. Swollen elongated cells with 2–4 nuclei per cell (Fig. 1D) and containing disorganised cell plate material (Fig. 2D). These were classified as cell plate mutants blocked later in the process since they accumulated cell plate material.

Table 2. Mutants defective in early cell plate formation

Gene	Allele	Transition point	Notes
cdc 7	24	0.50	
cdc 11	119	0.80	
,,	123	0.80	
,,	136	0.81	
cdc 14	118	0.48	leaky[a]
cdc 15	127	0.72	
,,	140	0.81	

General features. Cells of all strains become elongated with 4–8 nuclei/cell after 7 h at 35° C. No cell plates or cell plate material is formed

[a] Shows some residual cell division at 35°C

Table 3. Mutants defective in late cell plate formation

Gene	Allele	Transition point	fgDNA/ nucleus on plateau of DNA[a]	pg protein/ nucleus on plateau of DNA	Notes
cdc 3	6	0.73	30.4	15.3	
,,	124	0.73	30.9	14.4	
cdc 4	8	0.80	31.1	15.9	intragenic
,,	31	0.78	–	–	complemen-
cdc 8	27	0.87	28.5	14.3	tation
,,	110	0.83	–	–	
,,	134	0.73	–	–	
cdc 12	112	0.82	30.7	15.1	

General features. Cells of all strains accumulate as enlarged dumbbell shapes with 2–4 nuclei/cell after 7 h at 35° C. Disorganised cell plate material is observed in all cells

[a] The 2C content of DNA in the wild type strain 972 h⁻ is 33.8 fg/cell (calculated from Nurse, 1975)

Table 1. Mutants defective in DNA synthesis or nuclear division

Gene	Allele	Transition point	fgDNA/nucleus after 5 h at 35° C[a]	Defect	Notes
cdc 1	7	0.69	32.6	Nuclear division	
,,	18	0.74	30.1	,,	
cdc 2	33	0,78	30.2	,,	
,,	56	0.69	–	,,	
,,	130	0.74	–	,,	
cdc 5	120	0.79	31.1	,,	leaky[b]
cdc 6	23	0.44	–	,,	leaky[b]
,,	121	0.38	32.1	,,	
cdc 10	129	−0.10	20.3	DNA Synthesis	
,,	28	−0.10	–	,,	
cdc 13	117	0.64	30.5	Nuclear division	Forms multiple cell plates
–	22	0.88	33.1	,,	Sterile

General features. Cells of all strains become elongated with a single nucleus after 5 h at 35° C. With the exception of *cdc* 13–117 no cell plates or cell plate material is formed

[a] The 2C content of DNA in the wild type strain 972 h⁻ is 33.8 fg/cell (calculated from Nurse, 1975)

[b] Shows some residual cell division at 35° C

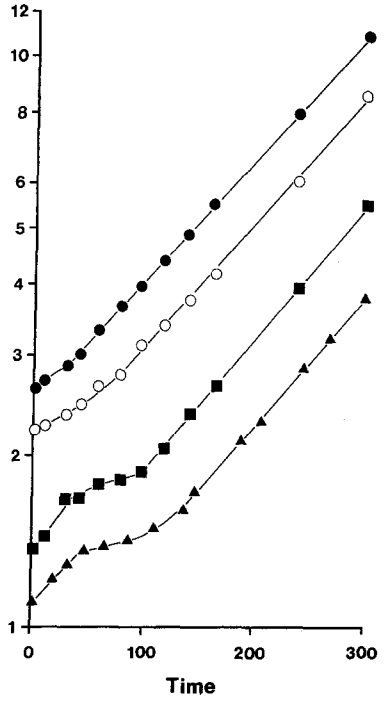

Fig. 3. Cell number, DNA, RNA and protein of 972 h^- wild type strain on shift from 25° C to 35° C. The culture was grown throughout on minimal medium and was shifted from 25° C to 35° C at 0 min. The experimental parameters are plotted per unit volume of culture on an arbitrary log scale. The real values of the experimental parameters per ml of culture equivalent to one unit on the arbitrary log scale, are given within the brackets in the symbol key. ● Protein (15.6 μg), ○ RNA (3.85 μg), ■ DNA (84.5 ng), ▲ Cell number (3.00×10^6)

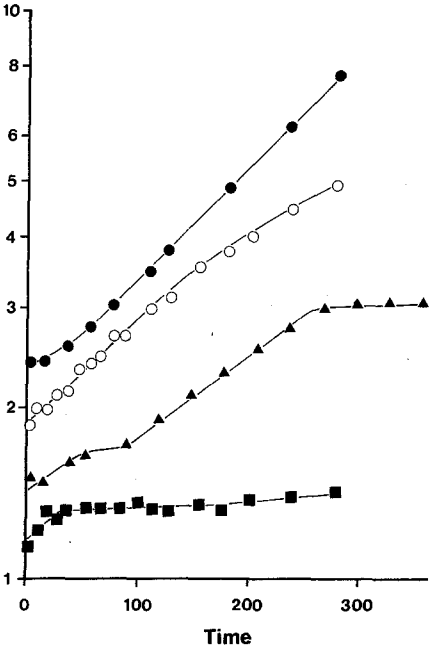

Fig. 4. Cell number, DNA, RNA and protein of DNA synthesis mutant *cdc* 10–129 h^- on shift from 25° C to 35° C. The experimental conditions and an explanation of the arbitrary log scale are given in the legend of Figure 3. ● Protein (25.4 μg), ○ RNA (6.87 μg), ▲ Cell number (3.45×10^6), ■ DNA (153 ng)

Distinction was made between the DNA synthesis and nuclear division mutants of the first category by determining their DNA content per nucleus after 5 h growth at 35° C (Table 1). DNA synthesis mutants completed nuclear division at 35° C, and so accumulated cells with the 1C content of DNA per nucleus, whereas nuclear division mutants were unable to complete nuclear division and so accumulated cells with the 2C content of DNA nucleus (Table 1).

DNA Synthesis Mutants

The two mutants defective in DNA synthesis were found to map in one gene *cdc* 10 (Table 1). On shift to 35° C DNA synthesis was rapidly inhibited whereas cell number doubled before reaching a plateau (Fig. 4). This gives a transition point of 0.90 in the *previous* cell cycle, that is 0.10 of a cell cycle before the start of the cell cycle in which the gene product of *cdc* 10 is required. Thus the gene product of *cdc* 10 appears to complete its function about 0.10 of a cell cycle earlier than DNA synthesis itself.

Cultures transferred to 35° C eventually stopped undergoing nuclear division and accumulated cells containing a single nucleus, showing that the inhibition of DNA synthesis eventually prevents nuclear division. The probable explanation for this is that the inhibition of DNA synthesis prevents two copies of the DNA from becoming available for segregation between the two nuclei.

Although this study revealed only two mutants which were defective in DNA synthesis, new mutants defining several further genes have recently been isolated (Nasmyth, unpublished).

Nuclear Division Mutants

Nine nuclear division mutants mapped in five genes, *cdc* 1, *cdc* 2, *cdc* 5, *cdc* 6 and *cdc* 13 (Table 1). A sixth gene may be represented by the sterile mutant strain 22 which is phenotypically different from all the other nuclear division mutants. At 35° C strain 22 accumulated cells with a dramatically elongated nucleus (Fig. 1E), whilst the other mutants accumulated cells with nuclei that were only slightly elongated (Fig. 1B). The asymetrical appearance of the nucleus in strain 22 suggests that nuclear elongation preparatory to

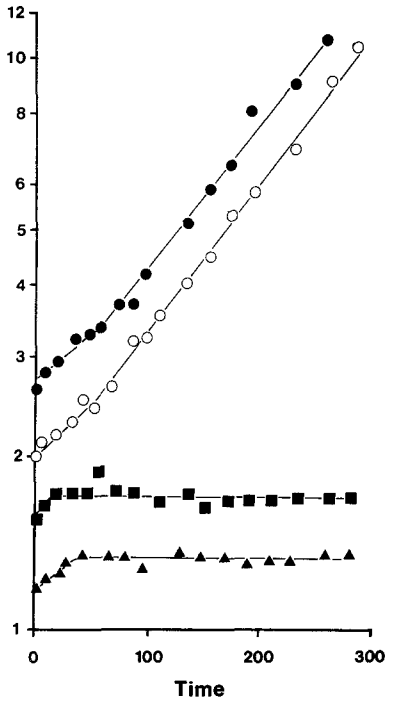

Fig. 5. Cell number, DNA, RNA and protein of nuclear division mutant *cdc* 2–33 h^- on shift from 25° C to 35° C. The experimental conditions and an explanation of the arbitrary log scale are given in the legend of Figure 3. ● Protein (16.3 μg), ○ RNA (4.19 μg), ■ DNA (68.3 ng), ▲ Cell number (2.74×10^6)

division has taken place, but that chromosome separation has not. Since the strain is sterile, the possibility that expression of this phenotype may require mutation in more than one gene, cannot be excluded.

Most of the mutants had transition points of about 0.70 to 0.80 of a cell cycle (Table 1), which is just before or coincident with nuclear division. The two mutants mapping in *cdc* 6 both had early transition points at about 0.40 of a cell cycle (Table 1). Therefore the gene product of *cdc* 6 probably completes its function about 0.35 of a cell cycle before nuclear division takes place. Strain 22 had a late transition point at 0.88 of a cell cycle (Table 1). Since it had a slow generation time at 25° C ($5^1/_2$ h), it seems probable that the mutant phenotype is partially expressed at 25° C. Cells would tend to accumulate just before the block over nuclear division, which would distort the age distribution of cells throughout the cell cycle and tend to bias the transition point to later in the cycle.

In all nuclear division mutants the pattern of increase of DNA at 35° C was similar to the one illustrated for mutant *cdc* 2–33 (Fig. 5). DNA synthesis continued for a short time and then stopped. The time for which DNA continued to be synthesised was dependent upon the transition point of the particular mutant being investigated. Thus, inhibition of nuclear division prevents further DNA synthesis even though RNA and protein synthesis is unaffected. This dependency of DNA synthesis upon nuclear division was confirmed by measuring the DNA content per nucleus of representative mutants in each gene after 5 h growth at 35° C (Table 1). Cultures of each mutant accumulated cells with one nucleus containing the 2C content of DNA, indicating that no further rounds of DNA replication were taking place once nuclear division had been inhibited. This conclusion was supported by the pattern of increase of DNA seen in wild type on shift to 35° C (Fig. 3). The temporary inhibition of nuclear division immediately after the shift (Nurse, 1975) resulted in a short plateau in the increase of DNA 50 min later (Fig. 3).

In nine of the ten nuclear division mutants, cell plate formation and cell division were prevented once nuclear division became inhibited. Thus nuclear division usually has to take place before a cell plate can be formed. The exceptional mutant was *cdc* 13–117. In this mutant multiple cell plates were formed which must be defective in some way since cell separation never took place. The nucleus eventually disintegrated revealing Giemsa stained fragments which could be condensed chromosomes. It is possible that this mutant is blocked at a late stage in nuclear division when the cell is already committed to cell plate formation. This possibility is at present under investigation.

It should be noted that other nuclear mutants which still allow cell plate formation and cell division to take place, may have been missed in the screening proceedure as they would not have produced elongated cells.

Early Cell Plate Formation

The seven early cell plate mutants mapped in four genes *cdc* 7, *cdc* 11, *cdc* 14 and *cdc* 15 (Table 2). The transition points for most of the mutants was at about 0.80 of a cell cycle, which is just before cell plate formation. Two mutants *cdc* 7–24 and *cdc* 14–118 have earlier transition points at about 0.50 of a cell cycle. The early transition points however are difficult to interpret, since the two genes concerned are each represented by only the single alleles.

All of the mutants accumulated 4–16 nuclei per cell at 35° C (Fig. 1C, 1E), and none of them formed cell plates or accumulated cell plate material (Fig. 2C). In representative mutants from each gene, protein, RNA and DNA increased similarly to wild type, whilst cell number increased to a plateau determined by the transition point characteristic for that strain (Fig. 6). Thus DNA synthesis and nuclear divi-

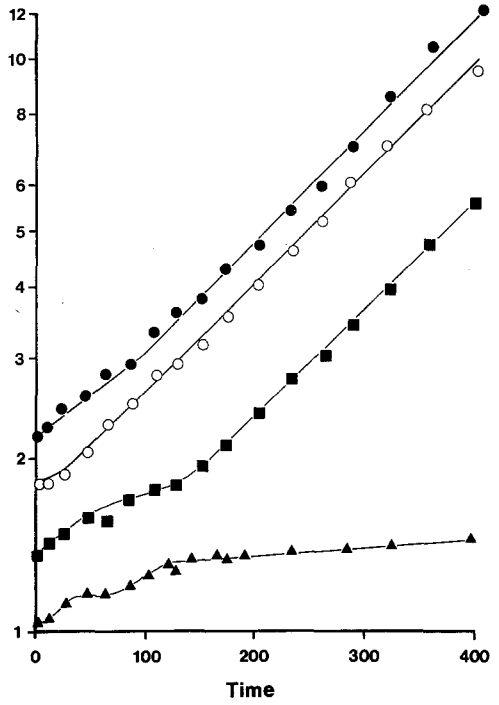

Fig. 6. Cell number, DNA, RNA and protein of early cell plate mutant *cdc* 7–24 h^+ on shift from 25° C to 35° C. The experimental conditions and an explanation of the arbitrary log scale are given in the legend of Figure 3. ● Protein (11.5 μg), ○ RNA (2.84 μg), ■ DNA (56.5 ng), ▲ Cell number (2.20×10^6)

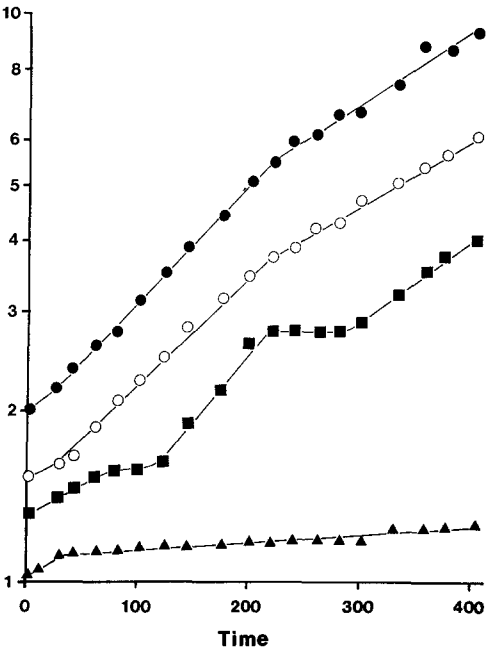

Fig. 7. Cell number, DNA, RNA and protein of late cell plate mutant *cdc* 3–6 h^- on shift from 25° C to 35° C. The experimental conditions and an explanation of the arbitrary log scale are given in the legend of Figure 3. ● Protein (18.0 μg), ○ RNA (4.83 μg), ■ DNA (76.9 ng), ▲ Cell number (2.92×10^6)

sion can continue in the absence of cell plate formation and cell division.

Late Cell Plate Mutants

Eight mutants mapping in four genes *cdc* 3, *cdc* 4, *cdc* 8 and *cdc* 12 were defective in late cell plate formation (Table 3). They had similar transition points at about 0.80 of a cell which is just before cell plate formation (Table 3). At 35° C they all accumulated 2–4 nuclei per cell as well as substantial quantities of cell plate material as judged by primulin staining (Figs. 1D, 2D). This cell plate material was completely disorganised and frequently a strand of cytoplasm was seen running through it in the Giemsa stained preparations (Fig. 1D). Thus cell plate formation appears to be initiated but cannot be completed and so these mutants were classified as defective at a late stage of cell plate formation.

The cells of these mutants frequently increased in width at 35° C (Figs. 1D, 2D), becoming swollen and dumbbell like. It is possible that these cells were blocked in the "constant volume" stage that has been observed close to cell plate formation (Mitchison, 1970) which could result in a reduced rate of cell elongation leading to some cell swelling. This mode

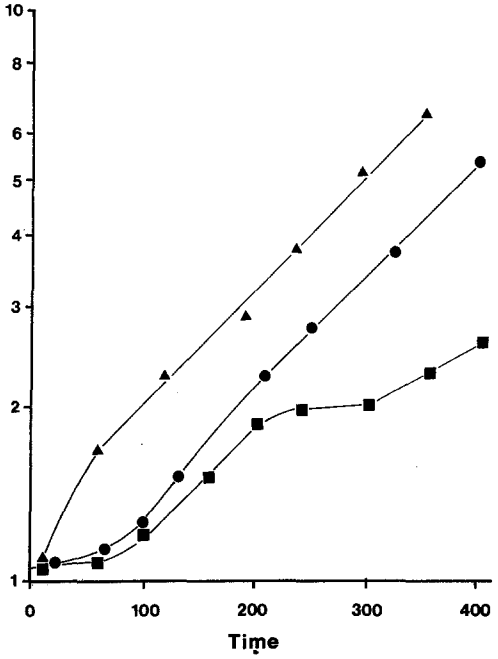

Fig. 8. Average number of nuclei per cell of cdc^- mutants on shift from 25° C to 35° C. The cultures were grown throughout on minimal medium and were shifted from 25° C to 35° C at 0 min. The average number of nuclei per cell was calculated from counts made on Giemsa stained cells at the various times, and is plotted using a log scale ordinate. ▲ *cdc* 3–6 *wee* 1–50 h^- double mutant in late cell plate formation and in the size control over nuclear division. ● *cdc* 7–24 h^+ early cell plate mutant. ■ *cdc* 3–6 h^- late cell plate mutant

of cellular growth may impose a restriction on macromolecular synthesis since RNA and protein synthesis progressively slowed down after 200 min at 35° C (Fig. 7). At about the same time there was an even more dramatic effect on DNA synthesis (Fig. 7) and nuclear division (Fig. 8), which both stop for about 100 min before increasing again at a reduced rate. Similar results were obtained for representative mutants of each gene. These patterns should be compared to the continuous increases in DNA and nuclei per ml observed for the early cell plate mutants (Figs. 6 and 8).

These patterns of increase in DNA and nuclei may be understood in terms of a size control over nuclear division. The inhibition of DNA synthesis is due to an inhibition of nuclear division rather than *vice-versa*, since at the time of the plateau in DNA the nuclei in all mutants have the 2C content of DNA (Table 3). The inhibition of nuclear division in these cells could be the consequence of a control which prevents nuclear division until the cell has achieved a critical size (Nurse, 1975). If the size control is measuring a nucleus to cytoplasmic mass ratio, then the transition from 2 to 4 nuclei will not take place until the cell is double this critical size. As protein and RNA synthesis is restricted in these cells (Fig. 7), the cells will be delayed in reaching the critical size required for further nuclear division. The population will then accumulate cells containing 2 nuclei. This explanation is consistent with the observation that at the plateau in nuclei per ml, the protein content per nucleus never exceeded 17 pg protein (Table 3) which is the mass at which nuclear division is triggered in wild type (calculated from Nurse, 1975). It is also fully supported by observations made with a double mutant *cdc* 3–6 *wee* 1–50. The allele *wee* 1–50 abolishes the size control acting over nuclear division. When combined with the late cell plate allele *cdc* 3–6, *wee* 1–50 also abolishes the restriction over the rate of increase of nuclei per ml (Fig. 8). This demonstrates that the plateau in nuclei per ml observed in the single mutant *cdc* 3–6 (Fig. 8) was due to the size control acting over nuclear division.

Thus, DNA synthesis and nuclear division can continue in the presence of a partially completed cell plate, though they may be eventually restricted by the size control acting over nuclear division.

It has so far been argued that strains such as *cdc* 3–6 are genuine cell plate mutants which do not elongate as rapidly as wild type because they are blocked in the "constant volume" phase which proceeds cell division. An alternative explanation is that they are defective in some aspect of cell wall metabolism which directly impairs cell elongation as well as cell plate formation. This was tested by constructing a double mutant *cdc* 3–6 *cdc* 11–119. *cdc* 11–119 is an early cell plate mutant which forms very elongated cells since cells are blocked before they enter the "constant volume" phase. If *cdc* 3–6 directly prevents cell wall elongation then the double mutant will not elongate as rapidly as the single mutant *cdc* 11–119. However if *cdc* 3 specifically controls a late function in cell plate formation and has no direct effect on cell wall elongation, then *cdc* 3–6 *cdc* 11–119 will elongate as rapidly as *cdc* 11–119 since the presence of the early cell plate mutant allele will prevent the cells from ever entering the "constant volume" phase of the cell cycle. This was observed and thus confirmed the identification of *cdc* 3–6 as specifically blocked in cell plate formation.

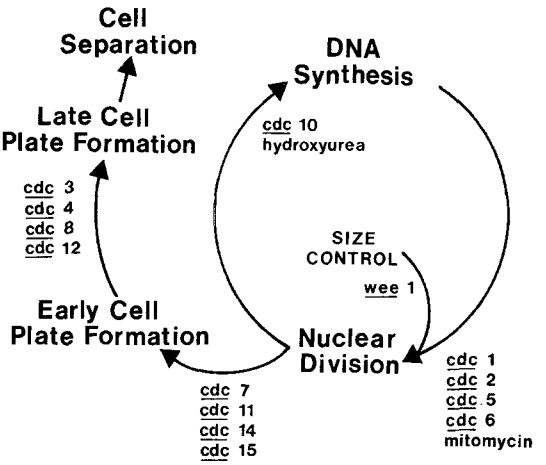

Fig. 9. Summary of the relationships between the various events of the cell cycle and the genes that control them. The *cdc* genes and the cell cycle inhibitors are placed just before the event in which they are involved. The connecting arrows are not analogous to a biochemical pathway but only formally represent the interdependence relationships of the various cell cycle events. Strain 22 has been omitted as its phenotype may be the result of a mutation in more than a single gene and *cdc* 13–117 has been omitted for reasons discussed in the text

Discussion

Twenty seven recessive temperature sensitive *cdc*-alleles have been isolated which define 14 unlinked genes involved with DNA synthesis (*cdc* 10), nuclear division (*cdc* 1, *cdc* 2, *cdc* 5, *cdc* 6 and *cdc* 13), early cell plate formation (*cdc* 7, *cdc* 11, *cdc* 14 and *cdc* 15), and late cell plate formation (*cdc* 3, *cdc* 4, *cdc* 8 and *cdc* 12). A further gene involved in nuclear division may be represented by a sterile strain 22 which is phenotypically different from the other nuclear division mutants.

Bonatti et al. (1972) isolated 71 temperature sensitive mutants of *S. pombe* with the intention of finding

mutants conditionally blocked in DNA synthesis. Eleven of these mutants, each defining a different gene, showed some elongation at the restrictive temperature and so may be *cdc*$^-$ mutants. However their analysis is not yet sufficiently advanced to establish which cell cycle events may be blocked. Stetten and Ledeberg (1973) have isolated a colcemid resistant mutant which may also be a *cdc*$^-$ mutant since it shows defective cell separation.

Cell Cycle Distribution of cdc$^-$ *Mutant Transition Points*

The transition point of a *cdc*$^-$ mutant indicates the stage in the cell cycle when the product of the particular *cdc*$^-$ allele completes its function. It should be stressed that a transition point does not give any information concerning the pattern of synthesis of the gene product. Thus *cdc* gene products could be made periodically or continuously throughout the cell cycle. The distribution throughout the cell cycle of the transition points of all the *cdc*$^-$ mutants is a measure of the periods in the cell cycle when cell cycle gene products complete their functions. Most of the *cdc*$^-$ mutants in *S. pombe* have transition points at a time close to the cell cycle events in which they were defective (Tables 1, 2, 3). Thus there appears to be a high density of development just before the major cell cycle events of nuclear division and cell plate formation, which probably reflects the large numbers of gene products that become involved at these "catastrophic" events (in the sense of Thom, 1972). The single gene involved in DNA synthesis (*cdc* 10) also completes its function close to the event in which it is defective.

Four of the mutants had transition points rather earlier than the cell cycle event in which they were involved. Two of them (*cdc* 7–24 and *cdc* 14–118) which are defective in cell plate formation, are the only representatives of their respective genes, and so it is not known whether the early transition point is gene specific rather than allele specific. This distinction is important since, if a transition point is only allele specific, it may reflect only the decay characteristics of the particular thermosensitive product of that allele. The other two mutants did have transition points which were gene specific. They were separate alleles of *cdc* 6, a gene involved in nuclear division, and both had similar transition points at about 0.40 of a cell cycle. This strongly suggests that this gene product completes its function about 0.35 of a cell cycle before nuclear division takes place.

These observations, that most of the *cdc* gene products complete their functions close to the event in which they are involved, contrasts with the situation in *S. cerevisiae*. In this organism it was reported that the products of all the seven genes involved in nuclear division completed their functions about a third of a cell cycle before nuclear division (Culotti and Hartwell, 1971). However later analysis has shown that the gene with the earliest transition point (*cdc* 7), was actually defective in DNA synthesis which is an early event (Hartwell, 1973). Also some of the other early transition points may be allele rather than gene specific, since later estimates of some of the transition points show considerable variation from the earlier estimates and between different alleles of the same gene (Hartwell, Mortimer, Culotti and Culotti, 1973). Thus, the cell cycle distribution of the times when *cdc* gene products complete their functions may not be so different for *S. cerevisiae* and *S. pombe* as would at first appear.

Dependency Relationships between Cell Cycle Events

The *cdc*$^-$ mutants defective in DNA synthesis and nuclear division have pleiotropic effects on cell cycle events subsequent to the one in which they are blocked. As pointed out by previous workers (Hirota et al., 1968; Hartwell, 1974), such pleiotropy suggests that there are dependency relationships between various events of the cell cycle. The relationships revealed by this study with *S. pombe* have been summarised in Fig. 9. DNA synthesis and nuclear division form a cycle of mutually dependent events. This confirms inhibitor studies using hydroxyurea and deoxyadenosine to inhibit DNA synthesis and mitomycin to inhibit nuclear division (Mitchison, 1974).

The behaviour of the *cdc*$^-$ mutants defective in cell plate formation has demonstrated that the DNA synthesis and nuclear division cycle can continue to operate in the absence of cell plate formation and cell separation (Fig. 9). Cell plate formation itself usually requires nuclear division to have been completed (Fig. 9). An exception to this was provided by only one mutant *cdc* 13–117. In this nuclear division mutant, several cell plates were formed and cell separation could not take place. Because the nucleus possibly contains condensed chromosomes, this mutant may be blocked at a late stage in nuclear division, after the cell has become committed to cell plate formation. Because of the uncertainty in interpretation of this mutant, it has been omitted from Fig. 9. However it is under current investigation and will be discussed in more detail in a later publication. These relationships between the various cell cycle events illustrated in Fig. 9 are very similar to those found for *S. cerevisiae* (Hartwell, 1974), except there is no

"bud emergence-nuclear migration pathway" which is unique to budding yeast. There is also an analogous system of relationships in *E. coli*, where DNA replication can occur independently of cell division, whilst cell division itself is dependent upon the completion of DNA replication (see review by Donachie, Jones and Teather, 1973). These controls over the relationships between the cell cycle events (Fig. 9) would prevent the formation of anucleated cells, and would also maintain a constant ploidy level. The possible basis of the dependency between the cell cycle events has been discussed by Hartwell (1974) and Mitchison (1974).

Concept of "Start"

From his analysis of the cell cycle in *S. cerevisiae*, Hartwell (1974) has proposed that the major control over the cell division cycle acts at a certain stage in G1 called "start". A molecular event involving a "start" gene *cdc* 28 is thought to integrate various cell cycle controls such as the availability of nutrients and sex hormones, and possibly also monitors cell size. Only if all these controls are satisfied does the cell "start", after which it is completely committed to finishing that mitotic cycle. The "start" event fulfills three distinct functions in the cell:

1. It initiates and integrates two otherwise independent developmental pathways in the cell cycle, namely DNA synthesis leading to nuclear division and bud emergence leading to nuclear migration.

2. It restricts exit from the mitotic cycle to begin alternative developmental programmes to cells in the G1 phase just before "start". The alternative developmental programmes available to yeast are conjugation and sporulation leading to meiosis.

3. It completely commits the cell to the mitotic cycle. Once a cell has passed "start" it can alsways complete the mitotic cycle even if it is deprived of various nutrients. Thus stationary phase cultures accumulate cells in the G1 phase of the cell cycle "before start".

It is important to establish whether the "start" hypothesis is applicable to the control of cell division in organisms other than *S. cerevisiae*. In *S. pombe* there is no equivalent to the bud emergence-nuclear migration pathway which diverges early in the cell cycle from the DNA synthesis-nuclear division pathway. Therefore the definition of a start gene analogous to *cdc* 28 as being necessary for the initiation of two otherwise independent developmental pathways in the cell cycle cannot as yet be applied to *S. pombe*.

The alternative developmental programmes of conjugation and sporulation are begun from the G1 phase (Egel and Egel-Mitani, 1974), which is consistent with the start hypothesis. However, it is possible that conjugation and sporulation can only be properly completed when they are initiated from cells that have not undergone DNA replication. If this is the case then there has to be a control which ensures that cells start these alternative programmes from the G1 phase. Such a control would have to exist, even if there was no cell cycle control in the G1 analagous to "start" which, once passed, committed the cell to completing the mitotic cycle. Indeed in *S. pombe*, cells which have left G1 are not necessarily committed to completing that mitotic cycle. Cells undergoing nutritional starvation may become blocked in the G1 or the G2 phase or even in both. Cells starved of phosphate are arrested in G1 (Bostock, 1970), and cells starved of nitrogen are predominantly so (Nurse and Thuriaux, unpublished), whilst cells starved of glucose are arrested in G2 (Bostock, 1970; Creanor, unpublished) and cells starved of sulphate are predominantly so (Nasmyth, unpublished). Therefore, whether a cell can complete the mitotic cycle or not once it has left the G1 phase, is very dependent upon its precise nutritional conditions.

Implicit in the "start" hypothesis is that there is only one major control over cell division which is located in the G1 phase. In *S. pombe* the controls over the cell cycle seem more complex. There is a major control over cell division which acts during the G2 phase just before nuclear division (Nurse, 1975). This control requires the cell to have attained a critical size before it can initiate nuclear division. There is some evidence for a second size control over the initiation of DNA synthesis (Nurse, 1975; Nurse and Thuriaux, unpublished). This control is usually cryptic in fast growing wild type cells and is only revealed under conditions when cells are very small such as during nutrient limitation and spore germination. Thus we would propose that there are potentially two major controls over the cell cycle of *S. pombe*, one acting at the G1/S boundary (which could be similar to start) and a second one acting at the G2/M boundary.

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