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Cell Cycle Phase Specificity of Putative Cyclin-Dependent Kinase Variants in Synchronized Alfalfa Cells

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The eukaryotic cell division cycle is coordinated by cyclin-dependent kinases (CDKs), represented by a single major serine/threonine kinase in yeasts (Cdc2/CDC28) and a family of kinases (CDK1 to CDK8) in human cells. Previously, two *cdc2* homologs, *cdc2MsA* and *cdc2MsB*, have been identified in alfalfa (*Medicago sativa*). By isolating cDNAs using a *cdc2MsA* probe, we demonstrate here that at least four additional *cdc2* homologous genes are expressed in the tetraploid alfalfa. Proteins encoded by the new *cdc2MsC* to *cdc2MsF* cDNAs share the characteristic functional domains of CDKs with the conserved and plant-specific sequence elements. Transcripts from *cdc2MsA*, *cdc2MsB*, *cdc2MsC*, and *cdc2MsE* genes are synthesized throughout the cell cycle, whereas the amounts of *cdc2MsD* and *cdc2MsF* mRNAs peak during G_2-to-M phases. The translation of Cdc2MsA/B, Cdc2MsD, and Cdc2MsF proteins follows the pattern of transcript accumulation. The multiplicity of kinase complexes with cell cycle phase–dependent activities was revealed by in vitro phosphorylation experiments. Proteins bound to p13^{suc1}–Sepharose or immunoprecipitated with Cdc2MsA/B antibodies from cells at G_1-to-S and G_2-to-M phase boundaries showed elevated kinase activities. The Cdc2MsF antibodies separated a G_2-to-M phase–related kinase complex. Detection of histone H1 phosphorylation activities in fractions immunoprecipitated with antimitotic cyclin (CyclinMs2) antibodies from G_2-to-M phase cells indicates the complex formation between this cyclin and a kinase partner in alfalfa. The observed fluctuation of transcript levels, amounts, and activities of kinases in different cell cycle phases reflects a multilevel regulatory system during cell cycle progression in plants.

INTRODUCTION

Cyclin-dependent kinases (CDKs) play a central role in regulation of the eukaryotic cell cycle. CDKs are specific serine/threonine kinases that are activated at defined cell cycle phases, including START, when cells become committed to the new division cycle and the entry into mitosis (reviewed in Morgan, 1995). Genetic studies with yeasts have revealed a single CDK gene (*cdc2* in *Schizosaccharomyces pombe* and CDC28 in *Saccharomyces cerevisiae*) required for cell cycle transitions (Norbury and Nurse, 1992; Nasmyth, 1993). In Drosophila, two CDK genes essential for G_1-to-S and G_2-to-M phase transitions have been identified (Stern et al., 1993). By contrast, in human cells, the cell cycle is controlled by a family of CDK1 to CDK8 kinases. Mammalian kinases Cdc2 (CDK1) and CDK2 are implicated in the regulation of G_2-to-M and G_1-to-S phase transitions, respectively, whereas CDK4 to CDK6 act at the G_1-to-S phase transition (reviewed in Pines, 1995, 1996). Other Cdc2-related kinases, such as PHO85 in yeast and CDK5 in mammals, are also involved in the regulation of functions other than the cell cycle (Hellmich et al., 1992; Kaffman et al., 1994; Tsai et al., 1994).

The activity of CDKs is controlled post-transcriptionally at multiple levels. CDKs form diverse complexes with cyclins, whose levels oscillate during the cell cycle (Pines, 1995). The cyclin–CDK complexes are activated by phosphorylation of Thr-160 and Thr-161 residues of the kinase subunits, whereas their activity is inhibited by phosphorylation of CDK residues Thr-14 and Tyr-15 as well as by binding of CDK inhibitors (reviewed in Peter and Herskowitz, 1994).

Cell division is one of the major driving forces in completion of the ontogenic program during the life cycle of flowering plants. Meristems are the primary foci of cell divisions. Cell division activity in plant meristems is regulated through a complex control system influenced by hormonal and

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developmental signals as well as environmental stimuli (reviewed in Jacobs, 1992; Dudits et al., 1997). In contrast to cell division in animals, meristematic cell division activity contributes continuously to the formation of the plant body after embryogenesis and persists. Therefore, plant cell division control probably involves some regulatory features that are unique.

Results of cloning work and characterization of the identified plant *cdc2* homologous genes clearly demonstrated that plant cells—similar to animal cells—possess more than one *cdc2*-related gene. Highly homologous *cdc2* gene pairs (exhibiting 80 to 90% amino acid identities) have been identified from several plant species, including maize, rice, alfalfa, soybean, Antirrhinum, and pea (Colasanti et al., 1991; Hashimoto et al., 1992; Hirt et al., 1993; Miao et al., 1993; Fobert et al., 1994; Jacobs 1995). The two alfalfa (*Medicago sativa*) *cdc2* homologous genes (designated *cdc2MsA* and *cdc2MsB*) display different abilities to complement yeast *cdc28* mutants blocked at G_1-to-S and G_2-to-M phases. This observation suggests that they have different roles in cell cycle regulation (Hirt et al., 1993). The two functionally similar *cdc2* genes of soybean appear to be regulated by different developmental programs in the shoot and root tissues (Miao et al., 1993). In Arabidopsis, two more distantly related *cdc2* homologous genes (*cdc2aAt* and *cdc2bAt*), with 56% identity at the amino acid level, have been identified (Ferreira et al., 1991; Hirayama et al., 1991). *cdc2aAt*, but not *cdc2bAt*, is functionally able to complement *cdc28* mutants in yeast (Ferreira et al., 1991; Imajuku et al., 1992). Moreover, the *cdc2c* and *cdc2d* genes of Antirrhinum and the closely related Arabidopsis *cdc2bAt* were suggested to be expressed in a cell cycle–regulated fashion (Fobert et al., 1994, 1996; Segers et al., 1996). In situ hybridizations and histochemical studies with *cdc2* promoter–driven β-glucuronidase reporter gene fusions have shown that the known *cdc2* genes are expressed in proliferating tissues (Martinez et al., 1992; Fobert et al., 1994, 1996; Segers et al., 1996) or in cells competent for division (Hemerly et al., 1993), such as alfalfa protoplasts whose division is activated by hormonal stimulation (Magyar et al., 1993).

The cloning data are supported by biochemical studies demonstrating the presence of $p34^{cdc2}$-related proteins in plants (John et al., 1989; Feiler and Jacobs, 1990; Hirt et al., 1991; Mineyuki et al., 1991; Colasanti et al., 1993) that cross-react with antibodies raised against the PSTAIRE peptide motif as well as bind to the yeast $p13^{suc1}$ protein and display histone H1 kinase activity. The quantity of p34 carrying the PSTAIRE motif was found to be higher in dividing cells of basal leaf segments of wheat than in differentiated cells (John et al., 1990), and a $p13^{suc1}$-binding p34 protein displayed histone H1 kinase activity in maize mitotic cells (Colasanti et al., 1991). Moreover, fluctuation of the activity of a p34 histone kinase during the cell cycle has been demonstrated in synchronized alfalfa cells (Magyar et al., 1993).

In this study, the repeated screening of alfalfa cDNA libraries with the alfalfa cdc2MsA gene probe resulted in the isolation of four new *cdc2* homologs that encode structurally related proteins carrying all functional domains of yeast, plant, and animal CDKs. Thus, it appears that alfalfa contains at least six different *cdc2* homologous genes. According to our data, two gene variants (*cdc2MsD* and *cdc2MsF*) show differential expression during cell cycle progression. Both $p13^{suc1}$ affinity binding and immunoprecipitation with anti-kinase or anti-cyclin peptide antibodies revealed a set of kinase complexes showing different histone H1 phosphorylation activities in cells synchronized for various cell cycle phases.

RESULTS

Identification of a Cyclin-Dependent Kinase Family in Alfalfa

Previous studies with CDKs of alfalfa led to the isolation of two cDNA clones encoding highly homologous Cdc2-related proteins (Cdc2MsA and Cdc2MsB; Hirt et al., 1991, 1993; Magyar et al., 1996). To search for novel Cdc2- or CDK-related transcripts in alfalfa, a fragment of *cdc2MsA* cDNA representing both the PSTAIRE and catalytic domains was used as a probe in low-stringency hybridization to screen different cDNA libraries prepared from auxin-treated microcallus suspensions (Györgyey et al., 1991) and somatic embryos of alfalfa.

Six alfalfa CDK-related kinases were identified. Alignment of the predicted gene product sequences of *cdc2MsA* to *cdc2MsF* indicates considerable variation even in the functionally important regions, such as the PSTAIRE element, the catalytic domain, and the T loop (Figure 1). The two previously characterized alfalfa kinases, Cdc2MsA and Cdc2MsB, with 91% amino acid identity to each other, show the highest homology to CDKs carrying the perfect PSTAIRE motif. CDKs possessing this motif form the major group of plant CDKs (Dudits et al., 1997).

The other four newly identified kinases (Cdc2MsC to Cdc2MsF) represent Cdc2 homologs. A full-length cDNA for *cdc2MsC* isolated by rapid amplification of 5′ cDNA ends (Frohman et al., 1988) was found to encode 509 amino acids of a protein of ~57 kD. The protein encoded by the *cdc2MsC* variant showed 94.5% amino acid identity with a partial protein sequence of a Cdc2-like pea kinase (Feiler and Jacobs, 1991). The unexpected size of this alfalfa kinase corresponds to a long transcript of ~2.0 kb, as determined by RNA gel blot analysis (data not shown). The Cdc2MsC protein has 52.8% identity with the human cholinesterase-related cell division control protein (CHED; Lapidot-Lifson et al., 1992) and 44% identity with a human PITALRE protein (Grana et al., 1994). Functional characterization of the two human homologs shows that the CHED is required during hematopoiesis and that the kinase with the PITALRE motif is a nuclear Cdc2-related protein that phosphorylates the retinoblastoma protein in vitro.

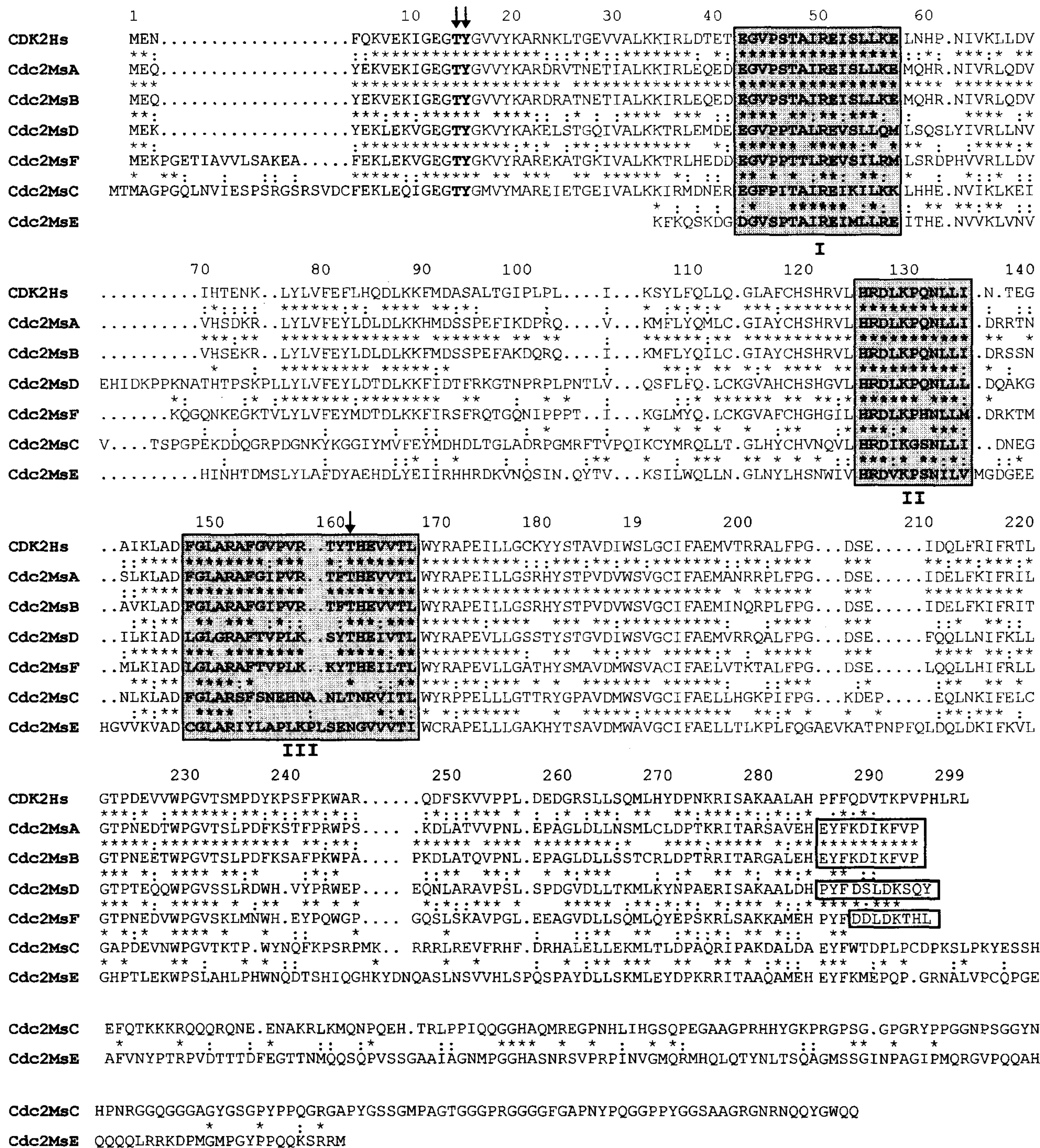

Figure 1. Sequence Alignment of the Predicted Six Alfalfa CDKs and the Human CDK2 Proteins.

Cdc2MsA corresponds to the full-length cDNA clone of the previously published gene (Hirt et al., 1991). The cdc2MsB gene product is identical to the CDKB variant published by Hirt et al. (1993). Identical amino acids are indicated by asterisks, and similar ones are marked by colons. Gaps introduced for the best fit are represented by dots. Three characteristic conserved regions of the CDK proteins, including the PSTAIRE motif (43 to 56), catalytic domain (125 to 135), and T-loop region (147 to 167), are boxed and shaded. The conserved phosphorylation sites are marked by arrows, and the C-terminal peptide regions used to generate antibodies are boxed. The human CDK2 sequence was published by Tsai et al. (1991). EMBL accession numbers of the new alfalfa cDNA clones are X97314 (*cdc2MsC*), X97315 (*cdc2MsD*), X97316 (*cdc2MsE*), and X97317 (*cdc2MsF*).

The missing 5′ ends of the truncated *cdc2MsD* and *cdc2MsF* cDNA clones were also obtained by rapid amplification of cDNA ends. The longest *cdc2MsD* and *cdc2MsF* cDNA sequences contain open reading frames coding for 311 and 316 amino acids of the predicted 35- and 36-kD proteins, respectively. These closely related proteins show 64.1% amino acid identity. The Cdc2bAt kinase of Arabidopsis (Hirayama et al., 1991), their closest homolog, shares 83.9 and 66.9% amino acid identity with the deduced sequences of Cdc2MsD and Cdc2MsF, respectively. The canonical PSTAIRE motif of Cdc2MsA and Cdc2MsB was represented by PPTALRE in Cdc2MsD and PPTTLRE in Cdc2MsF. Such modified PSTAIRE motifs have also been identified in two Antirrhinum Cdc2 kinases (Fobert et al., 1994). Finally, the *cdc2MsE* cDNA clone contains a partial open reading frame of 414 amino acids showing only 33 to 40% sequence identity with plant and animal Cdc2 or CDK kinases. The SPTAIRE sequence motif identified in Cdc2MsE has not been found in any of the Cdc2-like proteins in the data bases.

Differential Regulation of *cdc2Ms* Gene Expression during Cell Cycle Progression

We have previously shown that the transcripts of *cdc2MsA* are detectable in alfalfa cells throughout the cell cycle and that the accumulation of mRNAs is modulated by plant hormones (Hirt et al., 1991; Magyar et al., 1993). To monitor the levels of transcripts from alfalfa gene variants homologous to CDKs during cell cycle progression, we prepared mRNAs from aphidicolin-synchronized alfalfa cells passing through different phases of the cell cycle. The RNA gel blots were hybridized with the cloned cDNAs that showed no cross-hybridization under stringent conditions. In the case of the highly homologous *cdc2MsA* and *cdc2MsB* genes, gene-specific probes were used, representing only the 3′ ends of the cDNA clones (Figure 2; see Methods). The *cdc2MsA* to *cdc2MsC* mRNAs were detected in all cell cycle phases, although the level of *cdc2MsB* mRNA was significantly lower compared with those of *cdc2MsA* to *cdc2MsC*. Remarkably, an accumulation of *cdc2MsD* and *cdc2MsF* mRNAs was detected in samples in which the majority of cells were passing the G_2 and M phases. Repetition of the RNA blot experiment using an independent aphidicolin-synchronized alfalfa cell culture convincingly showed that the highest amounts of *cdc2MsD* and *cdc2MsF* transcripts are characteristic of cells in G_2-to-M phases (Figure 3).

The data also indicate that the level of *cdc2MsF* transcripts is considerably higher than the level of *cdc2MsD* mRNAs in G_2-to-M cells. Compared with all other *cdc2Ms* mRNAs, the *cdc2MsE* transcripts showed the lowest abundance but were present throughout the cell cycle, as were transcripts from the *cdc2MsA* to *cdc2MsC* genes. Here, we also show the accumulation of histone H3 mRNAs from an S phase–specific histone variant (Kapros et al., 1992, 1993).

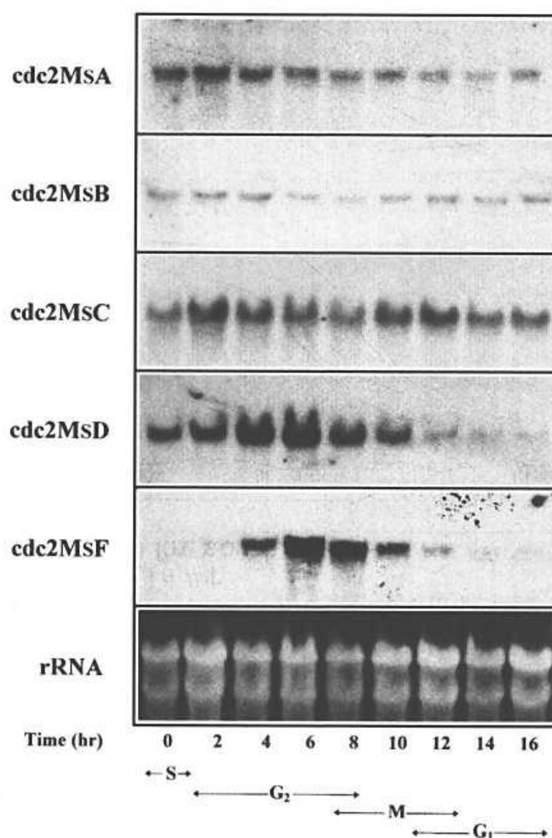

Figure 2. mRNA Levels of Alfalfa CDK Gene Variants in Cells from Various Cell Cycle Phases Synchronized by Treatment of Alfalfa (A2) Suspension Cultures with 20 μg/mL Aphidicolin.

Total mRNA of samples were isolated at indicated time intervals after release from the aphidicolin block and hybridized with gene-specific probes. The ethidium bromide staining of the rRNA after transfer to nylon filter indicates the relative amounts of RNA analyzed. Periods dominated by S, G_2, M, and G_1 phases are marked by arrows. In these experiments, the boundaries indicated for the various cell cycle phases are based on the data of flow cytometric analysis and determination of mitotic index shown in Figure 5C.

These data can also help to determine the extent of the S phase in this experiment.

Immunodetection of Cdc2Ms Proteins during the Cell Cycle

To monitor whether the translation of Cdc2Ms proteins follows the pattern of transcription observed during cell cycle progression, we prepared affinity-purified antibodies against C-terminal peptides of Cdc2MsA, Cdc2MsB, Cdc2MsD, and Cdc2MsF proteins. As shown in Figure 1, the Cdc2MsA and Cdc2MsB proteins possess identical C-terminal sequences, and therefore, the generated antibodies recognize both pro-

teins. To demonstrate that these affinity-purified antibodies specifically detect the corresponding Cdc2Ms proteins, we used the protein samples from nonsynchronized cells for competition experiments in which the antibodies were blocked by the corresponding Cdc2Ms peptides or mixed with peptides from other Cdc2Ms kinases. Thus, these antibodies were used to identify the Cdc2Ms kinases by immunoblotting of proteins from nonsynchronized cells (Figure 4A) as well as from cells accumulated in the G_2-to-M phase (Figure 4B).

The affinity-purified antibody raised against the common C terminus of Cdc2MsA and Cdc2MsB proteins detected a protein of 31 to 32 kD in nonsynchronized cells that was also present in all samples withdrawn from synchronized cells during the cell cycle (Figure 4C). The anti-Cdc2MsD antibody revealed a protein of 37 kD in nonsynchronized cells. The same protein was also present at reduced level in G_2-to-M phase–arrested cells in which the anti-Cdc2MsD antibody cross-reacted with a more abundant protein of 33 kD. These data suggested that p37 could correspond to a post-translationally modified form of the p33 Cdc2MsD protein but did not exclude that p37 may be a related Cdc2Ms kinase that escaped our detection during cDNA screening. In fact, in synchronized cells, p37 was found to be present throughout the cell cycle, whereas the accumulation of p33 Cdc2MsD was only detected in G_2-to-M phases (Figure 4C), as seen for the corresponding transcript (Figure 3). Finally, the anti-Cdc2MsF antibody specifically reacted with a protein of 34 kD in nonsynchronized cells. The p34 (Cdc2MsF) protein showed accumulation during late G_2-to-M phases but was also detectable in G_1, in which the levels of p33 (Cdc2MsD) were sharply reduced in comparison (Figure 4C).

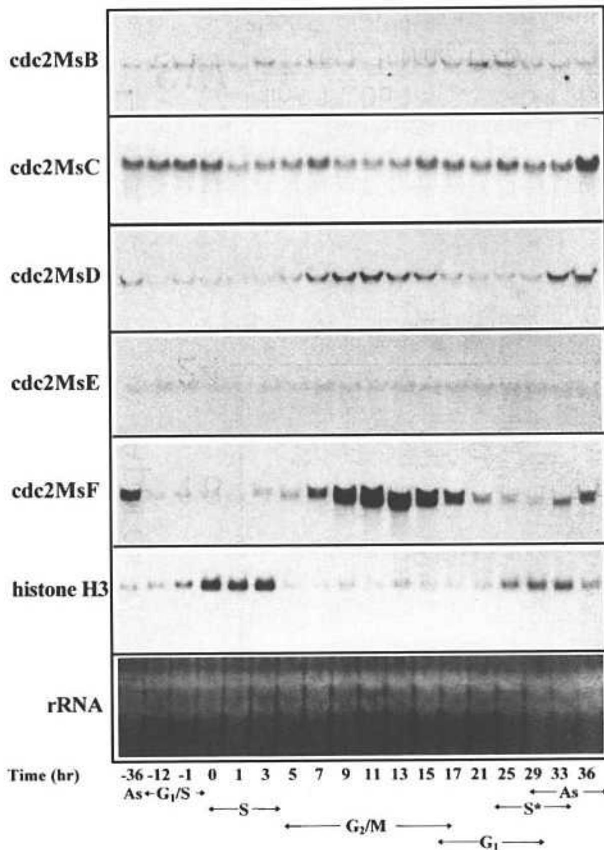

Figure 3. mRNA Levels of Alfalfa CDK Gene Variants in Cells from Various Cell Cycle Phases Synchronized by Treatment of Alfalfa (A2) Suspension Cultures with 10 μg/mL Aphidicolin.

Total mRNA of samples were isolated at indicated time intervals before and after release from the aphidicolin block and hybridized with gene-specific probes. The ethidium bromide staining of the rRNA after transfer to nylon filter indicates the relative amounts of RNA analyzed. In these experiments, the boundaries indicated for the various cell cycle phases are based on the data of flow cytometeric analysis shown in Figure 8B. Periods of the S, G_2/M, and G_1 phases are marked by arrows. S*, an increased S phase population in the second cell cycle with reduced synchrony; As, asynchronously dividing cells.

Cell Cycle Phase–Dependent Fluctuation of Kinase Activities in Alfalfa CDK Complexes

We have previously demonstrated that the yeast p13^{suc1} protein can interact with alfalfa kinases that show elevated phosphorylation activities in S and G_2-to-M phase cells after synchronization with hydroxyurea (Magyar et al., 1993). Here, we extend the search for phase-specific kinase complexes by using anti-CDK and anti-cyclin antibodies in immunoprecipitation studies. Protein samples from aphidicolin-synchronized cells were extracted by a p13^{suc1}–Sepharose matrix, and the activity of bound kinases was monitored by in vitro phosphorylation assays using histone H1 protein as the substrate (Figure 5A). Activity detected by this assay correlated with the enrichment of cells in S phase after the release from the aphidicolin block as well as the time of mid-G_2 and M phases. The flow cytometric data are shown in Figure 5C. The last samples (16 to 28 hr) represent the next cycle with reduced synchrony in which the increased number of S phase cells may partially account for the elevated phosphorylation activity.

To test to what extent the Cdc2MsA/B kinases could contribute to the observed changes, we immunoprecipitated the same protein fractions with the affinity-purified anti-Cdc2MsA/B antibody and used them analogously in histone H1 phosphorylation assays (Figure 5B). The pattern of histone H1 kinase activity obtained by the latter approach closely resembled that observed in the p13^{suc1}-bound kinase fractions. The immunoprecipitated kinase complexes, however, exhibited the highest histone H1 phosphorylation activity in the cell population representing the S phase. We could clearly recognize a mid-G_2 phase peak that was followed by reduced phosphorylation activity during early M phase. These differences may indicate the

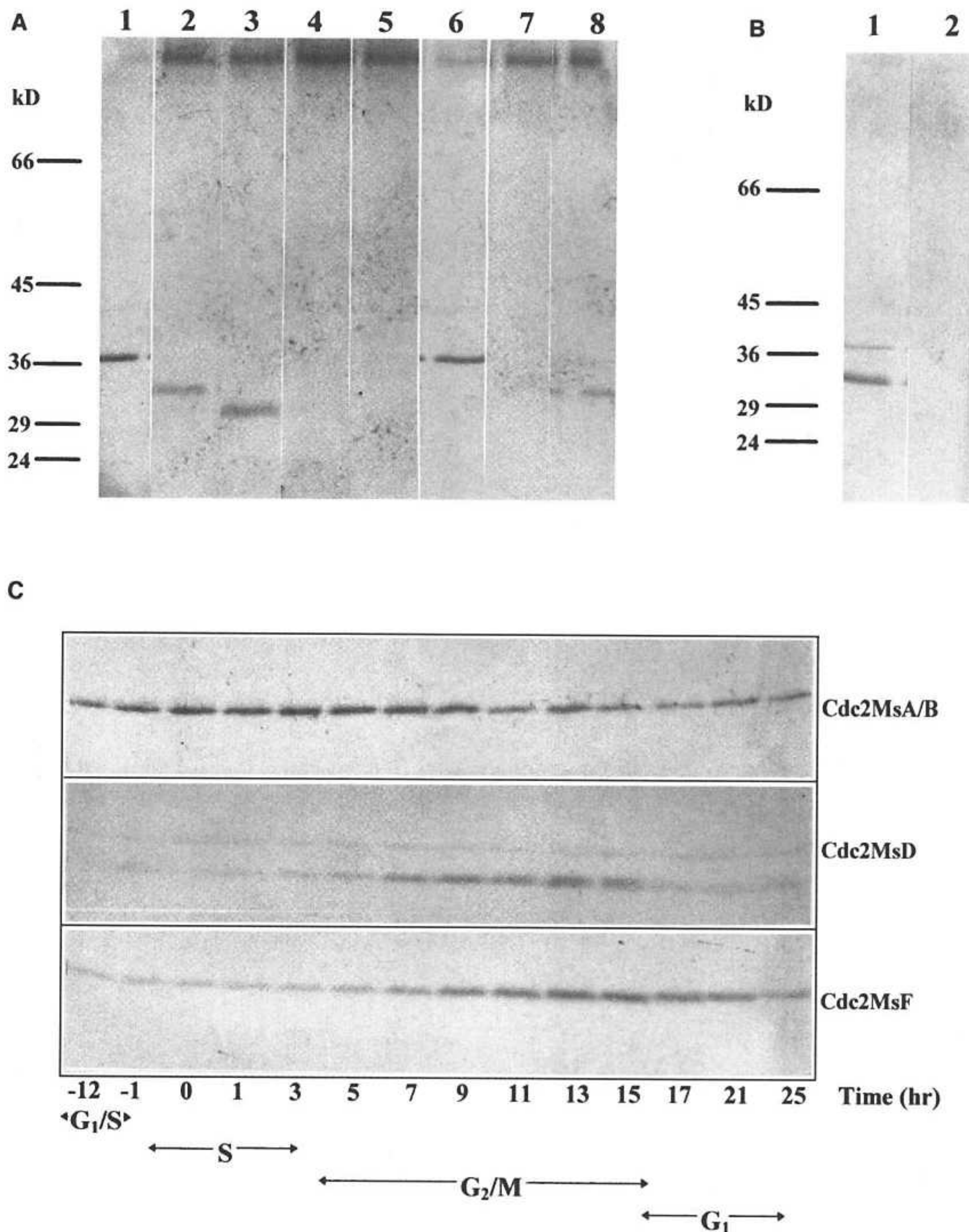

Figure 4. Immunological Detection of Cdc2MsA/B, Cdc2MsD, and Cdc2MsF Proteins at Different Phases of the Cell Cycle in Synchronized Alfalfa (A2) Cell Suspension.

(A) and **(B)** Protein extracts prepared from alfalfa cells dividing asynchronously or synchronized for the G_2-to-M phases, respectively, were used for immunoblotting with affinity-purified polyclonal anti-C-terminal (CT) Cdc2MsD (lanes 1), Cdc2MsF (lane 2 in **[A]**), and Cdc2MsA/B (lane 3 in **[A]**) antibodies. In the competition experiments, the anti−CT-Cdc2MsA/B antibodies were preincubated with Cdc2MsA/B synthetic peptides (lane 4 in **[A]**), the anti−CT-Cdc2MsD antibodies were preincubated with Cdc2MsD (lane 5 in **[A]** and lane 2 in **[B]**) or Cdc2MsF (lane 6 in **[A]**) synthetic peptides, and the anti−CT-Cdc2MsF antibodies were preincubated with Cdc2MsF (lane 7 in **[A]**) or Cdc2MsD (lane 8 in **[A]**) synthetic peptides before the immunostaining. Molecular mass markers are indicated at left.

(C) Equal amounts of total proteins extracted at indicated time points from aphidicolin-synchronized alfalfa cells were assayed by an immunoblotting method, using anti−CT-Cdc2MsA/B, anti−CT-Cdc2MsD, and anti−CT-Cdc2MsF antibodies. The G_1/S, S, G_2/M, and G_1 phases are marked by arrowheads or arrows. The boundaries indicated for the various cell cycle phases are based on the data of flow cytometric analysis shown in Figure 8B.

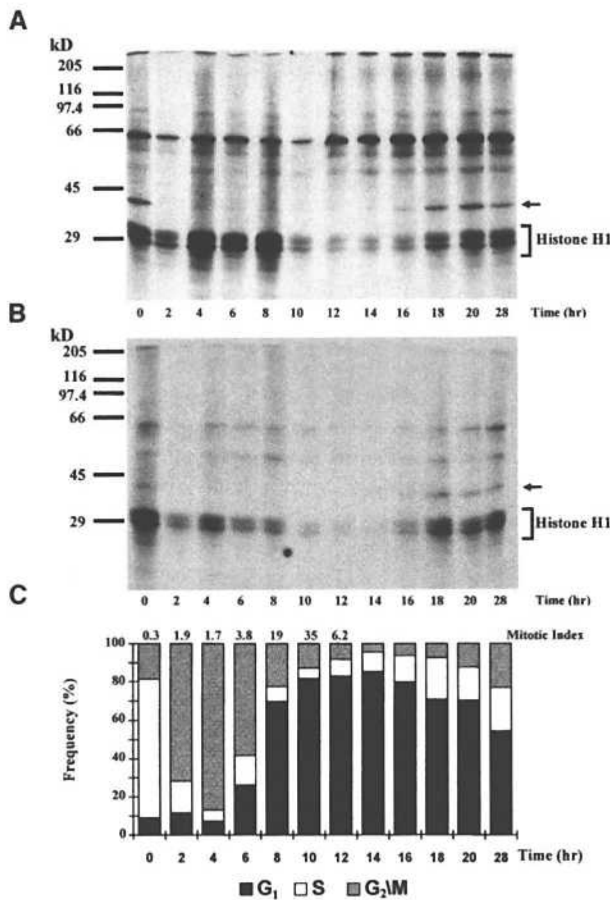

Figure 5. Cell Cycle–Dependent CDK Activities in Aphidicolin-Synchronized Alfalfa (A2) Cell Suspension.

In **(A)** and **(B)**, aphidicolin (20 μg/mL) was used as the blocking agent to synchronize the alfalfa suspension culture. Samples were taken at the indicated time points after release from the aphidicolin block.

(A) and **(B)** Equal amounts of extracted proteins were bound to p13^{suc1}–Sepharose beads or immunoprecipitated with anti–C-terminal–Cdc2MsA/B antibody, respectively. In both cases, the bound kinase activities were assayed in the presence of histone H1, and the phosphorylated proteins were resolved by SDS-PAGE. The arrows indicate an endogenous protein of ~42 kD, which is visible only in the S-phase fractions in the autoradiograms. Molecular mass markers are indicated at left.

(C) The relative frequency of cells in various cell cycle phases was determined by flow cytometry. The percentage of the mitotic cells was calculated at indicated time points.

presence of additional G_2-to-M phase kinase components in p13^{suc1}–Sepharose–bound fractions that can act together with Cdc2MsA/B kinases in mitotic cells. In both cases, endogenous proteins of similar molecular masses were phosphorylated, and one of them at ~40 to 42 kD was detected only at the S phase.

In a repeated experiment, we were able to analyze cell populations also during progression through the G_1-to-S phase boundary and early S phase, as indicated by the data of flow cytometry (see Figure 6B). The first peak of histone H1 kinase activity detected by immunoprecipitation with the anti-Cdc2MsA/B antibody could be clearly assigned to samples in which the majority of cells were passing G_1-to-S and/or early S phases (Figure 6A). In these samples, we could distinguish the 40- to 42-kD endogenous phosphoprotein that was also detected in the previous experiment. Through S phase, histone H1 kinase activity decreased but peaked again at the onset of the G_2 phase, occurring 12 to 14 hr after releasing the cells from the aphidicolin block.

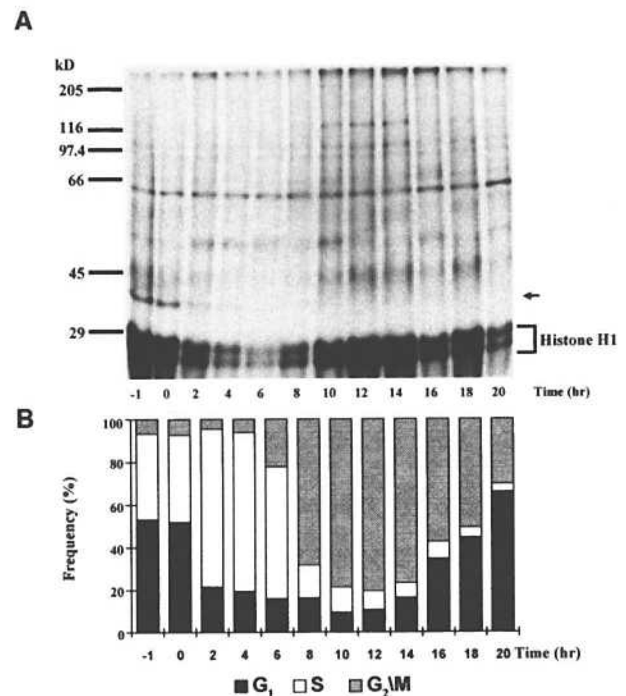

Figure 6. Increased CDK Activities Near the G_1-to-S and G_2-to-M Transition Points in Synchronized Alfalfa (A2) Cell Suspension.

Aphidicolin (20 μg/mL) was used as the blocking agent to synchronize alfalfa cultured cells. Samples were taken at indicated time points before (−1 hr) and after (0 to 20 hr) release from the aphidicolin block.

(A) Equal amounts of extracted proteins were precipitated with anti–C-terminal–Cdc2MsA/B antibody. The bound kinase activities were assayed in the presence of histone H1, and the phosphorylated proteins were resolved by SDS-PAGE. The arrow indicates an endogenous protein of ~42 kD, which is visible only in G_1/S– or early S-phase fractions in the autoradiogram. Molecular mass markers are indicated at left.

(B) The relative frequency of cells at various cell cycle phases was determined by flow cytometry.

To test whether the increased amount of the Cdc2MsF protein detected in G_2-to-M phase cells (Figure 4C) is accompanied by an elevated kinase activity, we immunoprecipitated proteins by using anti-Cdc2MsF peptide antibodies to search for the corresponding kinase complex (Figure 7A). The cell cycle progression after aphidicolin synchronization in this experiment was monitored by flow cytometry and determination of the mitotic index (Figure 7B). The histone H1 kinase activities were definitely restricted to samples with a high frequency of mitotic cells. Based on this strong correlation, we propose that Cdc2MsF is a mitotic kinase exhibiting characteristic phosphorylation of endogenous proteins in the complex.

In the above-described experiments, the protein complexes separated either by p13^{suc1}–Sepharose affinity binding or immunoprecipitation by kinase antibodies were found to be active in phosphorylation of histone H1 and several endogenous proteins. The active state of kinases in these fractions is expected to depend on the presence of a cyclin partner. To test the complex formation between alfalfa cyclin and kinase partners and to identify CDK complexes, we immunoprecipitated proteins from synchronized cells by anti-mitotic cyclin (CycMs2) peptide antibodies. We previously published that the *cycMs2* gene encodes a mitotic cyclin with elevated mRNA level in G_2-to-M phase cells (Hirt et al., 1992). As shown in Figure 8A, the kinase complexes precipitated with anti-cyclin antibodies are active in the phosphorylation of histone H1 and several alfalfa proteins. The histone H1 kinase activity fluctuated during the cycle, with cells at the G_2-to-M phase having increased activity. Several characteristic phosphoproteins can be detected in these complexes. These data demonstrate the formation of cyclin–CDK complexes in plants. Further studies are needed to identify the interacting cyclin and kinase partners in various phases of the cycle.

DISCUSSION

The current models of cell division control in eukaryotes stem primarily from genetic and molecular studies with yeast, Drosophila, Xenopus, and mammals. The major achievements in the discovery of basic regulatory components and their molecular structure support a unified view of the eukaryotic cell cycle control and emphasize the phylogenetic conservation of the key regulatory pathways (Murray and Kirschner, 1989; Nurse, 1990). In accordance with this concept, significant progress has been achieved in the isolation of plant genes encoding homologs of CDKs and cyclins (reviewed in Doerner, 1994; Jacobs, 1995). The results of gene isolation studies with a wide range of plant species have provided comprehensive amino acid sequence information about plant CDKs. An evolutionary tree based on the overall similarity between different CDKs from yeasts to man has grouped the majority of plant enzymes into a separate branch of the phylogram (see details in the review article by Dudits et al., 1997). The typical representatives of this group are those plant CDKs that exhibit the PSTAIRE element and frequently form closely related pairs as Cdc2MsA and Cdc2MsB in alfalfa. This group of plant kinases is located close to CDKs from animals in the similarity tree and shows a considerable distance from the yeast homologs.

The key determinants in the regulation of CDK functions have been extensively studied by using different approaches, such as mutant analysis, biochemical characterization, and recently, determination of the crystal structure of the human CDK apoenzyme and of the human cyclin A–CDK2–ATP complex (DeBondt et al., 1993; reviewed in Morgan, 1995). Based on the crystal structure, the cyclin A–CDK2 interface comprises elements including the α1-helix with the PSTAIRE sequence motif, the T loop with Thr-160 and Thr-161 as the phosphorylation site, and part of the

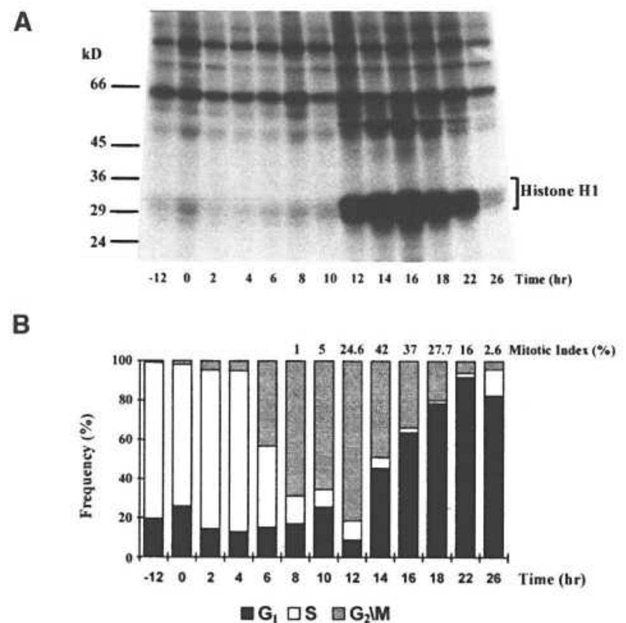

Figure 7. Elevated Histone H1 Kinase Activities in Protein Complexes Immunoprecipitated by Anti–C-Terminal-Cdc2MsF Antibodies from Mitotic Alfalfa Cells.

Aphidicolin (20 μg/mL) was used as the blocking agent to synchronize the alfalfa suspension culture. Samples were taken at the indicated time points before (−12 hr) and after (0 to 26 hr) release from the aphidicolin block.

(A) Equal amounts of extracted proteins were precipitated with anti–C-terminal-Cdc2MsF antibodies. The bound kinase activities were assayed in the presence of histone H1, and the phosphorylated proteins were resolved by SDS-PAGE. Molecular mass markers are indicated at left.

(B) The relative frequency of cells at various cell cycle phases was determined by flow cytometry and calculation of mitotic index.

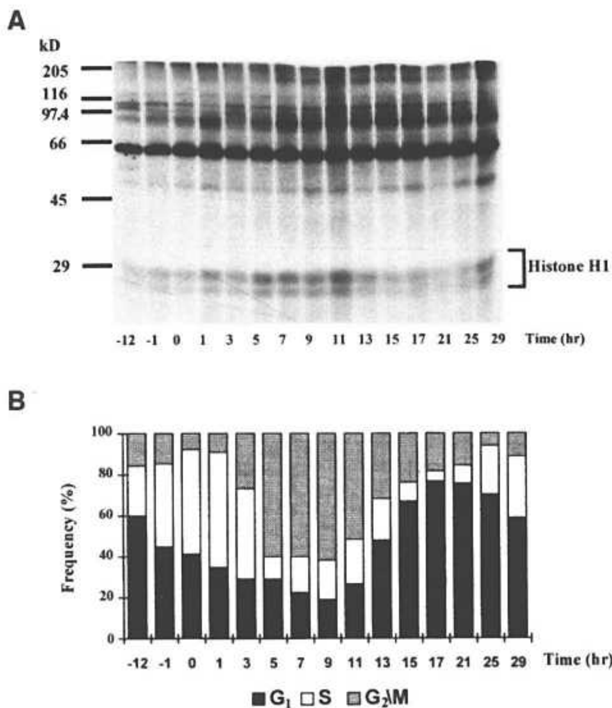

Figure 8. Elevated Histone H1 Kinase Activities at G_2-to-M Phase in Protein Complexes Immunoprecipitated by Anti–C-terminal-CycMs2 Antibodies.

Aphidicolin (10 μg/mL) was used as the blocking agent to synchronize the alfalfa suspension culture. Samples were taken at the indicated time points before (−12 and −1 hr) and after (0 to 29 hr) release from the aphidicolin block.

(A) Equal amounts of extracted proteins were precipitated with anti–C-terminal-CycMs2 antibodies. The bound kinase activities were assayed in the presence of histone H1, and the phosphorylated proteins were resolved by SDS-PAGE. Molecular mass markers are indicated at left.

(B) The relative frequency of cells at various cell cycle phases was determined by flow cytometry.

N-terminal β-sheet and C-terminal lobe from CDK2 (Jeffrey et al., 1995). The amino acid sequence in the PSTAIRE region has been correlated with the cyclin interaction (see Pines, 1994), and a mutation here abolished cyclin binding (Ducommun et al., 1991). The deduced amino acid sequence data in Figure 1 show that of six alfalfa kinase variants, only two exhibit the typical PSTAIRE element found in the majority of known plant CDKs. Similarly, high variation can be recognized in the T-loop region among the alfalfa CDKs. The functional significance of this extended variability in both regions of the alfalfa enzymes has not been studied experimentally. It may be the background of selectivity in binding cyclin partners, as has been shown in other cell systems (Sherr, 1993). The two G_2-to-M phase–dependent alfalfa kinases (Cdc2MsD and Cdc2MsF) exhibit a nontypical PSTAIRE motif: the PPTALRE and PPTTLRE sequence elements have only been identified in CDKs of other plant species, such as Arabidopsis and Antirrhinum (Hirayama et al., 1991; Fobert et al., 1994; Shaul et al., 1996). The PITALRE element detected here in the Cdc2MsC protein as well as in a pea kinase (Feiler and Jacobs, 1991) can be considered as a phylogenetically conserved domain because it has been found in two related human proteins, such as CHED and PITALRE with known functions in proliferating cells (Lapidot-Lifson et al., 1992; Grana et al., 1994).

Despite the growing number of cloned plant cDNAs homologous to cell cycle control genes, the biochemical and functional characterization of the related kinase complexes and cyclin partners is very limited. Expression of plant homologs of *cdc2* genes has primarily been analyzed by in situ hybridization and histochemical studies with *cdc2* promoter–driven β-glucuronidase reporter gene fusions (Martinez et al., 1992; Hemerly et al., 1993; Fobert et al., 1994). The results clearly indicate that the distribution of transcripts is according to the cell division activity in organs and tissues of Arabidopsis and Antirrhinum. Parallel with our experiments, further in situ hybridization analysis of Arabidopsis and Antirrhinum *cdc2* homologs suggested a cell cycle phase–specific expression pattern of *cdc2MsD*- and *cdc2MsF*-related genes (Fobert et al., 1996; Segers et al., 1996). In our study, we provide direct evidence supporting these observations based on the analysis of phase dependency during cell cycle progression in synchronized alfalfa cell culture. Our previous attempts to detect phase-dependent variation in transcript levels of the alfalfa *cdc2* gene revealed constant mRNA amounts in all cell cycle phases (Magyar et al., 1993). In this study, our RNA gel blot hybridization analyses indicated the constitutive pattern of expression for the *cdc2MsA*, *cdc2MsB*, *cdc2MsC*, and *cdc2MsE* gene variants and showed preferential transcript accumulation at G_2-to-M phases in the case of *cdc2MsD* and *cdc2MsF* genes. These essential differences in the activity among the individual CDK genes during cell cycle progression may reflect a complex regulatory system based on a set of kinase genes with characteristic expression profiles.

In this study, we not only demonstrate the existence of a multicomponent CDK family in a single plant species, alfalfa, but also show that the two CDK gene variants (*cdc2MsD* and *cdc2MsF*) exhibit both high transcript levels and elevated protein accumulation during the G_2-to-M phases. Furthermore, the presented phosphorylation experiments (Figure 7A) demonstrate that protein complexes immunoprecipitated from the G_2-to-M phase cells by anti–C-terminal Cdc2MsF antibodies exhibit high histone H1 phosphorylation activities. Considering the apparent specificity of the PPTALRE and PPTTLRE motifs to plants and the tight control of *cdc2MsD* and *cdc2MsF* transcript levels in the G_2-to-M phase, we propose that these CDKs represent a unique type of kinases not found in other eukaryotic organisms to date.

In yeast cells, the *cdc2* mRNAs are present at constant levels throughout the cell cycle and during exit from the cell

cycle (Durkacz et al., 1986; Simanis and Nurse, 1986). In mammalian cells, transcriptional control of CDK genes is an important component in regulating new synthesis of kinases (McGowen et al., 1990). The *cdc2* transcription and mRNA accumulation were highest in S and G_2 phases and decreased at late G_2 or early M phase in HeLa cells (Dalton, 1992). In contrast, we detected high *cdc2MsD* and *cdc2MsF* mRNA levels in late-G_2 and M phase alfalfa cells. The functional significance of the Cdc2MsD and Cdc2MsF proteins remains to be determined. We postulate that the described G_2-to-M phase kinases play a special role in plant cells accumulated at the G_2 phase of the cycle. As indicated by cytometric data from analyses of nuclei of dormant pea buds or of mesophyll cells from petunia leaves, plant cells can stop the cycle at the G_2 phase (Bergounioux et al., 1992; Devitt and Stafstrom, 1995). The reactivation of the cycle in these cells may rely on the function of these G_2-to-M kinases required for progression in cycling cells as well.

The hypothesis of different kinase complexes involved in different phases of the plant cell cycle is also supported by biochemical data. The results of in vitro phosphorylation studies (Figures 5 to 8) show the phase dependency for all analyzed complexes. Previous studies had already indicated peaks of activity in the S and the G_2-to-M phases (Magyar et al., 1993). The synchronization experiments in this study allowed us to analyze cell populations enriched in early S phase as well as G_2-to-M and the G_1-to-S phase transitions. Phosphorylation of both histone H1 and endogenous substrates indicated elevated kinase activities in protein fractions from cells at G_1-to-S or early S and G_2-to-M phases.

Our findings show that in the G_2-to-M phases, both the PSTAIRE kinases (Cdc2MsA/B) and the PPTTLRE kinase (Cdc2MsF) play roles. With the time resolution of these experiments, we recognized a certain sequence in activity peaks of the kinase variants. The Cdc2MsA/B kinases were already active in the mid-G_2 phase, and these activities lessened as the frequency of mitotic cells increased (Figure 5B). However, the Cdc2MsF kinase exhibited the highest histone H1 phosphorylation in mitotic cells (Figure 7A). Based on the diversity of the detected kinases with differential phosphorylation activities, further functional studies are needed to show their putative regulatory roles in the cell cycle of flowering plants.

Although the terminology of CDK is used in the case of plant kinases encoded by *cdc2/CDC28* homologs, none of the Cdc2-related plant kinases thus far has been shown to form complexes with cyclin and to possess cyclin-dependent activity. In general, these kinases are identified as CDKs on the basis of amino acid sequence identity (>40%), the presence of characteristic functional domains, and the ability to complement yeast cell cycle mutants. Our immunoprecipitation experiments with anti–C-terminal CycMs2 antibodies (Figure 8A) provide direct evidence for complex formation between this mitotic cyclin and a kinase with high histone H1 phosphorylation activity in cells at the G_2-to-M phases. Further experiments are in progress to identify the cyclin and CDK partners that preferentially interact at defined phases of the plant cell cycle.

METHODS

Plant Material

A highly homogenous, fast-growing cell suspension culture of *Medicago sativa* subsp *varia* (genotype A2; tetraploid) was established and maintained in the presence of 1 mg/L 2,4-D and 0.2 mg/L kinetin, according to Bögre et al. (1988).

Gene Isolation and DNA Sequencing

λ GEM2 (Promega) and λ ZAPII (Stratagene) cDNA libraries were constructed from mRNAs isolated from 2,4-D–induced alfalfa (RA3; tetraploid) suspension cultures (Dudits et al., 1991). A third library was made from mRNA of alfalfa somatic embryos, kindly provided by H. Hirt (Institute of Microbiology and Genetics, University of Vienna, Vienna, Austria). These three libraries were screened with an ~0.4-kb XmnI-KpnI fragment of the *cdc2MsA* cDNA clone (Hirt et al., 1991), containing the characteristic PSTAIRE and the catalytic kinase domains. Gel-purified inserts were labeled with α-^{32}P-CTP by the random priming method, and low-stringency hybridization was performed at 55°C. The cDNA inserts subcloned into pBluescriptII SK− plasmids (Stratagene) were sequenced by the dideoxy chain termination method (Sanger et al., 1977), using an automatic DNA sequencer (model No. 373; Applied Biosystem, Foster City, CA). The missing 5′ ends of the cDNA clones were isolated using the Marathon cDNA cloning kit (Stratagene).

Synchronization of the Cell Cycle, Flow Cytometric Analysis, and Determination of the Mitotic Index

For synchronization, the alfalfa cell suspension (A2) was treated with aphidicolin (Sigma) at a final concentration of 20 or 10 μg/mL for 36 hr. Thereafter, the cells were washed three times with fresh Murashige and Skoog medium (Murashige and Skoog, 1962) and cultured further for synchronous growth (Magyar et al., 1993). The isolation of nuclei and the flow cytometric analysis were performed according to Savouré et al. (1995). The mitotic index was determined microscopically on ethanol–acetic acid—fixed cells after carbol fuchsin staining.

RNA Preparation and Gel Blot Analysis

Total RNA was extracted according to the freezing phenol method (Maes and Messens, 1992), with a slight modification to scale the method to the volume of Eppendorf tubes. Frozen alfalfa suspension cells (A2) were homogenized under liquid nitrogen in a small mortar with 0.5 mL of phenol (equilibrated to pH 4.9 with 3 M K-acetate). After diluting with 0.5 mL of 1% SDS, the samples were incubated for 15 min at 65°C and then centrifuged for 10 min in an Eppendorf centrifuge at 4°C. The supernatant was extracted with phenol-chloroform twice and precipitated with one-quarter volume of 8 M LiCl for overnight incubation at 4°C. The precipitates were dissolved in 100 μL of diethyl pyrocarbonate–treated water, and a second LiCl pre-

cipitation was applied to remove residual DNA contamination. Total RNA was quantified by optical density at 260 nm (Sambrook et al., 1989). Twenty micrograms of total RNA was loaded on 1% formaldehyde gel containing 0.01% ethidium bromide. Transferring RNAs to Hybond N filters (Amersham) was performed with the capillary action technique (Sambrook et al., 1989), and the filters were examined under UV light to verify the efficiency of transfer and to test the quality and quantity of loaded RNA samples. Hybridization of the filters was performed in Rapid-hyb buffer (Amersham) at 65°C. Radiolabeled probes were generated by random-primed ^{32}P-labeling from the following: fragments containing the coding regions of *cdc2MsC*, *cdc2MsD*, *cdc2MsF*, and *cdc2MsE* genes and fragments containing the 3′ nontranslated regions of histone H3-1 (Kapros et al., 1992), *cdc2MsA*, and *cdc2MsB* (Hirt et al., 1991, 1993).

Protein Extraction, p13^{suc1}–Sepharose Affinity Binding, and Histone H1 Kinase Assays

Alfalfa cells (A2) were harvested at indicated time points and either used immediately or snap-frozen in liquid nitrogen and stored at −70°C. Proteins were extracted by grinding cells with quartz sand in homogenization buffer containing 25 mM Tris-HCl, pH 7.5, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 15 mM *p*-nitrophenylphosphate, 60 mM β-glycerophosphate, 1 mM DTT, 0.1% Nonidet P-40, 0.1 mM Na_3VO_4, 0.5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL each leupeptin, aprotinin, and antipain, and 5 μg/mL both pepstatin and chymostatin. Equal amounts of total protein were incubated with 100 μL of 25% (v/v) p13^{suc1}–Sepharose beads overnight at 4°C on a rotary shaker. After incubation, the p13^{suc1}–Sepharose beads were washed three times with washing buffer and once with kinase assay buffer, and the kinase reaction was initiated by the addition of a 30-μL reaction mixture containing a 1 mg/mL histone H1 substrate and 2.5 μCi of γ-^{32}P-ATP. Detailed descriptions of these methods have been given previously (Magyar et al., 1993).

Preparation of Antibodies, Immunoblotting, and Immunoprecipitations

Polyclonal antibodies raised against the C-terminal EYFKDIKFVP peptide of alfalfa Cdc2Ms A/B (common sequence motif; Hirt et al., 1991, 1993), the C-terminal CFLLENKNQP peptide of alfalfa CyclinMs2 (Hirt et al., 1992), as well as against either the C-terminal PYFDSLDKSQY peptide of Cdc2MsD or the C-terminal DDLDKTHL peptide of Cdc2MsF were raised in rabbits by using synthetic peptides coupled to Keyhole limpet hemocyanin through an additional cysteine residue at their N termini.

Crude IgG fractions were separated from whole sera by ammonium sulphate fractionation. The IgG fractions were further purified on peptide-coupled affinity columns as described previously (Harlow and Lane, 1988). For immunoblotting, the SDS–polyacrylamide gels were transferred onto polyvinylidene difluoride (Millipore, Bedford, MA) membranes in 50 mM Tris-base–50 mM boric acid buffer at a constant 30 V overnight. The filters were blocked in 5% milk powder–0.05% Tween 20 in TBS (25 mM Tris-Cl, pH 8.0, 150 mM NaCl) buffer for 2 hr at room temperature, reacted with the first antibody at a concentration of 1 μg of IgG per mL of blocking buffer for 2 hr at room temperature, washed three times with TBST (0.2% Tween 20 in TBS, pH 8.0), and reacted with alkaline phosphatase–conjugated goat anti-rabbit IgG by using the dilution recommended by the manufacturer (Sigma). Signals were developed by the standard nitro blue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate staining method.

Determination of the specificity of the purified antibodies was performed by competition experiments. In this case, the purified antibodies were preincubated with 10-fold molar excess of the relevant synthetic peptides for 2 hr on ice before the immunoreaction in the protein gel experiments. For immunoprecipitation, equal amounts of protein samples in homogenization buffer (see above) were precleared with 60 μL of 25% (v/v) protein A–Sepharose beads (Pharmacia) for 1 hr at 4°C on a rotary shaker. After a short centrifugation, the supernatants were transferred to new Eppendorf tubes containing 2 to 4 μg of anti–C-terminal alfalfa antibodies (anti-Cdc2MsA/B, anti-Cdc2MsF, or anti-CycMs2) and incubated at 4°C for 1 hr. In the following step, 50 μL of 50% (v/v) suspension of protein A–Sepharose was added, and the tubes were gently shaken for 1 hr at 4°C. Thereafter, the beads were washed three times with RIPA buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 300 μM phenylmethylsulfonyl fluoride, and 10 μg/mL both aprotinin and leupeptin, and the histone H1 kinase reaction was performed as described by Magyar et al. (1993).

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