

# Intramitotic Controls in the Fission Yeast *Schizosaccharomyces pombe*: The Effect of Cell Size on Spindle Length and the Timing of Mitotic Events

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**Abstract.** We have used a new cinemicroscopy technique in combination with antitubulin immunofluorescence microscopy to investigate the timing of mitotic events in cells of the fission yeast *Schizosaccharomyces pombe* having lengths at division between 7 and 60  $\mu\text{m}$ . Wild-type fission yeast cells divide at a length of 14  $\mu\text{m}$ . Separation of daughter nuclei (anaphase B) proceeds at a rate of  $1.6 \pm 0.2 \mu\text{m min}^{-1}$ , until the spindle extends the length of the cell. Coincident with spindle depolymerization, the nuclei reverse direction and take up positions that will become the center of the two daughter cells. This postmitotic nuclear migration occurs at a rate of  $1.4 \pm 0.5 \mu\text{m}^{-1}$ . In cells in which the *wee1*<sup>+</sup> gene is overex-

pressed fivefold and that have an average length at mitosis of 28  $\mu\text{m}$ , the rate of nuclear separation was only slightly reduced but, as spindles in these cells measure 20–22  $\mu\text{m}$ , the duration of anaphase B was extended by  $\sim 40\%$ . By contrast, in the mutant *wee1.50*, which divides at 7  $\mu\text{m}$ , both the rate and duration of anaphase B were indistinguishable from wild type. Nuclei reach the ends of these cells earlier but remain there until a point corresponding to the time of postmitotic nuclear migration in wild type. Thus, the events of mitosis can be extended but not abbreviated. These results are discussed in terms of a mitotic termination control that monitors many different events, one of which is spindle elongation.

**W**ILD-TYPE fission yeast cells divide at a length of 14  $\mu\text{m}$  (Mitchison, 1970; Nurse, 1975; Mitchison and Nurse, 1985). Initiation of mitosis is accompanied by the disappearance of interphase, cytoplasmic microtubules and the formation of an intranuclear mitotic spindle (Hagan and Hyams, 1988). A brief anaphase A (the movement of the chromosomes to the spindle poles) is followed by an extended spindle elongation (anaphase B), which forms the major observable component of mitosis (McCully and Robinow, 1971; Hiraoka et al., 1984; Tanaka and Kanbe, 1986; Hagan and Hyams, 1988; Robinow and Hyams, 1989). Anaphase B proceeds until the daughter nuclei reach the ends of the cell. Its completion is marked by the activation of two microtubule-organizing centers at the cell equator, forming the transient post-anaphase array (PAA)<sup>1</sup>, which precedes the reestablishment of interphase cytoplasmic microtubule network (Hagan and Hyams, 1988). Coincidentally, spindle breakdown occurs and the nuclei migrate away from the cell ends to points which will become the centers of the two daughter cells.

The timing of mitotic initiation in *S. pombe* is controlled

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1. *Abbreviations used in this paper:* DAPI, 4'-6-diamidino-2-phenylindole; PAA, postanaphase array.

by the antagonistic interaction of the genes *wee1*<sup>+</sup> and *cdc25*<sup>+</sup> with the major cell cycle control gene *cdc2*<sup>+</sup> (reviewed in Fantes, 1989). The *wee1*<sup>+</sup> gene product appears to be a dosage-dependent inhibitor of the *cdc2* protein kinase and the *cdc25*<sup>+</sup> gene product an activator, although the molecular basis of this control network remains to be determined (Russell and Nurse, 1986). Neither gene affects the execution of mitosis, merely the timing of its initiation. Mutations in *wee1*<sup>+</sup> can lead to cell size at division being reduced by as much as 50% (Nurse, 1975; Nurse and Thuriaux, 1988), whereas integration of extra copies of the *wee1*<sup>+</sup> gene leads to incremental increases in size at division (Russell and Nurse, 1987). Conversely, mutations in, or a deletion of *cdc25*<sup>+</sup> in the presence of wild-type copy of the *wee1*<sup>+</sup> gene lead to cell cycle arrest, whereas overexpression results in premature mitotic initiation.

In this paper we have exploited the ability to manipulate the *cdc25*<sup>+</sup>/*wee1*<sup>+</sup> mitotic control network to investigate the effect of altered cell size upon spindle elongation and the timing of mitotic events. We show that when cells initiate mitosis at lengths  $>21 \mu\text{m}$ , mitotic spindle length is increased. Cinemicroscopy of dividing cells shows that the rate of nuclear separation (anaphase B) is unaffected by manipulation of spindle length. Thus, in larger cells the mitotic period is increased. By contrast, when cell size is halved the mitotic period is not similarly reduced. Rather, nuclei remain at the

cell ends until the point corresponding to spindle depolymerization and postmitotic nuclear migration in wild-type cells. These results are discussed in terms of the termination of mitosis being controlled by a number of different events, one of which is spindle elongation.

## Materials and Methods

### Cell Culture

The strains *972h<sup>-</sup>*, *weel-50<sup>-</sup>* (Nurse, 1975), *cdc25-22h<sup>-</sup>* (Nurse et al., 1976; Thuriaux et al., 1980), *ade6-210 ura4-d18 : : weel<sup>+</sup> ura4<sup>+</sup>* ( $3 \times weel<sup>+</sup>$ ), *ade6-210 ura4-d18 : : weel<sup>+</sup>* ( $5 \times weel<sup>+</sup>$ ) (Russell and Nurse, 1987), were grown in minimal medium EMM2 (Nurse, 1975), supplemented in the case of the *weel<sup>+</sup>* integrant strains with adenine (250 mg·ml<sup>-1</sup>) to growth state described in the text.

### Immunofluorescence Microscopy

The technique of Hagan and Hyams (1988) was used to stain cells with the anti- $\alpha$  tubulin antibody YOL 1/34 (Kilmartin et al., 1982) (a generous gift from Dr. J. V. Kilmartin).

### Cinematic Microscopy

Yeast extract medium supplemented with adenine, uracil, leucine, histidine, glutamate, and lysine (all at 250  $\mu$ g·ml<sup>-1</sup>) (Gutz et al., 1974) containing 0.5% ultrapure agarose and 30% polyvinyl pyrrolidone (both from Sigma Chemical Co., St. Louis, MO) was maintained in the melted state in an incubator. 32-mm-diam glass coverslips were cleaned and rested on coins smaller than the glass, all placed on a tin lid for subsequent moving. Enough of the melted agarose medium was flooded onto the coverslips to just reach all edges of the glass. The lid was then covered with the upturned tin. When sufficiently set at room temperature to allow movement, the prepared coverslips were placed at 5°C to become firm. For use, the cover glasses were allowed to equilibrate to 35.5°C and then picked up at the edge with forceps.

A drop of early mid-log phase culture was placed on the base of a 5-cm hydrophilic Petriperm dish (Hereas Equipment Ltd., Brentwood, UK). An inverted coverglass with the nutrient gel on it was then placed over the drop and the dish lid was sealed with tape. To avoid the top coverslip becoming misted, a heat-absorbant filter was placed over the dish, thus keeping the top of the dish marginally warmer than the coverslip. The gas permeability of the dish base allowed satisfactory growth conditions for long periods. After 24 h on the microscope monolayer, individual cells had grown up into microcolonies.

Preparations were allowed to equilibrate on the microscope stage for  $\sim 1$  h, at a temperature of 35.5°C, during which time any tendency for displacement between the gel and membrane had ceased. Isolated groups of two or four cells were selected for filming.

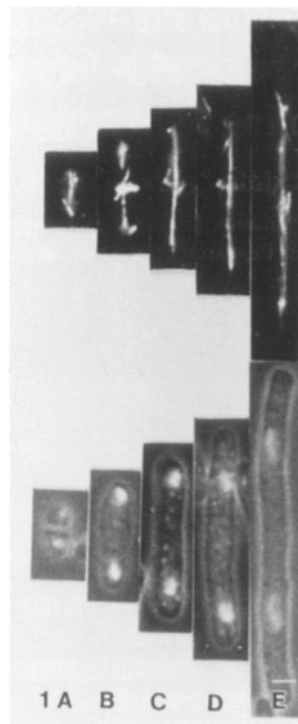
Cultures were filmed on an Olympus IMT microscope MkI with Olympus control units and a Bolex camera. The stage area was maintained at constant temperature as described in Riddle (1979). The thin membrane of the dishes allowed the use of Wild  $\times 50$  or  $\times 100$  Fluotor lenses and a Wild long-working distance condenser, which was substituted for the Olympus lenses. The ultimate field width was  $\sim 80$ – $160$   $\mu$ m, depending upon the objective. Accurate size measurements were recorded at the start of films by making a few exposures of a stage micrometer.

The film used was Kodak Infocapture AHU microfilm, which was developed in a laboratory bench top developing unit using Kodak Dektol or equivalent developers.

## Results

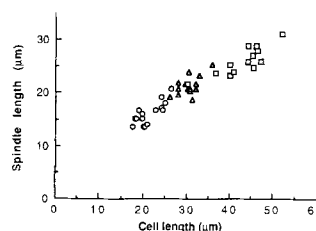
### Correlation of Cell Size with Spindle Length

By manipulating the *S. pombe* cell division control genes *weel<sup>+</sup>* and *cdc25<sup>+</sup>*, it was possible to create cells that enter mitosis at lengths ranging between 7 and 60  $\mu$ m (Fig. 1). The mutant *weel.50* divides at 7  $\mu$ m (Nurse et al., 1976) compared with the wild-type length of 14  $\mu$ m (Mitchison and Nurse, 1985). Strains containing three or five extra copies

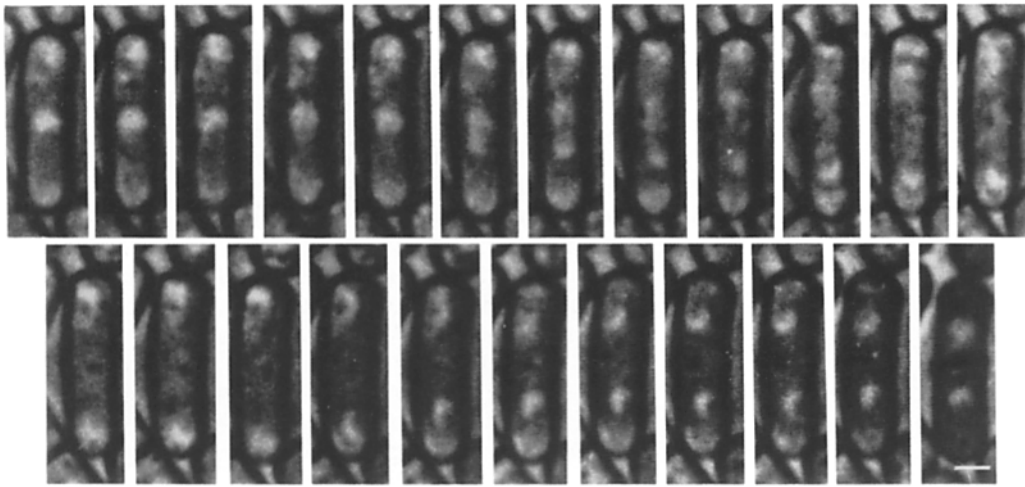


**Figure 1.** Spindle length in fission yeast strains of increasing cell length. (a) Anti-tubulin immunofluorescence and DAPI/phase images of late mitotic cells. The rather ragged appearance of the spindle and the presence of the post-anaphase array at the center of each spindle both mark the end of anaphase. (a) *weel-50h<sup>-</sup>*; (b) wild-type *972h<sup>-</sup>*; (c)  $3Xweel<sup>+</sup>$ ; (d)  $5Xweel<sup>+</sup>$ ; (e) *cdc-25,22h<sup>-</sup>*. a was grown at 36°C, b–d at 25°C. The cells in e were arrested at the G2/M boundary by growth at 36°C for 4.25 h then allowed to enter mitosis at 25°C. Bar, 10  $\mu$ m.

of the *weel<sup>+</sup>* gene divide at 21 and 28  $\mu$ m, respectively (Russell and Nurse, 1987). To generate cells of even greater length than the  $5 \times weel<sup>+</sup>$  integrants, cultures of the mutant *cdc25-22h<sup>-</sup>* were grown to early log phase at 25°C and shifted to 35.5°C. These cells arrest at the G2/M boundary but continue to elongate (Hagan and Hyams, 1988). By returning such cultures to the permissive temperature at the appropriate time, cells entering mitosis at 60  $\mu$ m were obtained (Fig. 1). All of these strains were prepared for immunofluorescence microscopy using an anti-tubulin antibody. In each case, cells were seen to contain all of the typical microtubule arrays observed in wild-type cells (Hagan and Hyams, 1988; data not shown). However, it was clear that when the longer cells underwent division, spindle length considerably exceeded that in wild-type cells. Correlation of spindle length with cell length requires an accurate marker of maximal spindle elongation. In wild-type cells, the activation of the PAA occurs when the spindle reaches its maximum length (14  $\mu$ m) and starts to exhibit a ragged appearance, indicative of the early stages of spindle breakdown (Fig. 1 b). In cells having longer spindles, the relationship of the PAA to spindle breakdown was clearly main-



**Figure 2.** Spindle length is extended in a cell volume-dependent fashion in cells longer than 21  $\mu$ m. Spindle and cell lengths were measured from projected immunofluorescence micrographs of cells containing intact late mitotic spindles and early PAAs.  $3Xweel<sup>+</sup>$  (triangles);  $5Xweel<sup>+</sup>$  (circles); *cdc25,22h<sup>-</sup>* (squares).



**Figure 3.** Mitosis in wild-type fission yeast cells. Negative prints of phase-contrast images made from 16-mm cinefilm records. Fifty seconds separates consecutive images. During interphase, the DNA-containing and nucleolar regions of the nucleus are easily distinguishable. Just before separation, the nucleus appears more amorphous and dynamic, moving in and out of the plane of focus. Immediately before nuclear separation a degree of chromosome condensation can be seen (*third panel*). This is followed by the typical U-shape of early anaphase (Toda et al., 1981). During their initial separation, daughter nuclei undergo a series of oscillations, appearing to twist back and forth until separation is complete. Bar, 2.5  $\mu\text{m}$ .

tained (Fig. 1, *c-e*). Thus the appearance of the PAA is progressively delayed in these strains.

Using the PAA as a marker of the end of mitosis, maximal spindle extension was compared with cell length, as shown in Fig. 2. This shows that in cells dividing at wild-type length or below, the spindle extends the entire length of the cell. In longer cells this is not the case, although, in cells longer than 21  $\mu\text{m}$ , spindle length exceeds the maximum value for wild-type cells in a manner related to total cell length.

#### ***The Mitotic Period Is Extended in Cells Containing Five Copies of the *wee1+* Gene***

We were interested to discover whether the rate of nuclear separation in cells having longer spindles was the same as in wild type, and thus the cell spends a correspondingly longer time in mitosis, or whether nuclear separation was accelerated so that the duration of mitosis remained unchanged. Wild-type cells and cells with five extra copies of the *wee1+* gene were analyzed during cell division by phase-contrast cinemicroscopy. Fig. 3 shows an example of the images obtained with wild-type cells. Nuclei moved to the cell ends at a rate of  $1.6 \pm 0.2 \mu\text{m} \cdot \text{min}^{-1}$  (Table I), often pausing briefly before moving back down the cell in a postanaphase movement, which is initiated  $513 \pm 14$  s after the first

**Table I.** Rates of Nuclear Movement in Various *S. pombe* Strains at 35.5°C

Strain	Nuclear separation rate	Postmitotic nuclear migration rate
	$\mu\text{m} \cdot \text{min}^{-1}$	$\mu\text{m} \cdot \text{min}^{-1}$
972 h <sup>-</sup>	$1.59 \pm 0.22$ ( $n = 9$ )	$1.36 \pm 0.48$ ( $n = 9$ )
5X <i>wee1+</i>	$1.39 \pm 0.10$ ( $n = 4$ )	No consistent movement
<i>wee1-50</i>	$1.68 \pm 0.31$ ( $n = 5$ )	$1.56 \pm 0.29$ ( $n = 4$ )

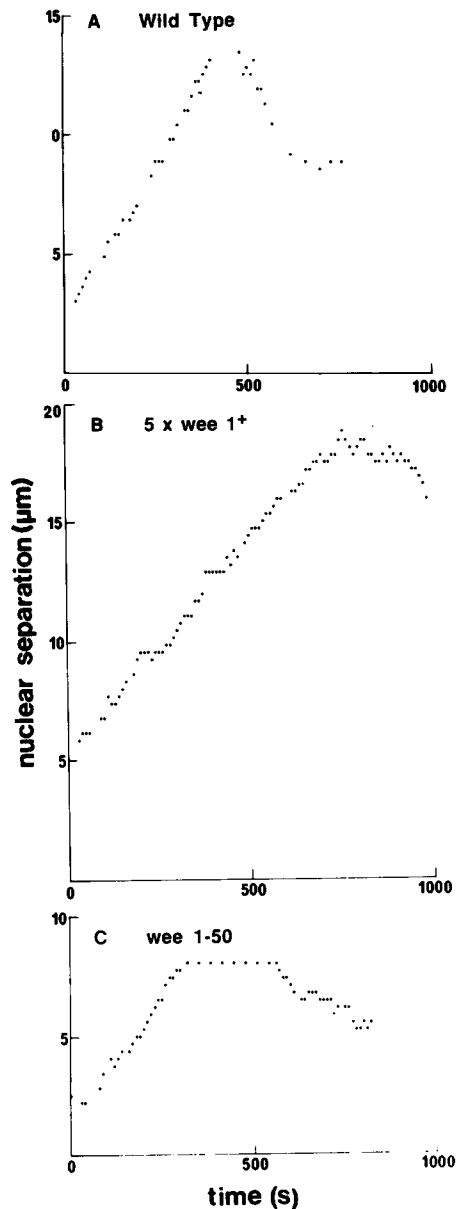
Rates of anaphase nuclear separation and postanaphase nuclear movement determined from graphs such as those shown in Fig. 3.  $n$  = number of determinations.

signs of nuclear separation and which returns the nuclei to the centers of the new daughter cells (Table II). The events are plotted graphically in Fig. 4 *a*.

Nuclear movement in the strain carrying five extra copies of the *wee1+* gene occurred at a similar speed (Fig. 4 *b*; Table I). Postanaphase nuclear movement was either greatly reduced or not observed at all in these cells, presumably because the separated nuclei are at, or very near to, their ultimate position at the center of the daughter cells. However, it is clear from Fig. 4, *a* and *b* that the duration of anaphase B ( $843 \pm 46$  s; Table II) is considerably extended in this strain. Nuclear separation is not a smooth, uninterrupted process but is punctuated by occasional pauses. The phases of movement were used to calculate the overall rate of  $1.4 \pm 0.1 \mu\text{m} \cdot \text{min}^{-1}$  (Table I). Interestingly, around half of the 5X *wee1+* cells initiated two septa, although these invariably collapsed into a single structure (not shown).

#### ***The Mitotic Period Is Not Reduced in *wee1-50h-* Cells That Initiate Mitosis at a Reduced Cell Size***

One prediction of a model in which the duration of mitosis was determined by cell volume would be that a reduced cell size should result in a corresponding reduction in mitotic time. To investigate this, cells containing a *ts* mutation in the *wee1+* gene, *wee1.50* were analyzed by cinemicroscopy. Fig. 4 *c* shows that there is no reduction in the period between the initiation of nuclear separation and postanaphase nuclear movement in *wee1.50*, despite the fact that nuclear separation again proceeds at a rate comparable to wild-type cells ( $1.7 \pm 0.3 \mu\text{m} \cdot \text{min}^{-1}$ ; Table I), and is therefore completed much earlier. This is accounted for by the fact that nuclei remain at the cell ends for longer than in wild-type cells before initiating spindle breakdown and postanaphase migration (Fig. 4 *c*). In some instances premature septation was observed in *wee1-* cells, in extreme cases before the initiation of mitosis, indicating that some level of premature mitotic initiation may be occurring (Hagan and Hyams, 1988).



**Figure 4.** Time-course of nuclear separation in *S. pombe*. (a) The wild-type strain 972h<sup>-</sup>; (b) 5Xwee1<sup>+</sup>; (c) wee1-50. Films of dividing cell were projected onto a desktop screen and the two most distal points of the nuclei, in every frame where a clear image could be obtained, were measured and plotted against time. Data shown are representative examples of each strain. Note that nuclear separation is completed earlier in wee1-50 than in wild type but that postanaphase nuclear movement occurs at about the same time (see also Table II).

## Discussion

In this paper, we exploit the *cdc25/wee1* mitotic control network in the fission yeast *S. pombe* to investigate the effect of cell length at the initiation of mitosis on spindle length and, hence, the duration of anaphase B. The product of the *cdc25*<sup>+</sup> gene is a predicted activator of the *cdc2* protein kinase and *wee1*<sup>+</sup> an inhibitor although direct tests of these predictions are still awaited (Fantes, 1989). Because cells undergo normal divisions in the absence of functional copies of either gene (Fantes, 1979; Russell and Nurse, 1986), it

is reasonable to assume that it is just the timing, and not the mechanical events, of mitosis that are perturbed by these manipulations. Increasing cell length at division either by increased expression of the *wee1*<sup>+</sup> inhibitor or by temporary inactivation of the *cdc25*<sup>+</sup> inducer, leads to increased maximum spindle length, in other words, an extended anaphase B, in cells longer than 21 μm. By contrast, in the mutant *wee1-50*, both cell length and spindle length are reduced to 7 μm.

Mitosis in living cells over a range of sizes was analyzed using a modification of methods first described by Robinow (1981) to visualize nuclei in living fission yeast cells using a high refractive index medium. This employed a growth chamber freely permeable to oxygen that supported repeated cell divisions. Our measured rate of nuclear separation (anaphase B) in wild-type *S. pombe* cells was in good agreement with that reported by others (Tanaka and Kanbe, 1986). In the longer spindles of the *wee1*<sup>+</sup> integrant strains the apparent mean rate was somewhat slower ( $1.39 \pm 0.1 \mu\text{m}\cdot\text{min}^{-1}$ ) and the nuclei repeatedly paused during separation. Video analysis of mitosis in the fungus *Fusarium* also shows non-continuous movement of nuclei (Aist and Bayles, 1988) and it may be that these long cells are revealing a normal feature of mitosis in *S. pombe*, which is undetectable in wild type. In these long cells, mitosis lasts ~6 min longer than in wild type at 35.5°C. If anything, this is likely to be a conservative estimate due to the lack of postanaphase movement of these nuclei, which acts as a clear marker for spindle breakdown in wild type cells.

Immunofluorescence microscopy shows that the appearance of the PAA is linked to the delayed spindle breakdown. Septation is also delayed, indicating that the extension of mitosis results in the coordinate delay of other cell cycle events. Further evidence for a correlation between mitotic progression and the status of the spindle is provided by the *nda3*, cold-sensitive β-tubulin mutants of *S. pombe* (Hiraoka et al., 1985). These cells arrest with condensed chromosomes indicating that mitotic processes have been initiated, however, they fail to septate. Because the only defect in these cells is the inability to form a spindle, the initiation of a set of late-division events evidently requires spindle formation.

In *wee1-50* cells, which divide at a reduced size, the mitotic period is the same as in wild type. This strongly suggests that although the mitotic period can be extended it cannot be abbreviated. Thus, the mitotic period may be determined by an internal "timer control" analogous to that controlling the minimum duration of G2 (Fantes and Nurse, 1978), G1 (Nasmyth et al., 1979; Thuriaux et al., 1979) and the initiation of bipolar growth (Mitchison and Nurse, 1985) in *S. pombe*.

Our observations further suggest that the duration of mito-

**Table II.** Duration of Anaphase B in Fission Yeast Strains having Different Cell Lengths

Strain	Mean cell length	Duration of Anaphase B
	μm	
<i>wee1-50</i>	7.0	570 ± 70 (n = 15)
972h <sup>-</sup>	14.0	513 ± 14 (n = 26)
5Xwee1 <sup>+</sup>	28.0	843 ± 46 (n = 12)

Measurements taken from cine records. n = number of determinations.

sis is a function of the coordinate and/or sequential execution of a number of events, the final one being the turning off of the mitotic state, resulting in the dissolution of the spindle and the movement of the nuclei back down the cell. In this case the minimum period is defined by the longest rate-determining step. We envisage that there are two classes of mitotic events, those whose completion is essential for the activation of a spindle dissolution signal and those which are not. Spindle elongation is an example of the former category and becomes the rate limiting step when driven, for example by increased levels of spindle components, for a longer time in cells  $>21 \mu\text{m}$ . Below this threshold, some other event is rate limiting and accounts for the maintenance of the mitotic period in small cells. The occurrence of aberrant mitoses in *wee1*<sup>-</sup> mutants (Hagan and Hyams, 1988) suggests that some mitotic processes are dependent upon the completion of G2 events that are not required for the initiation of mitosis but, if delayed, also have the potential to be rate limiting. Although much is known about the events mediating entry into mitosis in eukaryotic cells through the *cdc2/cyclin* mitotic kinase (Lohka, 1989), there is little information about how the process is terminated. The recent identification of mitotic genes encoding protein phosphatases in both fission yeast and *Aspergillus* (Booher and Beach, 1989; Ohkura et al., 1989; Doonan and Morris, 1989), however, suggests that the molecular basis of the control elements identified in this study might soon be revealed.

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