The EMBO Journal vol.6 no.11 pp.3507–3514, 1987

p13^{suc1} acts in the fission yeast cell division cycle as a component of the p34^{cdc2} protein kinase

L.Brizuela, G.Draetta and D.Beach

Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, NY 11724, USA

Communicated by J.D.Watson

$cdc2^+$ encodes a protein kinase that is required during both G_1 and G_2 phases of the cell division cycle in fission yeast. $suc1^+$ is an essential gene that was originally identified as a plasmid-borne sequence that could rescue certain temperature-sensitive *cdc2* mutants. To investigate the role of the $suc1^+$ gene product in the cell cycle p13^{suc1} has been expressed in *Escherichia coli* and purified. An immunoaffinity purified anti-p13^{suc1} polyclonal serum has been prepared and used to identify p13^{suc1} in fission yeast. The abundance of this protein did not alter either during the cell cycle or during entry into stationary phase. p13^{suc1} was found in yeast lysates in a complex with the $cdc2^+$ gene product. Approximately 5% of cellular p34^{cdc2} was associated with p13^{suc1}, and this fraction of p34^{cdc2} was active as a protein kinase. The stability of the complex was disrupted in yeast strains carrying temperature-sensitive alleles of *cdc2* that are suppressible by overexpression of $suc1^+$. The level of association between p13^{suc1} and p34^{cdc2} was not affected by cell cycle arrest in adverse nutritional conditions. p13^{suc1} is not a substrate of the p34^{cdc2} protein kinase. We propose instead that it acts as a regulatory component of p34^{cdc2} that facilitates interaction with other proteins.

Key words: fission yeast/protein kinase/cell cycle control/*suc1*/*cdc2*

Introduction

The cell cycle of the fission yeast, *Schizosaccharomyces pombe*, is regulated at two steps, one acting in G_1 sometimes referred to as cell cycle 'start', and the other in G_2 that controls the initiation of mitosis (Fantes and Nurse, 1977; Nasmyth, 1979). The existence of two independent points of cell cycle control is reflected in the ability of this yeast to enter stationary phase from either G_1 or G_2 (Costello *et al.*, 1986).

$cdc2^+$ is presently the only gene known to be required both in G_1 before DNA synthesis and also in G_2 before the initiation of mitosis. The $cdc2^+$ gene product shares 62% sequence homology with the product of the *cdc28* cell cycle 'start' gene of the distantly related budding yeast *Saccharomyces cerevisiae* (Hindley and Phear, 1984). Furthermore the *cdc28* gene can rescue *cdc2*ts mutants of fission yeast (Beach *et al.*, 1982), and $cdc2^+$ can rescue *cdc28*ts strains of budding yeast (Booher and Beach, 1986). Both genes encode protein products of ~34 kd that have protein kinase activity *in vitro* (Reed *et al.*, 1985; Simanis and Nurse, 1986).

We have undertaken a series of studies to identify genes encoding proteins that interact directly with p34^{cdc2}, either as positive or negative regulatory subunits or as substrates of the protein kinase. $suc1^+$ was the first candidate to be identified. During a screen for DNA sequences that confer a cdc^+ phenotype on a temperature-sensitive *cdc2.33* strain, not only $cdc2^+$ was isolated but also $suc1^+$, a previously unidentified gene (Hayles *et al.*, 1986a). $suc1^+$ carried on a multicopy plasmid vector rescued some but not all temperature-sensitive alleles of *cdc2*. Subsequently *suc1* mutants were found in a classical genetic screen for extragenic suppressors of *cdc2* mutants (Hayles *et al.*, 1986b). Evidence that $suc1^+$ plays a direct role in the division cycle was obtained by creating a null-allele of the gene by gene-replacement in yeast (Hayles *et al.*, 1986b; Hindley *et al.*, 1987). Spores lacking $suc1^+$ are capable of germinating, and sometimes undergo a few cell divisions, but eventually arrest with an elongated *cdc* phenotype. In the absence of convenient conditional alleles of *suc1* no analysis of the relative role of $suc1^+$ in G_1 and G_2 has yet been undertaken.

The nucleotide sequence of $suc1^+$ has been determined (Hindley *et al.*, 1987). The gene contains two intervening sequences and is predicted to encode a protein of 113 amino acids. In this study we have expressed this protein in *E. coli* and raised a polyclonal antiserum with which to study its properties in *S. pombe* and in particular its relationship to the p34^{cdc2} protein kinase.

Results

Purification of p13^{suc1} expressed in E. coli

Nucleotide sequencing and S1 nuclease mapping of the $suc1^+$ gene reveals that it contains a predicted coding region of 113 amino acids that is interrupted by two introns (Hindley *et al.*, 1987). In order to express the full length protein in *E. coli* these two introns have been deleted by oligonucleotide mutagenesis (Zoller and Smith, 1984; see also Materials and methods). Two oligonucleotides of 40 nucleotides were prepared. They consisted of the protein coding sequence that spanned each of the two introns, such that annealing of the oligonucleotides to the gene would cause the introns to be looped out. A 672 bp *ScaI*–*SspI* restriction fragment containing the entire gene was inserted into the *SmaI* site of pUC118, a vector that can be obtained in single-stranded form (Figure 1A). The introns were successively deleted in two separate rounds of mutagenesis (Figure 1A). The resulting plasmid was introduced into a *cdc2.33* strain of *S. pombe* (SP36) by cotransformation with pDB248 (Beach and Nurse, 1981) and was shown to retain the ability to rescue this mutant. This result confirms that the previous mapping of the two introns within the $suc1^+$ gene was correct and that their removal had been precisely achieved.

In a final round of mutagenesis the sequence surrounding the initiating methionine of the $suc1^+$ coding sequence was altered to create an *NdeI* site (CATATG; Figure 1A), and the full coding sequence of the gene was then inserted into pRK172, a bacterial expression vector that carries a promoter derived from the bacteriophage T7 (see Materials and methods). The resulting construction (Figure 1A) was introduced into a strain of bacteria BL21(DE3) that carries a chromosomal copy of the T7 RNA

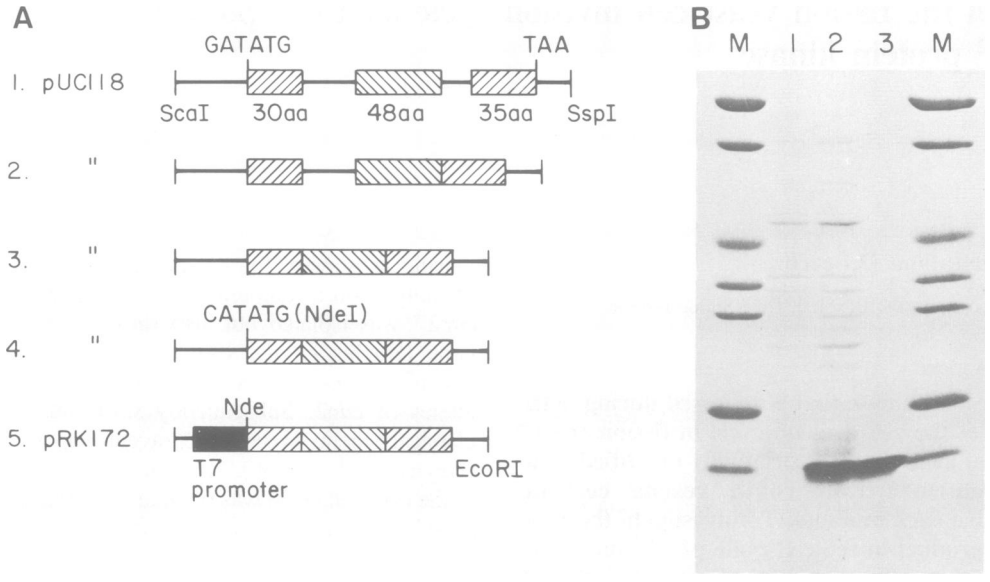

Fig. 1. Expression of p13^{suc1} in *E. coli*. **A**. Plasmid constructions of *suc*1$^+$ gene. (**1**) 672 bp *suc*1$^+$ *Sac*I–*Ssp*I fragment in *Sma* site of pUC118. The filled boxes indicate regions of coding sequence. (**2**) *suc*1 after removal of one intron. (**3**) *suc*1 after removal of both introns. (**4**) Insertion of *Nde*I site at the initiating methionine of coding sequence. (**5**) Insertion of *suc*1 coding sequence into bacterial expression vector pRK172. **B**. Coomassie-Blue stained protein gel. **Lane M**: Mol. wt markers of 97, 66, 45, 36, 29, 20 and 14 kd. (**1**) Whole cell lysate of *E. coli* carrying pRK172/*suc*1 construct before IPTG induction. (**2**) As above 3 h after IPTG induction. (**3**) p13^{suc1} purified from *E. coli* by Sepharose CL6B chromatography.

polymerase gene under control of the *lac*UV5 promoter (Studier and Moffat, 1986: see Materials and methods).

Induction of the T7 RNA polymerase was achieved by addition of IPTG to the bacterial culture medium. Whole cell protein lysates were prepared 3 h after induction and analyzed by SDS polyacrylamide gel electrophoresis (PAGE). A protein of approximately 13 kd accumulated massively following IPTG induction (Figure 1B, lanes 1 and 2). This protein, presumably the product of *suc*1$^+$, was found to be fully retained in the supernatant fraction (S100) following 100 000 *g* centrifugation of the whole cell lysate. p13^{suc1} was purified to virtual homogeneity by gel filtration chromatography of the S100 fraction through a Sepharose CL6B column (Figure 1B, lane 3: see Materials and methods). A yield of approximately 10 mg p13^{suc1} was obtained from 100 ml of bacterial culture.

Identification of p13^{suc1} in S. pombe

The purified bacterial p13^{suc1} protein was injected repeatedly into two rabbits (see Materials and methods) in order to obtain a polyclonal antiserum. One rabbit developed a strong immunological response that could be directly assayed by antigen precipitation in glass capillaries (see Materials and methods). Serum was taken from this rabbit in successive bleeds and was passed through a Sepharose column to which purified p13^{suc1} had been covalently attached (see Materials and methods). Approximately 13 mg IgG per ml of rabbit serum was retained on the column and recovered by elution at pH 11.5. This preparation of affinity purified anti-p13^{suc1} polyclonal IgG was used in each of the subsequent experiments.

A wild-type *S. pombe* strain (h^{-S}, 972) was metabolically labelled with [^{35}S]methionine. A whole cell lysate was prepared and reacted with either preimmune rabbit serum or purified anti-p13^{suc1} serum. Two bands were specifically precipitated by the immune serum. One was of precisely the same electrophoretic mobility as p13^{suc1} from *E. coli*, and the other was of much higher mol. wt (Figure 2). Confirmation that the 13 kd band represented yeast p13^{suc1} was obtained by repeating the immunoprecipitation using a strain that overexpressed the *suc*1$^+$

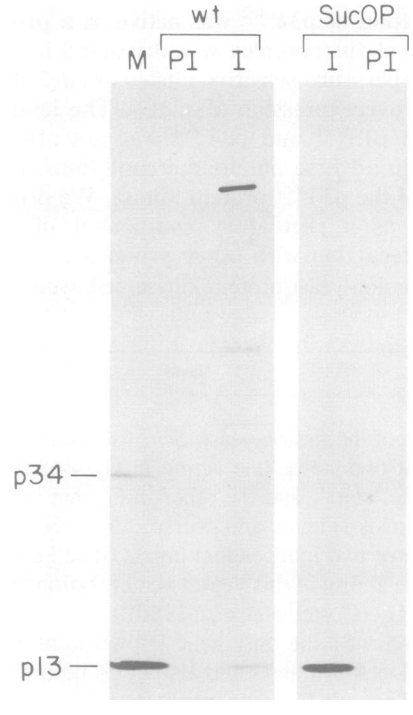

Fig. 2. p13^{suc1} in *S. pombe*. **M**: marker lane containing p34^{cdc2} and p13^{suc1} labelled with [^{35}S]methionine in *E. coli*; wt: immunoprecipitates of a [^{35}S]methionine labelled wild-type *S. pombe* strain with either preimmune (**PI**) or immunoaffinity purified anti-p13^{suc1} antibody (**I**). *suc*O.P. immunoprecipitates of a [^{35}S]labelled *S. pombe* strain that overexpresses the *suc*1$^+$ gene.

gene (*Suc*O.P.; see Materials and methods). The abundance of the 13 kd band was greatly increased and that of the higher mol. wt band was found to decrease (Figure 2). We conclude that the 13 kd band is the product of the *suc*1$^+$ gene and is recognized by the immuno-affinity purified rabbit antiserum. The band of higher mol. wt is presumably a protein that shares antigenic deter-

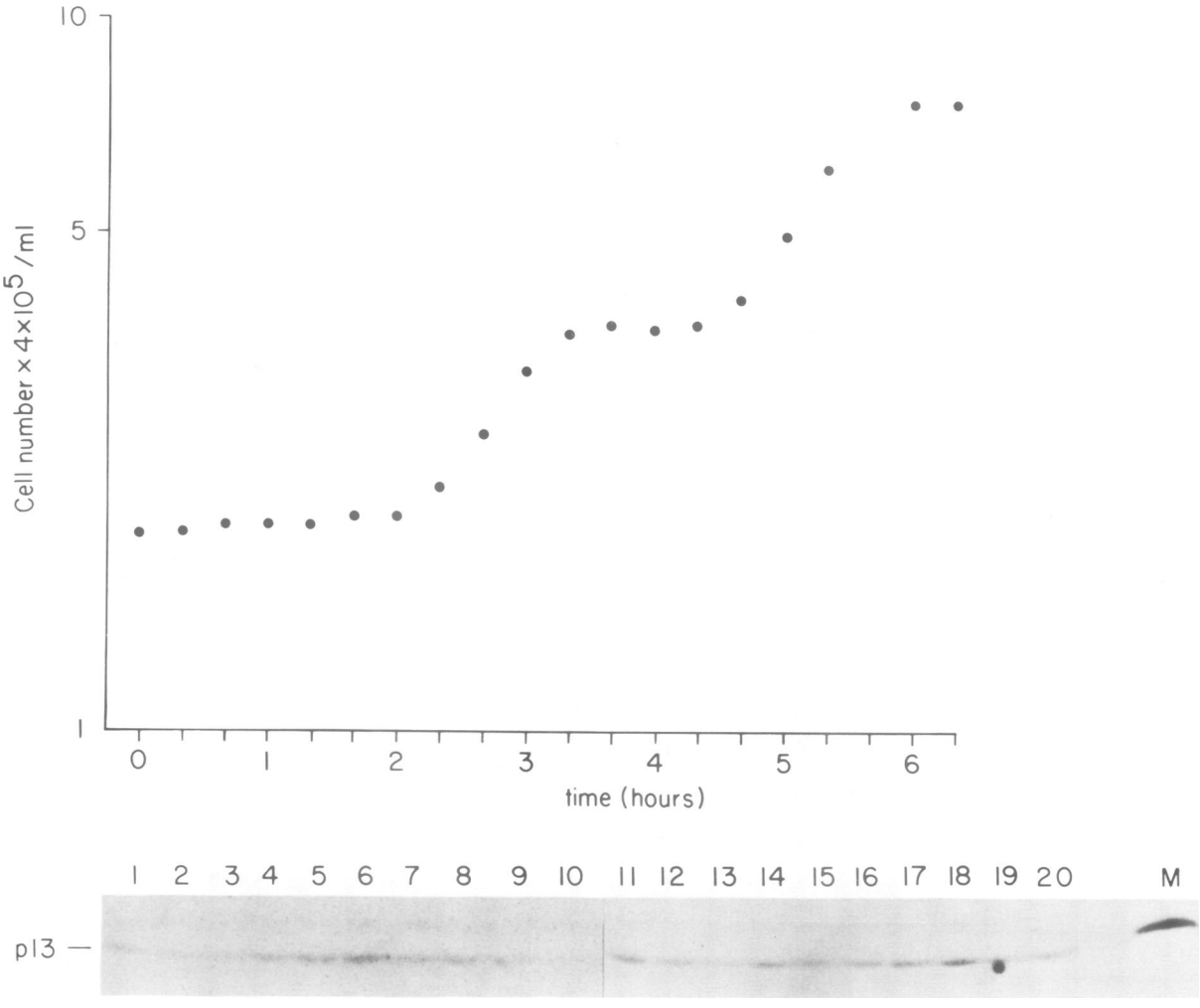

Fig. 3. p13^{suc1} levels during the cell cycle. A synchronous culture of *S. pombe* was obtained by elutriation centrifugation. The **upper panel** shows the cell density at intervals after elutriating the culture. The **lower panel** is an immunblot showing the level of p13 in cell extracts isolated at the same time intervals. Extracts from 2 × 10^7 cells were loaded for each time point. **M** indicates p13^{suc1} marker from *E. coli*.

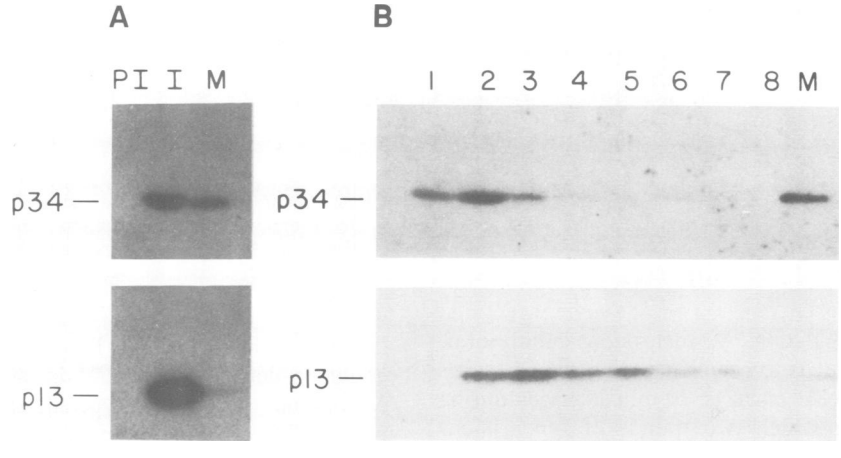

Fig. 4. Association between p13^{suc1} and p34^{cdc2}. **A.** Two columns, one of preimmune serum (**PI**) and the other of affinity-purified anti-p13^{suc1} IgG (**I**) were prepared. A yeast lysate was passed through each column, and retained material was eluted at pH 11.5. The figures show an immunblot of the pooled eluted fractions probed with either anti-p34^{cdc2} (**upper panel**) or anti-p13^{suc1} antibodies (**lower panel**). **M** indicates either p13^{suc1} or p34^{cdc2} from *E. coli* as marker. **B.** Profile of eluted fractions from the anti-p13^{suc1} immunoaffinity column. Part of each 0.5 ml fraction (1–8) was loaded on a gel and immunoblotted to detect p13^{suc1} or p34^{cdc2}.

minants with p13^{suc1}. In lysates prepared from a *suc1*$^+$ over-producing strain this protein appears to be out-competed in the immunological reaction by the high levels of p13^{suc1}.

Abundance of p13^{suc1} is not cell cycle regulated

Since the *suc1*$^+$ gene is required for cell cycle progression it was of interest to establish whether the level of p13^{suc1} might

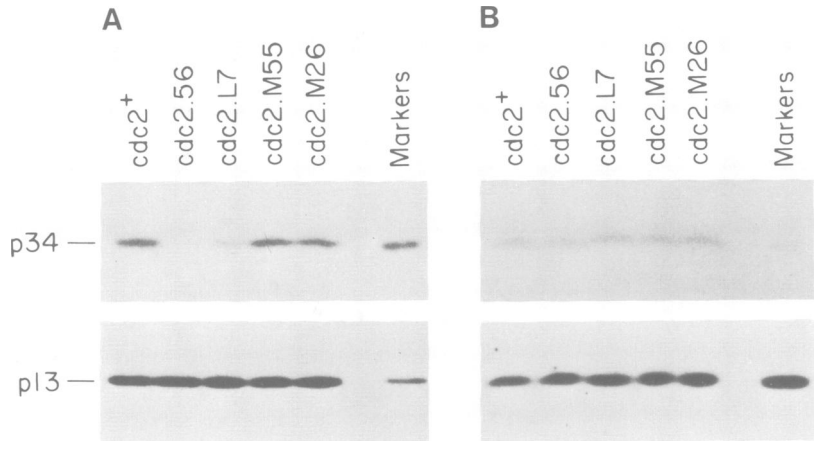

Fig. 5. Co-immunoprecipitation of $p13^{suc1}$ and $p34^{cdc2}$ in *cdc2ts* mutants. **A**. Lysates from *cdc2*$^+$-, *cdc2*.56-, *cdc2*.L7-, *cdc2*.M55- and *cdc2*.M26-carrying strains were reacted with anti-$p13^{suc1}$ immunoglobulins and precipitated with protein-A Sepharose. 90% of the material was subjected to SDS−PAGE, and an immunoblot was probed with anti-$p34^{cdc2}$ polyclonal serum (**upper panel**). The remaining 10% of the sample was run on a separate gel that was probed with anti-$p13^{suc1}$ (**lower panel**). The markers are $p34^{cdc2}$ and $p13^{suc1}$ from *E. coli*. **B**. Direct western immunoblots of whole cell lysate from the same strains as those in **A**, probed with the same antibodies.

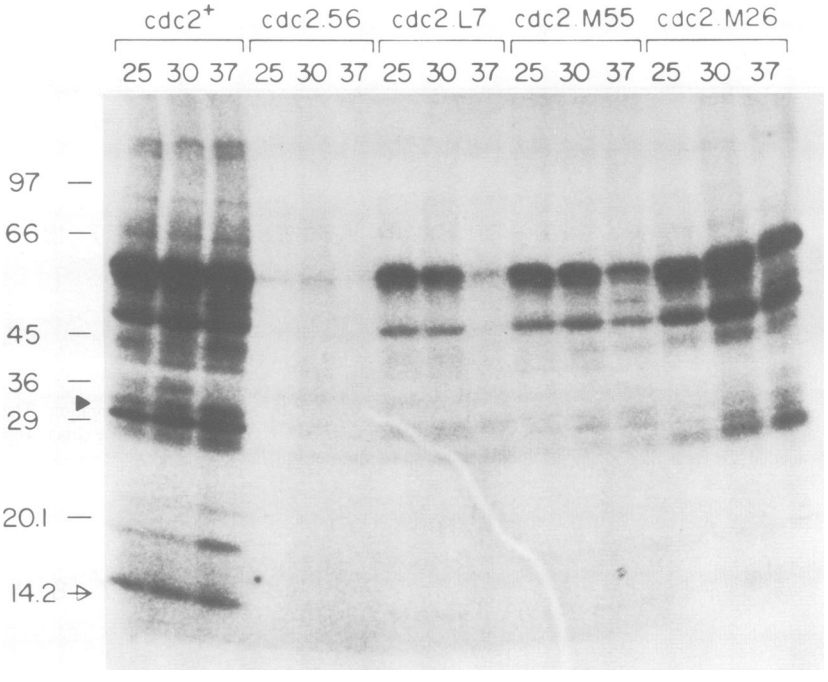

Fig. 6. Protein kinase activity in the $p34^{cdc2}/p13^{suc1}$ complex. Lysates from *cdc2*$^+$, *cdc2*.56-, *cdc2*.L7-, *cdc2*.M55- and *cdc2*.M26-carrying strains were reacted with anti-$p13^{suc1}$ immunoglobulins and precipitated with protein A-Sepharose. After several washes the material was incubated at 25, 30 or 37°C with 5 μCi $\gamma[^{32}P]$ATP for 10 min before being subjected to SDS−PAGE and autoradiography. Markers are of the mol. wt. indicated. The closed and open triangles indicate the positions of $p34^{cdc2}$ and $p13^{suc1}$ respectively.

be subject to cell cycle regulation. A synchronous culture of *S. pombe* was obtained by elutriation centrifugation (see Materials and methods). Samples were taken at 30 min intervals during the following 7 h during which two rounds of synchronous division occurred. The level of $p13^{suc1}$ in each sample was assayed by Western immunoblotting (see Materials and methods). The level of $p13^{suc1}$ did not vary significantly throughout the cell cycle (Figure 3).

$p13^{suc1}$ and $p34^{cdc2}$ are physically associated

One obvious possible role for $p13^{suc1}$ is that it might act as a substrate of the $p34^{cdc2}$ protein kinase. This possibility was investigated in two ways. Firstly yeast cells were metabolically labelled with inorganic $^{32}PO_4$ in order to test whether label could be detected in $p13^{suc1}$ immunoprecipitated from yeast. Making use of both wild-type and *suc1*$^+$ overexpressing strains, no evidence could be obtained that $p13^{suc1}$ is a phosphoprotein (data not shown). Secondly $p13^{suc1}$ purified from *E. coli* was added to an *in vitro* kinase reaction in which the activity of $p34^{cdc2}$ was assayed in immunoprecipitates from yeast extracts. Under conditions in which exogenously added casein was phosphorylated, no phosphorylation of $p13^{suc1}$ could be detected (data not shown). We conclude from these negative results that $p13^{suc1}$ is probably not a substrate of $p34^{cdc2}$ or of any other protein kinase.

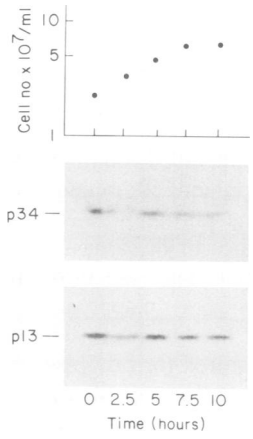

Fig. 7. p34^{cdc2}/p13^{suc1} association during entry into stationary phase. **Upper panel**: cell number during the hours following transfer of an *S. pombe* culture from a complete medium to a nitrogen-free medium. **Middle panel**: western immunoblot of anti-p13^{suc1} immunoprecipitates prepared from cells at each time interval. 90% of the immunoprecipitation was loaded on the gel. **Lower panel**: remaining 10% of the anti-p13^{suc1} immunoprecipitates were separately immunoblotted and probed with anti-p13^{suc1} antibody.

A second plausible role for p13^{suc1} is that it might act as a regulatory component of the p34^{cdc2} protein kinase. If this were the case the two proteins would be expected to be found in physical association with each other *in vivo*. Immunoprecipitation of [^{35}S]methionine-labelled p13^{suc1} did not result in detectable co-immunoprecipitation of a protein of 34 kd (Figure 2). However since the p13^{suc1} signal was not particularly strong in this experiment, a low level of association between p13^{suc1} and p34^{cdc2} would have been undetectable.

In order to specifically test whether a small fraction of p34^{cdc2} is associated with p13^{suc1} two immunoaffinity columns were prepared. Three milligrams of either rabbit preimmune immunoglobulins or immuno-affinity purified anti-p13^{suc1} immunoglobulins was covalently attached to protein A-Sepharose (see Materials and methods). A lysate of 5×10^{10} yeast cells was passed through each column and eluted at pH 11.5 after several washes in buffer 1 (see Materials and methods). The eluted proteins were subjected to gel electrophoresis and probed in a Western immunoblot for the presence of both p13^{suc1} and p34^{cdc2}. As expected the preimmune column did not retain detectable levels of either p13^{suc1} or p34^{cdc2}. However the immune column retained not only p13^{suc1} but also p34^{cdc2} (Figure 4A). Assay of the elution profile of the immune column revealed that as the pH was raised from 7.5−11.5 p34^{cdc2} was rapidly released, whereas p13^{suc1} was more gradually eluted (Figure 4B).

These results are open to two interpretations. Either p34^{cdc2} cross-reacts with the anti-p13^{suc1} antiserum and is thus retained on the immunoaffinity column but not the preimmune column, or 34^{cdc2} and p13^{suc1} exist as a complex in the yeast lysate, and p34^{cdc2} is therefore retained on the column by virtue of its association with p13^{suc1}. The latter explanation appears to be correct. Attempts to detect p34^{cdc2} with the immunoaffinity-purified anti-p13^{suc1} serum were entirely negative (data not shown). No such cross-reactivity would have been anticipated, because p13^{suc1} and p34^{cdc2} share no obvious amino acid sequence homology.

We have made rough estimates of the amount of p13^{suc1} and p34^{cdc2} in a wild-type *S. pombe* cell and also of the fraction of p34^{cdc2} that is associated with p13^{suc1}. These data were obtained by quantitative immunoblotting using known amounts of p13^{suc1} and p34^{cdc2} purified from *E. coli* as standards. An *S. pombe* cell cultured in complete medium is estimated to contain ~ 25 000 molecules of p34^{cdc2} and 5000 molecules of p13^{suc1}. No more than 5% of total cellular p34^{cdc2} was retained on the anti-p13^{suc1} immuno-affinity column under conditions in which the bulk of p13^{suc1} was cleared from a yeast lysate.

Instability of p13^{suc1}/p34$^{cdc2\text{-}ts}$ complex in vitro

If the observed apparent association between p34^{cdc2} and p13^{suc1} does not represent an unforeseen experimental artifact, and if furthermore this association exists *in vivo* and has physiological significance, a simple prediction follows. In a yeast strain carrying one of the temperature-sensitive alleles of *cdc2* that can be rescued by overexpression of *Suc1*$^+$ the association between p13^{suc1} and p34^{cdc2} might be expected to be altered.

The association between p13^{suc1} and p34^{cdc2} was therefore investigated in four *cdc2* mutant strains. Two of these strains (those carrying the *cdc2*.56 or *cdc2*.L7 alleles) are suppressible by overexpression of *suc1*$^+$, whereas the other two (carrying the *cdc2*.M55 or *cdc*M26 alleles) are not suppressible (Hayles *et al.*, 1986a). 10^{10} cells of each strain were cultured at a permissive temperature (25°C). Cell lysates were prepared and reacted in solution with 25 μg anti-p13^{suc1} immunoglobulins at 4°C. The immunocomplexes were precipitated with protein A-Sepharose at 4°C, subjected to SDS−PAGE and probed by immunoblotting for the presence of p13^{suc1} and p34^{cdc2}. The level of p34$^{cdc2.M55}$ and p34$^{cdc2.M26}$ that coprecipitated with p13^{suc1} was identical to that of the wild-type, whereas that of p34$^{cdc2.L7}$ was severely reduced and p34$^{cdc2.56}$ was undetectable (Figure 5A). In order to fully interpret this experiment it was essential to know whether each *cdc2* mutant strain had the normal level of p13^{suc1} and p34^{cdc2}. This was assayed by direct immunoblotting of a crude protein lysate of each strain (Figure 5B). The level of both proteins was identical in the wild-type and each of the four mutant strains. This experiment strongly supports the conclusion that the observed interaction between p13^{suc1} and p34^{cdc2} is of biological significance (see Discussion).

Protein kinase activity of p34^{cdc2}/p13^{suc1} complex

p34^{cdc2} displays protein kinase activity *in vitro*, and a variety of evidence suggests that this activity is essential to the biological role of the protein (Simanis and Nurse, 1986; Booher and Beach, 1986). We therefore tested whether the p34^{cdc2}/p13^{suc1} complex had such activity. The previous experiment was repeated precisely with the wild-type and with each of the *cdc2ts* strains, except that the material immunoprecipitated at 4°C from each cellular lysate was incubated with [γ-^{32}P]ATP in an appropriate reaction buffer (see Materials and methods) at 25, 30 or 37°C before being analyzed by gel electrophoresis. At all three temperatures the wild-type complex showed phosphorylation of a variety of polypeptides, whereas no phosphorylation occured at any temperature in the immunoprecipitate prepared from the *cdc2*.56 strain (Figure 6). This result demonstrates that the fraction of p34^{cdc2} that is associated with p13^{suc1} has enzymatic activity and furthermore that p34^{cdc2} is the only detectable protein kinase that is associated with p13^{suc1} under the experimental conditions employed.

The behavior of the other *cdc2ts* alleles is also informative. p34$^{cdc2.L7}$ is associated with p13^{suc1} although at a reduced level (Figure 5). The unbound form of this protein has previously been shown to have thermolabile kinase activity *in vitro* (Simanis and Nurse, 1986). The complexed p34$^{cdc2.L7}$ and also p34$^{cdc2.M55}$ displayed temperature-sensitive activity (Figure 6). On the other

hand, $p34^{cdc2.M26}$ showed only minimal temperature sensitivity (Figure 6). These data indicate that different t.s. alleles of *cdc*2 are defective for different biochemical functions.

The identity of the polypeptides phophorylated *in vitro* in the previous experiment is of considerable interest but has not been pursued here. This is considered in detail elsewhere (Brizuela, Draetta and Beach, in preparation). However it is notable that one of the polypeptides has exactly the electophoretic mobility of $p34^{cdc2}$, and also that even though sufficient $p13^{suc1}$ was immunoprecipitated to visualize by Coomassie Blue staining (data not shown), no phosphorylated band of the same mol. wt was observed (Figure 6).

$p34^{cdc2}/p13^{suc1}$ complex is retained in stationary phase cells

Neither the level of $p34^{cdc2}$ (Simanis and Nurse, 1986) nor of $p13^{suc1}$ (Figure 3) varies during the course of the cell cycle. It was of interest however to establish whether the degree of association between the two proteins fluctuates during the cycle. This experiment could not be undertaken with a culture synchronized by elutriation centrifugation because of the high number of cells required to detect the $p13^{suc1}/p34^{cdc2}$ complex. Instead a wild-type yeast strain was transferred, during exponential growth, from a complete medium to one lacking a nitrogen source (see Materials and methods). It has previously been shown that under these conditions 80% of the cells accumulate in $G_{0/1}$ and 20% in $G_{0/2}$ (Costello *et al.*, 1986). Following transfer from a complete to a nitrogen-free medium no change in the level of association between $p34^{cdc2}$ and $p13^{suc1}$ was observed (Figure 7).

Discussion

The *suc*1^+ gene has been predicted to contain two intervening sequences and to encode a polypeptide of 113 amino acids (Hindley *et al.*, 1987). These predictions have been fully confirmed. Removal of the two introns did not cause loss of biological activity of the gene. The resulting continuous *suc*1^+ coding sequence was expressed in *E. coli* and yielded a 13 kd protein (Figure 1). An affinity-purified polyclonal antiserum prepared against this protein recognized a fission yeast protein of exactly the same apparent molecular weight (Figure 2). This protein was more abundant in a strain that overexpressed the *suc*1^+ gene. We can therefore conclude that p13 is the product of the *suc*1^+ gene.

The level of $p13^{suc1}$ was unaltered during the cell cycle (Figure 3) and also during entry into stationary phase (Figure 6). It was estimated that each *S. pombe* cell contains approximately 5000 $p13^{suc1}$ molecules. The cell cycle control protein $p34^{cdc2}$ is present at a slightly higher level of 25 000 molecules per cell. Immunoprecipitation of $p13^{suc1}$ was shown to result in co-immunoprecipitation of approximately 5% of the cellular $p34^{cdc2}$ in a wild-type yeast strain, under the particular experimental conditions employed. However, in strains carrying either the *cdc*2.56 or *cdc*2.L7 temperature-sensitive alleles, the complex between $p13^{suc1}$ and $p34^{cdc2}$ was unstable *in vitro* at 4°C. This is significant because these two alleles are suppressible by overexpression of *suc*1^+, whereas in two strains carrying alleles that are not suppressible (*cdc*M.55, *cdc*2M26) the complex was stable *in vitro*. We take these data to imply that the temperature-sensitive defect of *cdc*2.56 and *cdc*2.L7, but not of *cdc*2.M55 or *cdc*2.M26, causes instability of the $p13^{suc1}/p34^{cdc2}$ complex both *in vitro* and *in vivo*, where the defect may be at least partially overcome by overexpression of $p13^{suc1}$.

The presence of $p34^{cdc2}$ in the complex with $p13^{suc1}$ was associated with a kinase activity that was entirely attributable to this protein. This is of interest because much of the cellular $p13^{suc1}$ appears not be associated with $p34^{cdc2}$ and might therefore be available for interaction with some other protein kinase. This seems not to be the case. However even though $p34^{cdc2}$ is the only detectable protein kinase associated with $p13^{suc1}$ it cannot be concluded, on the basis of the present data, that all or any of the proteins co-immunoprecipitated in this complex and that become phosphorylated *in vitro* by $p34^{cdc2}$ are physiological substrates of the kinase.

The preceding experiments are instructive about the role of the $p13^{suc1}/p34^{cdc2}$ complex. In approaching this question several relevant observations need to be considered: (i) $p34^{cdc2}$ is a protein kinase that is required for progression through both the G_1 and G_2 phases of the cell cycle. (ii) $p13^{suc1}$ is an essential protein, in the absence of which cell-cycle arrest occurs. The point or points of arrest have yet to be determined. (iii) Overexpression of *suc*1^+ rescues temperature-sensitive *cdc*2 strains that are defective in both G_1 and G_2 progression and in which the $p34^{cdc2}/p13^{suc1}$ complex is unstable *in vitro*. (iv) Only ~5% of $p34^{cdc2}$ is associated with $p13^{suc1}$, and this level appears to remain unaltered in stationary phase cells. (v) The fraction of $p34^{cdc2}$ that is associated with $p13^{suc1}$ is active as a protein kinase, and probably no other kinase is associated with $p13^{suc1}$. (vi) No evidence that $p13^{suc1}$ is a substrate of $p34^{cdc2}$ has been obtained.

Taken together these observations tend to suggest that $p13^{suc1}$ acts as a necessary and positive component of the $p34^{cdc2}$ protein kinase. $p13^{suc1}$ need not act directly as a kinase activator but perhaps might allow localization of $p34^{cdc2}$ to a particular cellular site or even act as a linker that allows binding of other stimulatory components or substrates or a multi-component enzyme complex.

Irrespective of the precise function of the $p13^{suc1}/p34^{cdc2}$ complex, it is clear that this complex exists in organisms other than fission yeast. Mammalian cell homologues of both $p34^{cdc2}$ (Draetta *et al.*, 1987; Lee and Nurse, 1987) and $p13^{suc1}$ (Draetta *et al.*, 1987) have recently been identified. In mammalian cells p34 and p13 also form a complex (Draetta *et al.*, 1987). Studies on the molecular basis of cell cycle control in fission yeast may thus have general relevance to all eukaryote cell types.

Materials and methods

Yeast strains and growth media

The *S. pombe* strains used in this study were the wild-type strains 972 h^{-s} and the temperature sensitive mutants h^{-s}*cdc*2.L7 (SP567), h^{-s}*cdc*2.M55 (SP575), h^{-s} *cdc*2.M26 (SP568) and h^{-s}*cdc*2.56 (SP570) previously described (Nurse *et al.*, 1976). The strain overexpressing *suc*1^+ (*suc*1OP) was created by integration of *suc*1^+ under the control of the *S. pombe ADH* promoter (Hindley *et al.*, 1987). SP36 (h^{-s} *leu*1.32 *cdc*2.33) was used to test the biological activity of the *suc*1^+ constructions. Transformation of *S. pombe* was as described in Beach *et al.* (1981).

Cells were cultured either in rich medium (YEA: 0.5% yeast extract, 3% glucose, 75 μg/ml adenine) or in minimal medium (Mitchison, 1970) buffered with pthalate rather than citrate at pH 5.6. For the nitrogen starvation experiment the cells were shifted from minimal medium to minimal medium without ammonium chloride.

To label proteins, exponentially growing cells cultured in minimal medium were exposed to 50 μCi/ml [^{35}S]methionine (1160 Ci/mmol, New England Nuclear) for 3 h at a density of 5×10^7 cells/ml.

Cell cycle synchronization

A synchronous culture of *S. pombe* was obtained by centrifugal elutriation of an exponentially growing culture using a Beckman JE-10X elutriator rotor (chamber volume: 73 ml) essentially as described by Creanor and Mitchison (1979). Cells were cultured in 2.5 l of minimal medium at 32°C to a density of 1.6×10^7 cells/ml and loaded into the elutriator at 2000 r.p.m. at the same temperature. The smallest 2% of cells was separated from the remainder by increasing the

flow rate while holding the rotor speed constant. The elutriated cells were recovered in a final volume of 1000 ml. Samples of 50 ml were taken every 20 min to determine cell number by means of a Coulter counter and to prepare cell extracts.

Oligonucleotide mutagenesis

The *suc1*$^+$ gene was inserted as a 672 bp *ScaI*–*SspI* restriction fragment into the unique *SmaI* site of pUC118, a vector that can be obtained as a single-stranded DNA following superinfection of a plasmid-bearing *E. coli* strain with M13K07 (Vieira, personal communication). The two introns of *suc1* were successively removed by oligonucleotide-directed mutagenesis as described by Zoller and Smith (1984) with the following modifications: only the mutagenic primer was used during the extension reaction, and the single-stranded template was obtained from a *ung*$^-$ strain of *E. coli* RZ1032B in order to enrich the mutant population following transformation into a *ung*$^+$ strain of *E. coli* (TG1).

The following two 40-mer DNA primers were used to remove the first and second introns of *suc1*$^+$.

5′ CCAATCCATTATTCTCCTAGATATGCTGATGATGAATATG 3′
5′ GAGGACTTGGAATTACTCAAAGTCTGGGATGGGAAATGTA 3′

After the introns were removed an *NdeI* site was created at the predicted initiating methionine of the gene using the following 21-mer:

5′ TTAAGGAGGACATATGTCGAA 3′

Expression of suc1$^+$ *in E. coli*

The intron-less form of *suc1*$^+$ was subcloned from pUC118 into the expression vector pRK172 (described in McLeod *et al.*, 1987) as a *NdeI*–*EcoRI* fragment rendering the expression of *suc1*$^+$ under the control of the promoter of gene 10 of the T7 bacteriophage (Studier and Moffatt, 1986). This construction was then introduced in the *E. coli* strain BL21(DE3)LysS, which contains a chromosomal copy of the T7 RNA polymerase under the control of the lacUV5 promoter (Studier and Moffatt, 1986) and also contains a chloramphenicol-resistant plasmid carrying the T7 lysozyme gene.

Cells carrying *suc1*$^+$ in pRK172 were cultured in LB broth in the presence of 50 mg/ml ampicillin and 30 mg/ml chloramphenicol until they attained an O.D.$_{600}$ nm of 0.4 mM. At this point expression of *suc1*$^+$ was induced by adding IPTG to the culture to a concentration of 0.4 mM. After 3 h the cells were harvested by centrifugation and the protein extracts prepared by sonication in buffer I (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 10% glycerol) in the presence of the following protease inhibitors: soybean trypsin inhibitor (10 μg/ml), aprotinin (1 mg/ml), leupeptin (1 μg/ml), tosyl-phenylalanine-chloromethyl ketone (TPCK, 10 μg/ml), phenylmethylsulphonylfluoride (75 μg/ml). The lysate was cleared by centrifugation at 100 000g for 30 min and the soluble and insoluble fractions were separated.

Purification of p13^{suc1}

The soluble fraction of a 400 ml bacterial culture, prepared as previously described, was loaded on a 1.5 × 80 cm Sepharose CL6B column (Pharmacia) and eluted with buffer I without glycerol, at a flow rate of 0.1 ml/min. Two milliliter fractions were collected and analyzed by SDS–PAGE.

Preparation of antibodies against suc1$^+$ *protein*

Two New Zealand white rabbits were immunized according to the following scheme: on day one 200 μg of the purified 13 kd protein was injected subcutaneously in complete Freund's ajuvant. The same dosage was applied 14 and 28 days later in incomplete Freund's adjuvant. The animals were bled two weeks after the last injections, and the serum was prepared and tested for its ability to precipitate soluble antigen in capillaries (Williams and Chase, 1967). The rabbits were boosted every six weeks with 200 μg of protein, and 30 ml of blood was obtained two weeks after the last injection.

The anti-p13^{suc1} immunoglobulin fraction of the immune serum was purified by affinity chromatography essentially as described by Schneider *et al.* (1982), using as immunoabsorbent 6 mg of purified p13^{suc1} protein coupled to 1 ml of CNBr-activated Sepharose 4B, prepared following the instructions of the manufacturer (Pharmacia).

Preparation of yeast extracts, immunoprecipitations and affinity chromatography

Cells were harvested by centrifugation and were broken by vortexing in the presence of an equal volume of cold 500 μm glass beads and 0.3 ml of buffer II (50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 150 mM NaCl plus protease inhibitors). After achieving approximately 90% cell disruption, 1.7 ml of buffer II, containing 1% Triton X-100, 0.1% SDS and 0.5% deoxycholate, was added to the lysate, and the soluble fraction was recovered by centrifugation and further clarified by a 13 000 *g* spin for 15 min. The protein content of the extracts was determined colorimetrically as described by Bradford (1976) using immunoglobulin as standard.

Immunoprecipitations were carried out by incubating the yeast extract with 25 μg purified anti-p13^{suc1} immunoglobulin for 1 h at 4°C in a rotator. The immune complex was brought down by a further 20 min incubation with 50 μl of protein A-Sepharose (Pharmacia) followed by a brief spin. At this point the immune complex was washed three times with buffer II containing detergents. The final pellet was resuspended in 50 μl of 2× protein sample buffer (0.1 M Tris pH 7.0, 4% SDS, 10% β-mercaptoethanol and 20% glycerol) and heated at 100°C for 2 min.

For determination of kinase activity, the protein A-Sepharose pellets were washed once in assay buffer (50 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT). Twenty-five microliters of the same buffer was then added and, after a 5 min preincubation at the indicated temperatures, the reaction was started by addition of 5μCi [γ-^{32}P]ATP (final concentration 1 μM). After 10 min, the incubations were stopped by addition of 30 μl of 2× protein sample buffer and analyzed by gel electrophoresis.

For affinity chromatography of the yeast extracts, an immunoabsorbant was prepared by incubating 3 mg of the purified immunoglobulin fraction with 0.8 ml of protein A-Sepharose and crosslinking it with dimethylpimelimidate dihydrochloride as described by Simanis and Lane (1985). The yeast extract was incubated batchwise with the immunoabsorbant for 2–6 h; the matrix was then washed extensively with buffer I and poured into a column. The proteins specifically bound to the column were eluted with 500 μl aliquots of elution buffer (50 mM diethylamine pH 11.5) and immediately neutralized with 1 M Tris–HCl pH 7.0.

Electrophoresis and immunoblots

Proteins were separated electrophoretically using either a 7.5–15% gradient SDS–polyacrylamide gel (Laemmli, 1970) or a 8–25% gradient SDS–polyacrylamide gel system (Fling and Gregerson, 1986). Gels were stained with colloidal Coomassie Blue R250.

The proteins were transferred from unstained gels to 0.1 μm nitrocellulose paper in a transblot cell (BioRad) for 5 h at 200 mA in transfer buffer (25 mM Tris–HCl pH 8.3, 192 mM glycine, 20% methanol). Subsequently the filters were blocked with phosphate-buffered saline (PBS) containing 3% ovalbumin for 1 h at 37°C. For p13^{suc1} immunoblots the filters were incubated with a 1:1000 dilution of the immune serum in PBS, 2% BSA for 2 h at room temperature. After three washes of 15 min each, one with PBS, 0.5% NP40 and one intermediate wash with 2 M urea, 100 mM glycine and 1% NP40 the filters were incubated with 1 μCi of [^{125}I]protein A (80 μCi/mg, ICN). After further washes in PBS, 0.5% NP40 the filters were exposed to photographic film.

p34^{cdc2} immunoblots were done using a monoclonal antibody (J8) described elsewhere (Potashkin *et al.*, in preparation).

Acknowledgements

We thank Mark Zoller for synthesis of oligonucleotides and providing RZ1032B, William Studier for providing BL21(DE3) and Diane Biedermann for performing the rabbit immunization. Susan Dembski is thanked for her technical assistance. Dave Green, Mike Ockler, Teri Katz and Carol Marcincuk are thanked for their assistance with the figures and manuscript. This work was funded by NIH grant GM34607 to D.Beach, a Cold Spring Harbor Robertson Fellowship to G.Draetta and an FGMA (Venezuela) Fellowship to L.Brizuela.

References

Beach,D. and Nurse,P. (1981) *Nature*, **290**, 140–142.
Beach,D., Durkacz,B. and Nurse,P. (1982) *Nature*, **300**, 706–709.
Booher,R. and Beach,D. (1986) *Mol. Cell Biol.*, **6**, 3523–3530.
Bradford,M. (1976) *Anal. Biochem.*, **72**, 248–254.
Costello,G., Rodgers,L. and Beach,D.H. (1986) *Curr. Genet.*, **11**, 119–125.
Creanor,J. and Mitchison,J. (1979) *J. Gen. Microbiol.*, **112**, 385–388.
Draetta,G., Brizuela,L., Potashkin,J. and Beach,D. (1987) *Cell*, **50**, 319–325.
Fantes,P. and Nurse,P. (1977) *Exp. Cell Res.*, **107**, 377–386.
Fling,S. and Gregerson,D. (1986) *Anal. Biochem.*, **155**, 83–88.
Hayles,J., Beach,D., Durkacz,B. and Nurse,P. (1986a) *Mol. Gen. Genet.*, **202**, 291–293.
Hayles,J., Aves,S. and Nurse,P. (1986b) *EMBO J.*, **5**, 3373–3379.
Hindley,J. and Phear,G. (1984) *Gene*, **31**, 129–134.
Hindley,J., Phear,G., Stein,M. and Beach,D. (1987) *Mol. Cell Biol.*, **7**, 504–511.
Laemmli,V. (1970) *Nature*, **227**, 680–685.
Lee,M. and Nurse,P. (1987) *Nature*, **327**, 31–35.
McLeod,M., Stein,M. and Beach,D. (1987) *EMBO J.*, **6**, 729–736.
Mitchison,J. (1970) In Prescott,D.M. (ed.), *Methods Cell Physiol.* Academic Press, New York, Vol. 4, 13–65.
Nasmyth,K. (1979) *J. Cell Sci.*, **36**, 155–168.
Nurse,P., Thuriaux,P. and Nasmyth,K. (1976) *Mol. Gen. Genet.*, **146**, 167–178.
Reed,S., Hardwiger,J. and Lorincz,A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4055–4059.
Schneider,C., Newmann,R., Southerland,R., Asser,U. and Greaves,M. (1982) *J. Biol. Chem.*, **257**, 10766–10769.
Simanis,V. and Lane,D. (1985) *Virology*, **144**, 88–100.
Simanis,V. and Nurse,P. (1986) *Cell*, **45**, 261–268.

Studier,F. and Moffat,B. (1986) *J. Mol. Biol.*, **189**, 113–130.
Williams,C. and Chase,M. (eds) (1967) *Methods in Immunochemistry and Immunology*. Academic Press, New York, Vol. 1, 238.
Zoller,M. and Smith,M. (1984) *DNA*, **3**, 479–488.

Received on June 9, 1987