

Complementation of a yeast cell cycle mutant by an alfalfa cDNA encoding a protein kinase homologous to p34^{cdc2}

(plant cell cycle/*Medicago sativa* L./plant cell culture/somatic embryogenesis/2,4-dichlorophenoxyacetic acid)

HERIBERT HIRT*†, ANIKÓ PÁY*, JÁNOS GYÖRGYÉY‡, LÁSZLÓ BAKÓ‡, KINGA NÉMETH‡, LÁSZLÓ BÖGRE‡, RUDOLF J. SCHWEYEN*, ERWIN HEBERLE-BORS*, AND DÉNES DUDITS‡

*Institute of Microbiology and Genetics, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria; and †Institute of Plant Physiology, Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, 6701 Szeged, POB 521, Hungary

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ABSTRACT The *cdc2* protein kinase plays a central role in control of the eukaryotic cell cycle of animals and yeasts. We have isolated a cDNA clone (*cdc2Ms*) from alfalfa (*Medicago sativa* L.) that is homologous to the yeast *cdc2/CDC28* genes. The encoded protein is 64% identical to the yeast and mammalian counterparts and shows all the prominent structural features known from these organisms. Antibody raised against a 16-amino acid synthetic peptide with crossreactivity against p34 proteins recognized a 34-kilodalton protein in extracts of alfalfa cells. When transferred into a fission yeast, the plant *cdc2* homolog can complement a temperature-sensitive *cdc2* mutant. Northern analysis revealed higher transcript levels in shoots and suspension cultures than in roots. In addition to the dominant transcript of 1.4 kilobases detected in the poly(A)⁺ fraction, 2.5- and 1.2-kilobase transcripts were detected in total RNA preparations from shoots or somatic embryos. Suspension cultures that were induced to form somatic embryos by an auxin (2,4-dichlorophenoxyacetic acid) showed fluctuations in transcription pattern during the induction period and embryogenesis.

The *cdc2* protein kinase plays an important role in regulation of the cell division cycle of animals and yeasts (for review, see refs. 1 and 2). In the fission yeast *Schizosaccharomyces pombe*, *cdc2* function is required for the transition from G₁ to S phase and from G₂ to M phase (3). In the budding yeast *Saccharomyces cerevisiae*, the homologous gene *CDC28* is required for the completion of "start," a G₁ control point for commitment to cell division (4, 5) and at a later time in the cell cycle (6). Homologs to the yeast *cdc2/CDC28* genes have been reported in a variety of animals (reviewed in ref. 7). Recently, a polymerase chain reaction (PCR) fragment has been isolated from pea that displays high homology to the yeast and animal *cdc2* genes (8). Both the human and the chicken *cdc2* homologs have been shown to complement a fission yeast *cdc2* mutation (9, 10). The *cdc2* protein kinase p34^{cdc2} has been shown to be a component of purified maturation (or M phase) promoting factor in both *Xenopus laevis* (11, 12) and starfish (13). The *cdc2* protein kinase activity, assayed by *in vitro* phosphorylation of histone H1, oscillates during the cell cycle and peaks in M phase both in starfish (14, 15) and in human cells (16). Therefore, it appears that the *cdc2* protein kinase is involved in cell cycle regulation in all eukaryotes so far investigated. The activity of the protein kinase is regulated at the level of phosphorylation (17, 18) and by interaction with other proteins (12, 18–21). Furthermore, by genetic analysis, a variety of genes have been identified that affect *cdc2* function (19, 22–26).

Here we report the isolation and characterization of the *cdc2/CDC28* homologous cDNA from alfalfa[§]. An antibody

directed against a synthetic peptide with amino acid sequence predicted from the cDNA sequence specifically recognizes a 34-kDa protein from alfalfa cells. Complementation of a fission yeast *cdc2* mutant suggests that the yeast and plant *cdc2* genes are functionally interchangeable. Furthermore, our results show that the *cdc2Ms* gene is expressed in a complex manner that is influenced by exogenous application of the synthetic auxin 2,4-dichlorophenoxyacetic acid.

MATERIALS AND METHODS

Amplification by PCR. Two degenerate oligonucleotides, A (GCTGAATTCGGNGAA/GGGIACITAT/CGGNGT) and B (GCGAAGCTTT/CTGIGGT/CTTIAA/GA/GTCNCG/TA/GTG) (where N = G, A, T, or C and I = inosine), were synthesized as primers. PCR was performed on an alfalfa cDNA library, constructed by GC tailing into the *Pst* I site of the vector pGEM2 (Promega), as described (27). After 40 cycles, the reaction products were phenol/chloroform extracted and then digested with *Eco*RI and *Hind*III. The products were separated on 1% agarose gels, and the amplified band was cut out, electroeluted, and ligated to *Eco*RI/*Hind*III-digested pTZ19 vector (Pharmacia). Sequence analysis was carried on both strands with a phage T7 sequencing kit (Pharmacia), according to the manufacturer's instructions.

Complementation of a Fission Yeast *cdc2* Mutant. A *Pst* I fragment containing the entire *cdc2Ms* cDNA was inserted into the *Pst* I site of the yeast/*Escherichia coli* shuttle vector pDB248 (28). In this construct the ATG of the vector sequence can serve as start of the coding sequence ATG CCT GCA GGG GGG GGG GGG GGG GGG GGC GAA AAT The first three codons are derived from pDB248, the seven GGG triplets represent the dG tail from the original cloning procedure, and they are followed by codons from the *cdc2Ms* sequence. The *Sc. pombe cdc2-33 leu1-32 h⁻* strain was transformed as described (29) and selected for leucine prototrophy at 23°C on minimal medium plates for 3 days. Colonies were then shifted to 25, 27, 29, 31, 33, or 35°C for 2 days.

Protein Extraction and Immunoblotting. Alfalfa cells were homogenized with quartz sand in extraction buffer (25 mM Tris-HCl, pH 7.4/15 mM MgCl₂/15 mM EGTA/100 mM NaCl/0.1% Tween 20/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/aprotinin (20 μg/ml)/1 mM Na₂P₂O₇/1 mM NaF/1 mM sodium vanadate), and centrifuged at 250,000 × *g*. The supernatant was applied to a Q-Sepharose column and proteins were eluted with a linear gradient of 0–0.65 M NaCl in chromatography buffer (25 mM Tris-HCl, pH 7.4/5 mM MgCl₂/5 mM EGTA/0.1% Tween 20/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/1 mM Na₂P₂O₇/1 mM NaF/1 mM sodium vanadate).

Abbreviation: PCR, polymerase chain reaction.

†To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M58365).

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Collected fractions were assayed for endogenous phosphorylation and purified further on a Superose 12 FPLC column in 20 mM Tris-HCl, pH 7.4/50 mM NaCl/5 mM EGTA buffer.

Proteins from a 10% gel were transferred to an NC-filter in 10 mM imidazole/30 mM glycylglycine/20% (vol/vol) methanol/0.5% SDS buffer at 2.5 mA/cm² for 2 hr. The filter was soaked first in a saturation buffer [0.1% Tween 20/2% bovine serum albumin/5% fetal calf serum in phosphate-buffered saline (PBS), pH 7.5] for 30 min at room temperature, then at 4°C overnight, and subsequently with the PSTAIR antibody at a 1:750 dilution in saturation buffer for 2 hr (directed against the synthetic peptide Glu-Gly-Val-Pro-Ser-Thr-Ala-Ile-Arg-Glu-Ile-Ser-Leu-Leu-Lys-Glu) then at 4°C overnight. The filter was rinsed three times with 0.1% Tween 20/PBS, then incubated in a 1:1000 dilution of goat anti-rabbit IgG-peroxidase conjugate as second antibody in saturation buffer for 2 hr. After five washes with 0.1% Tween 20/PBS, color was developed according to Wolff *et al.* (30).

Plant Tissue Culture. A suspension culture of the RA3 genotype of *Medicago sativa* L. was used (31). The suspension culture was established from a callus made from stem segments of alfalfa plants and was maintained on SH medium (32) supplemented with 15 μM naphthaleneacetic acid and 10 μM kinetin. For the induction of somatic embryogenesis, the suspension culture was transferred to medium containing 100 μM 2,4-dichlorophenoxyacetic acid instead of naphthaleneacetic acid. After 1 hr, the cells were washed twice and cultured in hormone-free liquid SH medium supplemented with 30 mM proline and 10 mM (NH₄)₂SO₄.

RNA Extraction and Northern Blotting. To isolate total RNA, cells or plant tissues were frozen in liquid nitrogen and extracted by grinding in a mortar with quartz sand and extraction buffer as described (33). Poly(A)⁺ RNA was isolated by two rounds of oligo(dT) column chromatography. Northern analysis was performed with 24 μg of total RNA or with 5 μg of poly(A)⁺ RNA in each lane as described (34). Probes for hybridization were the PCR fragment of *cdc2Ms* or the *Msc27* cDNA, which were labeled by the random primer method (35). The *Msc27* cDNA was used as internal control in quantification of RNA samples. Based on the

nucleotide sequence, the function of this gene cannot yet be determined. According to the Northern analysis data its expression is constant in the alfalfa tissues analyzed.

RESULTS

PCR Cloning of the Alfalfa *cdc2* Homolog. Two degenerate oligonucleotides were synthesized encoding two highly conserved amino acid sequences in all *cdc2/CDC28* homologs (indicated by arrows X and Z in Fig. 1, respectively). After PCR amplification of an alfalfa cDNA library, a fragment of 366 nucleotides was found to be specifically amplified. Cloning and sequencing revealed that the putative amino acid sequence encoded by the amplified fragment has highest similarity to the mammalian *CDC2* genes (64–65% identity; refs. 9 and 10) and the two yeast genes (63.5% identity to *Sc. pombe cdc2* and 64.7% identity to *S. cerevisiae CDC28*; refs. 36 and 37). We therefore call the corresponding gene *cdc2Ms* for *Medicago sativa* L. *cdc2*.

Isolation and Characterization of a *cdc2Ms* cDNA Clone. To isolate the full-length cDNA clone from the cDNA library, we used the amplified *cdc2Ms* fragment as a probe for colony hybridization. Screening of 250,000 colonies yielded five positive clones. The two longest clones, with inserts of ≈1.1 kb, were fully sequenced but were found to be identical. As shown in Fig. 1, the clones contained a perfect match of the fragment isolated by PCR. The 1.1-kb insert contains an open reading frame of 291 amino acids. Alignment of the putative *cdc2Ms* protein sequence with those from human and from fission, and budding yeast (Fig. 2) indicates the absence of the first few amino acids but nonetheless shows clear similarity to the most conserved amino acid regions of the mammalian and yeast *cdc2* proteins. The predicted 148-amino acid sequence from a *cdc2*-homologous PCR fragment of pea (8) differs from the corresponding region of *cdc2Ms* in only 4 amino acids. Specifically, in addition to the regions coding for the domains of the ATP-binding site and the catalytic site of the kinase (arrows A and C in Fig. 2, respectively), a stretch of 16 amino acids was found to comprise the PSTAIR region (arrow B in Fig. 2), which is highly conserved in all *cdc2* homologous genes so far found. Moreover, the functionally

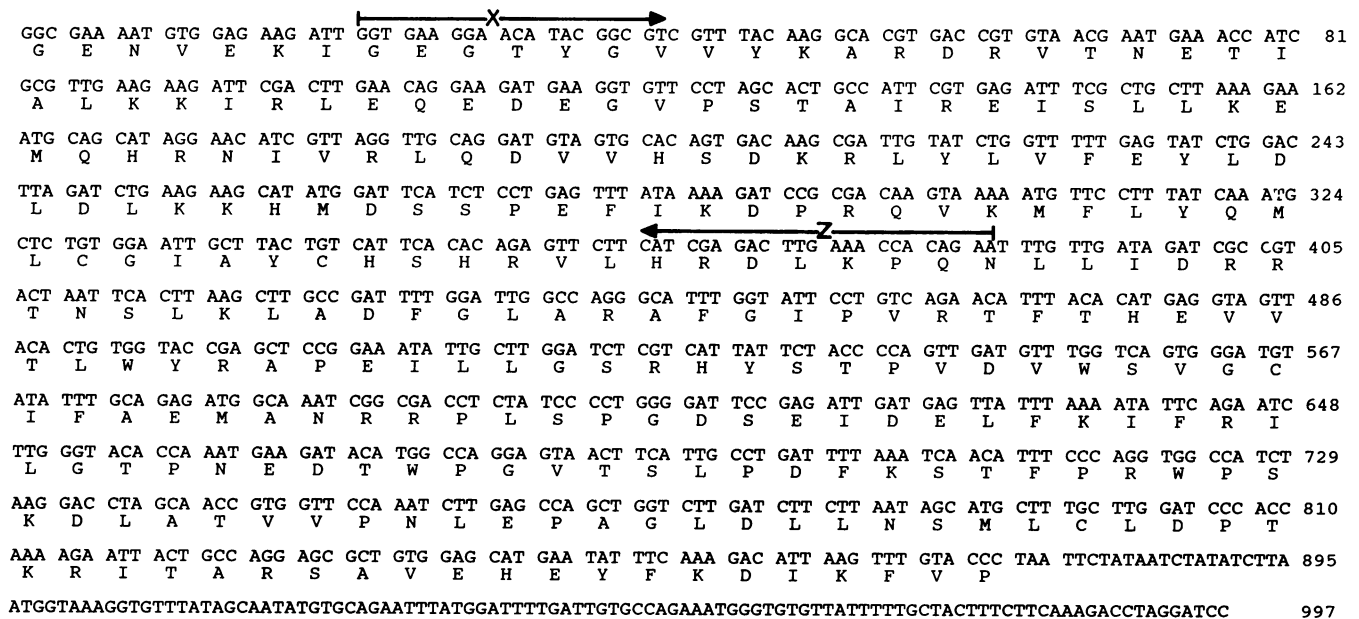


FIG. 1. cDNA and deduced amino acid sequences of alfalfa *cdc2Ms*. Using PCR with primers A and B (arrows X and Z), a 366-nucleotide fragment was isolated. By colony hybridization with the PCR fragment as a probe, a 1.12-kilobase (kb) cDNA clone was isolated. The cDNA sequence shown contains 997 nucleotides. The 3' end of the clone, with another 120 nucleotides, has not been sequenced and is not shown. The open reading frame extends from nucleotide 1 to nucleotide 873 and encodes 291 amino acids. The PCR fragment and the cDNA clone have identical sequences.

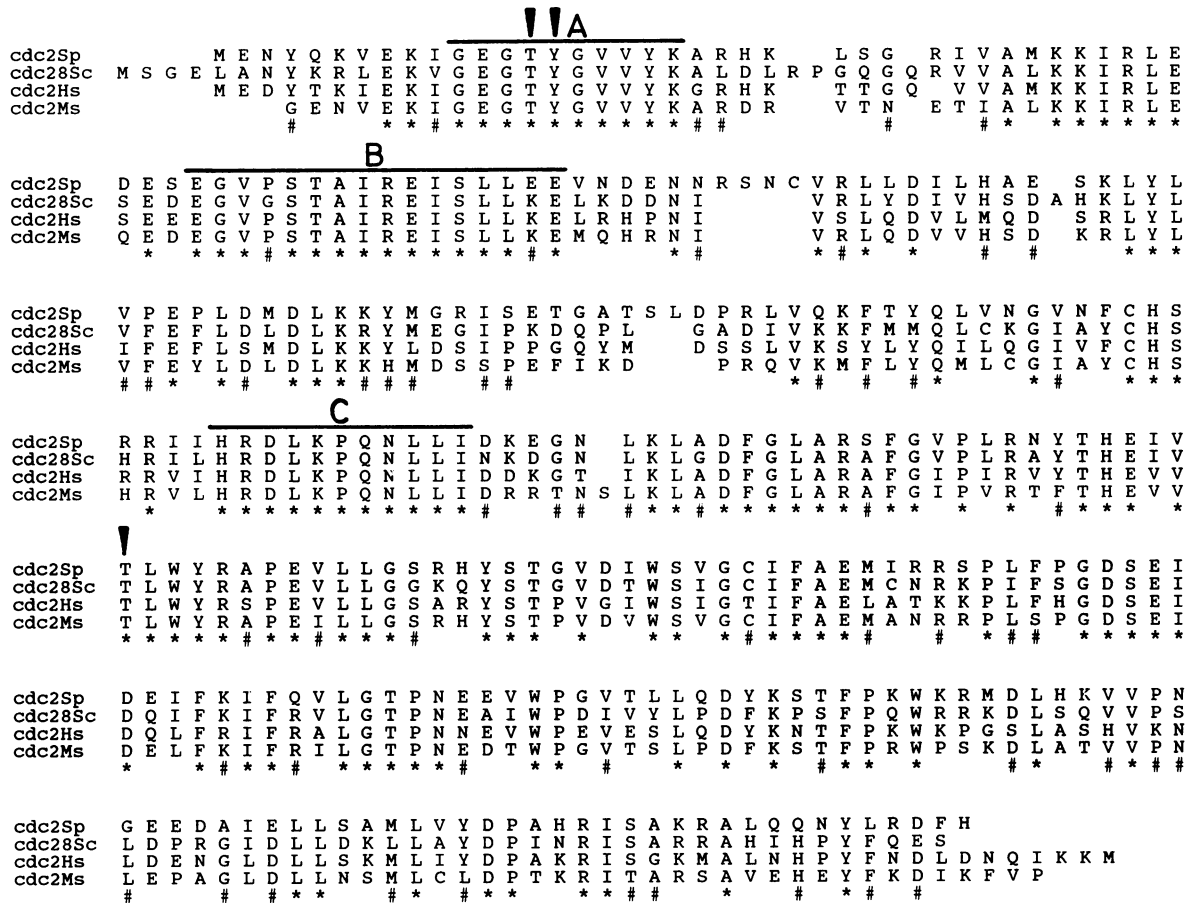


FIG. 2. Sequence alignment of alfalfa *cdc2Ms* with homologs from human and from fission and budding yeasts. Amino acids that are identical in all four or in three proteins are indicated by * and #, respectively. The highly conserved ATP-binding region, the kinase domain, the most-conserved 16-amino acid PSTAIR sequence element are indicated by overbars A, C, and B, respectively. Arrowheads indicate positions of conserved phosphorylation sites.

important phosphorylation sites at position Tyr-15 and Thr-167 in fission yeast and Thr-14 (arrowheads in Fig. 2) in human cells are also present in the *cdc2Ms* clone.

Identification of the Alfalfa p34^{cdc2Ms} Protein. Antibodies raised against the synthetic 16-amino acid PSTAIR peptide of *Sc. pombe* have been shown to identify *cdc2* proteins in a variety of organisms (9, 11). Furthermore, this antibody has been shown to react specifically with the plant *cdc2* homologs of several unrelated plant species but not with the PHO 85 sequence, which shows homology to the PSTAIR region (38). In Western blots of extracts prepared from alfalfa suspension cultures, the PSTAIR antibody recognized a double band of *M_r* 34,000 (Fig. 3, lane 1). Multiple bands with this molecular weight have also been reported by other investigators and have been attributed to different phosphorylated forms of the protein (16, 38). These results indicate that the *cdc2Ms* gene potentially encodes a *M_r* 34,000 protein highly similar to its counterparts in fungi and animals.

Rescue of a Temperature-Sensitive Fission Yeast *cdc2* Mutant by Complementation with the Alfalfa *cdc2Ms* Homolog. To examine whether the *cdc2Ms* gene can provide the functions of the fission yeast *cdc2* gene, we tried to complement a temperature-sensitive *Sc. pombe cdc2* mutant that has been used successfully for the complementation of both the human and the chicken homologs (9, 10). Unlike the vector alone (Fig. 4A), the yeast/*E. coli* shuttle vector carrying the *cdc2Ms* cDNA was able to complement the *cdc2* mutant phenotype at the restrictive temperature (Fig. 4B). Complementation was found up to 33°C, indicating a certain thermosensitivity of the plant *cdc2* protein. When transformants were grown on yeast extract/peptone/dextrose plates for 3

days, plasmid loss became apparent at the restrictive temperature, showing behavior identical to that of the nontransformed fission yeast *cdc2* mutant.

We conclude that the *cdc2Ms* gene is the plant homolog of the *cdc2* gene. It appears that in alfalfa cells, the *cdc2* protein kinase functions in a way similar to those of its yeast and animal counterparts.

Variation in Transcript Levels of the *cdc2Ms* Gene. Northern analysis of total RNAs from an alfalfa cell suspension culture and from different plant organs revealed a very low level of expression of *cdc2Ms* transcripts in comparison to that of the constitutively expressed *Msc27* (Fig. 5A). In shoots, three equally well expressed transcripts of 1.2, 1.4, and 2.5 kb (lanes 1) were present while in roots, transcript levels were considerably lower, with the 1.4-kb transcript being the most prominent band (lanes 2). Suspension cultures

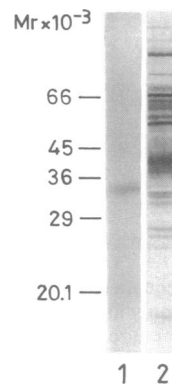


FIG. 3. Expression of alfalfa p34^{cdc2Ms} in suspension culture cells. Protein extracts were chromatographed and separated by SDS/PAGE and analyzed by immunoblotting with PSTAIR antibodies (lane 1) or by silver staining (lane 2).

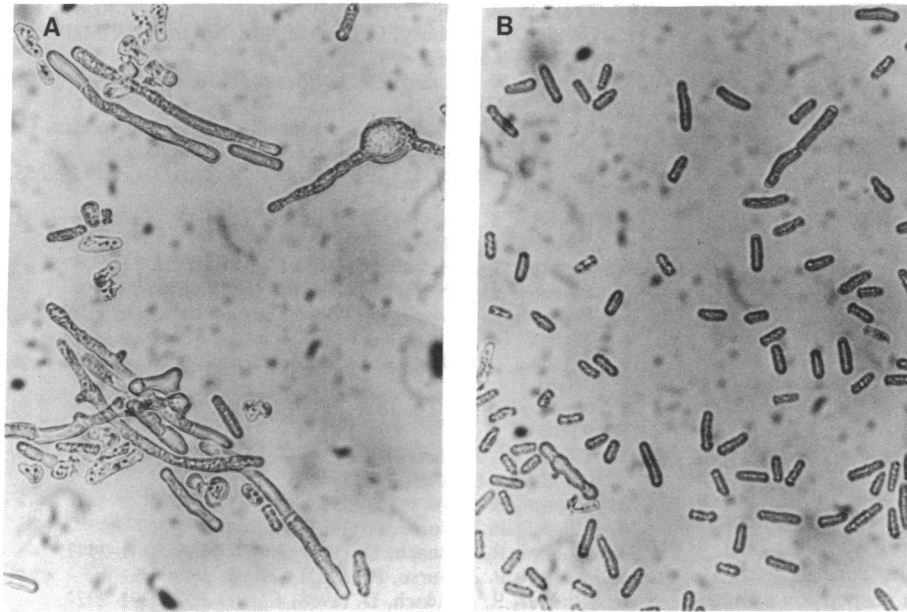


FIG. 4. Complementation of fission yeast *cdc2* mutation by alfalfa *cdc2Ms*. *Sc. pombe cdc2-33 leu1-32 h⁻* cells, transformed with pDB248 (A) or with pDB248 containing the *cdc2Ms* gene (B) were grown at the restrictive temperature of 33°C. Colonies transformed with vector alone grew normally at 23°C but showed the typical elongated form at 33°C, whereas cells transformed with the vector construct containing the *cdc2Ms* gene grew normally at both temperatures. When pDB248/*cdc2Ms* transformants were grown at 33°C on yeast extract/peptone/dextrose plates for 3 days a mixed population of normal and elongated cells, indicating plasmid loss, was observed.

revealed an expression pattern similar to that of roots, but the 1.2-kb fragment could not be detected (lanes 3). Northern analysis of poly(A)⁺ RNAs from the same organs revealed only a single transcript of 1.4 kb (Fig. 5B).

The transcript levels of *cdc2Ms* were also analyzed during auxin-induced somatic embryogenesis. Somatic plant cells have the striking ability to form embryos in culture (39, 40). In the alfalfa suspension cultures described above, after a 1-hr pulse treatment with 2,4-dichlorophenoxyacetic acid and subsequent culture in hormone-free medium, a high percentage of cells form somatic embryos that regenerate into whole plants (J.G., unpublished data). In these cultures, the cells change from an unorganized type of growth to well-organized embryos followed by the formation of shoot and root poles. Since cell division takes place in both callus and embryos, we investigated expression of the cell-cycle controlling *cdc2Ms* gene. Although the control gene *Msc27* did not show any changes in transcript levels, a complex transcription pattern was observed for the *cdc2Ms* transcripts. The 1.4-kb transcript decreased over the first 8 hr after the auxin pulse (Fig. 6, lanes 2–4), and then peaked between 24 and 72 hr (lanes 5 and 6). The 2.5-kb transcript showed the reversed pattern. Over the next 18 days, *cdc2Ms* transcripts decreased to the levels seen during the first 8 hr (lanes 7 and 8). The 1.2-kb transcript appeared during early embryogenesis when globular embryos had formed (lane 9). During progression of embryogenesis, the levels of all three transcripts decreased (lanes 10 and 11).

DISCUSSION

We have isolated a cDNA clone encoding the alfalfa homolog of the *cdc2* protein kinase genes. Since protein kinase gene

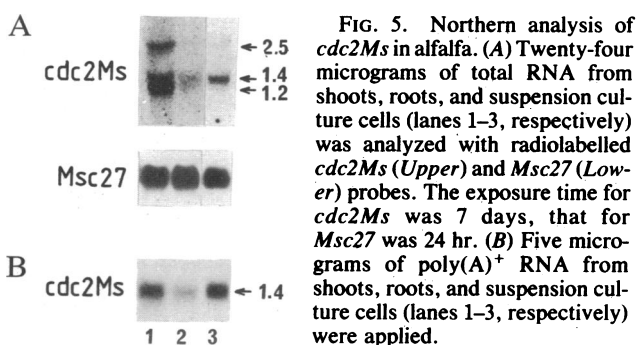


FIG. 5. Northern analysis of *cdc2Ms* in alfalfa. (A) Twenty-four micrograms of total RNA from shoots, roots, and suspension culture cells (lanes 1–3, respectively) was analyzed with radiolabelled *cdc2Ms* (Upper) and *Msc27* (Lower) probes. The exposure time for *cdc2Ms* was 7 days, that for *Msc27* was 24 hr. (B) Five micrograms of poly(A)⁺ RNA from shoots, roots, and suspension culture cells (lanes 1–3, respectively) were applied.

families have been isolated with amino acid homologies to the *cdc2/CDC28* gene products (41–44), several criteria must be fulfilled to define a gene as a *cdc2/CDC28* homolog. We demonstrate this by the following results. First, analysis of the amino acid sequence deduced from the cDNA clone revealed significant similarity to the mammalian and yeast *cdc2/CDC28* sequences. Structural conservation extends over the full length of the protein, including the ATP-binding region, the PSTAIR-region, and the catalytic kinase domain. All putative phosphorylation sites are perfectly conserved. An antibody raised against the 16-amino acid synthetic PSTAIR peptide was shown to recognize two proteins with apparent molecular weight of 34,000. Since all homologous *cdc2/CDC28* proteins from mammals and yeasts have the same molecular weight, we assume that we have identified the alfalfa p34^{cdc2} protein. A similar protein has been identified by Feiler and Jacobs (8) using an anti-*cdc2* mouse monoclonal antibody. This protein had histone H1 kinase activity. Complementation of the temperature-sensitive fission yeast *cdc2* mutant by the *cdc2Ms* cDNA is further proof for the identification of a plant *cdc2/CDC28* homolog. In interpretation of the results of the complementation experiments, we have to consider the fact that the presently available *cdc2Ms* cDNA clone lacks few codons at the 5' end of the coding region. We suppose that synthesis of a functional *cdc2* product became possible by insertion of the *cdc2Ms* cDNA clone into the yeast/*E. coli* shuttle vector pDB248. In this case an artificial fusion protein could be

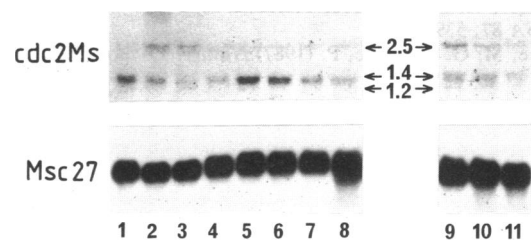


FIG. 6. Northern analysis of suspension culture cells induced by a 1-hr 2,4-dichlorophenoxyacetic acid treatment (100 μ M) to form somatic embryos in hormone-free medium for 0, 1, 3, and 8 hr and 1, 3, 7, and 21 days (lanes 1–8, respectively). After 3 to 4 weeks, globular, heart-shaped, and torpedo-shaped embryos were isolated and analyzed (lanes 9–11, respectively). The exposure time for *cdc2Ms* was 7 days, that for *Msc27* was 24 hr.

obtained through inclusion of three amino acids from the pDB48 vector. Expression of the transcripts can occur from fortuitous promoter sequences that are part of the pBR322 sequence (H.H., unpublished; ref. 45).

By Northern analysis of shoots, roots, and suspension culture cells from alfalfa plants, we have detected variations in the transcript pattern of the *cdc2Ms* gene. Shoots and roots showed very different amounts of three transcripts of 1.2, 1.4, and 2.5 kb. In contrast suspension culture cells contained only transcripts of 1.4 and 2.5 kb. The occurrence of distinct *cdc2* transcripts has also been reported in human cell lines but has been attributed to growth in tissue culture (45). Since we have found different *cdc2Ms* transcripts not only in suspension cultures but also in whole plants, we think that these transcripts might represent a type of regulation at the level of RNA processing.

Unlike in the chicken where expression of the *cdc2* kinase seems to be switched off in the adult animal (10), the expression pattern of the *cdc2Ms* gene could reflect the typical organization in plants with dividing cells, in particular primary and secondary meristematic regions.

To address the question of whether the *cdc2Ms* gene is developmentally regulated, alfalfa cells in suspension culture were induced to form somatic embryos. Northern analysis of the time course after treatment with 2,4-dichlorophenoxyacetic acid showed a complex pattern for the *cdc2Ms* transcripts. The 1.4-kb transcript was found to be maximally induced between 1 and 3 days after induction. This time course coincides with expression of the histone H3 major transcript, which has been found to be under cell cycle control in synchronized cultures of an alfalfa cell line (T. Kapros, personal communication). Experiments with mammalian tissue culture cells suggest that short-term changes in *cdc2* kinase activity are probably mediated by postranslational mechanisms whereas long-term changes (such as differentiation) may be regulated at the transcriptional level (46). However, a fluctuating transcript pattern of the *cdc2* kinase has not been reported. Whether these fluctuations represent regular cell cycle-dependent changes remains to be seen.

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