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cycMs3, a Novel B-Type Alfalfa Cyclin Gene, Is Induced in the G_0-to-G_1 Transition of the Cell Cycle

Irute Meskiene,[a,1] László Bögre,[a,1] Marlis Dahl,[a] Manfred Pirck,[a] Dang Thi Cam Ha,[a,2] Ines Swoboda,[a] Erwin Heberle-Bors,[a] Gustav Ammerer,[b] and Heribert Hirt [a,3]

[a] Vienna Biocenter, Institute of Microbiology and Genetics, Dr. Bohrgasse 9, A-1030 Vienna, Austria
[b] Vienna Biocenter, Institute of Biochemistry and Molecular Cell Biology, Dr. Bohrgasse 9, A-1030 Vienna, Austria

Cyclins are key regulators of the cell cycle in all eukaryotes. We have previously isolated two B-type cyclin genes, *cycMs1* and *cycMs2*, from alfalfa that are primarily expressed during the G_2-to-M phase transition and are most likely mitotic cyclin genes. Here, we report the isolation of a novel alfalfa cyclin gene, termed *cycMs3* (for cyclin *Medicago sativa*), by selecting for mating type α-pheromone–induced cell cycle arrest suppression in yeast. The central region of the predicted amino acid sequence of the *cycMs3* gene is most similar to the cyclin box of yeast B-type and mammalian A- and B-type cyclins. In situ hybridization showed that *cycMs3* mRNA can be detected only in proliferating cells and not in differentiated alfalfa cells. When differentiated G_0-arrested cells were induced to reenter the cell cycle in the G_1 phase and resume cell division by treatment with plant hormones, *cycMs3* transcript levels increased long before the onset of DNA synthesis. In contrast, histone H3-1 mRNA and *cycMs2* transcripts were not observed before DNA replication and mitosis, respectively. In addition, *cycMs3* mRNA was found in all stages of the cell cycle in synchronously dividing cells, whereas the *cycMs2* and histone H3-1 genes showed a G_2-to-M phase– or S phase–specific transcription pattern, respectively. These data suggest that the role of cyclin CycMs3 differs from that of CycMs1 and CycMs2. We propose that CycMs3 helps control reentry of quiescent G_0-arrested cells into the G_1 phase of the cell cycle.

INTRODUCTION

A highly refined picture of eukaryotic cell cycle regulation has emerged in recent years. At the center of this regulatory network is the $p34^{cdc2}$ protein, the catalytic subunit of a conserved serine/threonine protein kinase, which was originally identified in fission and budding yeast. In these yeasts, a single kinase allows both the G_1-to-S and G_2-to-M phase transitions (for reviews, see Nurse, 1990; Nasmyth, 1993), whereas several related kinases, called cyclin-dependent protein kinases (CDKs), have evolved in animals (for review, see Pines, 1993) and higher plants (for review, see Hirt and Heberle-Bors, 1994). CDKs are not active by themselves (Poon et al., 1993) but become activated by association with regulatory subunits termed cyclins. Cyclins are cell cycle stage–specific activators of CDKs that appear to alter their substrate specificity (Peeper et al., 1993), their availability to upstream regulators such as the Wee1 kinase (Booher et al., 1993), and their intracellular localization (Maridor et al., 1993). Cyclins act in different phases of the cell cycle, due primarily to stage-dependent regulation of their synthesis (Amon et al., 1994) and specific degradation (Glotzer et al., 1991; Tyers et al., 1992), whereas other cyclins do not vary in abundance during the cell cycle (Bueno et al., 1991; Leopold and O'Farrell, 1991; Lew et al., 1991; Xiong et al., 1991; Tamura et al., 1993; Tyers et al., 1993).

Cyclins can be grouped according to sequence similarities that were originally believed to reflect different functional properties: the A-type cyclins acting in S and G_2 phases; B-type cyclins acting at the G_2-to-M phase transition; and D- and E-type cyclins acting at the G_1-to-S phase transition (for reviews, see Pines, 1993; Sherr, 1993). However, recent findings in fission and budding yeasts have challenged this view: B-type cyclins, such as $Cig1^+$ and $Cig2^+$ of *Schizosaccharomyces pombe*, act exclusively in the G_1 phase (Bueno et al., 1991; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994); CLB5 and CLB6 of *Saccharomyces cerevisiae* function predominantly in S phase (Schwob and Nasmyth, 1993). It was therefore proposed that B-type cyclins represent a prototype from which the other more specialized cyclins evolved with roles in, for example, budding in *S. cerevisiae* or coupling to developmental signals, such as growth factors, in animals (Schwob and Nasmyth, 1993).

To date, most plant cyclins have been isolated based on sequence similarities with animal A- and B-type cyclins (Hata et al., 1991; Hemerly et al., 1992; Hirt et al., 1992; Day and

[1] I.M. and L.B. have contributed equally to this work.
[2] Current address: Institute of Biotechnology, National Center for Natural Science and Technology, Nghia do Tu'liem, Hanoi, Vietnam.
[3] To whom correspondence should be addressed.

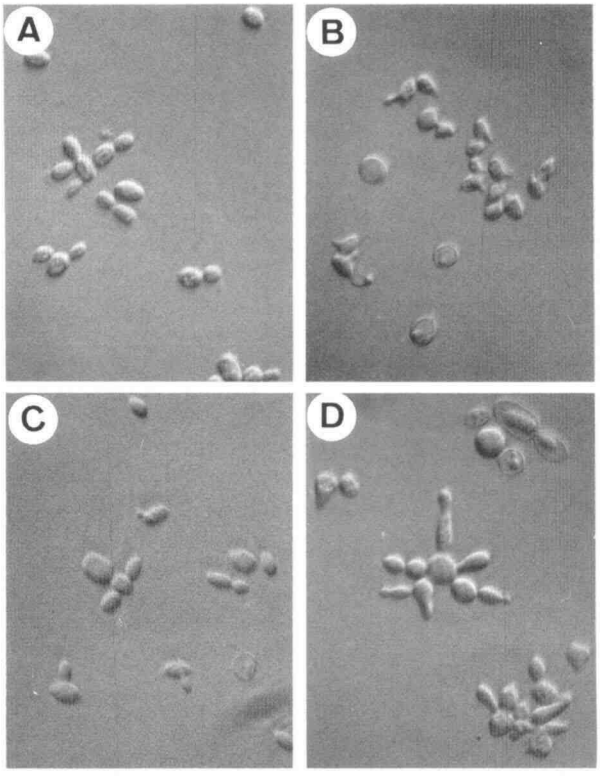

Figure 1. Alfalfa *cycMs3* Overrides Pheromone-Induced Cell Cycle Arrest in Yeast.

(A) In the absence of α-pheromone, *S. cerevisiae* GA2201 cells divide normally.
(B) Four hours after addition of α-pheromone, *S. cerevisiae* GA2201 cells show elongated morphology (shmoo phenotype) and are G_1 arrested.
(C) In the absence of α-pheromone, *S. cerevisiae* GA2201 cells containing ectopically expressed *cycMs3* divide normally.
(D) Four hours after the addition of α-pheromone, *S. cerevisiae* GA2201 cells containing ectopically expressed *cycMs3* show shmoo phenotype but still divide.

Reddy, 1994; Ferreira et al., 1994; Fobert et al., 1994; Renaudin et al., 1994). In functional Xenopus oocyte injection assays, Arabidopsis, maize, and soybean cyclin clones were found to induce oocyte maturation and were therefore reported to be mitotic cyclins (Hata et al., 1991; Hemerly et al., 1992; Renaudin et al., 1994). Expression analysis by RNA gel blot hybridization using synchronized alfalfa suspension-cultured cells and in situ hybridization with Antirrhinum floral meristems have shown that certain cyclin transcripts are restricted to cells in the G_2-to-M phase transition, indicating a mitotic function (Hirt et al., 1992; Fobert et al., 1994).

G_1 cyclins are much less conserved than A- and B-type cyclins, which have diverged very little in their central domain. To identify such G_1 cyclins that might act earlier than the G_2-to-M transition, we have used a yeast selection system that requires the functional interaction of heterologous plant cyclins with the yeast Cdc28 protein kinase (Whiteway et al., 1992) at the G_1-to-S phase transition. This approach exploits the fact that haploid yeast cells are prepared for mating by cessation of cell division due to the binding of a secreted peptide pheromone to cell surface receptors. We assumed that expression of an alfalfa G_1 cyclin in yeast would override the cell cycle block imposed by α-pheromone and allow continued growth. Here, we report a novel alfalfa cyclin gene, *cycMs3* (for cyclin *Medicago sativa*), whose product enables yeast cells to overcome this pheromone-induced cell cycle arrest. In contrast to the transcript levels of the mitotic cyclin genes *cycMs1* and *cycMs2*, which appear transiently in the G_2 and M phases, *cycMs3* mRNA was found in all cell cycle stages of proliferating alfalfa cells but was barely detectable in differentiated cells. When differentiated cells were induced to reenter the G_1 phase of the cell cycle from a G_0-arrested state, *cycMs3* mRNA levels increased well before the onset of DNA replication, suggesting a role early in the cell cycle.

RESULTS

Selection of Alfalfa Genes That Overcome Pheromone-Induced Cell Cycle Arrest in Yeast

To effectively select higher plant genes that might be involved in the G_1 phase of the cell cycle, we sought genes that when cloned in yeast would override the α-pheromone arrest of MATa yeast cells and allow normally pheromone-sensitive yeast cells to form colonies on medium containing the α-pheromone. To eliminate most spontaneous mutations that might result in α-pheromone resistance in the yeast host, we constructed a homozygous yeast diploid strain, GA2201. This strain is hypersensitive to pheromone because it lacks the major α-pheromone–degrading enzyme, Bar1, and contains *MATa* at both the silent and expressed mating type loci: thus, it cannot become *MATα* by mating type switching, which would lead to α-pheromone resistance. A cDNA library was prepared from an alfalfa cell suspension culture in a galactose-inducible yeast expression vector; the vector was used to transform this library into yeast strain GA2201. Transformants were selected for colony formation on media containing both the α-pheromone and galactose. Eight cDNA clones conferred galactose-regulated pheromone resistance. None of these affected the yeast phenotype in the absence of the α-pheromone (Figures 1A and 1C). However, yeast cells expressing these cDNA clones continued to grow and divide in the presence of up to 50 μg/mL α-pheromone, whereas cells transformed with the empty yeast–*Escherichia coli* shuttle vector were completely blocked by 1 μg/mL.

Normally, the addition of a pheromone has multiple effects on yeast: it results in the activation of a transcriptional program with characteristic morphological changes (projection formation, called "shmoo") and arrest in the G_1 phase of the

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cycMs1   MK-FSEEKNVSNN-PTNFEGGLD------SRKVGENRRALGVINQNLVVEGRPYP
cycMs2   .VNT...N.SNAVM.RK.Q..MNQVGHGGG.I..Q.R.ALGGI..NF.H-.....
cycMs3   ..EVAPGTQDQKQPLRANSKRAL---SDVTYLPHIKRAILQDVTNNCGVNTKRSC

cycMs1   CVV-----NKRALSERNDVCEKKQADPVHRPITRRFAAKIANTKTTNAEGTT---
cycMs2   ...-----H..V...KHEI.......LG.............GSQQSY..K.----
cycMs3   LNPTEIQAK..KVAKPAQPHVSN-EV.SAAE-LPP.I.DSKPVSSMEMRLRSSED

cycMs1   -------KRSNLAKSSSNGFGDFIFVDDEHKPVEDQPVPMALEQTEPMHSESDQM
cycMs2   -------.N..PL--NL.E..NS.AI...L.SP....E..T..H......DPLE.
cycMs3   FRCLDDLEDNAPFRM...QC.TNNNLLQS-QTSRISAR.LSSQKKASQIVAAKKG

cycMs1   EEVEMEDIMEEP-VMDIDTPDANDPLAVAEYIEDLYSYYRKVESTSCVSPNYMAQ
cycMs2   ........EG.M-IL...SC...NS...V......HA....I.YLG....T..DE
cycMs3   NIS.LL.VSKH.D.A...A-.FE..QLCSH.AA.I.DHL.VA.LSRRPY..F.ET

cycMs1   -QFDINERMRAILVDWLIEVHDKFDLMHETLFLTVNLIDRFLEKQSVVRKKLQLV
cycMs2   -.L.L.....................Q.............A..N.........
cycMs3   V.Q.ITPS.........V..SEGYK.QAN..S...Y...W..S.NCIE.ER...L

cycMs1   GLVAMLLACKYEEVSVPVVGDLILISDRAYTRKEVLEMEKVMVNALKFNISVPTA
cycMs2   ..................S...H.A.......DI.....L.L.T.QY.M.L...
cycMs3   .ITC..I.T....INA.RIK.FCF.Q.NT..KE..VKL.SLVLKSSSYQLFA..T

cycMs1   YVFMRRFLKAA-----QADRKLELLAFFLIELSLVEYAMLKFSPSQLAAAAVYTA
cycMs2   ..........-----...K....V....VD......E....P..LV........
cycMs3   KT.L....R.GTSFYKRPSIE..Y..NY.A..T.MN.GF.N.L..MV..SS.FL.

cycMs1   QCTMYGVKQ-WSKTCEWHTNYSEDQLLECSS-LMVDFHKKAGTGKLTGAHRKYCT
cycMs2   ...VS.F.H-.N.................M-...G..Q...A.....V....GS
cycMs3   RW.LDQSSHP.NP.L.HYAS.KASD.KATVLA.QDLQLNSNDDCP..TIRK..TQ

cycMs1   SKFSYTAKCEPASFLLENEL
cycMs2   A...F.......C.....KNQP
cycMs3   D.LNCV.-ALSSPK...TLF
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Figure 2. Alignment of Predicted Protein Sequences of Alfalfa CycMs1, CycMs2, and CycMs3.

Dashes indicate gaps introduced for optimal alignment. Amino acid residues that are identical to the CycMs1 sequence are shown by dots. The destruction box motifs at the N terminus are shown in boldface letters. The cyclin box region is indicated by black bars underlining the sequence. The PEST-rich regions of CycMs1, CycMs2, and CycMs3 are found at positions 137 to 180, 123 to 180, and 171 to 187, respectively. The *cycMs1*, *cycMs2*, and *cycMs3* nucleotide and predicted amino acid sequences were submitted to GenBank, EMBL, and DDBJ and have accession numbers X82039, X82040, and X85783, respectively.

cell cycle, as shown for the untransformed GA2201 cells (Figure 1B). Expression of any of the eight alfalfa cDNAs still allowed yeast cells to shmoo but not to arrest their cell cycle (Figure 1D). These data show that the products of these alfalfa cDNAs specifically interfere with the yeast cell cycle machinery.

cycMs3 Is a Novel Alfalfa Cyclin Gene

Partial sequencing, restriction mapping, and hybridization revealed four different classes of inserts. One cDNA clone containing a 1.5-kb insert exhibited strong homology with cyclins and was therefore selected for further study. The translational start codon of the longest open reading frame of 1143 nucleotides in this clone is not preceded by an in-frame stop codon, suggesting that it is incomplete. To obtain a full-length cDNA, another cDNA library that had been derived from somatic alfalfa embryos was hybridized with this 1.5-kb cDNA. The longest hybridizing clone, designated *cycMs3*, contains an insert of 1.9 kb that perfectly matched the open reading frame of the previously isolated cDNA and extended this open reading frame by 220 bp. Several in-frame stop codons upstream of the first possible ATG indicated that this clone does indeed represent a full-length cDNA sequence. This was supported by alfalfa RNA gel blot analysis, which revealed a single 2-kb hybridizing transcript (data not shown). The open reading frame in this clone could encode a 452–amino acid polypeptide (Figure 2).

Previously, we had isolated truncated cDNA clones encoding the majority of two different alfalfa cyclins, CycMs1 and CycMs2 (Hirt et al., 1992). To compare the *cycMs3* sequence with *cycMs1* and *cycMs2*, the same alfalfa embryo cDNA library was also screened with radiolabeled *cycMs1* and *cycMs2* cDNA fragments. The longest cDNA isolated, which corresponds to *cycMs1*, is 1.7-kb long, and it contains an open reading frame of 1287 nucleotides with upstream stop codons and a 281-nucleotide-long 3′ nontranslated region with a short poly(A)$^+$ tail, indicating that it is also full length. The predicted CycMs1 protein is 428 amino acids long (Figure 2). Similarly, the *cycMs2* cDNA clone recovered by hybridization is 1.75 kb and is identical to the published *cycMs2* cDNA in the overlapping region (Hirt et al., 1992). The first translational start codon of the open reading frame of *cycMs2* is preceded by several in-frame stop codons, indicating that this cDNA carries the entire open reading frame. The CycMs2 protein is predicted to be 434 amino acids long (Figure 2).

Alignment of the three cyclins over their entire lengths (Figure 2) shows 72% identity between CycMs1 and CycMs2 and 28 to 29% identity between these cyclins and CycMs3. Considering only the 154–amino acid cyclin box, CycMs1 and CycMs2 show 84% identity with each other and 41 and 42% with the CycMs3 sequence, respectively. Gel blot analysis with genomic DNA from two different alfalfa lines, *Medicago sativa* ssp *varia* cv Rambler lines RA3 and A2, indicated that the *cycMs1*, *cycMs2*, and *cycMs3* genes are each present in one copy per haploid alfalfa genome (data not shown).

CycMs1, CycMs2, and CycMs3 Belong to Different Cyclin Classes

The cyclin boxes of CycMs1, CycMs2, and CycMs3 show 33 to 45% identity with the cyclin boxes of yeast B-type and mammalian A- and B-type cyclins. A comparison of CycMs1 and CycMs2 with the currently known sequences from other plant species shows the highest identity (65 to 72%) with maize Cyc3 (Renaudin et al., 1994) and Arabidopsis Cyc2a and Cyc2b (Ferreira et al., 1994). For CycMs3, the highest identity (49 to 55%) is with Arabidopsis Cyc3a and Cyc3b (Ferreira et al., 1994), carrot Cyc1 (Hata et al., 1991), and maize Cyc2 (Renaudin et al., 1994). Despite these considerable similarities in the cyclin boxes, no homology was observed in the N-terminal 180 amino acids, except for a short nonameric motif that is conserved in all plant cyclins known to date (see later discussion).

Yeast B-type and mammalian A- and B-type cyclins contain a highly conserved proteolytic "destruction" box motif near their

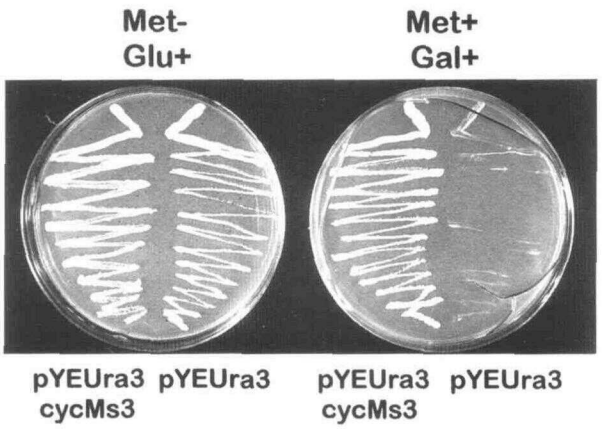

Figure 3. Alfalfa *cycMs3* Complements the Function of G_1 Cyclins in Yeast.

S. cerevisiae K3414 (*cln1 cln2 cln3 MET-CLN2*), containing pYEUra3, can divide only on methionine-free medium (Met−). *S. cerevisiae* K3414, containing pYEUra3–*cycMs3*, can grow in methionine-containing (Met+) and methionine-free medium. Glu+, glucose-containing medium; Gal+, galactose-containing medium.

N termini; it is used for ubiquitin-mediated degradation of these cyclins at the end of mitosis (Glotzer et al., 1991). This destruction box contains the nine–amino acid consensus motif RXA/VLGXIX(X)N (X stands for any amino acid). CycMs3 contains the sequence RAILQDVTN at its N terminus. This sequence resembles the consensus motif, but only three amino acids are identical. PEST-rich regions, enriched in proline, glutamic acid, serine, and threonine, are also found in the N-terminal regions of the alfalfa CycMs1, CycMs2, and CycMs3 protein sequences. The presence of a PEST-rich sequence renders a protein proteolytically unstable (Rogers et al., 1986); these sequences are found at the N or C terminus of many G_1 cyclins (Tyers et al., 1993).

cycMs3 Cyclin Can Replace Yeast G_1 Cyclin

Because the yeast pheromone response pathway acts through G_1 cyclins, we tested whether *cycMs3* could complement the function of the *S. cerevisiae* G_1 cyclins (CLNs). A truncated version of CycMs3, which was missing the first 73 amino acids (constituting the mitotic destruction box and part of the PEST region) and a full-length version of CycMs3 (pYEUra3-CycMs3 and pYEUra3-CycMs3FL, respectively) were expressed in the *S. cerevisiae* K3413 strain (Amon et al., 1993). This strain lacks genes for the G_1 cyclins CLN1 and CLN3 and carries CLN2 under the control of the *met3* promoter, thus allowing its expression on methionine-free medium. The absence of all three cyclins is lethal in yeast. Growth of K3413 cells carrying the truncated *cycMs3* cDNA clone on methionine-containing medium depended on galactose induction of the cloned gene (Figure 3). This indicated that CycMs3 can perform the CLN function and promote the G_1-to-S phase transition in the yeast cell cycle. Expression of the full-length *cycMs3* version under the same conditions led to slightly weaker growth (data not shown). We postulated that this reflects the importance of the N terminus of CycMs3 for its stability.

cycMs3 mRNA Is Predominantly Present in Actively Dividing Tissues

The cell cycle phase–regulated genes histone H3-1 (Kapros et al., 1992) and *cycMs2* (Hirt et al., 1992) are expressed exclusively in actively dividing plant tissues. To study the expression of the *cycMs3* gene in different organs, the transcript levels of this gene were compared with those of the histone H3-1 and *cycMs2* genes. mRNA was isolated from different alfalfa organs and used in RNA gel blot analysis using radiolabeled fragments from *cycMs3*, histone H3-1, *cycMs2*, and *Msc27* (Figure 4). As with the other cell cycle–regulated histone H3-1 and *cycMs2* genes, *cycMs3* was expressed predominantly in plant tissues containing dividing cells, such as root, flower bud, young leaf, and suspension-cultured cells. However, compared with *cycMs2*, *cycMs3* showed high expression in root.

To compare these data with the expression pattern of the *cycMs3* gene in different tissues and developmental stages, alfalfa plants were analyzed by in situ hybridization. All experiments were performed with sense and antisense probes for the histone H3-1, *cycMs2*, and *cycMs3* genes. In all cases, and under the conditions used, hybridization signals were obtained only with the antisense probes. In general, and in agreement with RNA gel blot analysis, expression of the *cycMs3* gene was detected mainly in actively dividing tissues, such as in young leaf and the root tip shown in Figures 5A and 5G, respectively. No tissue-specific expression pattern was observed for any of the three genes in the investigated material. In young

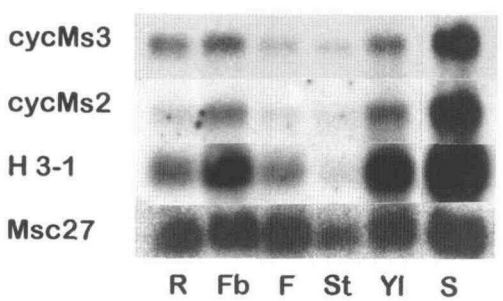

Figure 4. Expression of Alfalfa *cycMs2*, *cycMs3*, and Histone H3-1 Genes in Different Plant Organs.

RNA gel blot analysis of *cycMs3*, *cycMs2*, and histone H3-1 gene expression in alfalfa roots (R), flower buds (Fb), flowers (F), stems (St), young leaves (Yl), and suspension-cultured cells (S) is shown. Poly(A)$^+$ RNA that had been prepared from 100 μg of total RNA was loaded in each lane. As a control, a radiolabeled fragment of the *Msc27* gene was hybridized to the same blot.

leaves, only a subset of cells was found to contain *cycMs2* (Figure 5B) or histone H3-1 (Figure 5C) transcripts, representing the fraction of cells that were entering mitosis or undergoing DNA replication, respectively. This pattern of histone H3-1 and *cycMs2* gene expression resembles that seen for histone H4 and the mitotic cyclin 1 and cyclin 2 genes in Antirrhinum (Fobert et al., 1994). In contrast, the majority of young leaf cells showed *cycMs3* mRNA, although at varying levels (Figure 5A). This expression pattern is typical for genes that show little cell cycle phase–specific regulation, such as the *cdc2* gene (Martinez et al., 1992). In mature leaves, in which DNA replication and cell division had stopped, no *cycMs3*, *cycMs2*, or histone H3-1 transcripts were detected (Figures 5D, 5E, and 5F, respectively). These results indicate a proliferation-dependent expression of the histone H3-1, *cycMs2*, and *cycMs3* genes. The cell-specific pattern of the histone H3-1 and *cycMs2* genes correlates with the specific expression of the genes during S phase and the G_2-to-M phase transition, respectively. In contrast, the presence of *cycMs3* mRNA in most cells of young leaves but the complete absence in mature leaves suggest a relatively constitutive expression in all phases of the cell cycle but repression under nondividing conditions.

The root tip of alfalfa contains the most active center of dividing cells but also regions with no mitotic activity, such as the root cap and elongation zone. Therefore, it presents an ideal organ to study cell cycle phase–specific gene expression. In situ hybridization of root tips with either histone H3-1 (Figure 5I), *cycMs2* (Figure 5H), or *cycMs3* (Figure 5G) probes revealed expression of these genes in the actively dividing cells only. In some cases, histone H3-1 (Figures 5I and 5L) or *cycMs2* (Figure 5H) mRNA was detected in files of meristem cells, indicating a certain degree of synchrony in the cell cycles of the cells that were derived from a common founder cell. In differentiating cells of the elongation zone (data not shown) or the root cap, where cells had stopped dividing, no *cycMs3*, *cycMs2*, or histone H3-1 transcripts were detected (Figures 5G, 5H, and 5I, respectively). In contrast to the spotty cell-specific histone H3-1 and *cycMs2* transcript pattern in the root meristem, the majority of dividing cells were found to have considerable *cycMs3* transcript (Figure 5G), suggesting that *cycMs3* gene expression is restricted to proliferating cells but not to a certain cell cycle phase. These conclusions are corroborated by analyzing in situ hybridized root tip sections at high magnification after staining with diamidinophenylindole. The highest *cycMs2* transcript levels were found in cells that were in prophase of mitosis (Figures 5K and 5N). Histone H3-1 transcripts were observed only in cells that were in interphase (Figures 5L and 5O). In contrast, *cycMs3* transcript was found in cells of all cell cycle stages (Figures 5J and 5M). In summary, the data from leaves and root tips are consistent with the notion that all three investigated genes are expressed primarily in dividing tissues, but that in contrast to the *cycMs2* and histone H3-1 genes, which show a cell cycle phase–dependent expression pattern, *cycMs3* gene expression underlies minor phase-specific regulation.

cycMs3 mRNA Is Present in All Phases of the Cell Cycle in Proliferating Cells

The expression of *cycMs3* was studied in cells that were arrested at specific cell cycle stages because many cyclins show a cell cycle phase–specific transcription pattern corresponding to their function. For this purpose, suspension-cultured alfalfa cells were treated for 24 hr with hydroxyurea or oryzalin. Hydroxyurea blocks the G_1-to-S phase transition, and oryzalin prevents the completion of metaphase (Figure 6). RNA gel blot analysis of hydroxyurea- and oryzalin-blocked cells and of cells that were allowed to progress into S phase after removing hydroxyurea showed that *cycMs3* mRNA was present at comparable levels in all three stages. By contrast, histone H3-1 (the S phase–specific probe; Kapros et al., 1992) and *cycMs2* (the G_2-to-M phase–specific probe; Hirt et al., 1992) transcripts were found predominantly in S and M phase cells, respectively. These results are not in agreement with those from inhibitor studies by Ferreira et al. (1994). However, their use of a 2-day treatment of root tips with hydroxyurea or oryzalin and the lack of data on cell cycle parameters make it difficult to evaluate fully their report.

To exclude the possibility that these inhibitors influence transcript levels by processes other than the cell cycle, RNA gel blot analysis was performed with synchronous cell populations generated by first arresting cell division (G_1-to-S phase transition) with aphidicolin and then removing aphidicolin to allow synchronous entry into S phase and subsequent cell cycle steps. The level of synchrony was monitored by flow cytometric analysis (Figure 7A) and ^{3}H-thymidine incorporation into newly replicated DNA (Figure 7C) as well as by determining the percentage of cells in mitosis (Figure 7C). A radiolabeled *cycMs3* probe as well as histone H3-1 and *cycMs2* were hybridized to the same filter prepared from poly(A)$^+$ RNA purified from the synchronized cells at different time points (Figure 7B). In agreement with the aforementioned experiments in which we found that *cycMs3* mRNA was present in equal amounts in cells arrested by drug treatments at S and M phases, *cycMs3* transcripts could also be detected in cells passing synchronously through the S, G_2, and M phases of the cell cycle. The *cycMs3* transcript level decreased somewhat after cells had exited M phase. However, at the 27-hr time point, when cells were still in the G_1 phase and no DNA synthesis was apparent, *cycMs3* mRNA levels had increased to levels comparable to those of cells at the G_1-to-S phase transition (Figure 7B, 0 hr). By contrast, the *cycMs2* mRNA level correlated with the increase in the percentage of cells entering mitosis, with a peak value at 12 hr after release from the aphidicolin block. The expression of the histone H3-1 gene is coupled to DNA synthesis, which was high after release from the aphidicolin block and then at 30 hr, when cells reenter the S phase in the second synchronous cycle. The regulated expression of the M phase–specific *cycMs2* and the S phase–specific transcript H3-1 levels convincingly demonstrates the synchronous division cycle of the suspension culture after

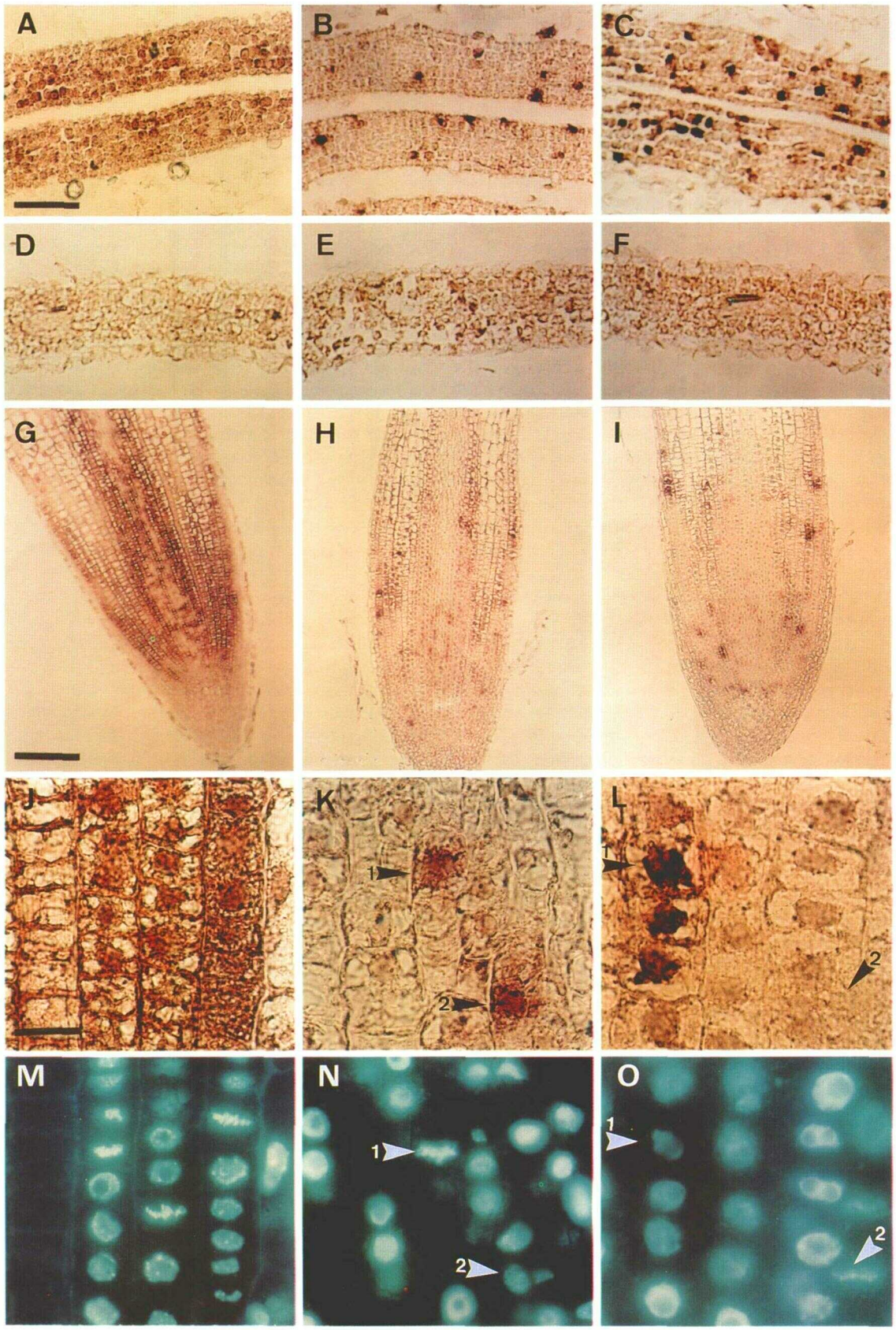

Figure 5. In Situ Hybridization of Alfalfa Leaves and Root Tips.

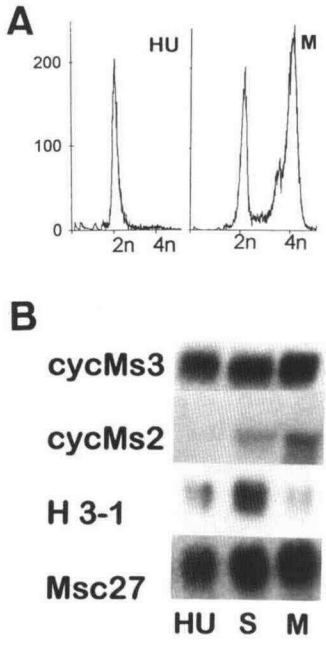

Figure 6. Alfalfa *cycMs3* Transcripts Are Equally Present in G_1-to-S Phase Transition and in S and M Phase Cells.

Alfalfa cells were arrested at the G_1-to-S phase transition with 4 mM hydroxyurea (HU) for 24 hr, released from the block for 3 hr into S phase (S), or arrested in metaphase (M) with 1 μM oryzalin for 24 hr.
(A) Flow cytometric analysis. Cells with 2n and 4n DNA content are in G_1 and G_2 phases, respectively. Intermediate DNA values represent cells in S phase.
(B) RNA gel blot analysis of *cycMs3*, *cycMs2*, histone H3-1, and *Msc27* genes.
Twenty micrograms of total RNA was loaded in each lane.

release from the aphidicolin block. These data indicate that, in contrast to histone H3-1 and *cycMs2*, the *cycMs3* transcript is present during all phases.

To achieve synchrony without any drug use, suspension cells were transiently arrested in G_1 by phosphate starvation and then allowed to enter S phase by restoring phosphate. RNA gel blot analysis of these cells showed an increase of histone H3-1 mRNA (Figure 8A). Twenty hours later, the cells had moved through the S and G_2 phases (Figure 8B), as indicated by the strong increase in the *cycMs2* transcript levels (Figure 8A). *cycMs3* mRNA levels in phosphate-starved G_1 phase cells (Figure 8A at 0 hr) were slightly lower than those in the S and G_2 phases (6 and 20 hr, respectively). The apparent increase in *cycMs3* transcript levels shortly after refeeding of the cells (Figure 8A at 0.2 hr) might indicate mRNA degradation during phosphate starvation or transcriptional activation when cells reentered the cell cycle from the G_0 phase.

In summary, *cycMs3* expression was abundant in all phases of the cell cycle but only if the cells were proliferating. In contrast, *cycMs2* transcript levels oscillated strongly during the cell cycle and were abundant only in the G_2 and M phases.

Figure 5. (continued).

Longitudinal sections (10 μm thick) of young and mature alfalfa leaves and root tips were hybridized with digoxigenin-labeled antisense and sense fragments of *cycMs3*, *cycMs2*, and histone H3-1.
(A) Bright-field microscopy of a young leaf that was hybridized with a *cycMs3* antisense fragment.
(B) Bright-field microscopy of a young leaf that was hybridized with a *cycMs2* antisense fragment.
(C) Bright-field microscopy of a young leaf that was hybridized with a histone H3-1 antisense fragment.
(D) Bright-field microscopy of a mature leaf that was hybridized with a *cycMs3* antisense fragment.
(E) Bright-field microscopy of a mature leaf that was hybridized with a *cycMs2* antisense fragment.
(F) Bright-field microscopy of a mature leaf that was hybridized with a histone H3-1 antisense fragment.
(G) Bright-field microscopy of a root tip that was hybridized with a *cycMs3* antisense fragment.
(H) Bright-field microscopy of a root tip that was hybridized with a *cycMs2* antisense fragment.
(I) Bright-field microscopy of a root tip that was hybridized with a histone H3-1 antisense fragment.
(J) Bright-field microscopy and high magnification of the same root tip section shown in **(G)** that was hybridized with a *cycMs3* antisense fragment.
(K) Bright-field microscopy and high magnification of the same root tip section shown in **(H)** that was hybridized with a *cycMs2* antisense fragment. Arrowheads 1 and 2 indicate stained metaphase and prophase cells, respectively.
(L) Bright-field microscopy and high magnification of same root tip section shown in **(I)** that was hybridized with a histone H3-1 antisense fragment. Arrowhead 1 indicates a stained interphase cell; arrowhead 2 indicates a nonstained metaphase cell.
(M) Epifluorescence microscopy of the same longitudinal section shown in **(J)** that was stained with 4′,6-diamidino-2-phenylindole.
(N) Epifluorescence microscopy of the same longitudinal section shown in **(K)** that was stained with 4′,6-diamidino-2-phenylindole. Arrowheads indicate stained metaphase (1) and prophase (2) cells.
(O) Epifluorescence microscopy of the same longitudinal section shown in **(L)** that was stained with 4′,6-diamidino-2-phenylindole. Arrowhead 1 indicates a stained interphase cell; arrowhead 2 indicates a nonstained metaphase cell.
Bar in **(A)** = 50 μm for **(A)** to **(F)**. Bar in **(G)** = 100 μm for **(G)** to **(I)**. Bar in **(J)** = 10 μm for **(J)** to **(O)**.

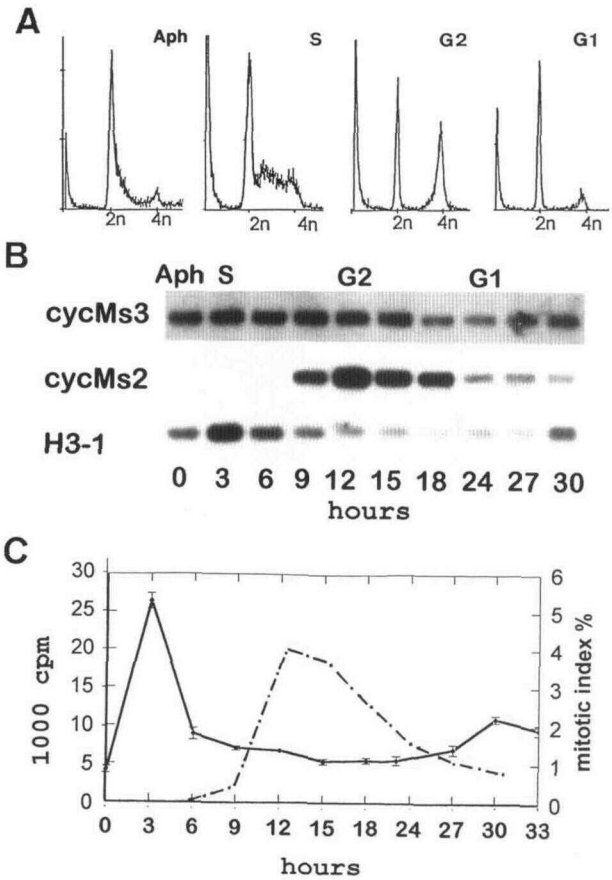

Figure 7. Alfalfa *cycMs3* Transcripts Are Present in All Phases of Synchronously Dividing Cells.

Aphidicolin-arrested cells (0 hr) were released and grown synchronously for one complete cell cycle (33 hr).

(A) Flow cytometric analysis of aphidicolin-arrested cells (Aph), S phase cells 3 hr after their release (S), G_2 phase cells 12 hr after their release (G_2), and G_1 phase cells 24 hr after their release (G_1). Definitions of 2n and 4n are as given in the legend to Figure 6A.

(B) RNA gel blot analysis of *cycMs3*, *cycMs2*, and histone H3-1 genes in synchronously growing cells after their release from G_1-to-S phase transition–arrested cells. One microgram of poly(A)$^+$ RNA was loaded in each lane.

(C) ^{3}H-thymidine incorporation analysis (closed line) and mitotic index (dashed line) of a synchronized cell culture.

cycMs3 Gene Expression Is Induced before DNA Synthesis in Mitogenically Stimulated Leaf Cells

To investigate *cycMs3* gene expression during reentry into the cell cycle from a quiescent G_0 state, pieces of fully differentiated leaves were incubated with mitogenic concentrations of auxin and cytokinin. Flow cytometric analysis (Figure 9B) showed that more than 95% of the cells had a 2C DNA content, indicating that they had exited the cell cycle in G_1. In situ ^{3}H-thymidine labeling showed that DNA replication resumed 3 days after hormone treatment (Figure 9A). RNA gel blot analysis at different time points over a period of 6 days (Figure 9C) showed that *cycMs3* mRNA was already present in low amounts in noninduced tissues and increased within 1 hr of incubation and reached a plateau of maximal expression after 24 hr. Histone H3-1 mRNA appeared only at the time that DNA replication started. *cycMs2* mRNA became detectable only when mitosis began. At later time points when cells were proliferating, *cycMs3* mRNA levels remained constant (Figure 9C at 3 and 6 days). The induction of *cycMs3* gene expression within 1 hr after mitogen stimulation, which was days before the onset of S phase and histone H3-1 expression, also suggests that *cycMs3* may help to regulate the reentry of quiescent cells into the G_1 phase of the cell cycle.

DISCUSSION

Conservation of the basic mechanisms of the eukaryotic cell cycle has enabled the isolation of components that regulate

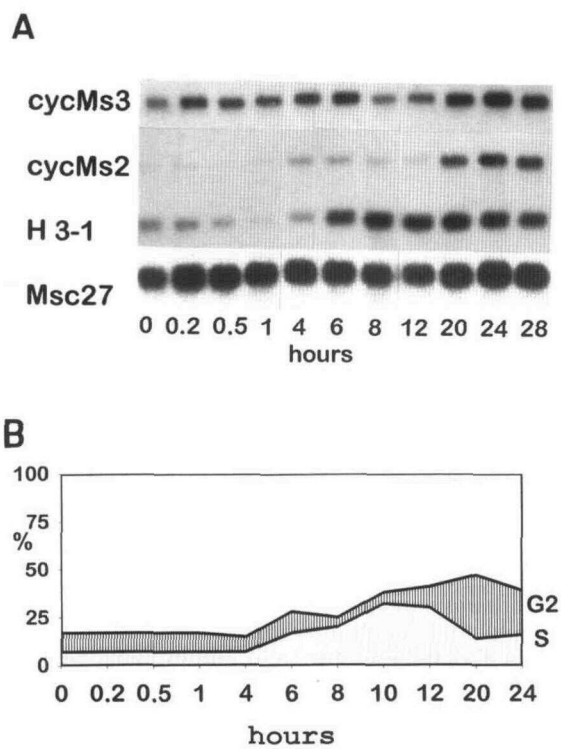

Figure 8. Alfalfa *cycMs3* Transcripts Are Induced in Phosphate-Starved G_1 Phase Cells and Subsequent Stages of the Cell Cycle.

(A) RNA gel blot analysis of *cycMs2*, *cycMs3*, histone H3-1, and *Msc27* genes in synchronously dividing cells after release from G_1 arrest. Poly(A)$^+$ RNA was prepared and loaded as given for Figure 4.

(B) The percentage of cells after release from G_1 arrest in S phase (shaded) and G_2 phase (hatched) by flow cytometry.

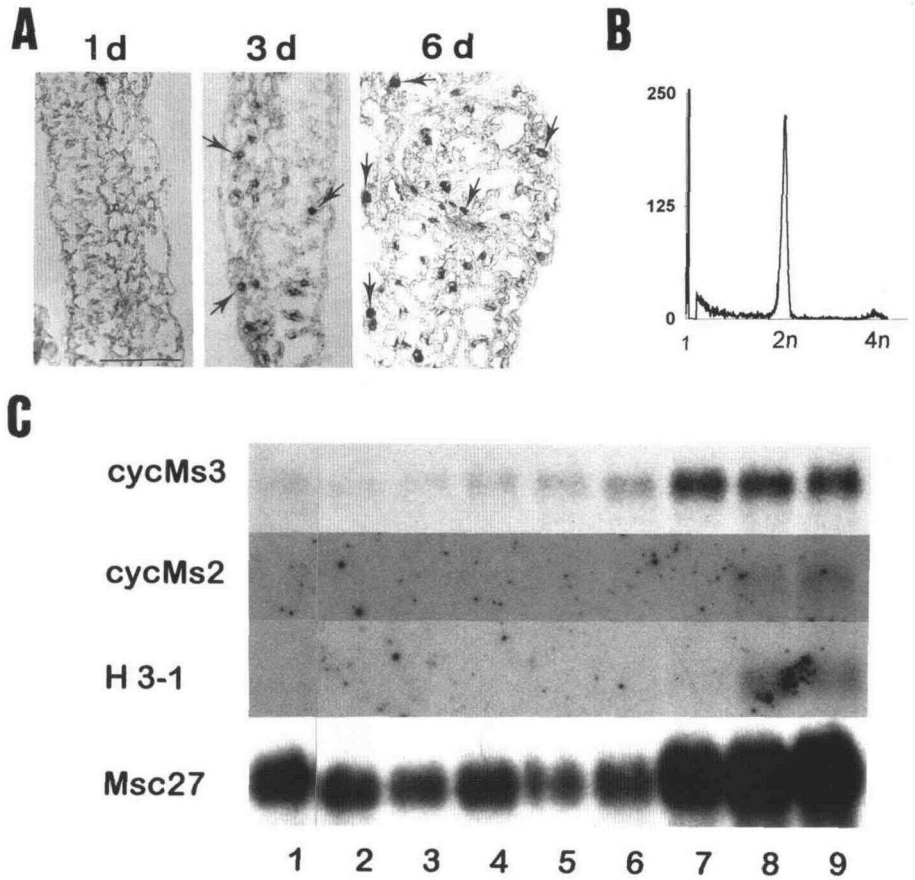

Figure 9. Alfalfa *cycMs3* Is Induced Long before DNA Synthesis in Mitogen-Stimulated, Fully Differentiated Alfalfa Leaf Cells.

Alfalfa leaf pieces were incubated in Murashige and Skoog medium containing 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.2 mg/L kinetin.
(A) Autoradiography of ^{3}H-thymidine pulse-labeled leaf pieces after 1, 3, and 6 days (d). Arrows indicate cells in S phase. Bar = 75 μm.
(B) Flow cytometric analysis of mature leaf. Definitions of 2n and 4n are as given in the legend to Figure 6A.
(C) RNA gel blot analysis of *cycMs3*, *cycMs2*, histone H3-1, and *Msc27* genes in mitogen-stimulated leaf pieces. Lanes 1 to 9 correspond to 0, 10, and 30 min, 1, 4, and 12 hr, and 1, 3, and 6 days.

the cell cycle from a wide range of organisms. On the basis of sequence conservation, two cyclin-dependent protein kinases and two mitotic cyclin genes have been isolated from alfalfa (Hirt et al., 1991, 1992, 1993). To isolate cell cycle regulatory components with little sequence conservation, we used a selection system based on the pheromone-induced G_1 phase arrest of the yeast cell cycle. Expression of several alfalfa cDNAs led to pheromone-resistant cell division. One of the cDNA clones encoded a novel alfalfa cyclin, *cycMs3*, that structurally resembles B-type yeast cyclins but is potentially involved in the reentry of G_0 phase–arrested alfalfa cells into the cell cycle.

Sequence comparison of CycMs3 with other cyclins in protein data banks revealed the highest similarity to be with mammalian A- and B-type and yeast B-type cyclins. However, these similarities do not extend beyond the generally conserved cyclin box. Known plant cyclin sequences can be divided into four groups according to sequence similarity. One class contains the alfalfa mitotic cyclins CycMs1 and CycMs2 (Hirt et al., 1992; this study), Cyc2a and Cyc2b from Arabidopsis (Ferreira et al., 1994), and the Cyc3 cyclins from maize (Renaudin et al., 1994), whereas the Arabidopsis Cyc1 (Hemerly et al., 1992), maize Cyc1a and Cyc1b (Renaudin et al., 1994), and soybean (Hata et al., 1991) and Antirrhinum (Fobert et al., 1994) cyclins belong to a second class of mitotic cyclins. Alfalfa CycMs3, Arabidopsis Cyc3a and Cyc3b (Ferreira et al., 1994), maize Cyc2 (Renaudin et al., 1994), and a carrot cyclin (Hata et al., 1991) form a third class. All of these cyclin classes show highest identity scores with yeast B-type cyclins and with animal A- and B-type cyclins. The fourth class of cyclins consists of the Arabidopsis δ-type (Soni et al., 1995) and alfalfa CycMs4 cyclins (M. Dahl, L. Bögre, I. Meskiene, D.T.C.

Ha, I. Swoboda, H. Hirt, and E. Heberle-Bors, manuscript in preparation), which are most similar to mammalian D-type cyclins.

Until recently, it was assumed that new cyclins with homology to mitotic cyclins would function at the G_2-to-M phase transition. This idea has been questioned because B-type cyclins with destruction box motifs from *S. cerevisiae* (Epstein and Cross, 1992; Schwob and Nasmyth, 1993) and *S. pombe* (Bueno et al., 1991; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994) were found to function at earlier steps in the cell cycle. Although the CycMs3 sequence is most similar to B-type cyclins, the analysis of *cycMs3* gene expression in suspension-cultured cells and plants showed a different pattern compared with other mitotic plant cyclins, suggesting a different function. In actively dividing meristems and in synchronized alfalfa cells, the histone H3-1 and the *cycMs2* genes showed oscillating transcript levels in different phases of the cell cycle. In contrast, *cycMs3* mRNA levels stayed relatively constant and were present in all phases of the cell cycle. As with the *cycMs2* gene transcripts, *cycMs3* transcripts were found predominantly in dividing tissues. However, high *cycMs3* transcript levels were also found in roots, suggesting that the *cycMs3* gene might also be expressed in nondividing tissues. RNA gel blot analysis of leaves supports this idea because low *cycMs3* transcript levels in mature tissues were detected.

Evidence for a role of CycMs3 in preparing cells to reenter the cell cycle was provided by studying the process of inducing differentiated cells to resume cell division. Whereas *cycMs3* transcript levels started to accumulate within 1 hr, histone H3-1 transcript levels increased only after 3 days and in conjunction with the onset of DNA replication. *cycMs2* expression became detectable slightly later, before the appearance of the first mitoses 3 days after mitogen stimulation. Therefore, induction of the *cycMs3* gene is one of the earliest indications that cells are preparing to reenter the cell cycle program. Supporting evidence for a role of *cycMs3* in the reentry of G_0 cells into G_1 comes from a comparison with the transcript pattern of *cycMs4* that is preceded by expression of *cycMs3.* The immediate transcriptional activation of the *cycMs3* gene in the G_0-to-G_1 transition is compatible with a function of *cycMs3* in the preparation of these cells for cell division. However, because *cycMs3* is expressed during all stages of the cell cycle in actively dividing cells, our current results do not indicate whether *cycMs3* might also function elsewhere in the division cycle.

Differentiated cells of many plants have remarkable totipotency. The majority of mature leaf cells are arrested with a G_1 DNA content but readily reenter the cell cycle after wounding or hormone treatment. Other studies have indicated that *cdc2* expression and the ability to resume cell division are correlated (Martinez et al., 1992; Hemerly et al., 1993). In this respect, it is interesting to note that the expression pattern of *cycMs3* in mitogen-stimulated leaf cells is most similar to that of the *cdc2* gene (data not shown). The low level of expression of the *cycMs3* gene in nonstimulated differentiated leaf cells is reminiscent of the situation found with the yeast *CLN3* cyclin. *CLN3* is also constitutively expressed during the cell cycle and appears to have the special function of monitoring the nutritional state and the size of the cells in G_1 (Tyers et al., 1993). Under appropriate conditions, formation of an active Cln3–Cdc28 kinase complex is required to start a new cell cycle. We propose that the complex of CycMs3 with Cdk determines the competence for division. As in yeast, this cyclin-dependent kinase complex would initiate synthesis of other cyclins and subsequently activate other Cdk–cyclin complexes. Studies of the activation of the CycMs3–Cdk complex are in progress and should clarify the role of CycMs3 in the activation of the cell cycle.

METHODS

Construction of a cDNA Expression Library

cDNA was generated with a Stratagene cDNA kit using 5 μg of poly(A)$^+$ RNA that was isolated from suspension-cultured alfalfa (*Medicago sativa* ssp *varia* cv Rambler, line A2) cells, according to the manufacturer's recommendations (Stratagene). The cDNA was ligated into the EcoRI site of the yeast–*Escherichia coli* shuttle vector λ-Max (Clontech, Palo Alto, CA). After in vitro packaging and transformation into *E. coli*, the phage library was excised in vivo as pYEUra3 plasmids, which were subsequently used for yeast transformation.

Strains and Culture Methods

The parental strains CY2 (*MATα::sup40 HMLa HMRa ho–β-gal ura3 HIS4 ade6-O ade2-1 can1-100 met his3 leu2-3 112 trp1-1 swi6::TRP1 bar1::HISG*) and K2149 (*MATa HMLa HMRa ho–β-gal ura3 HIS4 ade2-1 can1-100 met his3 leu2-3 112 trp1-1 bar1::HISG*) were provided by K. Nasmyth and C. Koch (Institute of Molecular Pathology, Vienna, Austria). Strain K2149, which contained the plasmid containing a *GAL1* promoter–*HO* fusion gene and *URA3*, was mated with strain CY2 to produce the diploid strain GA2201, carrying the *MATa* and silent a mating loci. GA2201 was used for transformation with the alfalfa cDNA expression library.

Standard methods were used for culturing and manipulating yeast. Yeast cell transformation was performed according to Gietz et al. (1992). After transformation, cells were plated onto uracil-free medium (to select the vector marker) containing 2% glucose, and after 2 days, they were replica plated onto uracil-free but galactose-containing medium. After 12 to 16 hr, cells were replica plated onto galactose-containing uracil-free medium containing 1 μg/mL α-pheromone. Resistance to pheromone was selected by growing colonies for 6 days on pheromone-containing medium and streaking out on fresh pheromone-containing plates.

Cloning and Sequencing

Most molecular techniques were performed as described by Sambrook et al. (1989). To isolate full-length cyclin *cycMs1, cycMs2,* and *cycMs3* clones, a cDNA library prepared from somatic alfalfa (*M. sativa*) embryos (Hirt et al., 1993) was screened with the *cycMs1* and *cycMs2* cDNA fragments as previously described (Hirt et al., 1992) or with the *cycMs3*

cDNA fragment that was isolated by the pheromone arrest screen (see earlier discussion). Plasmid pTZ19U was used for subcloning, and a Pharmacia T7 sequencing kit was used for sequence determination (Pharmacia, Uppsala, Sweden). Sequence comparisons were performed at the National Center for Biotechnology Information using the BLAST network service.

Plant Cell Culture, Synchronization, Flow Cytometry, and ^{3}H-Thymidine Incorporation

A suspension culture of alfalfa (*M. sativa* ssp *varia* cv Rambler, line A2) was used (Bögre et al., 1988). A2 plants (Bögre et al., 1990) were propagated under sterile conditions on hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962). Propagation of suspension-cultured cells and induction of leaf explants were performed in Murashige and Skoog medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.2 mg/L kinetin.

Synchronization of cultured cells was performed by treatment with aphidicolin (Sigma) for 24 hr or by phosphate starvation. Aphidicolin was added after 0 and 12 hr to a final concentration of 10 μg/mL at a cell density of 5×10^5 cells per mL. Then cells were washed five times with fresh medium and allowed to grow to the same density for the indicated times. Phosphate starvation of cells was performed as described by Amino et al. (1983). ^{3}H-thymidine incorporation and quantification were done as described by Kapros et al. (1992). Flow cytometric analysis was performed as described by Pfosser (1989) with slight modifications. Cells (10^5) were collected by centrifugation at 200*g* for 2 min, and the pellet was suspended in 0.2 mL of enzyme solution (2% [w/v] cellulase [Onozuka R10, Serva], 10% [v/v] pectinase [Sigma P5146], 0.6 M mannitol, 5 mM $CaCl_2$, 3 mM 2-[*N*-morpholino] ethane sulfonic acid, pH 5.7) and incubated for 1 hr at 37°C to digest the cell walls. Released protoplasts were stained without washing by mixing 0.1 mL of resuspended protoplast suspension in 0.4 mL of staining solution (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 4 μg/mL 4′,6-diamidino-2-phenylindole). Nuclei were released by passage through a needle, and the samples were analyzed for DNA content using a PAS2 flow cytometer (Partec, Münster, Germany).

RNA Extraction and RNA Gel Blot Analysis

Total RNA was isolated from 0.3 to 1 g of suspension-cultured cells or plant parts according to Cathala et al. (1983), and poly(A)$^+$ RNA was isolated from 100 μg of total RNA with poly(T)-containing Dynabeads according to the instructions of the manufacturer (Dynal, Oslo, Norway). Formaldehyde–agarose gel electrophoresis and RNA gel blot analysis were performed using standard protocols (Sambrook et al., 1989). Radiolabeled probes were generated from gene fragments containing coding regions of the *cycMs3* gene, *cdc2A* gene (Hirt et al., 1991), and fragments containing the 3′ nontranslated regions of the histone H3-1 gene by random primed ^{32}P labeling (Hirt et al., 1992; Kapros et al., 1992; S.-C. Wu, unpublished results). As a control, a radiolabeled fragment of the entire *Msc27* cDNA (Pay et al., 1992) was similarly generated.

In Situ Hybridization

For preparation of hybridization probes, a 320-bp EcoRI-HindIII fragment of the 5′ coding region of *cycMs3* and a 132-bp EcoRI-XhoI fragment of the 3′ nontranslated histone H3-1 cDNA (S.-C. Wu, unpublished results) were cloned into pBluescript SK+ vectors. Digoxigenin labeling of sense and antisense probes by in vitro transcription, tissue preparation, and in situ hybridization were as described by Bradley et al. (1993), including modifications by Fobert et al. (1994).

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