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The *Arabidopsis* Functional Homolog of the p34^{cdc2} Protein Kinase

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The p34^{cdc2} protein kinase is a key component of the eukaryotic cell cycle, which is required for G1 to S-phase transition and for entry into mitosis. Using a 380-base pair DNA fragment obtained by polymerase chain reaction amplification from an *Arabidopsis thaliana* flower cDNA library as a probe, we isolated and sequenced a *cdc2*-homologous cDNA from *Arabidopsis*. The encoded polypeptide has extensive homology with *cdc2*-like kinases. Furthermore, when expressed in a *CDC28*^{ts} *Saccharomyces* strain, it partially restores the capacity to grow at 36°C, indicating that the plant cDNA is a functional homolog of the p34^{cdc2} kinase. Genomic hybridization demonstrated that there is one copy of the *cdc2* gene per *Arabidopsis* haploid genome. Using RNA gel blot analysis, we found that *cdc2* mRNA is present in all plant organs.

INTRODUCTION

The genetic and biochemical controls that regulate the eukaryotic cell cycle have been studied in considerable detail in yeast and animals (for reviews, see Draetta, 1990; Nurse, 1990; Pines and Hunter, 1990). A key component of this mechanism is a 34-kD protein kinase encoded by the *cdc2* gene, known as p34^{cdc2} (Hindley and Phear, 1984). Genetic analysis has shown that p34^{cdc2} is required at two control points in the *Schizosaccharomyces pombe* cell cycle: in late G1 and in G2 to mitosis transition (Nurse and Bisset, 1982). Likewise, the *cdc2*-homologous gene of *Saccharomyces cerevisiae CDC28* (Lörincz and Reed, 1984) is also essential for the transition from G1 to S-phase (Reed, 1980) and for the entry into mitosis (Piggott et al., 1982; Reed and Wittenberg, 1990). In amphibia, starfish, and mammals, a 34-kD protein homologous to p34^{cdc2} was found to be part of the maturation-promoting factor, a biochemical activity that triggers the events that bring oocytes into nuclear division (Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1988; Langan et al., 1989; for a review, see Murray and Kirschner, 1989).

Although the *cdc2* kinase is also required during the G1 to S-phase transition, the regulation of the p34^{cdc2} mitosis-specific kinase (M-phase kinase) is better understood. It has been found that the M-phase kinase is only active after forming a complex with cyclins and after dephosphorylation of tyrosine and threonine residues (Draetta and Beach, 1988; Gould and Nurse, 1989; Moria et al., 1989; Pondaven et al., 1990). Moreover, in the yeasts, mutants have been used to demonstrate that a number of regulatory proteins control the M-phase activity of the *cdc2*. These studies have revealed the presence of both positive regulators, such as *cdc25*$^{+}$ and *nim1*$^{+}$ (Russell and Nurse, 1986, 1987a), and negative ones, such as *wee*$^{+}$ (Russell and Nurse, 1987b).

Genes homologous to *cdc2* have been isolated from humans (Lee and Nurse, 1987), chickens (Krek and Nigg, 1989), mice (Cisek and Corden, 1989), and *Drosophila* (Jimenez et al., 1990; Lehner and O'Farrell, 1990). Despite the evolutionary divergence of these species, an extensive degree of structural similarity is present in the reported sequences. Some of these similarities are shared with members of the serine/threonine protein kinase family (Hanks et al., 1988), and others are unique to *cdc2*-like kinases, including a perfectly conserved 16-amino acid region, the so-called "PSTAIR" domain.

In yeasts, the amounts of *cdc2* mRNA and p34^{cdc2} are constant during the cell cycle (Durkacz et al., 1986). However, McGowan et al. (1990) reported fluctuations in the abundance of *cdc2* mRNA and in the rate of p34^{cdc2} synthesis in synchronized HeLa cells. There is also evidence that the expression of *cdc2* is correlated positively with cell proliferation. For example, an increase in transcript levels was found when quiescent murine cells were shifted from stationary to the exponential phase (Lee et al., 1988). RNA gel blot analysis of chicken tissues has shown that the amounts of mRNA decrease continuously during embryonic development (Krek and Nigg, 1989), and similar results have been obtained from experiments with *Drosophila* (Jimenez et al., 1990; Lehner and O'Farrell, 1990). Moreover, in situ hybridizations to localize *cdc2* mRNA were performed with *Drosophila*, and the results

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confirmed that the level of expression was correlated with the proliferative state of the cell.

The patterns of plant development are very different from those of animals. Cell division in higher plants is confined largely to meristematic regions that retain their capacity for continuous growth and production of new organs. For example, the shoot apical meristem continuously forms a succession of leaf and bud primordia and, likewise, the root apical meristem forms an expanded root system. Furthermore, the shoot apical meristem can change its programmed activity from vegetative growth to reproductive growth, thereby producing flowers. Another unique property of plant cells is their totipotency. Under many natural and artificial conditions, a fully differentiated plant cell can dedifferentiate and eventually can be regenerated into a whole plant. In spite of the fact that mitosis in plants is mechanistically very similar to mitosis in other eukaryotes, very little is known about the biochemical components that regulate the cell cycle in plants, and the genes involved in the control of cell division have not yet been identified. Immunological detection using *cdc2*-specific antibodies, however, has shown cross-reactivity with a protein of 34 kD in the green alga *Chlamydomonas* and in several plant species, including *Arabidopsis* (John et al., 1989; Feiler and Jacobs, 1990). Recently, the cloning by the polymerase chain reaction (PCR) of part of a pea cDNA homologous to *cdc2* has been reported (Feiler and Jacobs, 1990).

As a first step for studying the regulation of cell division in plants, we cloned and characterized a *cdc2*-homologous cDNA from *Arabidopsis*. In addition, we showed that when the *Arabidopsis* cDNA is expressed in a temperature-sensitive *CDC28 S. cerevisiae* mutant strain, it can complement partially the cell division deficiency at a restrictive temperature, thus confirming that the gene identified here is a *cdc2/CDC28* functional homolog. These results provide strong evidence that the basic mechanisms that regulate cell division in plants are similar to those already described in animals and yeast.

RESULTS

Cloning of a *cdc2*-Homologous cDNA from *Arabidopsis*

A comparison of all known *cdc2* protein sequences revealed the presence of several highly conserved regions. Two such amino acid stretches were chosen to design degenerate oligonucleotides (see Methods), which were subsequently used as primers for a PCR with DNA prepared from a flower cDNA library of *Arabidopsis*. The first primer was based on the peptide sequence EGVPSTA, containing a part of the PSTAIR domain (indicated in Figure 2), and the second was based on the sequence HEV(I)VTLW. (For details of the PCR amplification, see

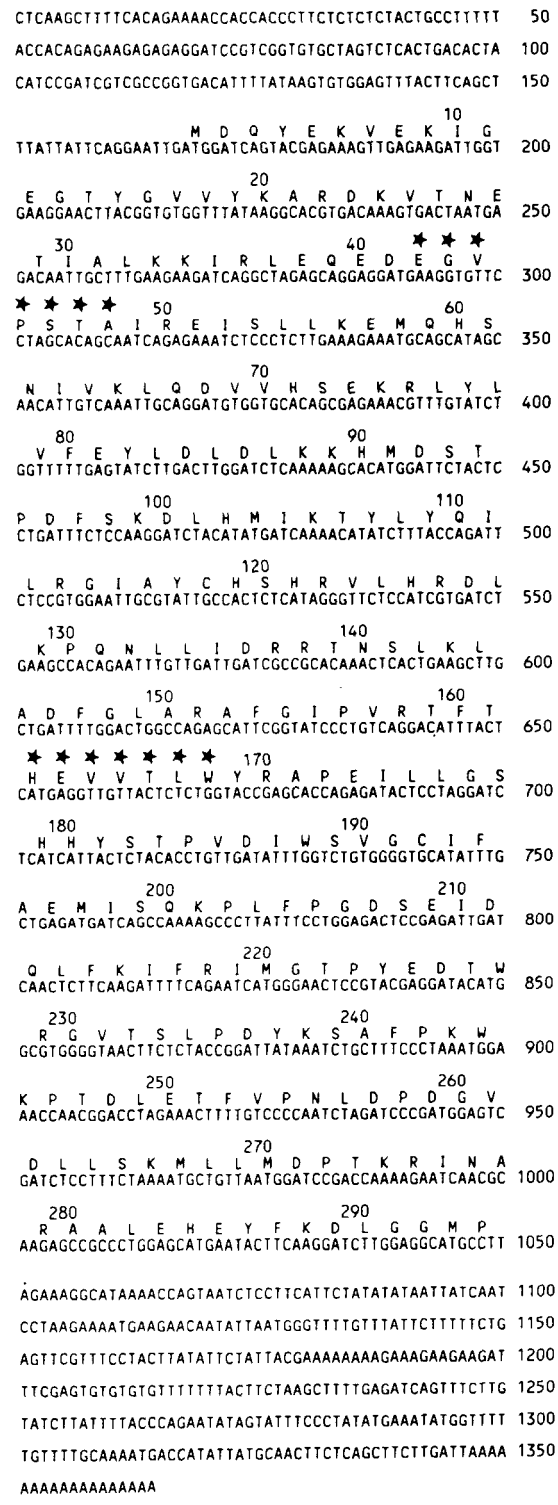

Figure 1. Nucleotide Sequence of the *Arabidopsis cdc2* cDNA.

The deduced amino acid sequence is indicated above the nucleotide sequence. The asterisks indicate the regions that were chosen to design the PCR primers.

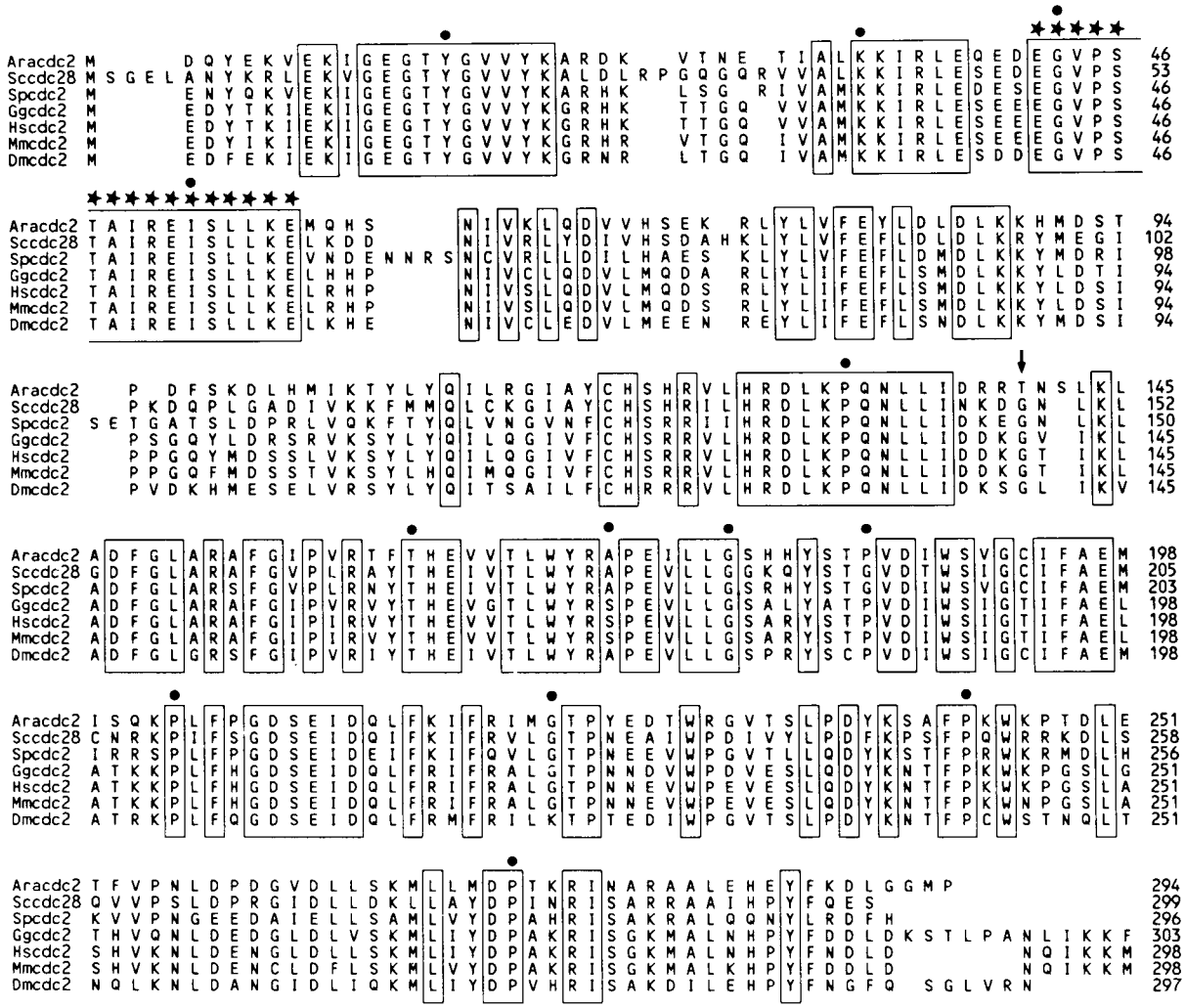

Figure 2. Sequence Comparison of the Functional *cdc2* Homologs.

The *Arabidopsis* (Ara) *cdc2* sequence is aligned with the *cdc2* homologs of *S. cerevisiae* (Sccdc28; Lörincz and Reed, 1984), *Sch. pombe* (Spcdc2; Hindley and Phear, 1984), *Gallus gallus* (Ggcdc2; Krek and Nigg, 1989), *Homo sapiens* (Hscdc2; Lee and Nurse, 1987), *Mus musculus* (Mmcdc2; Cisek and Corden, 1989), and *D. melanogaster* (Dmcdc2; Jimenez et al. 1990; Lehner and O'Farrell, 1990). Amino acids conserved in all sequences are boxed. Asterisks indicate the PSTAIR region, a conserved stretch of amino acids unique to $p34^{cdc2}$ kinases. Black dots designate residues that when substituted caused mutant phenotype. The arrow indicates a threonine in the *Arabidopsis cdc2* sequence that substitutes a highly conserved glycine (see Discussion).

Methods.) After the PCR, a band of the expected size—approximately 380 bp—was found to be amplified. Among other PCR products, this band hybridized best under moderate stringency with the human and chicken *cdc2* cDNAs (data not shown). After subcloning in pUC18, four independent clones were sequenced and found to be identical. The deduced amino acid sequence of the fragment strongly resembles those of *cdc2*-homologous proteins. Besides the presence of 16 amino acids of the PSTAIR domain, a comparison with published *cdc2* sequences shows approximately 60% homology.

To isolate a full-length cDNA, the 380-bp fragment was used as a probe to screen a flower cDNA library from *Arabidopsis*. One positive signal was identified out of 350,000 phages screened. The cDNA was subcloned as an EcoRI fragment in the Bluescript KS II +/−, yielding the plasmid pCDC2Ara. The nucleotide sequence of the 1.4-kb cDNA and the deduced amino acid sequence are shown in Figure 1. The cDNA contains an 882-bp open reading frame, encoding a polypeptide of 294 amino acids.

A comparison of the *Arabidopsis* sequence with published amino acid sequences of *cdc2*-homologous proteins

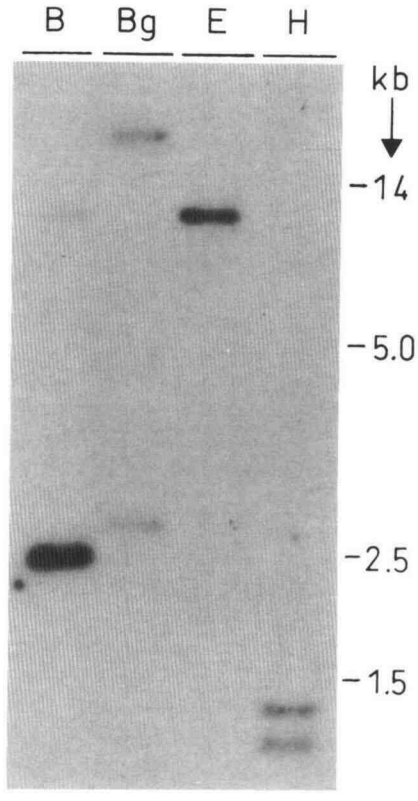

Figure 3. Evidence for the Fact That the *Arabidopsis cdc2* Is a Single-Copy Gene.

A genomic DNA gel blot of *Arabidopsis* DNA digested with BamHI (B), BglII (Bg), EcoRI (E), and HindIII (H) was hybridized with the 1.4-kb *cdc2* cDNA as a probe, as described in Methods.

from *S. cerevisiae*, *Sch. pombe*, chicken, humans, mouse, and *Drosophila* is shown in Figure 2. The highest homology is with the human sequence, 66%, followed by 65% with chicken, 64% with mouse, 62% with *Drosophila* and *Sch. pombe*, and 61% with *S. cerevisiae*. The homology of the *Arabidopsis cdc2* with the PCR product obtained from pea is 95% in the compared region. All highly conserved regions in known *cdc2* proteins are also present in the *Arabidopsis cdc2* sequence.

To determine whether the *cdc2* from *Arabidopsis* was encoded by a single copy gene, hybridization with total *Arabidopsis* DNA digested with four different restriction enzymes was performed. Figure 3 shows two hybridizing fragments with BamHI, two with BglII, one with EcoRI, and two with HindIII. A genomic clone subsequently isolated and sequenced confirmed the sizes and locations of the endonuclease restriction sites, revealing that there is only one copy of the *cdc2* gene per haploid genome of *Arabidopsis* (data not shown).

Complementation of an *S. cerevisiae CDC28* Mutant Strain

Although sequence comparison indicated clearly that the cloned *Arabidopsis* cDNA encodes a *cdc2* homolog, the complementation of a *CDC28 S. cerevisiae* mutant strain was undertaken to prove that the *Arabidopsis* cDNA was a functional homolog of *cdc2*. The 1.4-kb insert of pCDC2Ara was subcloned in the *Escherichia coli*-yeast shuttle vector pEMBLyex4 downstream from the *GAL-CYC1* hybrid promoter, yielding the plasmid pYCDC2Ara, as shown in Figure 4. This plasmid was then used to transform the *S. cerevisiae* strain 4078-21a, which is unable to grow at 36°C because it carries a temperature-sensitive mutation in the *CDC28* gene. When mutant cells grown at the permissive temperature (28°C) are shifted to the restrictive temperature, all of the cells will stop dividing and be arrested at the same point in the cell cycle in G1.

Cells transformed with pYCDC2Ara were grown overnight at 28°C in rich media containing glucose (YPD medium). The cells were pelleted down, resuspended in glycerol-containing media (YPGly medium), and grown further at 28°C. This step is necessary to permit expression from the galactose-inducible promoter that is tightly repressed on glucose-containing media. After 4 hr, galactose was added to the culture and the cells were incubated at 36°C. As controls, mutant cells and cells transformed with the cloning vector pEMBLyex4 and the wild-type strain 02431a were treated in the same way. Figure 5A shows that the mutant cells and cells carrying the vector did not divide at the restrictive temperature and that their viability decreased as a function of time. The strain carrying the

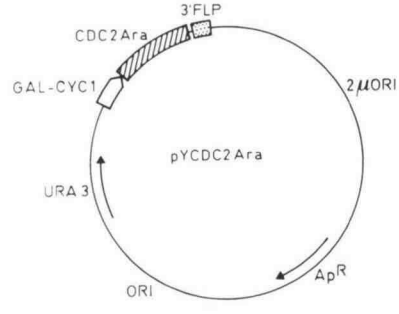

Figure 4. Structure of the Yeast Expression Vector pYCDC2Ara.

The *Arabidopsis cdc2* cDNA (CDC2Ara) ([////]) is flanked at the 5′ end by the galactose-inducible *GAL-CYC1* hybrid promoter (□) and at the 3′ end by the termination signal of the 2-μm FLP gene ([⁘⁘]). Abbreviations: Ap^R, ampicillin resistance gene; URA3, selection for uracil prototropy in yeast; ORI, pBR322-derived origin of replication; 2μORI, origin of replication of the yeast 2-μm plasmid. The construction of the plasmid is described in Methods.

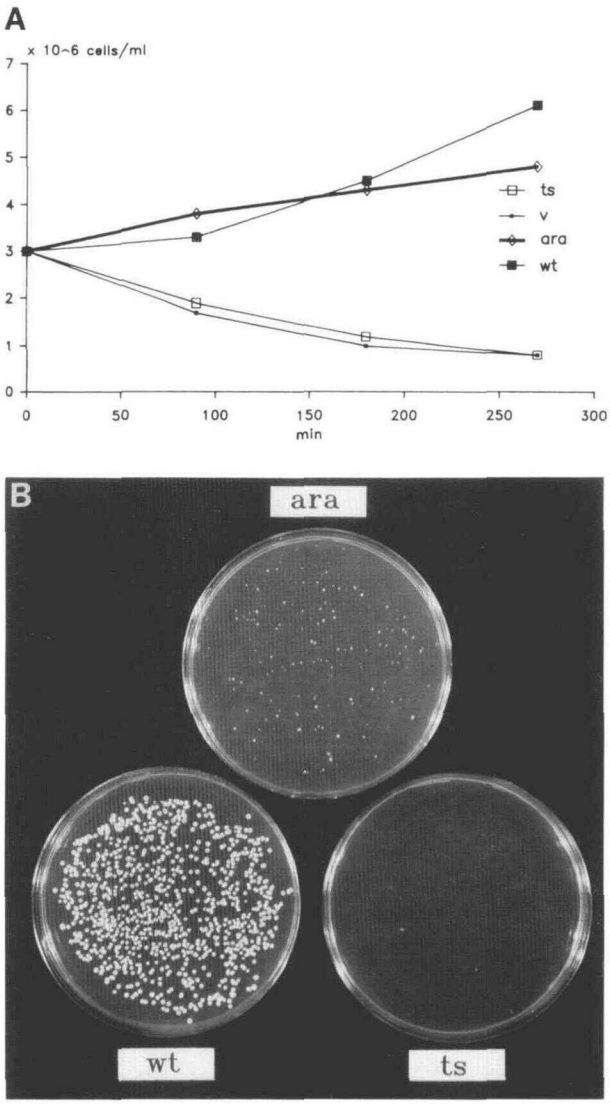

Figure 5. Growth of Mutant Cells Transformed with the *Arabidopsis cdc2* Gene.

(A) Growth curve. Samples from liquid cultures containing 2% galactose growing at 36°C were taken every 90 min, plated in YPD agar, and incubated at 28°C (see Methods). wt, *S. cerevisiae* strain 02431a; ts, *S. cerevisiae* strain 4078-21a; ara, *S. cerevisiae* strain 4078-21a transformed with pYCDC2Ara; and v, *S. cerevisiae* strain 4078-21a transformed with pEMBLyex4.
(B) Samples were taken after 1 hr of incubation at 36°C in liquid media containing galactose, plated onto YPG agar, and incubated 3 days at 36°C. ts, ara, and wt are defined in **(A)**. Whereas the mutant strain does not grow at 36°C, cells carrying the pYCDC2Ara plasmid can form colonies at the restrictive temperature. However, fivefold to sixfold more colonies are formed in plates of wild-type cells. Furthermore, these colonies are clearly larger.

Arabidopsis cdc2 continued to divide although slower than the wild-type strain. In the first 6 hr of incubation at 36°C, the ratio between cells with small buds, large buds, and unbudded cells was kept approximately constant (one-third of the cells had each form). Because an increase in the number of viable cells was observed (Figure 5A), we can conclude that, at least during that period, the *S. cerevisiae* mutant strain carrying the *Arabidopsis cdc2* was able to divide at the restrictive temperature. Furthermore, mutant cells transformed with pYCDC2Ara formed colonies in galactose-containing media at 36°C, whereas untransformed mutant cells did not (Figure 5B).

Figure 6A reveals that the mutant cells stopped cell division but continued to elongate when shifted to 36°C. The strain transformed with pYCDC2Ara, however, could grow at 36°C, and the cells divided normally (Figures 6B and 6C). This functional complementation of the *CDC28* mutation was dependent upon transcription of the plant *CDC2* cDNA because cell division was arrested in the same way as in the mutant strain when glucose was added instead of galactose to cells containing the pYCDC2Ara plasmid and the cultures were shifted from 28°C to 36°C (Figure 6C). However, prolonged (longer than 8 hr) incubation in galactose at 36°C resulted in reduced viability and an increasing number of cells with aberrant morphology, indicating that the plant *cdc2* does not restore complete wild-type behavior. Curing the cells of the recombinant plasmid confirmed further that the ability to grow at 36°C was conferred by the plant-derived *cdc2* cDNA. Cells containing pYCDC2Ara were grown at 28°C in YPD without selection for several days and then replica plated onto minimal media with and without uracil. (The inability to grow in the absence of uracil indicated plasmid loss.) All colonies that were not able to grow without uracil failed to grow at 36°C in galactose-containing media.

Expression Pattern

In higher eukaryotes, the expression of the *cdc2* gene is, at least in part, transcriptionally regulated. To investigate *cdc2* gene expression in plants, RNA blot hybridizations were used to study the abundance of *cdc2* transcripts in different *Arabidopsis* tissues. Total RNA from entire flowering plants, roots, stems, leaves, flowers, and actively dividing callus were probed with the *cdc2* cDNA. One unique band of approximately 1.4 kb hybridized with the probe in all samples. The result is shown in Figure 7. In comparison with stems and callus, slightly higher levels of mRNA were found in roots. The levels of *cdc2* transcripts in flower RNA were about 50% of those in stems and callus; the amounts in leaf and total plant RNA were approximately twofold lower.

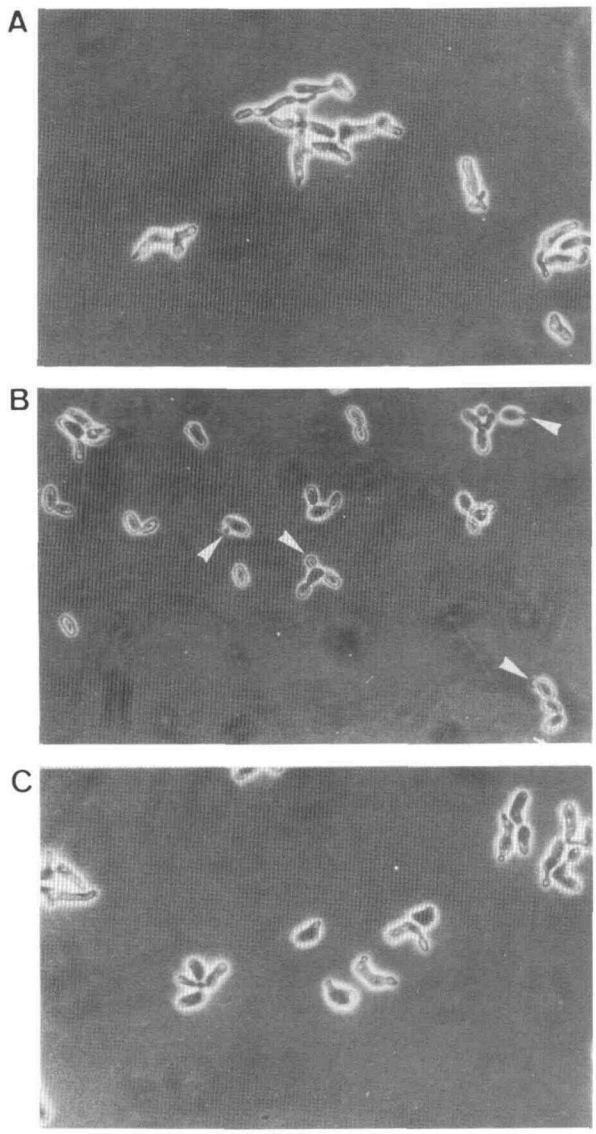

Figure 6. Complementation of the *S. cerevisiae CDC28* Mutant by the *cdc2 Arabidopsis* Gene Product.

(A) The *S. cerevisiae* strain 4078-21a 3 hr after the temperature shift to 36°C in medium containing 2% galactose. The *S. cerevisiae cdc28* strain 4078-21a contains a temperature-sensitive mutation in the *cdc28* gene (Hereford and Hartwell, 1974). When grown at restrictive temperature (36°C), cell division stops. No buds are present but continued growth of the cells produces an irregular cylindrical outline.

(B) Strain 4078-21a transformed with pYCDC2Ara 3 hr after the temperature shift to 36°C in medium containing 2% galactose. The arrows show the presence of small and large buds, indicating that cells expressing the *Arabidopsis cdc2* cDNA are able to progress through the cell cycle.

(C) The *S. cerevisiae* strain 4078-21a carrying the pYCDC2Ara 3 hr after the temperature shift to 36°C in medium containing 2% glucose. Cell division has not occurred, buds are absent, and cells resemble those seen in **(A)**.

DISCUSSION

The demonstration that some of the molecular components of cell cycle control are conserved in organisms that are very distant evolutionarily implies the existence of a mechanism of regulation that is common to all eukaryotic cells (Nurse, 1990). The results reported here suggest that this mechanism of regulation also embodies the plant kingdom. We used PCR to amplify a 380-bp DNA fragment from an *Arabidopsis* flower cDNA library that is homologous to known *cdc2* sequences. Using this probe, a nearly full-length cDNA clone was isolated subsequently from the cDNA library, and sequence determination revealed that the clone was indeed a *cdc2* homolog.

Expression of the *Arabidopsis cdc2* cDNA in a strain of *S. cerevisiae* that has a temperature-sensitive mutation in its *CDC28* gene can promote cell division at the restrictive temperature, showing that the protein encoded by the *Arabidopsis* cDNA is a functional homolog of $p34^{cdc2}$. However, like the human and *Drosophila cdc2* homologs, the plant *cdc2* does not restore complete wild-type behavior. This is not surprising because the *cdc2* gene product interacts with many components of the cell cycle control apparatus (e.g., cyclins, $suc1^+$, wee^+, $cdc25^+$, phosphatases, and numerous substrates), and it has been suggested that these interactions might be species specific (Lehner and O'Farrell, 1990).

In contrast, expression of *cdc2*-related cDNAs from *Drosophila* and frogs in *cdc2/CDC28* temperature-sensitive strains does not restore cell division even though they have a high degree of homology with *cdc2*-functional homologs, and they possess most of the conserved motifs characteristic of *cdc2*-related kinases (Lehner and O'Farrell, 1990; Paris et al., 1991). To define which amino acids are absolutely required for the biological activity of $p34^{cdc2}$, extensive in vitro mutagenesis of conserved amino acid residues in the *cdc2* genes is necessary. However, by examining the data accumulated from complementing

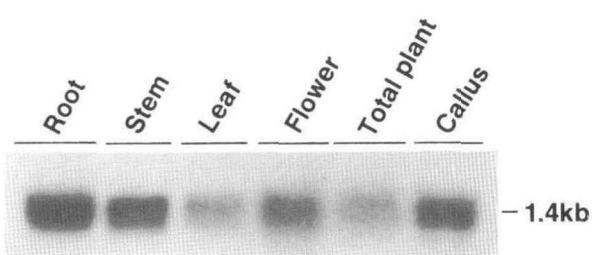

Figure 7. Expression Analysis of the *Arabidopsis cdc2* Gene.

RNA gel blot with 10 μg of total RNA from different organs of *Arabidopsis* hybridized with the 1.4-kb *cdc2* cDNA as a probe, as described in Methods.

cdc2 sequences, from the isolation of *cdc2* mutant alleles, and from the limited in vitro mutagenesis that has already been done, a rough picture of the amino acid residues and domains important for biological activity can be drawn. Carr et al. (1989) have identified the amino acid substitutions that resulted in temperature-sensitive mutations within the *cdc2* gene of *Sch. pombe*, and a temperature-sensitive *cdc2* allele caused by substitutions in two conserved amino acids was isolated recently from a mouse cell line (Th'ng et al., 1990). Additionally, Booher and Beach (1986) have identified mutations within the conserved ATP binding domain and in a putative phosphorylation domain that abolish biological activity. Gould and Nurse (1989) have shown that a tyrosine residue in the ATP binding domain is phosphorylated in vivo and that its dephosphorylation activates the *cdc2* kinase. Although some of these residues are absolutely conserved in all sequences published to date, others are not.

In the *Arabidopsis* sequence, one of the invariant residues whose substitution resulted in a temperature-sensitive phenotype in *Sch. pombe* is changed at position 139, where threonine occurs in the plant sequence and a glycine is found in all the other functional homologs (Figure 2). Moreover, other invariant amino acids are also replaced in the *Arabidopsis* gene. For example, a histidine replaces tyrosine at position 97, isoleucine replaces valine at position 111, arginine replaces proline at position 228, methionine replaces tyrosine at position 270, and asparagine replaces serine at position 277 (Figure 2). Because the *cdc2* gene product physically interacts with other proteins—cyclins (Booher and Beach, 1987; Draetta et al., 1989; Hadwiger et al., 1989; Gautier et al., 1990) and the *suc1*$^+$ product (Hayles et al., 1986a, 1986b; Brizuela et al., 1987; Hindley et al., 1987; Dunphy and Newport, 1989; Moreno et al., 1989; Richardson et al., 1990)—to have mitotic kinase activity, it might be possible to define the domains that are required for the interaction (reviewed in Draetta, 1990). Substitutions within these domains could have some degree of tolerance, provided the conformational change in the structure is not too drastic. For example, our results suggest that a threonine residue in place of a glycine at position 139 can be tolerated, but a substitution to aspartic acid cannot, as was shown by Booher and Beach (1986) and Carr et al. (1989). However, substitutions in residues that are important for catalytic activity or in amino acids that are phosphorylated presumably could not be tolerated.

RNA blot analysis revealed the presence of the *cdc2* transcript in all tissues examined, although different amounts were observed. It is possible that the *cdc2* gene is expressed preferentially in dividing cells. This interpretation is supported by the finding that the histones H3 and H4, which in *Arabidopsis* are expressed preferentially in tissues with meristematic activity, have a similar pattern of expression as observed by RNA gel blots (C. Gigot, personal communication). On the other hand, we cannot exclude the possibility that the *cdc2* gene is expressed uniformly throughout the entire plant. To answer this question, we are currently performing in situ hybridization with different *Arabidopsis* tissues and constructing promoter/β-glucuronidase fusions to analyze the tissue-specific expression in transformed plants.

METHODS

PCR Amplification

The sequences of the oligonucleotide primers used for amplification were: (1) 5′ TCCTGCAGGA(AG)GGIGTTCCIAG(CT)ACIGC 3′, (2) 5′ GAGGATCCCCA(CT)AAIGTIACIA(CT)(CT)TC(GA)TG 3′. The underlined sequences denote restriction sites (PstI and BamHI, respectively) that were subsequently used for convenient cloning of PCR products. DNA samples of a λgt10 cDNA library constructed with poly(A)$^+$ RNA from flowers of *Arabidopsis thaliana* ecotype Columbia (D.E. de Oliveira, personal communication) were used for the PCR and were prepared by using the following procedure: 100 μL of the library (approximately 1.7×10^8 plaque-forming units/mL) was mixed with 1 volume of a solution of 20% PEG 6000, 2 M NaCl and incubated for 1 hr on ice. The phage particles were collected, resuspended in 10 μL of λ storage buffer (Maniatis et al., 1982) and heated at 95°C for 10 min. PCR was carried out essentially as described by Saiki et al. (1985), except that a higher concentration of each primer (100 pM) was used because of primer redundancy. Conditions for the reactions were as follows: 10 μL of the *Arabidopsis* flower cDNA library prepared as above, 1 μg of each primer, 200 μM each deoxynucleotide triphosphate, in a final volume of 100 μL. The reaction mix also contained 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; and 0.01% (w/v) gelatin. Reactions were cycled automatically through time/temperature cycles as follows: denaturation 94°C/1 min, annealing 45°C/1 min, and extension 72°C/3 min, with the final extension cycle increased to 10 min. Ten microliters of the reaction mix was loaded on a 1% agarose gel and the gel was blotted to a nylon filter. The filter was then probed with both the chicken and the human *cdc2* cDNA clones (data not shown). A fragment of the expected size, 380 bp, that hybridized with both probes, was isolated from a low melting point agarose gel, digested with PstI and BamHI, and subcloned in pUC18. Four clones were sequenced by the dideoxy method of Sanger et al. (1977).

Library Screening and DNA Sequence

Approximately 350,000 plaque-forming units from the *Arabidopsis* flower library were screened under high stringency conditions following the procedure recommended by Amersham. Only one positively hybridizing phage could be identified. DNA from this phage was prepared using Qiagen tips according to the manufacturer's recommendations and digested with EcoRI to release a 1.4-kb insert, and the insert was subcloned in Bluescript KS II +/−. The resulting plasmid, pCDC2Ara, was sequenced by the method of Sanger et al. (1977) using T3 and T7 commercial primers (Boehringer Mannheim) and internal primers derived from the internal sequence.

Plant Material

All plant manipulations were carried out using *Arabidopsis* plants grown as described previously (Valvekens et al., 1988). Briefly, seeds of *Arabidopsis* ecotype C24 were grown in sterile conditions in germination medium (Valvekens et al., 1988). Tissues were collected after 2 months of growth and frozen at −70°C immediately. To obtain callus, roots from 1-month-old plants were placed on callus-inducing media (Valvekens et al., 1988). After 2 weeks, the resulting calli were collected and frozen at −70°C.

RNA and DNA Blot Hybridization Analyses

Total RNA was isolated from callus, roots, stems, leaves, flowers, and from total plants of *Arabidopsis* ecotype C24 as described (Logemann et al., 1986). Ten micrograms of each sample was electrophoretically separated on 1% agarose gels and transferred to nylon filters (Hybond-N, Amersham). Hybridization was done in 50% formamide, 10% dextran sulfate, 0.25% nonfat dry milk, 0.5% SDS, 3 × SSC, and 20 μg/mL denatured salmon sperm DNA at 42°C for 24 hr using the *Arabidopsis cdc2* cDNA probe labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983). The filters were washed twice with 3 × SSC, 1% SDS at 42°C; twice with 1 × SSC, 1% SDS at 42°C; twice with 0.1 × SSC, 0.1% SDS at 42°C; and twice with 0.1 × SSC, 0.1% SDS at 55°C, for 15 min each wash. These experiments were done twice with independently extracted RNA samples.

Total DNA from *Arabidopsis* ecotype C24 was prepared as described (Pruitt and Meyerowitz, 1986) and restricted with BglII, BamHI, EcoRI, and HindIII, respectively. Restriction fragments were separated on 0.8% agarose gels and transferred to nylon membranes (Hybond-N, Amersham). Hybridization was carried out using a ^{32}P-labeled *cdc2* cDNA in 3 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 20 μg/mL denatured salmon sperm DNA at 65°C for 24 hr. The filter was washed twice at 65°C with 2 × SSC, 15 min each time; once with 2 × SSC, 0.1% SDS for 30 min; and once with 0.1 × SSC, 0.1% SDS for 15 min.

Complementation of a *Saccharomyces cerevisiae* Mutant Carrying a Temperature-Sensitive Mutation within the *CDC28* Gene

The 1.4-kb cDNA insert of pCDC2Ara was isolated by digestion with restriction endonucleases SstI and SalI and ligated into the yeast shuttle vector pEMBLyex4 (Cesarini and Murray, 1987) downstream from the *GAL-CYCL1* hybrid promoter. The resulting plasmid, pYCDC2Ara (Figure 4), was transformed into *S. cerevisiae CDC28* mutant strain 4078-21a (*mat-a, cdc28-1, leu2-3, leu112, ura3-52, his7, can1*) (Hereford and Hartwell, 1974). Yeast transformation was carried out by the lithium chloride method (Ito et al., 1983). After 2 days of growth at 28°C, the resulting *ura*$^+$ colonies were inoculated in liquid-rich media containing glucose as carbon source (YPD) and grown overnight at 28°C. The cells were pelleted and resuspended in media containing 2% glycerol (YPGly) and grown further at 28°C. After 4 hr, galactose was added to the culture (2% final concentration) and the cells were shifted to the restrictive temperature of 36°C. Samples were taken every 90 min, plated onto YPD agar, and incubated at 28°C. The *S. cerevisiae* strain 02431a, which grows normally at 36°C, was used as control. The growth curve was constructed by counting the number of resulting colonies at each time point. YPD medium contains 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose; YPG contains 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% galactose; YPGly contains 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glycerol.

DNA Procedures and Yeast Manipulations

DNA procedures were carried out as described by Maniatis et al. (1982), and yeast manipulations were done as described by Sherman and Fink (1986).

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While this work was in press, similar results were reported with an alfalfa *cdc2* DNA: **Hirt, H., Páy, A., Györgyey, J., Bakó, L., Németh, K., Bögre, L., Schweyen, R. J., Heberle-Bors, E., and Dudits, D.** (1991). Complementation of a yeast cell cycle mutant by an alfalfa cDNA encoding a protein kinase homologous to $p34^{cdc2}$. Proc. Natl. Acad. Sci. USA **88,** 1636–1640.

REFERENCES

Booher, R., and Beach, D. (1986). Site-specific mutagenesis of *cdc2*$^+$, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. Mol. Cell. Biol. **6,** 3523–3530.

Booher, R., and Beach, D. (1987). Interaction between *cdc13*$^+$ and *cdc2*$^+$ in the control of mitosis in fission yeast; dissociation

of the G_1 and G_2 roles of the $cdc2^+$ protein kinase. EMBO J. **6,** 3441–3447.

Brizuela, L., Draetta, G., and Beach, D. (1987). $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. EMBO J. **6,** 3507–3514.

Carr, A.M., MacNeill, S.A., Hayles, J., and Nurse, P. (1989). Molecular cloning and sequence analysis of mutant alleles of the fission yeast *cdc2* protein kinase gene: Implications for $cdc2^+$ protein structure and function. Mol. Gen. Genet. **218,** 41–49.

Cesarini, G., and Murray, J.A.H. (1987). Plasmid vectors carrying the replication origin of filamentous single-stranded phages. In Genetic Engineering, Principles and Methods, Vol. 9, J.K. Setlow, ed (New York: Plenum Press), pp. 135–154.

Cisek, L.J., and Corden, J.L. (1989). Phosphorylation of RNA polymerase by the murine homologue of the cell-cycle control protein cdc2. Nature **339,** 679–684.

Draetta, G. (1990). Cell cycle control in eukaryotes: Molecular mechanisms of *cdc2* activation. Trends Biochem. Sci. **15,** 378–383.

Draetta, G., and Beach, D. (1988). Activation of *cdc2* protein kinase during mitosis in human cells: Cell cycle-dependent phosphorylation and subunit rearrangement. Cell **54,** 17–26.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). *cdc2* protein kinase is complexed with both cyclin A and B: Evidence for proteolytic inactivation of MPF. Cell **56,** 829–838.

Dunphy, W.G., and Newport, J.W. (1989). Fission yeast p13 blocks mitotic activation and tyrosine dephosphorylation of the Xenopus *cdc2* protein kinase. Cell **58,** 181–191.

Dunphy, W.G., Brizuela, L., Beach, D., and Newport, J. (1988). The Xenopus *cdc2* protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell **54,** 423–431.

Durkacz, B., Carr, A., and Nurse, P. (1986). Transcription of the *cdc2* cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. EMBO J. **5,** 369–373.

Feiler, H.S., and Jacobs, T.W. (1990). Cell division in higher plants: A *cdc2* gene, its 34-kDa product, and histone H1 kinase activity in pea. Proc. Natl. Acad. Sci. USA **87,** 5397–5401.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene $cdc2^+$. Cell **54,** 433–439.

Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J.L. (1990). Cyclin is a component of maturation-promoting factor from Xenopus. Cell **60,** 487–494.

Gould, K.L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast $cdc2^+$ protein kinase regulates entry into mitosis. Nature **342,** 39–45.

Hadwiger, J.A., Wittenberg, C., Mendenhall, M.D., and Reed, S.I. (1989). The *Saccharomyces cerevisiae CKS1* gene, a homolog of the *Schizosaccharomyces pombe* $suc1^+$ gene, encodes a subunit of the Cdc28 protein kinase complex. Mol. Cell. Biol. **9,** 2034–2041.

Hanks, S.K., Quinn, A.M., and Hunter, T. (1988). The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science **241,** 42–52.

Hayles, J., Aves, S., and Nurse, P. (1986a). *suc1* is an essential gene involved in both the cell cycle and growth in fission yeast. EMBO J. **5,** 3373–3379.

Hayles, J., Beach, D., Durkacz, B., and Nurse, P. (1986b). The fission yeast cell cycle control gene *cdc2*: Isolation of a sequence *suc1* that suppresses cdc2 mutant function. Mol. Gen. Genet. **202,** 291–293.

Hereford, L.M., and Hartwell, L.H. (1974). Sequential gene function in the initiation of *S. cerevisiae* DNA synthesis. J. Mol. Biol. **84,** 445–461.

Hindley, J., and Phear, G.A. (1984). Sequence of the cell division gene *CDC2* from *Schizosaccharomyces pombe*; patterns of splicing and homology to protein kinases. Gene **31,** 129–134.

Hindley, J., Phear, G., Stein, M., and Beach, D. (1987). $Suc1^+$ encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of *Schizosaccharomyces pombe*. Mol. Cell. Biol. **7,** 504–511.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153,** 163–168.

Jimenez, J., Alphey, L., Nurse, P., and Glover, D.M. (1990). Complementation of fission yeast $cdc2^{ts}$ and $cdc25^{ts}$ mutants identifies two cell cycle genes from *Drosophila*: A *cdc2* homologue and *string*. EMBO J. **9,** 3565–3571.

John, P.C.L., Sek, F.J., and Lee, M.G. (1989). A homolog of the cell cycle control protein p34*cdc2* participates in the division cycle of *Chlamydomonas*, and a similar protein is detectable in higher plants and remote taxa. Plant Cell **1,** 1185–1193.

Krek, W., and Nigg, E.A. (1989). Structure and developmental expression of the chicken *CDC2* kinase. EMBO J. **8,** 3071–3078.

Labbe, J.C., Lee, M.G., Nurse, P., Picard, A., and Doree, M. (1988). Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene $cdc2^+$. Nature **335,** 251–254.

Langan, T.A., Gautier, J., Lohka, M., Hollingsworth, R., Moreno, S., Nurse, P., Maller, J., and Sclafani, R.A. (1989). Mammalian growth-associated H1 histone kinase: A homolog of $cdc2^+$/*CDC28* protein kinases controlling mitotic entry in yeast and frog cells. Mol. Cell. Biol. **9,** 3860–3868.

Lee, M.G., and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell control gene *cdc2*. Nature **327,** 31–35.

Lee, M.G., Norbury, C.J., Spurr, N.K., and Nurse, P. (1988). Regulated expression and phosphorylation of a possible mammalian cell-cycle control protein. Nature **333,** 676–679.

Lehner, C.F., and O'Farrell, P.H. (1990). *Drosophila cdc2* homologs: A functional homolog is coexpressed with a cognate variant. EMBO J. **9,** 3573–3581.

Logemann, J., Schell, J., and Willmitzer, L. (1986). Improved method for the isolation of RNA from tissues. Anal. Biochem. **163,** 16–20.

Lörincz, A.T., and Reed, S.I. (1984). Primary structure homology between the product of yeast cell division control gene *CDC28* and vertebrate oncogenes. Nature **307,** 183–185.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

McGowan, C.H., Russell, P., and Reed, S.I. (1990). Periodic biosynthesis of the human M-phase promoting factor catalytic subunit p34 during the cell cycle. Mol. Cell. Biol. **10,** 3847–3851.

Moreno, S., Hayles, J., and Nurse, P. (1989). Regulation of $p34^{cdc2}$ protein kinase during mitosis. Cell **58,** 361–372.

Moria, A.O., Draetta, G., Beach, D., and Wang, J.Y.G. (1989). Reversible tyrosine phosphorylation of *cdc2*: Dephosphorylation accompanies activation during entry into mitosis. Cell **58,** 193–203.

Murray, A.W., and Kirschner, M.W. (1989). Dominoes and clocks: The union of two views of the cell cycle. Science **246,** 614–621.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature **344,** 503–507.

Nurse, P., and Bissett, Y. (1981). Gene required in G_1 for commitment to cell cycle and in G_2 for control of mitosis in fission yeast. Nature **292,** 558–560.

Paris, J., Le Guellec, R., Couturier, A., Le Guellec, K., Omilli, F., Camonis, J., MacNeill, S., and Phillipe, M. (1991). Cloning by differential screening of a *Xenopus* cDNA coding for a protein highly homologous to *cdc2*. Proc. Natl. Acad. Sci. USA **88,** 1039–1043.

Piggott, J.R., Rai, R., and Carter, B.L.A. (1982). A bifunctional gene product involved in two phases of the yeast cell cycle. Nature **298,** 391–393.

Pines, J., and Hunter, T. (1990). $p34^{cdc2}$: The S and M kinase? New Biologist **2,** 389–401.

Pondaven, P., Meijer, L., and Beach, D. (1990). Activation of M-phase-specific histone H1 kinase by modification of the phosphorylation of its $p34^{cdc2}$ and cyclin components. Genes Dev. **4,** 9–17.

Pruitt, R.E., and Meyerowitz, E.M. (1986). Characterization of the genome of *Arabidopsis thaliana*. J. Mol. Biol. **187,** 169–183.

Reed, S.I. (1980). The selection of *S. cerevisiae* mutants defective in the start event of cell division. Genetics **95,** 561–577.

Reed, S.I., and Wittenberg, C. (1990). Mitotic role for the Cdc28 protein kinase of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **87,** 5697–5701.

Richardson, H.E., Stueland, C.S., Thomas, J., Russell, P., and Reed, S.I. (1990). Human cDNAs encoding homologs of the small $p34^{Cdc28/Cdc2}$-associated protein of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Genes Dev. **4,** 1332–1344.

Russell, P., and Nurse, P. (1986). $cdc25^{+}$ functions as an inducer in the mitotic control of fission yeast. Cell **45,** 145–153.

Russell, P., and Nurse, P. (1987a). The mitotic inducer $nim1^{+}$ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. Cell **49,** 569–576.

Russell, P., and Nurse, P. (1987b). Negative regulation of mitosis by $wee1^{+}$, a gene encoding a protein kinase homolog. Cell **49,** 559–567.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230,** 1350–1354.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Sherman, F., Fink, G.R., and Hicks, J.B. (1986). Laboratory Course Manual for Methods in Yeast Genetics. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Th'ng, J.P.H., Wright, P.S., Hamaguchi, J., Lee, M.G., Norbury, C.J., Nurse, P., and Bradbury, E.M. (1990). The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive *CDC2* gene product. Cell **63,** 313–324.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* root explants using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.