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The size control of fission yeast revisited

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SUMMARY

An analysis was made of cell length and cycle time in time-lapse films of the fission yeast *Schizosaccharomyces pombe* using wild-type (WT) cells and those of various mutants. The more important conclusions about 'size controls' are: (1) there is a marker in G_2 in WT cells provided by a rate change point (RCP) where the linear rate of length growth increases by ~30%. The period before this RCP is dependent on size and can be called a 'sizer'. The period after the RCP is nearly independent of size and can be called a 'timer'. The achievement of a critical threshold size is at or near the RCP which is on average at about 0.3 of the cycle (halfway through G_2). This is much earlier than was previously believed. (2) The RCP is at about the time when H1 histone kinase activity and the B type cyclin cdc13 start to rise in preparation for mitosis. The RCP is also associated with other metabolic changes. (3) In *wee1* mutants, the mitotic size control is replaced by a G_1/S size control which is as strong as the mitotic control. As in WT cells, there is a sizer which precedes the RCP followed by a timer but the RCP is at about the G_1/S boundary and has a larger increase (~100%) in rate. (4) cdc25 is not an essential part of the size control at mitosis or at the G_1/S boundary. (5) Three further situations have been examined in which the mitotic size control has been abolished. First, induction synchronisation by block and release of *cdc2* and *cdc10*. In the largest oversize cells which are produced, the RCP is pushed back to the beginning of the cycle. There is no sizer period but only a timer. Second, when both the antagonists wee1 and cdc25 are absent in the double mutant *wee1-50 cdc25Δ*. In this interesting situation there is apparently no mitotic size control and the cycle times are quantised. Third, in *rum1Δ wee1-50* where the normal long G_1 in *wee1* is much reduced, there is probably no size control either in G_1 or in G_2 causing a continuous shortening of division length from cycle to cycle.

Key words: Cell cycle, Cell cycle mutant, Cell growth, Cell length, Fission yeast, Homeostasis, Mitotic control, Rate change point, *Schizosaccharomyces pombe*, Size control, Time-lapse film

INTRODUCTION

The co-ordination of growth and division is an important part of the cell cycle controls, though it has been somewhat neglected in recent years. Such a control is needed to maintain homeostasis of cell size (Fantes and Nurse, 1981).

In a classic paper, Fantes (1977) analysed the results from time-lapse films of individual cells of the wild-type (WT) fission yeast *Schizosaccharomyces pombe*. He showed that there was a homeostatic mechanism which maintained a constant average cell size in growing cultures. This mechanism worked through a 'size control' by which cells large at birth had less total growth in size and a shorter cell cycle than the average. The reverse was true for cells small at birth. This size control worked by altering the time to mitosis (and division) rather than the *rate* of growth. It was assumed that cells reached a critical threshold size which then allowed mitosis to proceed and it was also believed that this critical size was reached just before mitosis (Fantes and Nurse, 1981). 'Size' was measured as cell length which is proportional to volume in this yeast.

At the same time, Nurse and Thuriaux (1977) examined the situation in the small *wee1* mutant. Using synchronous cultures, spore germination and growth after nitrogen starvation, rather than films, they found a critical size (protein content) for the initiation of DNA synthesis. In a later paper, Fantes and Nurse (1978) showed that the mitotic size control was absent in *wee1* cells, so identifying *wee1*$^+$ as a genetic element of this control. Thus there were two size controls in fission yeast. In *wee1*, the mitotic size control was replaced by a G_1/S size control, whereas WT had a mitotic size control but the G_1/S control was cryptic since the cells were always larger than the critical size for this control.

This description of the size controls has remained more or less unchanged for 20 years. We decided it was worth re-examining the situation using the same film technique of Fantes (1977), partly because much more is now known about the biochemistry of mitosis and partly because more mutants are available than in the early days. We show that the critical size for this control is reached much earlier in the cycle than was believed before and it is at about the same time as metabolic changes and the start of the build-up of the regulatory network of mitotic enzymes.

MATERIALS AND METHODS

Strains and growth

The wild-type (WT) strain 972h⁻ of *Schizosaccharomyces pombe* was originally obtained from Professor Urs Leupold, Bern. The mutants used are listed in Table 1 (except for *rum1Δ wee1-50* in Table 2 and *cdc10-129* in Table 3) and were kindly provided by Drs Paul Nurse, Peter Fantes, Sergio Moreno and Jonathan Millar.

The cultures were grown up overnight to ~2×10^6 cells/ml at 35°C or 29°C in a shaking water bath in minimal medium EMM3 (Creanor and Mitchison, 1982), supplemented with leucine at 100 μg/ml where necessary. Before filming, the cultures were lightly sonicated to break up clumps. The procedures for induction synchrony are described in Table 3.

Time-lapse films

Time-lapse films were made of cells growing between a coverslip and a pad of nutrient agar. It is worth going into some detail about this since making these pads is an art rather a science and the exact technique may influence the growth patterns (May and Mitchison, 1995).

The agar was made at 2% (w/v) using 'conditioned' medium filtered off from the culture. Two strips of plastic (0.5 mm thick) were put 20 mm apart on a slide placed on a hotplate at ~60°C. 2 or 3 drops of hot agar were put between the strips and a silicone coated coverslip put on top of the agar and supported by the two strips. The slide was cooled at 4°C for 2-3 minutes and then left to 'cure' for 30 minutes at room temperature. The coverslip and strips were removed and a fine glass capillary (<0.5 mm diameter) sealed on the slide with one end near the pad and the other ~20 mm away. This was to prevent CO_2 from lifting the final coverslip during the subsequent growth of the cells. Then, 1-2 drops of culture were placed on top of the pad and left for ~2 minutes for the cells to sediment. Most of the liquid was then sucked off with tissue. This was tricky since if too much liquid was left, the cells moved, and if it was all removed, the pad could contract after an hour or two. Finally, another coverslip was placed over the pad and sealed with paraffin wax. The slide was placed under the microscope which was in a hotbox and usually left for an hour before filming to recover from possible temperature shocks.

Most of the photographs were taken with a Zeiss Photomicroscope with a Planapochromat objective ×10 (NA 0.32) and a darkground condenser, using an automatic timer to take a frame every 5 minutes for up to 8 hours. Fine grain 35 mm film (Ilford Pan F) was used with a fine grain developer. Up to about 100 cells could be included in the initial field.

Analysis

The negative films were projected onto a screen and measurements made of birth length (BL), division length (DL) and cycle time (CT) of cells which completed a cycle. When possible, the analysis was extended to daughters and granddaughters. In all cases normality of these variables was tested by Minitab version 7.2 on a computer. In all the steady state cultures, the distributions were normal (except the bimodal BL and DL distributions in *wee1-6* and the trimodal cycle time distribution in *wee1-50 cdc25Δ*, described later), but often not after temperature shifts. The length extension was Ext=DL-BL and the extension rate was Ext/CT, or in a corrected version Ext/(CT-CL) where CL was the constant length period at the end of the cycle.

The length of some cells (~40-80 in a film) were measured in every frame from birth to division to get growth patterns. These patterns were smoothed by using the 'rsmooth' command in Minitab. In these smoothed patterns, the position of rate change points (RCP) and CL was determined by eye. Regressions were made on the smoothed linear segments before and after an RCP and the extent of rate change calculated.

The corrected extension rates per cell were measured in successive cycles of a culture. In steady state cultures, these rates were very similar, indicating balanced growth, but were not exactly the same. As an example, the rate in WT cells was 102% of the rate in the preceding cycle. This figure was used to determine the extent of the 'cryptic' rate change at cell division (RCP1), see later in Table 2A and text. The measured rate change in WT at RCP2 was 31%, so the rate change at division was 102-31=71%.

In presenting the plots, the individual cells were grouped but the regression lines were made from the data before grouping. The significance of the slopes of these lines from zero or from −1.0 was determined by a *t*-test with α=0.05.

The asymmetry between daughters was derived from the ratio of each daughter's BL to the sum of both daughter's BLs (Tyson, 1989). With symmetric division, this value is 0.5 for each daughter. As asymmetry increases, the difference between this value in the two daughters increases and is given as a coefficient of variation in column 11 of Table 1 (the mean value remains as 0.5).

In *wee1-6* mutants, there was a significant number of diploid cells which had to be distinguished from the haploids. Since the diploids were larger, there was a bimodal BL distribution. At the local minimum, these bimodal histograms were cut into two parts, and the smaller cells were regarded as haploids and the larger ones as diploids. With induction synchrony of *cdc2-33 wee1-6* this procedure was done in each cycle.

RESULTS

Evidence for a size control from steady-state cultures

As described above, the primary data on individual cells from time-lapse films taken every 5 minutes were birth length (BL), division length (DL) and cycle time (CT) from one division to the next. In some cases, described later, cell length was also measured throughout the cycle, including the constant length (CL) period which lasts from about mitosis until division.

Two important kinds of plot are shown in the examples in Fig. 1. One plot is total extension (Ext) through the cycle (i.e. DL-BL) against birth length. This linear Ext/BL plot had a negative slope and is the evidence of a size control. Small cells at birth extended more than large cells with the implication that they all reached a critical length. A strong size control has in principle a slope of −1.0, irrespective of the average length, which means that deviations from the average are corrected within one cycle. Fantes (1977) showed an Ext/BL plot for wild-type (WT) cells with a slope of −0.76. We have confirmed this in Fig. 1A, though with a slightly higher slope of −0.89 which was not significantly different from −1.0. Fig. 1A also shows similar plots for two other cells of different mean sizes. *wee1Δ* had a slope of −1.01 so the size control was also strong but it was weaker in WT diploids with a slope of −0.37. The strength of the size control in *wee1* is interesting and novel since these cells have replaced the mitotic size control in G_2 by another size control operating in G_1.

The other kind of linear plot is CT/BL which brings in time and shows that the main way in which size is controlled is by altering cycle time. Small cells at birth have longer cycle times than large cells. Fantes (1977) showed this plot for WT cells. Our results were very similar but we also present in Fig. 1B plots for the same cells of different mean lengths as in Fig. 1A. Unlike Ext/BL plots, the slopes were markedly different. The reason for this is that it is easy to show that the slope of a

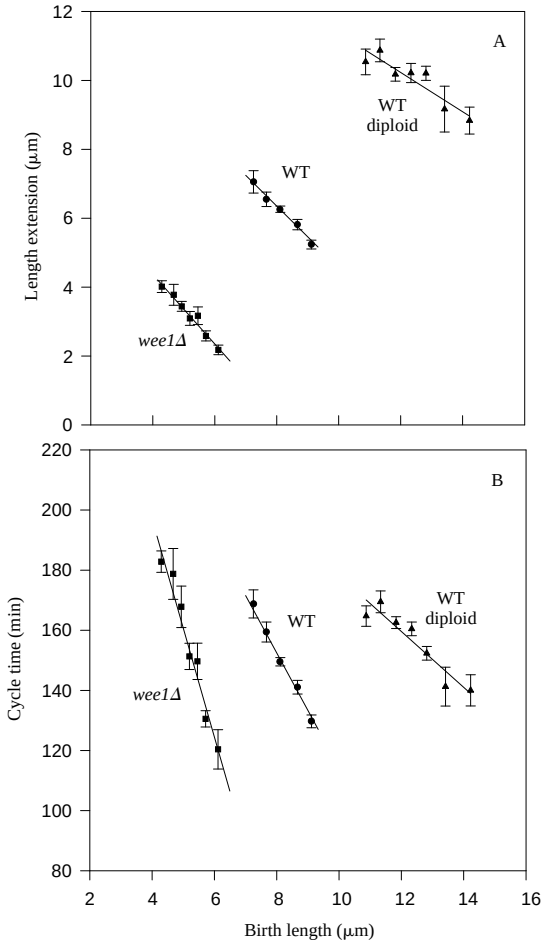

Fig. 1. (A) Extension/birth length, and (B) cycle time/birth length for WT, WT diploid, and *wee1Δ*. Mean values of groups + bar showing s.e.m.

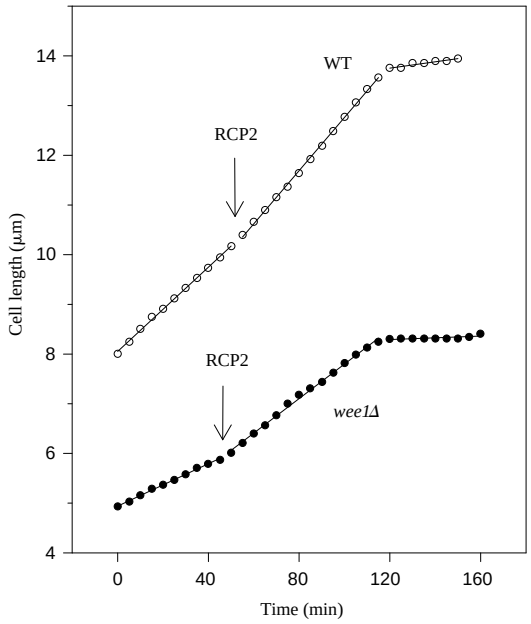

Fig. 2. Growth in length of two representative single cells of WT and *wee1Δ*. RCP2, rate change point. The reason for calling it RCP2 in *wee1* is given later in the Discussion.

CT/BL plot is the reciprocal of the rate of length growth and this rate was less in smaller cells.

The results from a number of steady-state cultures are summarised in Table 1. Some particular points about these results deserve comment. The variation in CT was about twice the variation in DL, which is consistent with the major effect of the size control being on DL. However, these variations were about twice as large in *wee1* as in WT. These variations in other cell systems are discussed by Mitchison (1977) and Tyson (1985). The smaller Ext/BL slopes showed a less efficient size control not only in the large cells of WT diploids and *cdc2-M35* but also in the smaller cells of *cdc2-33* and *cdc2-33 wee1-6*. These last three mutants, however, were grown at the semi-permissive temperature of 29°C which might cause a weakening of the size control. The difference in daughter's cycle times in *wee1* was larger than in WT, except for the *wee1-6* diploids. This is likely to be a reflection of the increased variation in division asymmetry shown in column 11 (which is also visible after DAPI staining of anaphase nuclei; data not shown). Small amounts of asymmetry have also been found in other strains of *S. pombe* (Johnson et al., 1979; May and Mitchison, 1995). Rates of length growth for the strains in Table 1 are not presented since for *steady state* cultures they did not show any significant correlation with BL because of the large variability in growth rate and the narrow range of BL. However, there was a strong positive correlation between strains. These rates ((DL-BL)/CT) can easily be found from the data in Table 1. We also found positive correlations in cases of broader BL ranges (induction synchrony, see below).

Some strains are of particular interest. *wee1-50 cdc25Δ*, with the large variation in cycle times, is discussed later. *cdc2-3w cdc25Δ* had a large amount of cell death but the living cells showed a good size control in the Ext/BL slope. The same happened in *cdc2-3w cdc25Δ pyp3Δ* where the back-up phosphatase of cdc25, pyp3 was also absent. Since the double and triple mutants with *cdc2-3w* are above WT size, the G_1/S control will be cryptic, so we conclude that *cdc25* is not an essential genetic element of the mitotic size control.

Location of the size control

There is a major change in the rate of length growth when WT cells start growing again after division. We call this the first rate change point (RCP1). After this they grow until the start of the constant length (CL) period at about time of mitosis, when length growth nearly ceases until division. However, Mitchison and Nurse (1985) found that the growth period in WT cells was not exponential but was rather made up of two linear segments with a second rate change point (RCP2) between them. This RCP2 was coincident with new end take off (NETO), when the new end formed at division starts growing, and was, on average, at 0.39 of the cycle. They also found that the time to NETO was very variable but they did not exploit this observation because of the limited number of cells which were analysed.

We have extended this analysis to a larger number of cells and of mutants and two examples of single cell growth curves are shown in Fig. 2. Two points should be noted and will be

Table 1. Steady state cultures

1. Strain	2. No. of cycles analysed	3. BL (μm)	4. DL (μm)	5. CT (min)	6. CV_{CT} (%)	7. CV_{DL} (%)	8. Slope Ext/BL (μm/μm)	9. Slope CT/BL (min/μm)	10. CT difference between daughters (min)	11. CV_{DA} (%)
1. WT (972)	164	$8.2^{0.52}$	$14.4^{0.85}$	148^{16}	10.8	5.9	−0.89	−19.1	12^{10}	3.2
2. WT (972 - diploid)	190	$12.4^{0.88}$	$22.2^{1.60}$	159^{16}	10.4	7.2	−0.37	−8.8	15^{12}	3.0
3. *wee1-6*	105	$4.7^{0.58}$	$7.9^{0.83}$	172^{34}	19.6	10.5	−0.97	−35.3	25^{29}	7.1
4. *wee1-6* (diploid)	105	$6.8^{0.59}$	$11.9^{1.00}$	160^{23}	14.4	8.4	−0.77	−19.9	14^{13}	3.9
5. *wee1-50*	156	$5.0^{0.56}$	$8.2^{0.85}$	146^{30}	20.4	10.4	−0.55	−33.9	20^{21}	6.6
6. *wee1Δ*	129	$5.0^{0.56}$	$8.4^{0.86}$	155^{30}	19.5	10.2	−1.01	−36.3	21^{19}	5.8
7. *cdc2-3w*	137	$5.4^{0.48}$	$9.0^{0.69}$	143^{18}	12.4	7.7	−0.62	−20.9	16^{12}	6.1
8. *cdc2-1w*	107	$5.5^{0.51}$	$9.1^{1.00}$	157^{27}	17.4	9.9	−0.46	−18.0	21^{15}	5.9
9. *cdc2-33*	148	$8.5^{0.48}$	$15.2^{0.76}$	197^{21}	10.5	5.0	−0.40	−26.5	18^{13}	3.2
10. *cdc2-33 wee1-6*	40	$5.2^{0.42}$	$8.5^{0.56}$	217^{31}	14.3	6.6	−0.27	−46.1	36^{26}	5.4
11. *cdc2-3w cdc25Δ*	115	$9.7^{0.77}$	$17.6^{1.26}$	159^{24}	15.3	7.2	−0.87	−21.3	24^{16}	4.3
12. *cdc2-3w cdc25Δ pyp3Δ*	80	$11.8^{1.10}$	$21.3^{1.97}$	153^{31}	20.0	9.2	−1.20	−17.8	27^{23}	4.0
13. *wee1-50 cdc25Δ*	465	$9.9^{1.93}$	$18.2^{3.71}$	131^{47}	35.8	20.3	*	*	37^{37}	5.1
14. *cdc2-M35*	116	$11.0^{0.96}$	$20.1^{1.51}$	219^{24}	10.8	7.5	−0.32	−15.1	20^{18}	4.7

Mean values plus s.d. as superscript. BL, birth length; DL, division length; CT, cycle time; CV, coefficient of variation; Ext, total extension (DL-BL); DA, asymmetry between daughter cells (see Analysis). All at 35°C except for strains 9,10,14 at 29°C. Disruptions (Δ), were *::ura4⁺ ura4-D18* in strains 6,11,12,13, which also had a *leu1* marker. Strains 3 and 4 were from one film and separated by BL in each cycle (see Analysis). All strains were h^-. Since BL and DL were measured in some incomplete cycles, the s.e.m. will be less than the value to be derived from the s.d. and the number of the complete cycles in column 2. The number of daughter cells in column 10 is less than that in column 2. For * in columns 8 and 9, see Fig. 6.

discussed below. First, that the change in rate at RCP2 and the duration of the CL period was larger in *wee1Δ* than in WT. Second, that these were only two individual cells and other cells differed especially in the time before RCP2.

Further analysis has revealed an important new relationship. The CT/BL plot in Fig. 3B shows that in WT the period before RCP2 (pre-RCP2) was dependent on BL, being shorter in the larger cells, whereas the post-RCP period did not vary with BL. The Ext/BL plot in Fig. 3A also shows the same behaviour. The mitotic size control therefore operates in the pre-RCP2 period. For *wee1Δ*, there was a small but significant negative slope in the post-RCP2 period in the CT/BL plot (Fig. 3D). This slope could be eliminated if the increasing length of the CL period in *wee1*, shown later, was taken into account (data not shown). Also the slope was not significantly different from zero in the Ext/BL plot (Fig. 3C). We conclude that the size control is similar to WT in operating before RCP2, though it is a different G_1/S size control.

Numerical data for steady state cultures are given in rows 1-8 in Table 2A and rows 1-3 in Table 2B. Some particular points should be made. *wee1* cells have effectively only one RCP with a large rate change whereas the other strains have two. RCP1 at cell division is a special case. It is a requirement of balanced growth that the total rate change/cell over the cycle increase by 100%. When all the RCPs occur during the growth period (as in *wee1* and *cdc2-3w*, see below) the rate change should be 100%, but it is a special case when an RCP occurs at division. Although such an RCP would be seen in a synchronous culture where cell numbers double at each division, it is 'cryptic' and not directly visible at the single cell level. The method for determining the rate change at this RCP is given in Analysis. It also provides a way of testing for balanced growth in the steady state cultures, since under these conditions, the total rate changes per cycle should be 100%. It can be seen by adding together columns 4 and 7 in Table 2A that the total rate changes were near to 100% so we conclude that these cultures were effectively in balanced growth.

The length of the CL period was longer in *wee1* than in WT. This was also found for the septated period in Creanor and Mitchison (1994). Mitosis must be earlier in *wee1* than in WT (Creanor and Mitchison, 1996). We also found that half the cells, both of WT and *wee1Δ* showed a small amount of growth during the CL period, mostly near division in the case of *wee1Δ* but mostly earlier in the case of WT. If the tip growth was at both ends, this could account for the appearance of NETO in the CL period in *wee1-1* (Mitchison and Nurse, 1985).

cdc2-3w is an interesting case since there were two RCPs and presumably two controls during the growth phase. The first was at 0.12 of the cycle with a rate change of 60%. We regard this as the large rate change at division (RCP1) in WT cells but which has been delayed until after division. The S period is also delayed since appreciable, but different, numbers of 1C cells have been found in two FACS analyses (10%, by Enoch et al. (1992); 18%, by Martin-Castellanos et al. (1996)). The 2nd RCP was at 0.43 of the cycle with a rate change of 40%. We presume that this is the RCP2 associated with the mitotic size control, though its late timing suggests that the 'timer' (see below) has been shortened.

It is useful here to resurrect two older terms (Fantes and Nurse, 1981). A 'sizer' is a period of the cell cycle which is strongly dependent on size, whereas a 'timer' is not. Our results show a sizer followed by a timer both in WT and in *wee1*, though the sizer finishes in mid-G_2 in WT and at the end of G_1 in *wee1*. The implication is that cells achieve a critical threshold size by, at most, the end of the sizer period. Although this locates the critical size in the broad range of the early half of G_2, it does not locate it precisely. This size could be reached earlier than RCP2 and be followed by a timer before this RCP. A more precise location comes from the experiments in the next section. Even so, location in the first half of G_2 is different from the earlier model for WT where the critical size was believed to be near mitosis. The reasons for this discrepancy will be discussed later.

It is a little dangerous to extrapolate, but it is worth pointing

Table 2. Analysis of rate change points (RCPs)

A

1. Strain	BL	2. Position of RCP1 (fraction of cycle)	3. Size at RCP1 (μm)	4. Increase of rate at RCP1 (%)	5. Position of RCP2 (fraction of cycle)	6. Size at RCP2 (μm)	7. Increase of rate at RCP2 (%)	8. CL period (fraction of cycle)
1. WT 972		0	8.2	71	0.34	10.4	31	0.21
2. WT (972 - diploid)		0	12.4	52	0.30	15.2	54	0.19
3. *wee1-6*		0	4.7	1	0.26	5.2	101	0.27
4. *wee1-6* (diploid)		0	6.8	1	0.27	7.8	97	0.22
5. *wee1-50*		0	5.0	3	0.21	5.5	98	0.31
6. *wee1Δ*		0	5.0	–5	0.27	5.8	100	0.27
7. *cdc2-3w*		0.12	6.0	60	0.44	7.3	40	0.27
8. *cdc2-M35*		0	11.0	77	0.34	14.3	21	0.26
9. *cdc2-33*	<12 μm	0	9.6	43	0.28	11.5	28	0.28
	>12 μm	0	14.8	2	0.05	15.1	71	0.40
10. *cdc2-33 wee1-6*	<7 μm	0	5.9	–4	0.30	6.9	75	0.31
	>7 μm	0	8.4	–1	0.03	8.6	72	0.31
11. *wee1-50*		0	5.7	–2	0.21	6.2	71	0.28
12. *rum1Δ wee1-50*		0	5.9	1	0.10	6.1	103	0.31

B

1. Strain	BL	2. No. of cycles analysed	3. Slope Ext/BL preRCP2 (μm/μm)	4. Slope Ext/BL postRCP2 (μm/μm)	5. Slope preRCP2/BL (min/μm)	6. Slope postRCP2/BL (min/μm)
1. WT (972)		55	$-0.77^{(S)}$	$-0.12^{(NS)}$	$-18.8^{(S)}$	$1.8^{(NS)}$
2. *wee1-6*		37	$-0.59^{(S)}$	$-0.08^{(NS)}$	$-40.1^{(S)}$	$-4.0^{(NS)}$
3. *wee1Δ*		56	$-0.79^{(S)}$	$-0.20^{(NS)}$	$-33.7^{(S)}$	$13.9^{(S)}$
4. *cdc2-33*	<12 μm	72	$-0.50^{(S)}$	$-0.07^{(NS)}$	$-17.8^{(S)}$	$-1.4^{(NS)}$
	>12 μm	28	$-0.08^{(NS)}$	$-0.01^{(NS)}$	$-1.6^{(NS)}$	$-2.4^{(S)}$
5. *cdc2-33 wee1-6*	<7 μm	31	$-0.70^{(S)}$	$0.36^{(S)}$	$-62.2^{(S)}$	$-9.6^{(S)}$
	>7 μm	63	$-0.05^{(NS)}$	$0.10^{(NS)}$	$-10.4^{(S)}$	$0.1^{(NS)}$
6. *wee1-50*		51	$-0.66^{(S)}$	$-0.18^{(NS)}$	$-30.7^{(S)}$	$-15.9^{(S)}$
7. *rum1Δ wee1-50*		58	$-0.07^{(NS)}$	$0.13^{(NS)}$	$-10.5^{(S)}$	$-6.2^{(S)}$

(A) Rows 1-8, steady state cultures; rows 9 and 10, induction synchrony (IS); rows 11 and 12, after temperature shift. For method of analysing RCP1, see text. For temperature regimes, see Tables 1 and 3. Strain 12 was *h^{90} rum1::ura4^{+} ura4-D18 wee1-50 leu1-32*.

(B) Slopes before and after RCP2. Rows 1-3, steady state cultures; other rows as in 2A. Superscripts give the significance (S) or not (NS) of the slope deviating from zero.

out that, if the pre-RCP2 line is extrapolated to time zero (i.e. the sizer is eliminated), the BLs were 10.9 μm for WT and 6.5 μm for *wee*. These are close to the values of ~12 μm and ~7 μm for the BLs at the breakpoints in the next section.

Abolishing the size control

We consider here three situations in which the size control was abolished.

(1) Induction synchrony

When a *cdc* mutant is shifted up to the restrictive temperature, division stops but growth continues and produces oversize cells. If the culture is then shifted down to the permissive temperature, the cells go through a series of rapid synchronous divisions as they slowly return to normal size. We refer to such a block and release experiment as induction synchrony or IS.

Fantes (1977) did such an experiment with *cdc2-33* and found a biphasic CT/BL curve. The smaller cells showed a size control up to a breakpoint whereas the larger cells showed a CT/BL segment with a much reduced negative slope. He argued that the breakpoint marked the end of the size control and that it had been abolished in the oversize cells.

In Fig. 4A and B, we show Ext/BL and CT/BL curves in IS both for *cdc2-33* and for the smaller cells of *cdc2-33 wee1-6*. In all 4 curves, there was a marked change from a steep negative slope, similar to those for the steady state cultures of WT and *wee1Δ* in Fig. 1A and B, to a much lower negative slope or a low positive one for Ext/BL in the double mutant. After the breakpoint these slopes were not significantly different from zero in the case of the Ext/BL plots, but the low negative slopes were significant in the case of the CT/BL plots. The breakpoints were at a smaller size in the *wee1* background and they were less sharply defined in the CT/BL curves.

Why are there small but definite CT/BL slopes after the breakpoint? The simplest explanation is that large cells could have a faster growth rate (Ext/CT) and so complete their cycle in a shorter time. Unlike Fantes (1977), we have found this to be so. Fig. 4C shows faster growth rates with increasing birth length. The rates, however, were less for cells of the same BL in *cdc2-33 wee1-6* than in *cdc2-33*. A major reason for this is that growth rate was calculated by simply dividing extension by cycle time. Allowance can be made for this by measuring rate during the growing phase before the CL period rather than over the whole cycle (Fig. 4E, see also D for the dependence of CL period on BL). As described in Analysis, the corrected growth rate was calculated as Ext/(CT-CL). The rates were higher than in Fig. 4C but were nearly the same in the two mutants.

Numerical data for the two IS situations described above are presented in the first two rows of Table 3 in two different ways. In Table 3A, the cells are grouped by cycles starting after the first synchronous division. This allows comparison with

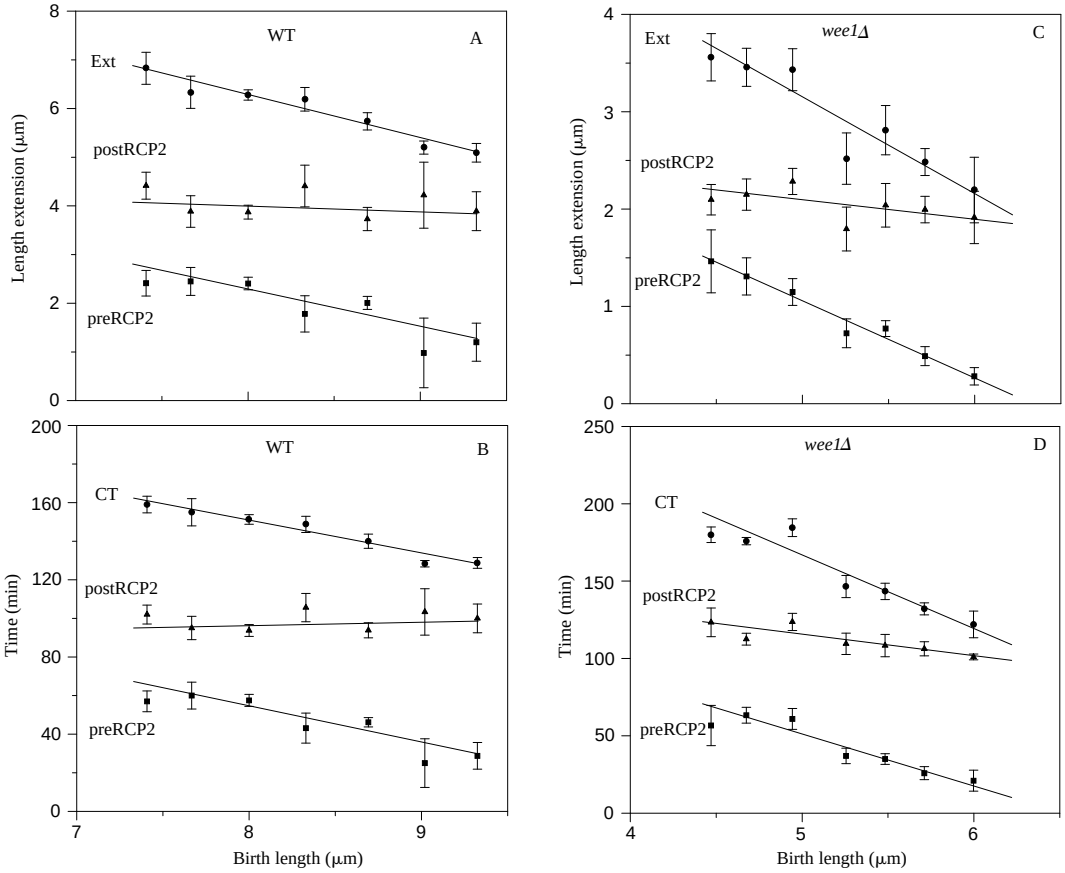

Fig. 3. (A) Extension/birth length in WT over the whole cycle and in the periods before and after RCP2. (B) A similar plot for time/birth length in WT. C and D are similar plots for *wee1Δ*. Mean values of groups + bar showing s.e.m.

previous IS measurements (e.g. Novak and Mitchison, 1990; Creanor and Mitchison, 1996). It also shows the unbalanced growth with decreasing BL and increasing CT. In Table 3B, the cells are grouped by size before and after the breakpoint, irrespective of cycles, and is more relevant to the present analysis. The third row in Table 3 shows the results with IS of *cdc10-129*. It has not been shown as a figure, since it is similar to *cdc2-33*, but is important in establishing that the same biphasic pattern was shown after release from a block at a different stage of the cycle (at Start) and which did not involve any mutation in the *cdc2* gene.

In contrast to the CT/BL curves, there was no significant slope to the Ext/BL curves after the breakpoint, although by eye it appears that in Fig. 4A the slope was negative for *cdc2-33* and positive for *cdc2-33 wee1-6*. At first, it is somewhat puzzling that these large cells should have had a constant extension irrespective of size, especially since growth rate was increasing with size (see columns 4 and 5 in Table 3B). However, CT was also shortening and it was the balance between these that gave the constant extension. This balance could have been accidental but it is also possible that the constant extension was due to the need to synthesise a constant amount of some component, perhaps a protein, before mitosis and this was rate-limiting in the absence of size control. Since the pre-RCP2 period in steady state WT cells shortens with increasing length, it is reasonable to suppose that it would be even shorter, and might disappear in the oversize cells in IS. That this is so is shown in Fig. 5. At the breakpoint in the CT/BL curve, the pre-RCP2 period was very short and vanished completely in cells with slightly longer BLs. The sizer period and the size control had been abolished since the cells had already reached the critical size at birth. Numerical data on this are shown in rows 9-10 in Table 2A and rows 4-5 of Table 2B. Another point from these results is that we have said earlier that the pre- and post-RCP2 periods only locate the critical size in the first half of G_2 and it might be earlier than the RCP, and followed by a timer running to the RCP. But if so, then the pre-RCP period would not fall to zero after the breakpoint. So we conclude that the critical size is reached at or very near RCP2.

(2) *wee1-50 cdc25Δ*

This steady state culture reveals a very interesting situation. The CT/BL curves of individual cells in Fig. 6A show four distinct groups. The smallest cells (<7 μm BL) had a definite size control which is very probably the G_1/S control seen in *wee1* cells, so *cdc25* is not a genetic element of this size control. Each of the other three groups of larger cells had a large variation of BL but lacked a size control since their Ext/BL slopes were not significant (data not shown). Both the CTs and the difference between daughters had polymodal distributions with three peaks (Fig. 6B and C) which differed in CT by rather less than the ratios 1:2:3. An important point was that individual daughter cells moved more or less randomly in both directions from one group to another. It was this movement which reduced the drift towards smaller cells which would be expected in a system lacking a size control, as was found in *rum1Δ wee1-50* (see below).

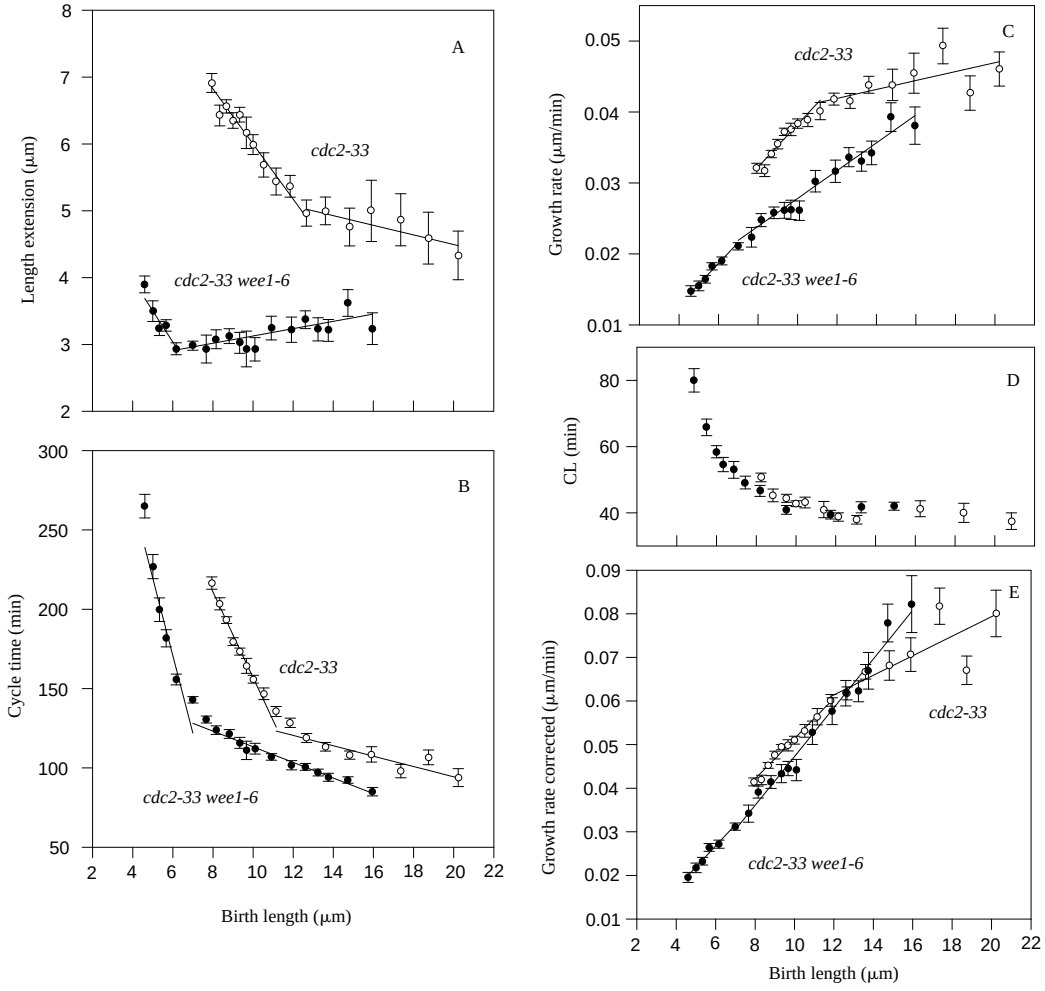

Fig. 4. Induction synchrony (IS) with *cdc2-33* and *cdc2-33 wee1-6*. (A) Extension/birth length. (B) Cycle time/birth length. (C) growth rate (extension rate)/birth length. (D) time of constant length period (data from both mutants). (E) Growth rate corrected by excluding CL period, see text. See Table 3 for temperature regimes (both long and short block periods used for data for double mutant). Mean values of groups + bar showing s.e.m.

The absence of these two important antagonists wee1 and cdc25 is clearly effective not only in abolishing the mitotic size control (which is dependent on *wee1*$^+$), but breaking down the total mitotic control system. The reason for the polymodal CTs needs further analysis. One clue may come from the 'period doubling' that occurs when a control system is breaking down and is on the way to 'chaotic' behaviour (Olsen and Degn, 1985).

(3) *rum1Δ wee1-50*

rum1$^+$ is an important regulator of progression through G_1 (Moreno and Nurse, 1994; Moreno et al., 1994). When it is disrupted in a *wee1-50* background and the cells shifted to the restrictive temperature, the normal long G_1 in *wee* cells is sharply reduced and perhaps eliminated. Both the G_1/S size control (probably dependent on *rum1*$^+$) and the mitotic size control (dependent on *wee1*$^+$) should therefore be absent. We have found this to be the case. Table 3 shows that the Ext/BL curve in *rum1Δ wee1-50* had no significant slope, unlike the control of *wee1-50* shifted up in temperature in the same way, which had a large significant slope. The lack of any size control in the double mutant results in a continuous decrease in cell length. We can also conclude that G_1/S size control is dependent on *rum1*$^+$ (but see Discussion). There is a significant CT/BL slope in the double mutant but this is presumably due to the effect of growth rate on CT which has been mentioned above for IS.

DISCUSSION

Sizers and timers

In the simplified models to explain our data in Fig. 7, those for *wee1* and for IS do not differ significantly from earlier models (Nurse and Fantes, 1981). The earlier model for *wee1* was based on experiments defining a critical size for entry into the S period and the apparent absence of a mitotic size control, but time-lapse films on individual cells were not made. Our films show a sizer operating in the pre-RCP period of the same 'strength' as in WT followed by a post-RCP timer. The RCP (0.21-0.27 of the cycle) is only marginally earlier than the mid-point of S at 0.29 (Nurse and Thuriaux, 1977), so the sizer is operating in G_1. The earlier model for IS, though based on fewer cases, came from films similar to ours. Both models assume that there is only a timer in the larger oversized cells.

The major and important difference between the models comes in WT cells. We find a sizer operating in early G_2 followed by a timer after mid-G_2. The earlier model for WT (Fig. 7) involved reaching a critical threshold size just before mitosis. There was therefore a sizer but it was preceded by a

Table 3. Cultures after temperature shifts

A

1. Strain	2. No. of cycle	3. No. of cycles analysed	4. CT (min)	5. BL (μm)	6. DL (μm)
	1	68	109^{22}	$16.2^{2.96}$	$21.0^{2.83}$
1. *cdc2-33*	2	151	131^{25}	$11.8^{1.69}$	$17.2^{1.62}$
	3	119	161^{18}	$9.8^{0.87}$	$16.0^{1.01}$
	1	63	115^{16}	$9.8^{1.20}$	$12.8^{1.34}$
2. *cdc2-33 wee1-6*	2	86	137^{19}	$7.5^{1.05}$	$10.6^{1.29}$
	3	83	149^{18}	$6.5^{0.70}$	$9.5^{0.94}$
	1	71	141^{30}	$11.1^{2.10}$	$15.4^{1.76}$
3. *cdc10-129*	2	128	167^{28}	$9.3^{1.29}$	$14.1^{1.25}$
	3	–	–	$8.1^{0.71}$	–
	1	80	127^{19}	$5.8^{0.43}$	$8.6^{0.78}$
4. *wee1-50*	2	147	156^{24}	$5.3^{0.47}$	$8.5^{0.95}$
	3	–	–	$5.1^{0.56}$	–
	1	99	117^{19}	$6.3^{0.66}$	$9.0^{1.07}$
5. *rum1Δ wee1-50*	2	158	124^{23}	$5.4^{0.69}$	$8.4^{1.02}$
	3	25	122^{19}	$5.1^{0.65}$	$8.2^{0.87}$

B

1. Strain	BL	2. Slope Ext/BL (μm/μm)	3. Slope CT/BL (min/μm)	4. Slope GR/BL (10^{-3}×min^{-1})	5. Slope GR_{corr}/BL (10^{-3}×min^{-1})
1. *cdc2-33*	<12 μm	$-0.41^{(S)}$	$-26.9^{(S)}$	$2.98^{(S)}$	$4.62^{(S)}$
	>12 μm	$-0.07^{(NS)}$	$-3.3^{(S)}$	$0.63^{(S)}$	$2.24^{(S)}$
2. *cdc2-33*	<6 μm	$-0.46^{(S)}$	$-48.8^{(S)}$	$2.94^{(S)}$	$4.83^{(S)}$
wee1-6	>6 μm	$0.05^{(NS)}$	$-4.9^{(S)}$	$1.96^{(S)}$	$5.59^{(S)}$
3. *cdc10-129*	<12 μm	$-0.30^{(S)}$	$-17.4^{(S)}$	$1.21^{(S)}$	–
	>12 μm	$-0.11^{(NS)}$	$-4.4^{(S)}$	$0.41^{(NS)}$	–
4. *wee1-50*		$-0.65^{(S)}$	$-33.5^{(S)}$	$0.42^{(NS)}$	$3.42^{(S)}$
5. *rum1Δ wee1-50*		$-0.01^{(NS)}$	$-16.6^{(S)}$	$2.43^{(S)}$	$7.07^{(S)}$

(A) An analysis by cycles with mean values plus s.d. as superscripts. Cycle 1 is the first full cycle between 2 synchronous divisions. (B) An analysis by cell length with superscripts of slope significance as in Table 2B. Strains 1-3 are with *cdc* induction synchrony when cultures growing at 29°C were shifted up to 35°C for a period and then shifted back to 29°C before filming. The period was 205 minutes for strain 1, 265 minutes for strain 2 and 180 minutes for strain 3. In B, however, the results for strain 2 were added to another film taken with a block period of 380 minutes so as to get more longer cells. Strains 4 and 5 were shifted from 25°C to 35°C before filming so as to inactivate wee1. GR is growth rate (Ext/CT) and GR_{corr} is corrected as in Analysis. Other abbreviations are as in Table 1.

timer (the 'incompressible' part of G_2). The evidence came from ingenious experiments in which cell size was diminished either by shifting *wee1-50* to the restrictive temperature (Nurse, 1975) or by nutritional shifts (Fantes and Nurse, 1977). Cell size diminished rapidly so it was argued that size was monitored near to mitosis. This was in contrast to bacterial experiments in which the change in cell size was delayed because of a timer running between the threshold size and division. The problem of these earlier experiments is that they involve transient effects and assume that the shifts do not shorten a timer. The modelling analysis of Novak and Tyson (1995) show that this assumption is unjustified. Our evidence for a timer, on the other hand, comes from steady state cultures using an RCP marker which had not been discovered in the 1970s.

What is happening during the sizer period? The simplest assumption is that growth continues until the cell reaches a

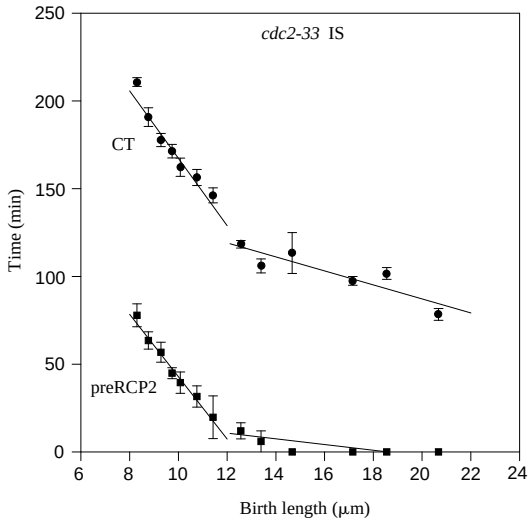

Fig. 5. Induction synchrony (IS) with *cdc2-33*. Upper plot is total cycle time/birth length and lower plot is time before RCP2. The temperature regime is given in Table 3. Mean values of groups + bar showing s.e.m.

critical size at the RCP and this triggers the start of the timer. But we know so little about the molecular mechanisms involved either in the G_1/S or the mitotic size control that it may not be an instantaneous effect and there might be preceding molecular changes. These changes, however, cannot last for long since in IS in the largest cells the RCP2 is pushed back to the start of the cycle.

What happens during the timer period? Growth continues from an average length in WT of 10.4 μm to 14.4 μm (60% of total growth through the cycle). But what is more significant is the start of the molecular 'preparations for mitosis', to use an old phrase. The start of the rise of both histone kinase activity and cdc13 cyclin in WT is at about 0.3 of the cycle (Creanor and Mitchison, 1994, 1996). Considering the approximate value of this figure, it is about the beginning of the timer at 0.36. In the same way, the beginning of the molecular preparations in *wee1* at about 0.3 are near the beginning of the G_2 timer (0.21-0.27), as they also are in the largest cells in IS (0.0). These preparations take time. To quote Novak and Tyson (1995): 'The mitotic control system is not so much like squeezing the trigger of a pistol as it is like lighting a fuse on a stick of dynamite. There is a considerable time lag after the decision...'. Lighting the fuse appears to be passing the critical size and this is the first *identifiable* event in the pathway leading to mitosis. Using a motor car analogy, this could be releasing the brake but it could equally well be the starter of the engine.

Size at division

In the earlier model for WT, size at division was determined only by the critical threshold size for mitosis. With our new model for WT, division size is dependent both on the critical size and also on the length of the timer. In a recent review on fission yeast, MacNeill and Fantes (1995) discuss a number of genes which affect division size, which is the only easy measurement that can be made. The question here is whether they are affecting critical size or the length of the timer (or both).

In the limited number of cases that we have examined, critical size for cells larger than WT (WT diploid and *cdc2-M35*) increases but the timer period remains the same. In *cdc2-3w*, however, both the critical size and the timer appear to be reduced. The timer is also variable in *wee1-50 cdc25Δ* where the sizer is lacking in most cells. For cells smaller than WT, e.g. *wee1*, the emergence of the G_1/S sizer complicates the picture. In much of the recent literature about division size, cells smaller than WT are described as being 'advanced' into mitosis, or when larger as being 'delayed'. This is entirely correct when dealing with the transients in the earlier shift experiments, and it is a convenient shorthand. But it is confusing when applied to steady state conditions. It is true that mitosis is earlier in the cycle in *wee1* cells but this is because they spend longer after mitosis in the septated period (Creanor and Mitchison, 1994, 1996). In the transient conditions in IS, mitosis is even earlier than in *wee1* (Creanor and Mitchison, 1996) but the cells are much larger than WT.

It is worth making a point here about variability. In the earlier models with the WT critical size near to mitosis, it has always been an embarrassment that size at division was so variable. This has led people to postulate a 'sloppy' size control. This may well be true, but, with our new model, extra and important contributions to the variation of division size can come from variation both in the length of the timer and in the growth rate during this period.

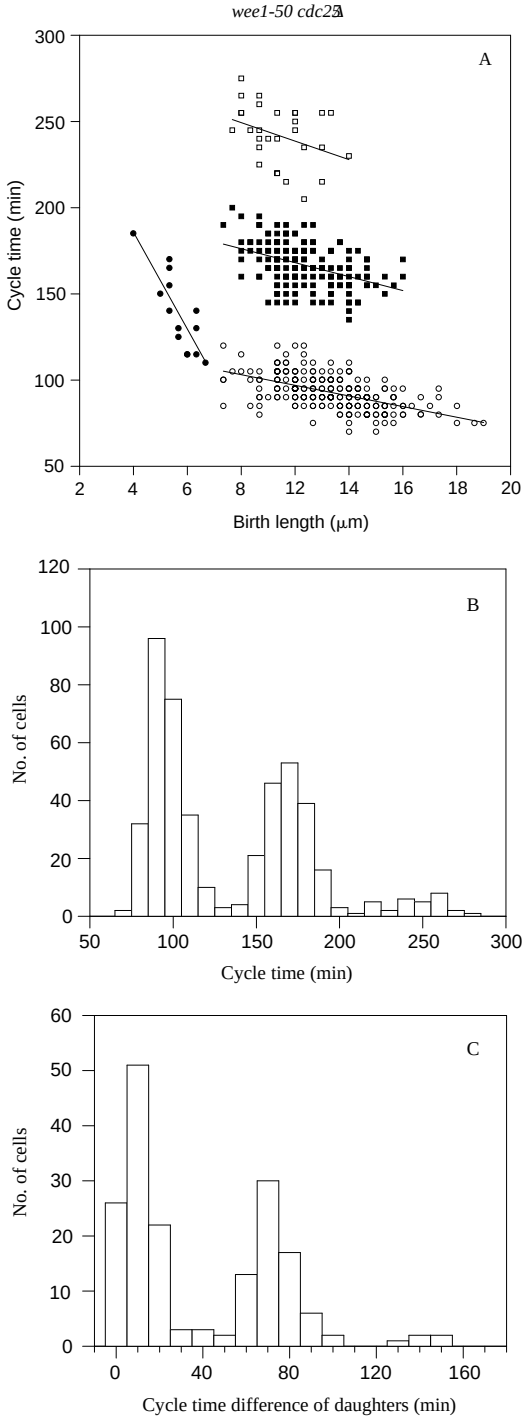

Fig. 6. Steady state culture of *wee1-50 cdc25Δ*. (A) Cycle time/birth length. (B) Cycle time distribution. (C) Distribution of cycle time difference between daughters. Single cell data ungrouped.

Genetic elements

Fantes and Nurse (1978) produced evidence that *wee1*$^+$ was an essential element of the mitotic size control and that in its absence the mitotic size control was abolished and it was replaced by a G_1/S size control. We have confirmed this in our analysis of single *wee1* cells.

Bearing in mind the important antagonism of wee1 and cdc25 in the tyrosine phosphorylation of cdc2, it was important to test whether *cdc25*$^+$ is also an essential element of the mitotic size control though it is more difficult since *cdc25* mutants only complete the cell cycle in *wee1* or *cdc2-3w* backgrounds. We have found a mitotic size control in *cdc2-3w* mutants which also have disruptions in both *cdc25* and its back-up phosphatase *pyp3* (Millar et al., 1992), though many cells die. So *cdc25* is not an essential part of the size control. However if *cdc25* is disrupted in a *wee1* background, so that both these antagonists are removed, the mitotic size control is abolished because of the absence of wee1 but there is an effect of the absence of cdc25 in the quantisation of cycle times which may presage the breakdown of the mitotic control. Although the G_1/S size control is working here in the smallest

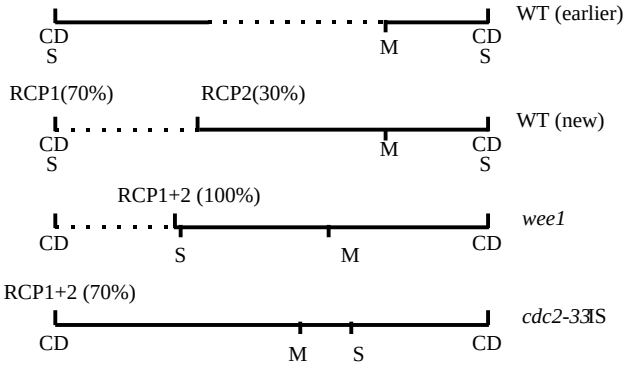

Fig. 7. Cell cycle models for the *average* single cell. Dotted line, sizer period varying with cell size. Continuous line, timer period invariant with size. RCP, rate change point with average increase in rate. CD, cell division; M, mitosis; S, mid point of S period (timings from Creanor and Mitchison, 1996). Earlier model for WT from Nurse and Fantes (1981).

cells, most of the cells do not have a *wee* phenotype. We conclude that *cdc25* is an element of the general mitotic control but not of the mitotic size control.

Further clues to the genetic elements of the mitotic size control may emerge from measurements of size at division and critical size together with their variability in a wider range of mutants than we have examined. Clues to other elements of the G_1/S control, which besides *rum1*, may come from situations where the absence of one of these elements would produce smaller and smaller cells and eventual death (as in *rum1Δ wee1-50*). From our results on this double mutant we conclude that *rum1*$^+$ is a genetic element of the G_1/S size control. The lack of both size controls can be explained the following way: by destroying *wee1*$^+$ the mitotic size control is removed, and by deleting *rum1*$^+$ the G_1/S size control is also removed. But there is an alternative hypothesis which should be borne in mind. The S period of this double mutant does not occur after cell division but its timing before this is unknown. If mitosis was at 0.67 of the cycle, as it is in other *wee* mutants (Creanor and Mitchison, 1996), and the S period was at the end of the cycle, as in WT, there could be a substantial G_1. Since this would lie in the CL period, a size control of cell length would not be observed but there could be a control affecting the length of G_1, as happens with the sizer in single *wee1* mutants. Such a control has been found in IS (Novak and Mitchison, 1990) and it should be remembered that cell mass increase (Mitchison, 1957) and protein synthesis (Creanor and Mitchison, 1982) continue during the CL period. So there might be a mass control at the G_1/S boundary in the double mutant.

Associations of the RCPs

So far we have mainly been concerned with RCP2 as a marker at about one third of the cycle between a sizer and a timer. Now we need to consider RCPs in more detail. RCP1 in WT is at cell division, has a rate increase of 70%, and is invariant in timing. RCP2 is later in the cycle, has a rate increase of 30%, and a large variation in timing between individual cells. In *wee1*, however, there is only one RCP in mid cycle with a rate increase of about 100% and with variable timing. The simplest assumption is that the two RCPs in WT are coincident in *wee1* but we have called it RCP2 since it is in the growth period of the cycle.

The second RCP in WT separates the sizer from the timer and we have argued that it marks the beginning of the molecular preparations for mitosis. What else happens at this time? One event is NETO when the 'new end' of the cell formed at the previous division starts growing (Mitchison and Nurse, 1985). But this association is not true in *wee1* where NETO is much later than the RCP. Mitchison and Nurse (1985) give a cell cycle timing of 0.87 which is well into the CL period. This may be because a proportion of the cells starts growing at both ends near to division, as mentioned earlier.

Many of the other parameters of growth in fission yeast show linear patterns with RCPs. One might expect that changes in the rate of length growth would be mirrored in other bulk measurements of growth and metabolism. This is certainly true for RCP1 in WT. One of the earliest measurements of growth in fission yeast showed a linear pattern of total dry mass with an RCP at 0.91 of the cycle, though in strain 132 (Mitchison, 1957, 1989). The rate of CO_2 evolution (a measure of about half the energy production) has an RCP at 0.0 of the cycle (Hamburger et al., 1977; Novak and Mitchison, 1986; Creanor, 1978, in enriched medium but not in minimal). These RCPs are approximately coincident with the S period. When the S period is later in the cycle in *wee1* cells, the length growth RCP moves later by the same amount, as does the CO_2 RCP (Novak and Mitchison, 1986). In the first cycle in IS, the CO_2 rate RCP is at about 0.72 and the S period at 0.67 (Novak and Mitchison, 1990). The first length growth RCP is delayed until the start of the next cycle but it cannot be expressed during the CL period where there is no length growth. Protein continues to be synthesised during this period (which, in IS, is in G_2) and it might be that the molecular preparations for mitosis could be started then. But they are also delayed until division which suggests a novel dependency relation discussed by Creanor and Mitchison (1996).

What happens to these bulk growth parameters at RCP2 in WT? A second RCP was not detected in the CO_2 measurements but this may be due to smoothing. However, one of the most accurate measures of growth comes from pulse labelling synchronous cultures with amino acids to determine the rate of total protein synthesis (Creanor and Mitchison, 1982). When corrected for partial asynchrony, the pattern for the *average* single WT cell was not a simple step (as would be caused by a single invariant RCP) but rather a smeared out step with a rapid rise after division followed by a slower rise and then a plateau. For this reason the pattern was called 'quasi-linear'. But with our present knowledge of two RCPs, this pattern is what would be expected from a large RCP at division followed by a smaller RCP about one third of the cycle later. Before correction, both WT and *wee1* started the rise in rate (the 'acceleration point' in Creanor and Mitchison, 1982) at about the same time late in the cycle. But synchronous cultures of *wee1* cells have worse synchrony than WT. The correction process to allow for this in *wee1* was not done in the original paper, but we have done so (data not shown) using the different correction process of Creanor and Mitchison (1994). This gives a pattern for *wee1* with a single broad step with a mid-point at 0.3 of the cycle. This is what would be expected from a single large RCP on average at this stage of the cycle. Hence there is close coincidence between the RCPs in protein synthesis and in length growth.

Alcohol dehydrogenase activity has only one RCP (with 100% increase) which is nearly coincident with the CO_2 RCP in WT, *wee1* and IS (Vicente-Soler et al., 1991). This is not, however, a measure of bulk growth and it may be that individual components only change their rates once per cycle.

These coincidences do not tell us about causation. It may be that the prime cause of the major changes in growth and metabolism at RCP1 is the S period. It could also be passage through Start though the exact timing of Start is unknown and, in addition, there might be a delay in establishing the RCP. RCP2 in WT marks the achievement of a critical size and the beginning of the molecular preparations for mitosis. It also marks NETO although, since in *wee1* there is a large RCP without NETO, NETO itself is unlikely to be a cause of the RCP. The real mystery is why there are these major changes in growth rate and what controls them. These are important problems for the future.

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