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Interaction of *cdc2* and *rum1* regulates Start and S-phase in fission yeast

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SUMMARY

The p34^{cdc2} kinase is essential for progression past Start in the G_1 phase of the fission yeast cell cycle, and also acts in G_2 to promote mitotic entry. Whilst very little is known about the G_1 function of *cdc2*, the *rum1* gene has recently been shown to encode an important regulator of Start in fission yeast, and a model for rum1 function suggests that it inhibits p34^{cdc2} activity. Here we present genetic data suggesting that rum1 maintains p34^{cdc2} in a pre-Start G_1 form, inhibiting its activity until the cell achieves the critical mass required for Start, and find that in the absence of *rum1* p34^{cdc2} has increased Start activity in vivo. It is also known that mutation of *cdc2*, or overexpression of *rum1*, can disrupt the dependency of S-phase upon mitosis, resulting in an extra round of S-phase in the absence of mitosis. We show that *cdc2* and *rum1* interact in this process, and describe dominant *cdc2* mutants causing multiple rounds of S-phase in the absence of mitosis. We suggest that interaction of *rum1* and *cdc2* regulates Start, and this interaction is important for the regulation of S-phase within the cell cycle.

Key words: cdc2, rum1, cell cycle, Start, *Schizosaccharomyces pombe*

INTRODUCTION

Progression through the eukaryotic cell cycle is controlled at two major points - in G_1 before the initiation of S-phase, and in G_2 before entry into mitosis. In yeast the G_1 control is called Start, and represents the point of commitment to the mitotic cell cycle, so that cells are only able to initiate alternative developmental programs such as conjugation and meiosis from pre-Start G_1 (Hartwell, 1974). In fission yeast the products of the *cdc2*, *cdc10* and *res1/sct1* genes are essential for progression past Start (Nurse and Bissett, 1981; Tanaka et al., 1992; Caligiuri and Beach, 1993). The *cdc10* and *res1/sct1* gene products form a transcriptional complex essential for the expression of genes required for S-phase, suggesting that the activation of such cell cycle regulated transcription is an important aspect of Start (Lowndes et al., 1992; Tanaka et al., 1992; Caligiuri and Beach, 1993). The role of the p34^{cdc2} kinase at Start remains unclear, although it appears that the p85^{cdc10} containing transcriptional complex is unable to bind DNA in the absence of *cdc2* function (Reymond et al., 1993).

To understand how progression through G_1 is regulated, it is necessary to identify those gene functions that determine the timing of Start, in order to identify the rate limiting step. The *rum1* gene product has recently been shown to play a critical role in determining the timing of Start in the fission yeast cell cycle (Moreno and Nurse, 1994). Whilst a wild-type cell is able to arrest or delay progression past Start, in conditions such as nutrient limitation where the cell must remain in G_1 until it has grown sufficiently to achieve the critical cell mass required to pass Start (Nurse and Thuriaux, 1977; Nasmyth et al., 1979), cells lacking the *rum1* gene undergo Start immediately after completing mitosis (Moreno and Nurse, 1994). *rum1* function is therefore required to maintain the cell in the pre-Start G_1 interval, and overexpression of the *rum1* gene product resets a G_2 cell back to pre-Start G_1, suggesting that *rum1* may define a cell as being before Start (Moreno and Nurse, 1994).

A model for *rum1* function has been proposed, in which *rum1* acts by maintaining p34^{cdc2} in a pre-Start G_1 form, inhibiting its activity until the critical mass required for Start is reached, at which point *rum1* inhibition is released, and the cell passes Start (Moreno and Nurse, 1994). It is not yet possible to test this model directly in vitro, since we still do not know in what form or complexes p34^{cdc2} is active at Start in fission yeast, whilst the detectable p34^{cdc2} kinase activity against the standard histone H1 substrate is very low in G_1 (Booher et al., 1989; Moreno et al., 1989; Creanor and Mitchison, 1994). However, it has recently been shown that bacterially produced rum1 protein does potently inhibit mitotic p34^{cdc2} kinase activity in vitro, associated with the B-type cyclin p56^{cdc13} (J. Correa and P. Nurse, unpublished). Here we describe a physiological approach to studying *cdc2* Start activity in vivo, and provide genetic evidence suggesting that rum1 acts as an inhibitor of p34^{cdc2} at Start, and is necessary to maintain p34^{cdc2} in a pre-Start G_1 form.

Both *cdc2* and *rum1* have also been implicated in the controls restricting S-phase to one round per cell cycle, since

subjection of *cdc2*^{ts} mutants to a brief heat shock treatment causes G_2 cells to undergo an extra round of S-phase in the absence of mitosis, whilst overexpression of rum1 causes multiple rounds of S-phase without mitosis (Broek et al., 1991; Moreno and Nurse, 1994). In both cases rereplication requires passage of Start, suggesting that regulation of Start may form the basis of the dependency of S-phase upon the completion of mitosis. We provide genetic data showing that *rum1* and *cdc2* interact in this aspect of cell cycle control, and describe new dominant *cdc2* mutants that cause overreplication when overexpressed in a wild-type cell, resembling overexpression of *rum1*.

MATERIALS AND METHODS

Fission yeast strains and methods

The following strains were used: ade6-704 ura4-D18 leu1-32 h$^-$, cdc2-33 h$^-$, cdc2-33 rum1Δ h$^-$, cdc2-M26h$^-$, cdc2-M26 rum1Δ h$^-$, cdc2-56h$^-$, cdc2-56 rum1Δ h$^-$, cdc10-129 ade6-704 h$^-$, rum1Δ::ura4$^+$ ura4D-18 leu1-32 ade6-M210 h$^+$. Basic fission yeast methods were as described by Moreno et al. (1991).

Integrants were obtained in the wild-type strain *ade6-704 ura4-D18 leu1-32 h*$^-$, using the *ade6-704* / *sup3-5* system (Hofer et al., 1979; Carr et al., 1989) and the lithium acetate transformation method (Okazaki et al., 1990). Southern blotting confirmed that integration occurred at some site other than the *cdc2* locus, allowing integrants to be crossed to *cdc2*^{ts} alleles, as described below (see 'N152/I172 induced overreplication'). Crosses involving integrants of *nmt1* constructs were performed on malt extract plates containing 5 μg/ml thiamine, to repress expression from *nmt1*. Since deletion of *rum1* causes sterility, double mutants involving *rum1*Δ were made by crossing *rum1*Δ*::ura4*$^+$ *ura4D-18 leu1-32 ade6-M210 h*$^+$ transformed with pREP3X *rum1*$^+$, so that *rum1* is expressed from the plasmid, and the double mutants were subsequently checked to ensure that the plasmid had been lost.

All experiments in liquid culture were carried out in minimal medium, starting with a cell density of 2-8×10^6 cells/ml, corresponding to mid-exponential phase growth. Temperature shift experiments were carried out using a water bath at 36.5°C, and the temperature was checked carefully, since the G_1 arrest of *cdc2*^{ts} mutants is weaker at lower temperatures.

To induce expression from the *nmt1* promoter, cells were grown in minimal medium containing 5 μg/ml thiamine to mid-exponential phase, then spun down and washed three times with water, before resuspending in fresh medium lacking thiamine at a density calculated to produce 4×10^6 cells/ml at the time of peak expression from the *nmt1* promoter.

To measure cell number, cell were fixed in 0.9% saline/3.7% formaldehyde and sonicated before analysis with a ZM Coulter counter (Coulter Electronics Ltd).

Plasmids

cdc2-N152 and *cdc2-I172* were originally analysed using pREP41, a multicopy vector in which expression is driven by the medium strength version of the *nmt1* promoter (Basi et al., 1993). To obtain integrants, the 0.9 kb *cdc2* cDNA was cloned as an *Nde*I-*Bam*HI fragment into the integrative plasmids pRIP45 and pREP5, which are identical except that the former contains the medium strength *nmt1* promoter, and the latter wild-type *nmt1*. pRIP45 and pREP5 were made by eliminating the 2.2 kb *LEU2* containing *Hin*dIII fragment from pREP41 and pREP1, respectively (Maundrell, 1993), and then inserting a 0.5 kb *Pst*I fragment containing the nonsense suppressor *sup3-5*, which suppresses the *ade6-704* mutation and so can be used to isolate integrants as described above. pREP5 was made by Jacqueline Hayles, and pRIP45 by K.L.

Flow cytometry and microscopy

About 10^7 cells were spun down, washed once with water, then fixed in 70% ethanol and processed for flow cytometry or DAPI staining, as detailed previously (Sazer and Sherwood, 1990; Moreno et al., 1991). A Becton-Dickinson FACScan was used for flow cytometry. Overreplicating cells expressing *cdc2-I172* are osmotically sensitive, and so were washed with 1 M sorbitol instead of water. DNA contents were assigned by reference to 1C, 2C and 4C controls, prepared using nitrogen starved haploid cells, exponentially growing haploid cells and exponentially growing diploid cells, respectively.

For anti-α-tubulin immunofluorescence, cells were fixed with paraformaldehyde and glutaraldehyde and stained with TAT1 primary antibody (Woods et al., 1989), followed by Goat α-mouse Texas Red secondary antibody (Jackson Immunoresearch Laboratories), essentially as described (Moreno et al., 1991). Cells were viewed with a Zeiss Axioskop microscope.

N152/I172 induced overreplication

I172 induced rereplication was difficult to analyse genetically, since although p34$^{cdc2\text{-}I172}$ overexpression causes overreplication at 32°C, rereplication is largely inhibited at the higher temperatures needed to arrest ts mutants, even in the presence of 1 M sorbitol. In addition, crossing the pREP5 *cdc2-I172* integrant to cell cycle mutants such as *cdc25* and *cdc2* produced strains that were too sick to work with even at the permissive temperature, whilst the need to include sorbitol in the medium to observe I172 induced rereplication further complicated such experiments. For these reasons we concentrated on N152.

N152 is more amenable to study, since lower levels of expression produce rereplication, and crossing the pRIP45*cdc2-N152* integrant to *cdc10-129* produced a double mutant that grows well at 25°C and so could be used to show N152 induced rereplication requires *cdc10* function. However, combining the pRIP45*cdc2-N152* integrant with *cdc2*^{ts} or *cdc25*^{ts} mutants produced strains that were very sick even at 25°C, and could not be analysed.

Immunoblots and H1 kinase assays

Cell extracts were made as described using HB15 buffer (Moreno et al., 1989, 1991) and spun at 4°C in a microfuge for 15 minutes, before assaying the protein concentration of the supernatant using the BCA assay kit (Pierce), and adjusting the sample volumes to give a uniform concentration in each case. p34^{cdc2} or p56^{cdc13} were immunoprecipitated from 1.3 mg of protein extract, using affinity purified anti-C-terminal antibody C2, or anti-p56^{cdc13} antibody SP4, respectively, at 0°C for 1.5 hours. Protein A-Sepharose was then added for 30 minutes at 4°C and the immuneprecipitates washed 5 times with HB15 buffer, before splitting in two. One half was heated to 99°C for 2 minutes in 1× SDS-PAGE sample buffer and used for immunoblotting with 'Immobilon-P' transfer membrane (Millipore), according to the manufacturer's instructions. The other half was resuspended in 10 μl of HB15 containing 200 μM ATP, 1 mg/ml histone H1 (Boehringer Mannheim) and 40 μCi/ml [γ-^{32}P] ATP, and incubated at 32°C for 20 minutes. The reaction was stopped with 10 μl of 2× sample buffer, denatured at 99°C for 2 minutes and the samples run on an 11% SDS-PAGE gel. Phosphorylated histone H1 was detected by autoradiography.

RESULTS

Absence of rum1 causes increased p34^{cdc2} Start activity in vivo

If rum1 functions specifically at Start as an inhibitor of p34^{cdc2}, cells lacking the *rum1* gene should have increased p34^{cdc2} Start activity, whilst p34^{cdc2} activity in G_2 should remain unchanged. Since we do not yet know in what complexes

p34^{cdc2} functions at Start in fission yeast, and in vitro can only detect an increase in p34^{cdc2} kinase activity once cells have entered G_2 (Moreno et al., 1989; Creanor and Mitchison, 1994), we developed an in vivo approach to test this idea. When a *cdc2^{ts}* mutant is placed at the restrictive temperature, cells become arrested in G_1 and G_2, and then either remain arrested upon continued incubation, or leak past one or both of the two block points. The latter depends on the particular allele chosen, and probably reflects the amount of residual activity at the restrictive temperature. If cells lacking *rum1* have increased p34^{cdc2} Start activity, shifting a *cdc2^{ts} rum1*Δ double mutant to the restrictive temperature should result in fewer cells becoming arrested in G_1, and a more transient arrest for those cells that do arrest before Start. In contrast, the ability of such *cdc2^{ts}* mutants to arrest in G_2 should remain unchanged.

cdc2-33 is typical of many *cdc2^{ts}* mutants in causing a very tight G_2 arrest, such that cells never enter mitosis upon prolongued incubation at 36.5°C, whilst the G_1 arrest is transient, and cells undergo S-phase after several hours at the restrictive temperature. As shown in Fig. 1A, incubation of *cdc2-33 rum1*Δ at the restrictive temperature causes fewer cells to become arrested before S-phase, and those cells that do arrest initially leak past the G_1 block point a generation time earlier than the *cdc2-33 rum1$^+$* control. Cell number increase is identical for both strains, and has ceased after 1 hour at 36.5°C, whilst DAPI staining shows that both strains remain arrested and elongate with a single interphase nucleus, demonstrating that deletion of *rum1* has no effect on G_2 arrest (not shown).

Fig. 1B shows a quantification of the percentage of G_1 arrested cells, based on the data in Fig. 1A, together with data from equivalent experiments with other alleles of *cdc2*. *cdc2-M26* causes an extremely tight arrest in G_1 and G_2 at 36.5°C, and the arrested cells never leak past Start or undergo S-phase. However, deletion of *rum1* in this strain again causes fewer cells to arrest in G_1, and those cells that do arrest leak through and undergo S-phase by 4 hours (Fig. 1B, left hand panel). We also examined *cdc2-56*, a rather leaky *cdc2^{ts}* mutant, where even the G_2 block is weak, and arrest at Start is very transient.

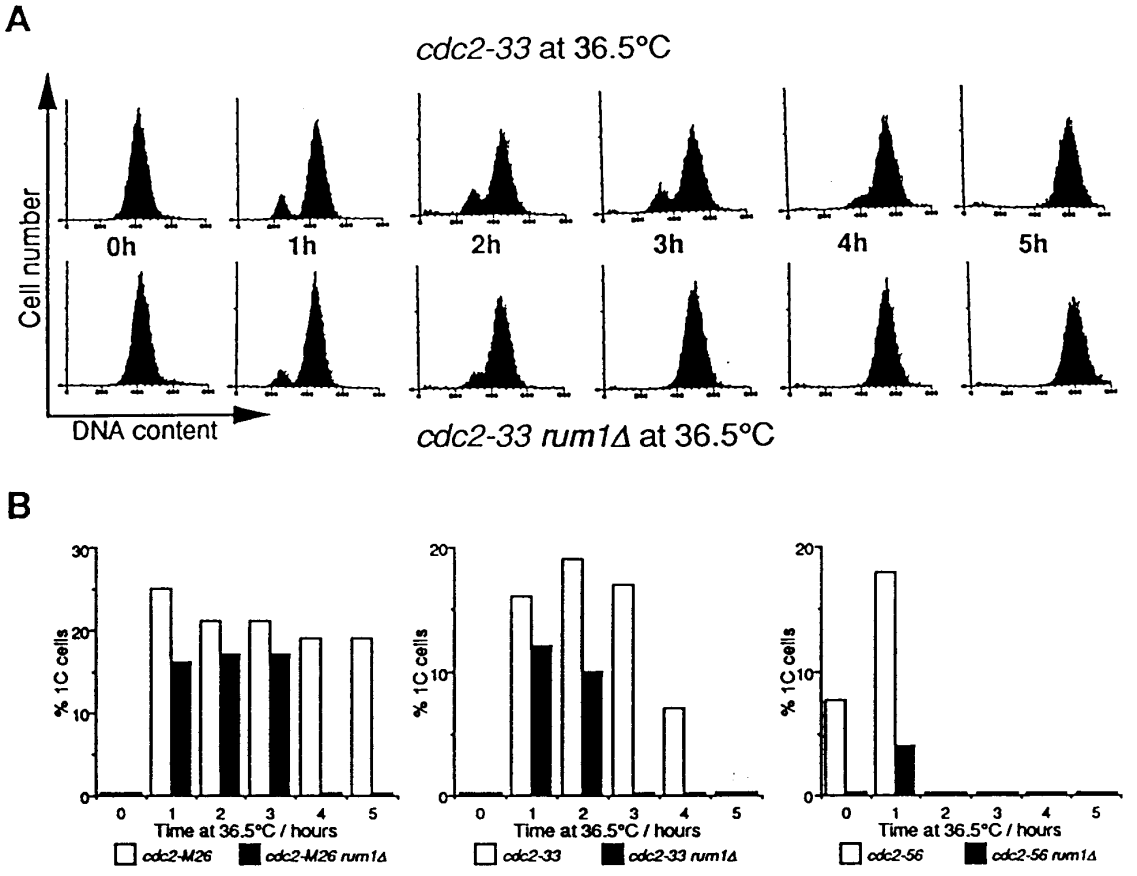
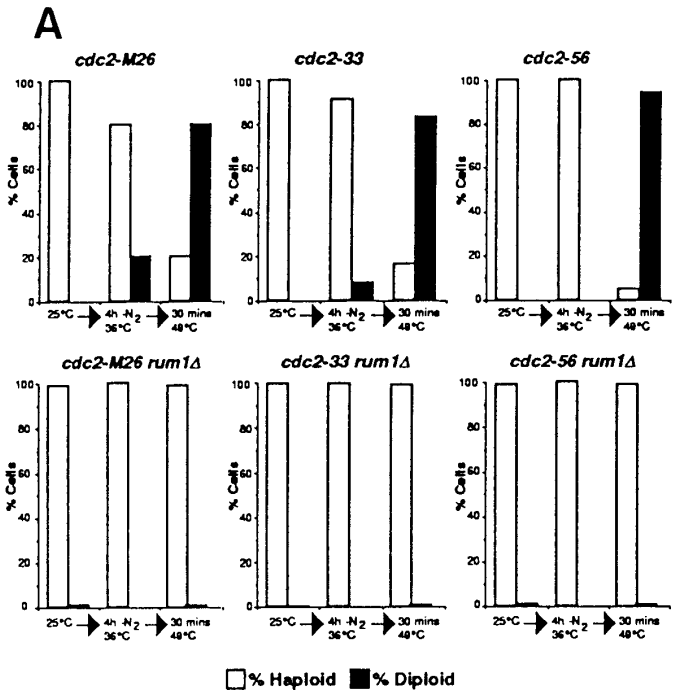
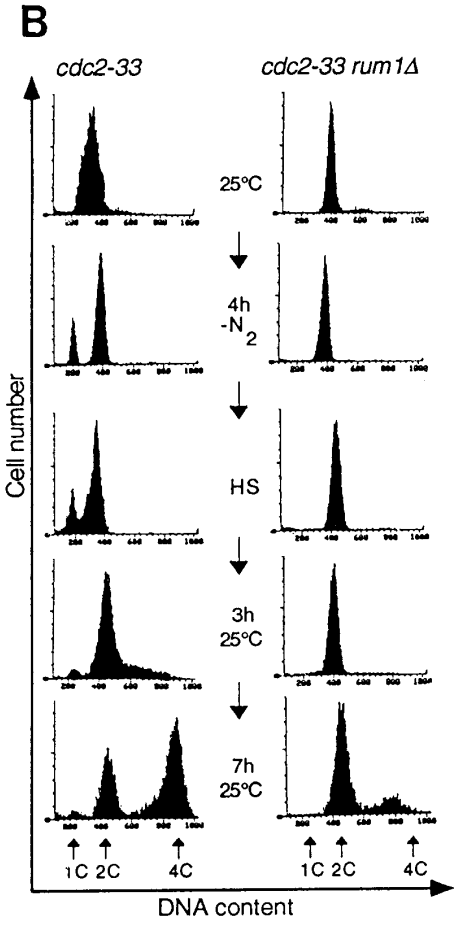


Fig. 1. p34^{cdc2} has increased Start activity in the absence of *rum1*. (A) *cdc2-33* and *cdc2-33 rum1*Δ strains were grown at 25°C in minimal medium to a density of 2×10^6 cells/ml, then shifted to 36.5°C and samples taken every hour. Cells were fixed in 70% ethanol and processed for flow cytometry as described in Materials and Methods. At the start of the experiment a single peak of 2C cells is seen, since the majority are in G_2, whilst G_1 is completed rapidly after mitosis and S-phase begins before the daughter cells have separated, so that a newly born cell has a 2C DNA content. Cells arrest in G_1 or G_2 at the restrictive temperature, depending on their position in the cell cycle, and so a peak of 1C cells appears, and remains until cells leak past the G_1 block and undergo Start and S-phase, before arresting in G_2. In the absence of *rum1* fewer cells arrest in G_1 initially, whilst those that do so leak past the block point a generation time earlier than in the presence of *rum1*. (B) The percentage of 1C cells is shown throughout the experiment described in (A), and also for equivalent experiments involving *cdc2-M26* and *cdc2-56*. Note that *cdc2-56* has a small 1C population even at the permissive temperature, since this allele enters mitosis prematurely at 25°C, producing small daughter cells that must delay G_1 progression transiently until they achieve the critical mass necessary for Start. Cells arrest in G_1 and G_2 at 36.5°C, but the mutant is very leaky and the G_1 arrest extremely transient. Deleting *rum1* in this strain greatly reduces the ability to arrest in G_1.





Deletion of *rum1* dramatically reduces the ability of this strain to arrest in G_1 (Fig. 1B, right hand panel). These results show that deletion of *rum1* specifically affects the ability of *cdc2*^{ts} mutants to become arrested at Start, without affecting the G_2 arrest of such mutants, suggesting that in the absence of rum1 p34^{cdc2} has increased Start activity. The data indicate a close interaction between *cdc2* and *rum1*, and are consistent with a function for rum1 as an inhibitor of p34^{cdc2} at Start.

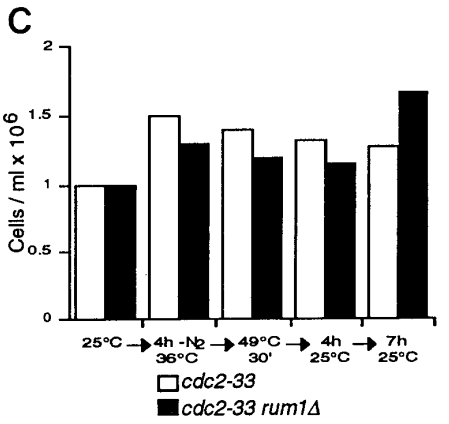
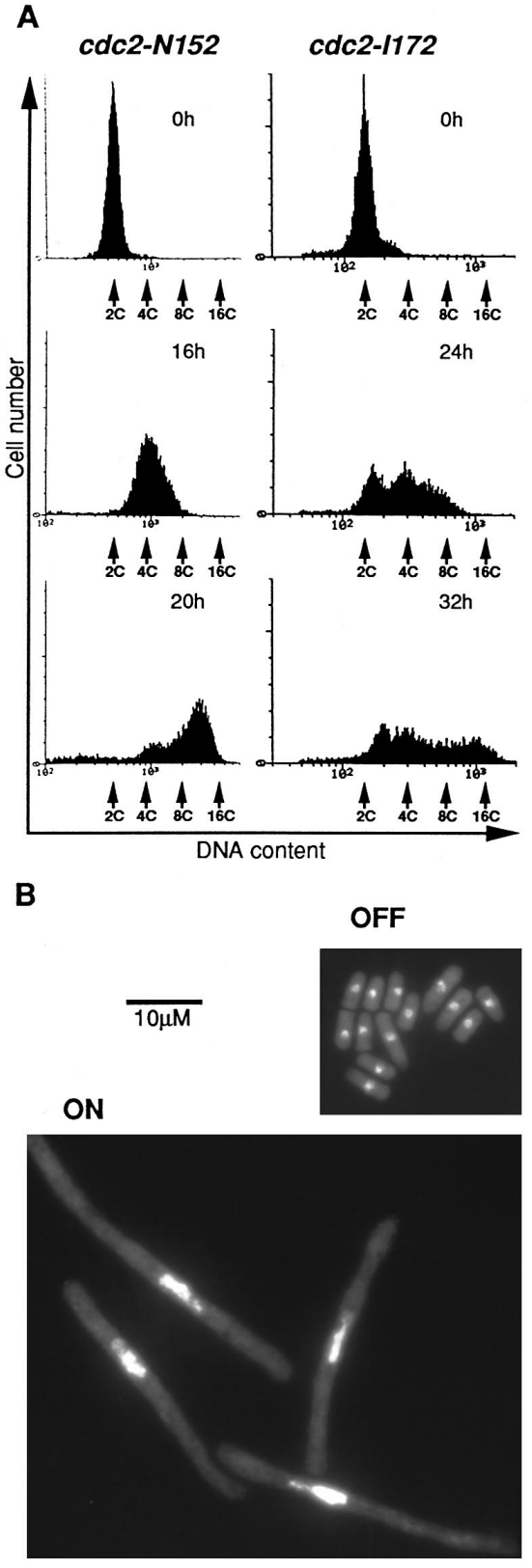


Fig. 2. Deletion of *rum1* abolishes the diploidization of *cdc2*^{ts} mutants upon heat shock. The same *cdc2* alleles described in Fig. 1B were grown at 25°C to mid-exponential phase in minimal medium, spun down and washed three times with water, then resuspended at a density of 10^6 cells/ml in minimal medium lacking a nitrogen source and incubated for 4 hours at 36.5°C. The nitrogen starvation was necessary to survive the subsequent heat shock, when cells were incubated at 49°C for 30 minutes. At each stage of the experiment cell number was measured, and an aliquot plated on a yeast extract plate containing 5 μg/ml phloxine B, and incubated at 25°C for 4 days to allow colony formation. In this way plating efficiency and the percentage diploids were calculated, since diploid colonies are easily identifiable by their dark pink colour on phloxine B containing medium. Whilst wild-type cells are unaffected by this treatment, heat shock of *cdc2*^{ts} mutants induces an extra round of S-phase in the absence of mitosis, resulting in diploidization. Deletion of *rum1* suppresses this effect completely, but does not affect the viability of cells at the end of the experiment (A). In a parallel experiment the nitrogen source was added back to the medium after heat shock, and samples taken for flow cytometry during continued incubation at 25°C. Whilst *cdc2-33* cells begin rereplicating after 3 hours and the majority have a 4C DNA content 7 hours after heat shock, deletion of *rum1* largely suppresses this effect (B). Cell number was also measured throughout the same experiment, showing that the failure of *cdc2-33 rum1*Δ cells to diploidize was not due to a failure to undergo cell cycle arrest during nitrogen starvation at 36°C (C).

rum1 is essential for rereplication of *cdc2*^{ts} mutants

Exposure of *cdc2*^{ts} or *cdc13*^{ts} mutants to a transient heat shock treatment causes resetting of G_2 cells back to pre-Start G_1, resulting in a single extra round of DNA replication in the absence of mitosis upon return to permissive conditions (Broek et al., 1991; Hayles et al., 1994). This suggests that the cdc2/cdc13 complex defines a cell as being in G_2, and so is important for the regulation of S-phase within the cell cycle (Hayles et al., 1994). Deletion of the *cdc13* gene causes multiple rounds of S-phase in the absence of mitosis, and over-expression of *rum1* in G_2 cells has the same effect (Hayles et al., 1994; Moreno and Nurse, 1994). It is possible that rum1 may inhibit cdc2/cdc13 complex formation, potentially explaining why pre-Start G_1 cells have very low levels of this complex (Hayles and Nurse, 1995). If rum1 maintains p34^{cdc2}

in a pre-Start G_1 form, preventing the formation of an active cdc2/cdc13 complex, deletion of the *rum1* gene might be expected to reduce the ability of *cdc2*^{ts} mutants to undergo rereplication when exposed to a transient heat shock.



As shown in Fig. 2, deletion of *rum1* completely abolishes the diploidization of *cdc2*^{ts} mutants upon heat shock, and the *rum1*Δ *cdc2*^{ts} double mutants remain viable haploids at the end of the experiment. The results again suggest a close interaction of *rum1* and *cdc2* in the regulation of Start and S-phase within the cell cycle, and are consistent with the proposed model, where rum1 is not simply a G_1 inhibitor of p34^{cdc2}, but is also necessary to stabilise a pre-Start form of the kinase (Moreno and Nurse, 1994). Possible explanations for these contrasting aspects of *rum1* function are discussed in more detail below (see Discussion).

Dominant *cdc2* mutants causing overreplication

A role for p34^{cdc2} in the controls regulating S-phase within the cell cycle, and thus in determining cell cycle 'memory', was inferred from the diploidization of *cdc2*^{ts} mutants after heat shock. Given the unphysiological nature of this treatment, together with the fact that in the absence of heat shock such *cdc2* mutants cause arrest in G_1 or G_2 at the restrictive temperature, we have searched for new *cdc2* mutants specifically defective in the regulation of S-phase, to facilitate study of this aspect of *cdc2* function. Our approach was to look for dominant mutants causing overreplication when overexpressed in a wild-type cell, to levels at which the wild-type protein has no effect on cell cycle progression. One such mutant, *cdc2-I172*, was found in a screen of dominant lethal *cdc2* mutants produced by chemical mutagenesis of a plasmid expressing the *cdc2* cDNA from a mutated version of the regulatable *nmt1* promoter (Labib et al., 1995). A second, *cdc2-N152*, had previously been shown to cause a dominant lethal phenotype when expressed in a wild-type strain either from *cdc2*'s own promoter, or from the thiamine repressible *nmt1* promoter, but even under repressed conditions was too lethal to allow analysis of the mutant phenotype (MacNeill and Nurse, 1993). We expressed N152 from a mutated version of the *nmt1* promoter (Basi et al., 1993), and found that cells grow well in repressed conditions, but undergo rereplication upon induction of p34$^{cdc2\text{-}N152}$.

As shown in Fig. 3, overexpression of I172 or N152 in a wild-type strain prevents cell division and causes a large increase in DNA content, from 2C typical of a normal G_2 cell up to 16C, resulting in highly elongated cdc$^-$ cells with very large nuclei. The phenotype resembles overexpression of rum1, though cells are unable to recover from expression of N152/172, presumably because p34^{cdc2} has essential functions

Fig. 3. N152 and I172 cause overreplication when overexpressed in wild-type cells. Single copy integrants of pRIP45 *cdc2-N152* and pREP5*cdc2-I172*, in which the mutants are expressed from the medium strength and wild-type *nmt1* promoters, respectively, were grown at 32°C in minimal medium containing thiamine. Expression of N152 or I172 was induced by washing the cells and incubating in thiamine free medium at 32°C. 1.2 M sorbitol was included in the medium for I172, since overreplicating cells expressing this mutant are osmotically sensitive. Samples were taken at the indicated times and fixed in ethanol before processing for flow cytometry (A) or DAPI staining to examine the nuclei (B). Expression from *nmt1* peaks after 12-14 hours at 32°C in thiamine free medium, and more slowly in the presence of 1.2 M sorbitol. Both mutants prevent mitotic entry and induce overreplication, resulting in highly elongated cells with large nuclei containing up to eight times the haploid DNA content for a G_2 cell.

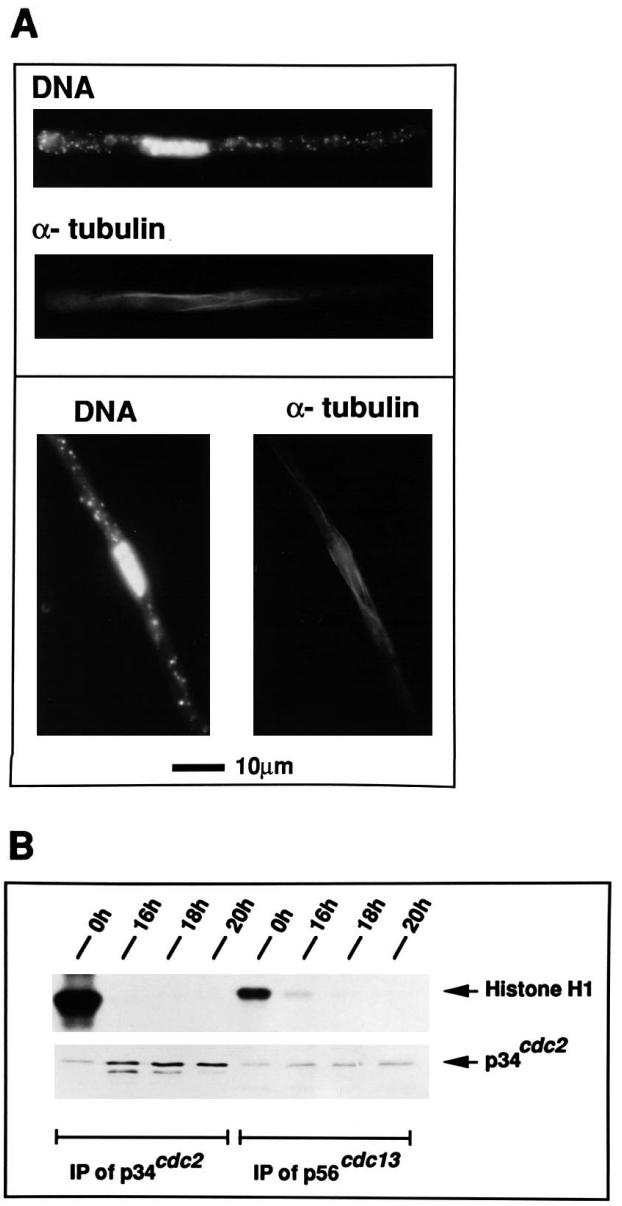


Fig. 4. N152 induces overreplication in the absence of mitosis. An integrant of pRIP45*cdc2-N152* was induced in thiamine free medium at 32°C and samples taken after 0 hours, 16 hours, 18 hours and 20 hours, and either fixed in ethanol for DAPI staining, fixed with formaldehyde/glutaraldehyde and used for anti-α-tubulin immunofluorescence, or used to make protein extracts.
(A) Microscopic examination of 200 cells for each time point showed that 100% of induced cells have decondensed chromatin and interphase microtubular arrays, implying that overreplication does not result from failed mitosis. Two examples are shown from the 20 hour timepoint. Similiar results were obtained for I172 in a parallel experiment. Note that mitochondrial DNA is more visible for these formaldehyde fixed cells, than for the ethanol fixed cells shown in Fig. 3. (B) Cell extracts were made and immunoprecipitated with affinity purified antibodies specific for $p34^{cdc2}$ or $p56^{cdc13}$. Half of each immunoprecipitate was used to assay H1 kinase activity, the other half being used to measure the level of $p34^{cdc2}$ by western blotting. Immunoprecipitation of $p56^{cdc13}$ shows that N152 induction results in loss of mitotic H1 kinase activity, whilst immunoprecipitation of $p34^{cdc2}$ shows that the mutant $p34^{cdc2\text{-}N152}$ protein lacks detectable kinase activity.

in other parts of the cell cycle which are inhibited by these two mutants. The two mutants produce a very similiar phenotype, although discrete rounds of DNA synthesis are more apparent for I172 than for N152, whilst N152 causes rereplication at lower levels of expression than I172.

No signs of chromosome condensation are seen in arrested cells during a timecourse of induction, whilst anti-α-tubulin immunofluorescence shows that rereplicating cells have interphase microtubules, indicating that repeated rounds of S-phase occur in the absence of mitosis (Fig. 4A, 200 cells were examined after 0 hours, 16 hours, 18 hours and 20 hours of N152 induction). This is further shown by the fact that N152 expression results in loss of endogenous mitotic $p34^{cdc2}/p56^{cdc13}$ kinase activity in overreplicating cells (Fig. 4B). N152 has proven much more amenable to further analysis (for technical reasons detailed in Materials and Methods), and the following analysis concentrates on this mutant.

Consideration of the mutated residues corresponding to N152 and I172 suggests that they are dominant negative mutants, inactive forms of $p34^{cdc2}$ causing rereplication when overexpressed by titrating some regulator of the wild-type protein. *cdc2-I172* represents mutation of Thr172 of $p34^{cdc2}$ to Ile172. Thr172 is conserved in domain VIII of almost all Ser/Thr specific protein kinases, incuding all $p34^{cdc2}$ homologues in other organisms, but is absent from protein tyrosine kinases, and is likely to have a role in accomodating Ser/Thr substrate sites for catalysis (Hanks et al., 1988). This is indicated by the fact that Thr172's side chain comes very close to that of the catalytic base in a proposed model for $p34^{cdc2}$ structure, based upon the crystal structure of cyclic AMP-dependent protein kinase (Knighton et al., 1991; Marcote et al., 1993; Endicott et al., 1994). *cdc2-N152* represents mutation of Asp152 of $p34^{cdc2}$ to Asn152. Asp152 is conserved in domain VII of all protein kinases, within the central catalytic domain, and the equivalent residue in cyclic AMP-dependent protein kinase is involved in binding MgATP and is thought to play a direct role in catalysis (Hanks et al., 1988; Knighton et al., 1991). Both N152 and I172 are therefore very likely to represent inactive forms of $p34^{cdc2}$, and Fig. 4B shows that indeed $p34^{cdc2\text{-}N152}$ lacks any detectable H1 kinase activity in vitro.

Rereplication induced by N152 overexpression requires *cdc10* function, indicating that overreplication involves resetting of G_2 cells back to pre-Start G_1 (Fig. 5). Overexpression of the inactive $p34^{cdc2\text{-}N152}$ protein therefore appears to titrate some regulator of wild-type $p34^{cdc2+}$ in G_2, causing cells to pass Start once again and reenter S-phase, without undergoing mitosis. Since rum1 is required for rereplication induced by heat shock of $cdc2^{ts}$ mutants, we investigated the effect of overexpressing N152 or I172 in a cell lacking the *rum1* gene. As shown in Fig. 6, *rum1* deletion greatly reduces overreplication produced by N152 overexpression, and has a more modest effect on I172 induced rereplication. In both cases, cell cycle arrest still occurs as in the presence of *rum1*, but the arrested cells are unable to rereplicate to the same degree, again suggesting that rum1 may be required to maintain $p34^{cdc2}$ in a pre-Start G_1 form. Whilst deletion of *rum1* completely suppresses the diploidization of $cdc2^{ts}$ mutants, where transient heat shock provides only one opportunity to rereplicate, it is perhaps not surprising that *rum1* deletion does not totally suppress the rereplication caused by

N152/I172, since the mutants are continually expressed and so provide a constant signal for rereplication.

Overreplication caused by N152/I172 overexpression is completely suppressed by co-overexpression of wild-type p34^{cdc2+} from the same *nmt1* promoter, restoring normal cell growth and division, confirming that the mutants produce rereplication by specifically interfering with p34^{cdc2} function, rather than by affecting a control in which the wild-type protein plays no part. We also tried to suppress N152 by co-overexpressing other genes involved in cell cycle control, and found that whilst multiple copies of the *suc1* gene, or co-induction of *suc1* or *cdc25* from the *nmt1* promoter, have no effect on overreplication, cells with multiple copies of the *cdc13* gene are still able to enter mitosis and cell division 24 hours after inducing N152 expression. This suppression could simply mean that N152 cannot prevent mitotic entry in the presence of increased levels of p56^{cdc13}, or could also imply that p56^{cdc13} specifically antagonizes resetting pre-Start and overreplication. We therefore used a lower level of *cdc13* expression, from the same mutated version of the *nmt1* promoter used to express N152, and found that cells inducing both N152 and p56^{cdc13} still undergo cell cycle arrest upon induction, but overreplication is dramatically reduced (Fig. 7). This shows that p56^{cdc13} can specifically antagonize rereplication induced by N152, and it is possible that titration of p56^{cdc13} by overexpression of N152/I172 may in part explain how these mutants cause rereplication, since deletion of the *cdc13* gene also leads to multiple rounds of S-phase in the absence of mitosis (Hayles et al., 1994).

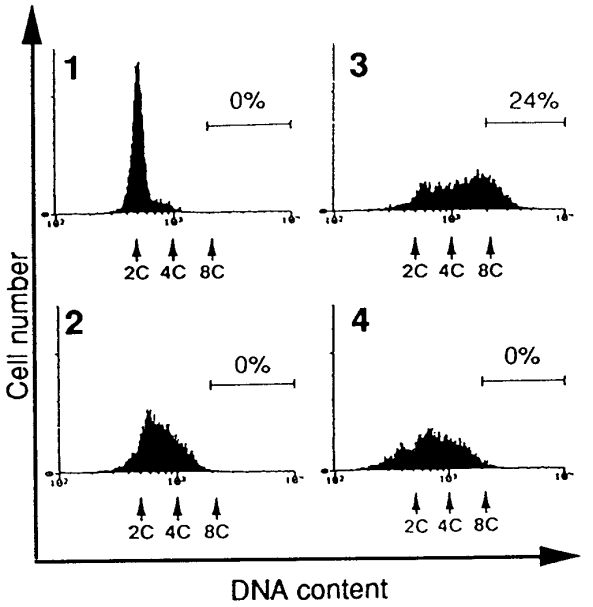


Fig. 5. N152 induced overreplication requires *cdc10* function and so involves resetting of G_2 cells back to Start. The pRIP45*cdc2-N152* integrant was crossed to *cdc10-129* and the double mutant grown at the permissive temperature of 25°C in minimal medium containing thiamine (1), before washing and continuing incubation at 25°C in thiamine free medium containing 1.2 M sorbitol for 39 hours to induce N152 expression and begin rereplication (2). At this point the culture was split in two and one half left at 25°C (3), whilst the other half was shifted to 36.5°C (4), the restrictive temperature for *cdc10*. Incubation was continued for a further 1.5 generation times and flow cytometry performed on samples from throughout the timecourse. Whilst 24% of cells left at 25°C attained a DNA content greater than 8C, inactivation of *cdc10* prevented this increase, showing that N152 induced rereplication requires passage of Start. Sorbitol was included as N152 overexpression does not induce rereplication at 36.5°C in its absence, and this explains the long induction time required to initiate rereplication at 25°C, since *nmt1* expression is induced more slowly at this temperature than at 32°C, and even more slowly in the presence of 1.2 M sorbitol.

DISCUSSION

In both fission yeast and budding yeast Start is known to involve the activation of a transcriptional program required for the initiation of S-phase, and in vertebrate cells transcription is also needed to pass the restriction point (Adolph et al., 1993). Both yeasts also require p34^{cdc2} activity at Start, although the relevant substrates remain unknown, whilst vertebrate cells require related CDKs (cyclin dependent kinases) for G_1 progression (Sherr, 1993, provides a review).

Whilst budding yeast G_1 cyclins that act in association with p34^{CDC28} are well characterised, no fission yeast cyclins have yet been shown to have a role in the G_1 phase of cycling cells. Apart from the mitotic cyclin *cdc13*, other cyclins have been identified such as *puc1* and *cig1*, but there is no evidence that these proteins act in the cell cycle at Start (Forsburg and Nurse, 1991; Bueno et al., 1991; Forsburg and Nurse, 1994, erratum in *Cell* 73 no. 5). The *cig2* gene is periodically expressed at the G_1/S boundary, but a role for this B-type cyclin in G_1 progression has yet to be demonstrated (Bueno and Russell, 1993; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994).

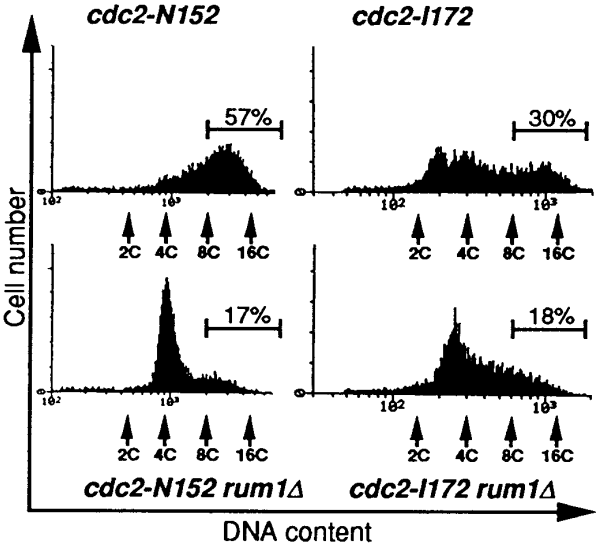


Fig. 6. Deletion of *rum1* inhibits N152/I172 induced rereplication. Strains containing integrants of pRIP45*cdc2-N152* or pREP5*cdc2-I172*, with or without *rum1*Δ, were induced at 32°C for 20 hours (N152) or 32 hours (I172), and samples fixed in ethanol and processed for flow cytometry. The percentage of cells with a DNA content greater than 8C DNA was quantitated, and shows that deletion of *rum1* severely inhibits N152 induced overreplication, and moderately inhibits rereplication induced by I172.

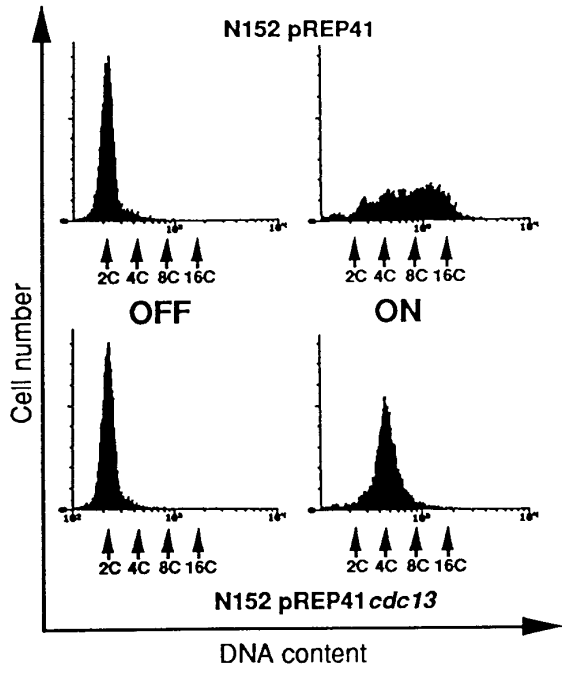


Fig. 7. Co-overexpression of p56^{cdc13} inhibits N152 induced rereplication. The pRIP45*cdc2-N152* integrant was transformed with pREP41, a vector containing the medium strength *nmt1* promoter, or pREP41*cdc13*, and transformants grown at 32°C in selective medium containing thiamine at 32°C, before inducing expression from *nmt1* for 18 hours in thiamine free medium. Samples were fixed in ethanol and processed for flow cytometry as before. Co-expression of p56^{cdc13} from *nmt1* did not prevent N152 induction from blocking mitosis and cell division, but specifically inhibited overreplication in the arrested cells. Overexpression of p56^{cdc13} from the same *nmt1* promoter does not block cell cycle progression in cells lacking the N152 integrant.

rum1 encodes a protein with a critical role in determining the cell cycle timing of Start that is proposed to maintain p34^{cdc2} in a pre-Start G_1 form, inhibiting its action until the cell has achieved the critical size required for progression past Start (Moreno and Nurse, 1994). Given the poorly characterized nature of fission yeast p34^{cdc2} Start activity, we used a physiological approach to show that in the absence of *rum1*, *cdc2^{ts}* mutants are less able to arrest in G_1 at the restrictive temperature, implying that p34^{cdc2} has increased Start activity in these cells, consistent with rum1 being an inhibitor of a pre-Start G_1 form of p34^{cdc2}. Recent work has shown that rum1 is indeed capable of inhibiting p34^{cdc2} activity, since bacterially produced rum1 protein inhibits mitotic p34^{cdc2}/p56^{cdc13} H1 kinase activity in vitro (J. Correa and P. Nurse, unpublished). This may explain why rum1 is also required to prevent entry into mitosis from pre-Start G_1 (Moreno and Nurse, 1994). It is therefore possible that our genetic data may reflect direct action of rum1 on p34^{cdc2} complexes in G_1, although the nature of such complexes remains to be determined.

We also describe new dominant *cdc2* mutants that cause overreplication when overexpressed, resetting a G_2 cell back before Start, and confirming the role of p34^{cdc2} in the regulation of S-phase within the cell cycle. We suggest that *cdc2* and *rum1* interact in such controls, since deletion of *rum1* abolishes the diploidization of *cdc2^{ts}* mutants and reduces the ability of p34$^{cdc2\text{-}N152}$ and p34$^{cdc2\text{-}I172}$ to cause overreplication when overexpressed in a wild-type cell. Our results indicate that rum1 is necessary to stabilize p34^{cdc2} in a pre-Start G_1 form, as opposed to forming the G_2 specific complex with p56^{cdc13}. It is possible that N152/I172 cause rereplication by efficient titration of p56^{cdc13}, since co-overexpression of *cdc13* suppresses N152 induced rereplication, whilst it has recently been shown that either deletion of *cdc13* or heat shock of *cdc13^{ts}* mutants produces overreplication (Hayles et al., 1994). Other *cdc2* dominant lethals have previously been described, one of which has been shown to be suppressed by *cdc13*, yet these mutants only cause G_2 arrest on overexpression (Fleig and Nurse, 1991; Fleig et al., 1992; MacNeill and Nurse, 1993). Either N152/I172 are particularly efficient at titrating p56^{cdc13}, or they titrate an additional factor that regulates p34^{cdc2} activity. It is of interest that in the predicted three-dimensional structure of p34^{cdc2}, the side chains of both Asp152 and Thr172 come very close to the catalytic base (Endicott et al., 1994), so that mutation to bulky residues such as Asn152 and Ile172 is likely to interfere directly with catalysis, and N152 and I172 probably act in a similiar manner. If N152/I172 cause rereplication by titrating p56^{cdc13}, allowing resetting of p34^{cdc2+} back to a pre-Start G_1 form, then it is interesting that rum1 is still required for efficient overreplication in these conditions.

Overexpression of p34$^{CDC28\text{-}N154}$ in budding yeast, or of p34$^{CDC2Hs\text{-}N146}$ in human cells, corresponding to p34$^{cdc2\text{-}N152}$ in fission yeast, has not been reported to cause rereplication in these systems (Mendenhall et al., 1988; Van den Heuvel and Harlow, 1993). This may reflect differences in p34^{cdc2} regulators in these organisms. For example, budding yeast B-type cyclins are required after Start for the onset of S-phase as well as for mitosis (Schwob et al., 1994). Titration of these proteins by a dominant negative p34^{cdc2} protein would therefore prevent overreplication. Overexpression of p34$^{cdc2\text{-}N152}$ in budding yeast causes cell cycle arrest with multiple elongated buds, equivalent to deletion of *CLBs1-6*, and overreplication does not occur (K.L., unpublished data).

Our data supports the proposed model for rum1 function, in which rum1 maintains p34^{cdc2} in a pre-Start G_1 form, inhibiting its activity until the critical mass needed for Start is reached, and rum1 inhibition is released. In this case, rum1 action at Start may be analogous to an archer's hand restraining an arrow in a bow - just as the archer's hand inhibits progression of the arrow, but also acts to stably maintain it in a potentially active form, so too rum1 may inhibit p34^{cdc2} at Start, but also be required to stably maintain the G_1 form in conditions where Start progression is delayed.

In order to directly test this model we need to know the nature of fission yeast cdc2/G_1 cyclin complexes. Until these are characterised various scenarios can be imagined for the role of rum1 in G_1 progression. rum1 may directly inhibit cdc2/G_1 cyclin complexes until the critical size required for Start is reached. Alternatively rum1 could have a more indirect role, preventing formation of an active cdc2/cdc13 complex in pre-Start G_1 cells and thereby allowing cdc2/G_1 cyclin complexes to form instead. These areas are currently under investigation.

A variety of inhibitors of cyclin dependent kinases have now been described in budding yeast and vertebrate cells (see reviews by Hunter, 1993; Nasmyth and Hunt, 1993; Pines, 1994). These proteins are required to inhibit cell cycle progression in response to diverse signals such as DNA damage,

contact inhibition, lack of nutrients, mating pheromones etc., and in mammalian cells their loss may be an important factor in the generation of tumours. p21 is one such inhibitor which appears to mediate p53 induced G_1 arrest in response to DNA damage (El-Deiry et al., 1993; Dulic et al., 1994), whilst it has been reported that the p16 inhibitor is mutated in many human cancers (Lamb et al., 1994; Nobori et al., 1994). In addition to inhibiting CDK/cyclin complexes, p21 also binds to and inhibits the DNA replication protein PCNA, showing that these small inhibitory proteins may have more than one function (Waga et al., 1994). It will be of interest to see how the functions of such proteins relate to rum1, and whether any is capable of complementing deletion of the *rum1* gene in fission yeast.

There is some evidence that p34^{CDC2} may also be involved in determining the dependency of S-phase upon mitosis in mammalian cells (Yoshida et al., 1990; Usui et al., 1991), just as p34^{cdc2} acts in fission yeast to determine cell cycle memory, and so it is possible that a rum1 like CDK-inhibitor may interact in this control in higher eukaryotic cell cycles.

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