**cdc2MsB**, a cognate cdc2 gene from alfalfa, complements the G1/S but not the G2/M transition of budding yeast cdc28 mutants

Heribert Hirt*, Aniko Páy, Laszlo Bögé, Irute Meskienė and Erwin Heberle-Bors  
Institute of Microbiology and Genetics, University of Vienna, Vienna Biocenter, Dr. Bohrgasse 9, 1030 Vienna, Austria

**Summary**

The product of the cdc2 gene encodes the p34cdc2 protein kinase that controls entry of yeast cells into S phase and mitosis. In higher eukaryotes, at least two cdc2-like genes appear to be involved in these processes. A cdc2 homologous gene has previously been isolated from alfalfa and shown to complement a fission yeast cdc2ts mutant. Here the isolation of cdc2MsB, a cognate cdc2 gene from alfalfa (Medicago sativa) is reported. Southern blot analysis shows that cdc2MsA and cdc2MsB are present as single copy genes in different tetraploid Medicago species. cdc2MsB encodes a slightly larger mRNA (1.5 kb) than cdc2MsA (1.4 kb). Both genes were found to be expressed at similar steady state levels in different alfalfa organs. Expression levels of both cdc2Ms genes correlate with the proliferative state of the organs. Complementation studies revealed that in contrast to cdc2MsA, cdc2MsB was not able to rescue a cdc2ts fission yeast mutant. cdc2MsB was also unable to rescue a G2/M-arrested cdc28ts budding yeast mutant which could be rescued by expression of the cdc2MsA gene. Conversely, cdc2MsB but not cdc2MsA was found to complement the G1/S block of another cdc28ts budding yeast mutant. These results suggest that cdc2MsA and cdc2MsB function at different control points in the cell cycle.

**Results**

Isolation and sequencing of cdc2MsB cDNA from M. sativa

To isolate cdc2-related genes, we hybridized 300,000 colonies of a eukaryotic cDNA library with a labeled cDNA fragment coding for cdc2. Positive colonies were isolated and sequenced. Three clones were found to have identical nucleotide sequences to cdc2MsA, the originally identified cDNA sequence (Hirt et al., 1991).

One clone showed differences at four positions in the ORF (data not shown). Three of these changes had occurred in third codon positions and did not change the protein sequence. Only one nucleotide difference is predic-
ted to cause an amino acid exchange at position 203 (see Figure 2). This resulted in the exchange of a serine in cdc2MsA by a phenylalanine which is the commonly found amino acid in most cdc2 proteins. Since the 3' nontranslated nucleotide sequence is also highly similar to cdc2MsA, this clone is assumed to be an allele of cdc2MsA.

One clone showed marked differences in the restriction pattern and the subsequently determined nucleotide sequence. This clone was denoted cdc2MsB. As shown in Figure 1, the cDNA sequence shows a nontranslated region of 69 nucleotides before the first ATG. An ORF of 879 nucleotides putatively encodes a protein of 293 amino acids. The cDNA has a 345 nucleotide long 3' end and contains a poly(A)+ tail of 12 nucleotides. Interestingly, all cdc2MsA clones were truncated at both the 5' and 3' ends (data not shown). This is surprising since four clones were found of the cdc2MsA type and only one for cdc2MsB. This possibly reflects different mRNA stabilities of the two cdc2Ms transcripts.

Sequence comparison of alfalfa cdc2MsA and cdc2MsB

Alignment of the predicted alfalfa cdc2MsA and cdc2MsB protein sequences as shown in Figure 2 revealed 89% identity. The amino acid exchanges did not appear to be particularly clustered in any one region, except possibly in the C-terminal half of the proteins. This reflects the motifs of a functional protein kinase and has a perfect copy of the PSTAIR sequence, which is a trademark of the cdc2 proteins. Comparison of cdc2MsB with the predicted protein sequences of the recently reported cdc2 cDNAs from Arabidopsis thaliana, maize and pea (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreira et al., 1991) resulted in scores similar to cdc2MsA. Alignment with the predicted yeast and animal cdc2 protein sequences yielded identity scores between 55 and 65%. Interestingly, homology to the two cdc2 proteins from Drosophila melanogaster, which are only 65% identical to each other, was 57 and 61% (Lehner and O'Farrell, 1990). Furthermore, 67% identity was observed to Eg1 from Xenopus (Paris et al., 1991). This cdc2-like gene was unable to complement budding and fission yeast cdc28/cdc2 mutants and is speculated to be involved in the control of the G1/S transition (Paris et al., 1991). In summary, on the basis of protein sequence homology, cdc2MsB cannot be classified to the mitotic or interphase subtypes.

cdc2MsA and B are not allelic with each other

Since M. sativa is an autotetraploid organism, it was important to determine whether cdc2MsA and cdc2MsB might represent alleles of the cdc2Ms gene. Therefore, lines of two different alfalfa species and a species hybrid were analysed by Southern blotting. The genomic DNAs were digested with HindIII and hybridized with 3'-specific probes of the cdc2MsA and cdc2MsB genes (Figure 3b and a, respectively). The restriction patterns for cdc2MsA and cdc2MsB were very different. All Medicago varia lines tested (Figure 3a and b, lanes 1–3) and also M. sativa (Figure 3a and b, lane 4) contained the cdc2MsA and the cdc2MsB genes. Since none of the probes contained HindIII sites within their nucleotide sequences, this indicates that four copies of the cdc2MsA, as well as of the cdc2MsB gene, are present per genome. In conclusion, cdc2MsA and cdc2MsB represent different genes and are present as single copy genes per haploid set of chromosomes.

Expression of cdc2MsA and cdc2MsB in alfalfa suspension-cultured cells and organs

To determine the transcript sizes of the cdc2MsA and cdc2MsB genes, total RNA was isolated from M. varia suspension-cultured cells and separated on a 1% agarose gel containing 1% formaldehyde. Hybridization with the gene-specific 3' probes revealed mRNA sizes of 1.5 kb for cdc2MsA and 1.4 kb for cdc2MsB (data not shown).

To investigate whether cdc2MsA and cdc2MsB are expressed in an organ-specific manner, we analysed the RNA from young (unfloral) adult internodes, stem, vegetative meristem, flower bud and suspension-cultured cells for steady state transcript levels of the two genes as shown in Figure 4, lanes 1–7, respectively. Since the use of total RNA resulted in hardly detectable hybridization signals, poly(A)+ RNA was isolated from each organ and analysed by Northern hybridization with cdc2MsA- and cdc2MsB-specific probes (Figure 4a and b, respectively). Considerable transcript levels of cdc2MsA and cdc2MsB were detected in most organs. However, in adult leaf hardly any mRNA of either cdc2Ms genes was observed (Figure 4a and b, lane 3). This transcriptional pattern correlates with the proliferative state of the organs. Comparison of transcript levels for cdc2MsA and cdc2MsB in each organ revealed no specific differences.

As control, the same filter was reprobed with a radio-labelled Arabidopsis thaliana 25S rRNA fragment as shown in Figure 4c (although undetectable by ethidium bromide staining, poly(A)+ RNA still contains sufficient amounts of rRNA to allow detection with radiolabelled probes). With the exception of flower bud (Figure 4c, lane 5) all organs showed similar 25S rRNA levels. This indicates that the lower cdc2MsA and cdc2MsB transcript levels in flower bud were due to the lower amounts of poly(A)+ in this organ. On the other hand, the 25S rRNA level in adult leaf was comparable with those of other organs and cannot explain the very low cdc2Ms mRNA levels.
cdc2MsB cannot complement a cdc2^ts fission yeast mutant

We have previously shown that cdc2MsA was able to complement a cdc2^ts fission yeast strain at the restrictive temperature (Hirt et al., 1991). To test whether the same is true for the cdc2MsB gene, we cloned the cdc2MsA and cdc2MsB genes into the yeast expression vector pSM-1. This plasmid contains the SV-40 promoter which was shown to be used efficiently in Schizosaccharomyces pombe (Jones et al., 1988). After transformation of the cdc2-33 fission yeast strain with either pSM-1/cdc2MsA, pSM-1/cdc2MsB or the empty vector pSM-1, cells were selected for leucine prototrophy at the permissive temperature of 25°C for 3 days. Transformants were plated on to fresh plates lacking leucine and grown at 25, 28, 30, 33, 35 and 37°C for 2 days. Fission yeast cells transformed with pSM-1, pSM-1/cdc2MsA and pSM-1/cdc2MsB were
found to grow on plates at 28°C as shown in Figure 5 (a–c), respectively. However, at temperatures above 30°C, only cdc2-33 mutant.

In budding yeast, the CDC28 gene is complements the CDC28 gene in the cdc2-33 mutant, which was introduced into the cdc28-1N strain by transformation with pYES2/cdc2MsA. The pYES2/cdc2MsA strain was used as a control. The cdc28-1N strain was grown at 28°C and 33°C in the presence of 'START', i.e. galactose. The other S. cerevisiae CDC28 mutants, which was introduced into the cdc28-1N strain by transformation with pYES2/cdc2MsA and pYES2/cdc2MsB, were determined to grow at 28°C and 33°C as shown in Figure 7 (a and b, respectively). The restrictive temperature of 28°C, S. cerevisiae cdc28-1N cells which were transformed with pYES2, pYES2/cdc2MsA or pYES2/cdc2MsB, grew equally well (Figure 7a). Under the restrictive conditions at 33°C, only pYES2/cdc2MsA-transformed cells showed an increase in cell number (Figure 7a). When the same analysis was performed with S. cerevisiae cdc28-4 cells that were transformed with the three constructs, incubation at the restrictive temperature resulted in a cell number increase only in pYES2/cdc2MsB-transformed yeasts (Figure 7b) but not in those that expressed the cdc2MsB gene (Figure 7b).

Discussion
In budding and fission yeasts, the induction of both DNA replication and mitosis requires the action of the p34cdc2 kinase (Hartwell, 1974; Lorincz and Reed, 1984; Nurse and Bissett, 1981; Reed, 1980). While yeasts contain only one cdc2 gene, several cdc2-like kinases have been cloned from S. cerevisiae strain was the cdc28-1N strain by transformation with pYES2/cdc2MsB construct (Figure 6c) but not alone (Figure 6a) were able to grow only at 25, 28, 30°C but not at 36°C. In contrast, pYES2/cdc2MsA-transformed cells were able to grow at 25, 28, 30°C but not at 36°C.
Since alfalfa is an autotetraploid plant, the possibility existed that the two cdc2Ms genes might be alleles. Considerable evidence argues against this possibility. First, we found a cDNA clone which is nearly identical to cdc2MsA. Although several nucleotide differences were observed, only one produced an amino acid substitution. Furthermore, the 3′ noncoding region was also highly similar to cdc2MsA. Therefore, this clone probably represents a true allele. In contrast, the cdc2MsB gene is only 99% identical to cdc2MsA at the amino acid level, and the 3′ nontranslated sequences of cdc2MsA and cdc2MsB differ considerably. Secondly, Southern blot analysis with the cdc2MsA and cdc2MsB genes showed that each of the two genes is present in four copies, i.e., one copy per haploid genome. Two different alfalfa species, *M. varia* and *M. sativa*, both contained cdc2MsA as well as cdc2MsB, therefore, we consider cdc2MsA and cdc2MsB to be different genes.

The presence of two different cdc2-like genes implies that the encoded kinases should perform different functions. A crucial test for the function of a cdc2 homologous gene is the complementation of temperature-sensitive yeast mutants that are defective in the function of the cdc2 kinase and cannot pass through the cell cycle at the restrictive temperature. When the two alfalfa cdc2 genes were tested for complementation of a fission yeast cdc21-ts mutant, only the cdc2MsA gene was able to substitute for the fission yeast cdc2 gene, suggesting that the cdc2MsB might have a different function in the cell cycle. When the same genes were tested in two budding yeast mutants, one of which arrests at the G1/S, the other at the G2/M transition point (Surana et al., 1991), cdc2MsB could only rescue the G1/S arrest but not the G2/M block while cdc2MsA was found to complement the G2/M but not the G1/S transition.

This is the first time that cdc2 cognates of eukaryotes have been shown to alternatively yeast mutants which are blocked at the two points of the eukaryotic cell cycle. These that cdc2MsA and cdc2MsB perform differently in the cell cycle of yeast cells and suggest that they have analogous functions in plant cells. Complementation in yeast is an indication, it is a sufficient proof for such a role in plants.

Using evidence implies the animal cdc2-like kinases are involved in the transition from G1 to S phase (Newport, 1989; Fang and Newport, 1991; Pines and Hunter, 1990). In contrast to cdc2Ms kinases (89% identity), the animal cdc2-like kinases show identities of 55–65%. In our efforts, no closer relatives could be identified (Meyerson et al., 1992). We have also tried to create distantly related members of the cdc2 family by the same method. However, no genes other than cdc2Ms genes could be obtained with a variety of higher eukaryotic genomes. cdc2-like genes have been reported (Colasanti et al., 1991; Feiler and Jacobs, 1991; Ferreira et al., 1991; Hata, 1991; Hirayama et al., 1991). Their functions are unclear. We have previously isolated a homologue of the fission yeast cdc2 gene from alfalfa (Hirt et al., 1991). Here, we report the isolation and characterization of cdc2MsB, a cognate cdc2 gene from *M. sativa*.
cdc2MsB from alfalfa complements G1/S transition

The high similarity of the cdc2-like genes in this species, in the absence of the nucleotide and amino acid changes to be found in the cdc2-like genes in other species, suggests that the encoded kinases may have different activities and perform distinct roles in specific tissues or cell types.

**Experimental procedures**

**Plant tissue culture**

A suspension culture of *M. varius* (line A2) was used (Bögre, 1998). The suspension culture was derived from callus in which originated from stem segments and was propagated on Murashige and Skoog medium (Murashige and Skoog, supplemented with 1 mg 1^-1^ 2,4 dichlorophenoxyacetic acid and 0.2 mg 1^-1^ kinetin.

**Library screening and sequence analysis**

Poly(A)^+ RNA was isolated from somatic embryos from infected alfalfa suspension-cultured cells (Györgyey et al., 1991). mRNA libraries were constructed according to the method described earlier (Hirt et al., 1991). One clone was found to be an allelic form of cdc2MsA. The fifth was found to encode cdc2MsB.

**Southern blot analysis**

DNA (10 μg) from different lines of *M. varius* cv. Ringuette, *M. sativa* cv. RA3 was digested with HindIII and separable 0.7% agarose gels. After blotting, the nylon filters were purified with cdc2MsA- or cdc2MsB-specific probes.

To generate cdc2MsA- or cdc2MsB-specific probes, the 3′ nontranslated regions of the two genes were synthesized: GGGGTACGAAATGAGAATGGAAAGCTTAAATGGTAAGGCGA for cdc2MsA; ATGACGTAATCAGCAGGAGTACGTTAAGG. Amplification of gene-specific fragments of cdc2MsA was carried out by PCR using the following primers: 5′-GATCCCTAAGATCTGGCAAGGAGTACGTTAAGGCGA-3′ and 5′-GATTCACGTTAAGGAGTACGTTAAGGCGA-3′.

**Complementation studies**

For complementation in fission yeast, the cdc2MsA and cdc2MsB cDNAs were inserted into the yeast/E. coli shuttle vector pSM-1. In this vector, the cdc2MsA genes are expressed by the SV-40 promoter which is a strong constitutive promoter in fission yeast (Jones et al., 1988). The cdc2MsA cDNA was amplified by PCR with the following primers: TCG TTG GAA GTT AAG AAG GAG TTT CAT.
TTC TTG ATG GAA CAG TAC GAA AAT GTG QAG AAG and ACC AAA TCT AGA AAA CTG AAG TTC CTG. After cloning into the plasmid pTZ19U, the cDNA was ligated into the E. coli yeast shuttle vector pSM-1. The cdc25msB cDNA was cloned directly into pSM-1. S. pombe cdc2-33, leu" cells (Nurse et al., 1976), were transformed by the UCI method (ito et al., 1983) and were maintained in YPD medium plates and grown at 23, 25, 28, 30, 33 and 36°C. Determination of cell numbers was performed by growing the cells under permissive temperatures to a cell density of 1 x 10⁶ cells ml⁻¹. Cells were then grown in galactose media for 12 h. At 2 h intervals, aliquots were counted under the microscope.

Identification of transcript levels

The plant tissue was frozen in liquid nitrogen and extracted by grinding in a mortar as described in 1983. Poly(A)⁺ RNA was isolated using the method of Dylon, Oslo, Norway. RNA was fractionated by exposure to Γ formaldehyde, 8% formaldehyde, and hybridized to 32P-labeled 3' probes or a [a-32P]-labeled 25S rRNA as described by Gerdau et al., 1990.

The S. pombe cdc2-33, leu" cdc28-1 mutation (Suran et al., 1991), were transformed with pYES2/cdc25msA, pYES2/cdc25msB or pYES2 as a control (ito et al., 1983). Transformants were first selected for uracil prototrophy and then confirmed as transformants by Southern analysis.

The S. cerevisiae W303 strains, carrying either the cdc28-4 or p34cdc2 relative protein kinase, CDK2, identified by complementation of a cdc28 mutation in Saccharomyces cerevisiae, is a homolog of human p34cdc2. Cells were grown either at 28°C or 30°C for 2 days. Cells were then grown in galactose media for 12 h. At 2 h intervals, aliquots were counted under the microscope.

RNA extraction and quantitation

Suspension-cultured cells on the 1gN medium were harvested and total RNA extracted as described by Cathala et al., 1987. RNA from 100 μg total RNA with DNase treatments of the manufacturer (Ges, 1994) was separated on 1% agarose gels, transferred to nylon membranes, incubated with cdc25msA- and cdc2-5msB-specific probes, blotted to nylon membranes, and hybridized to 32P-labeled 25S rRNA as described by Gerdau et al., 1990.

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EMBL Data Library accession number X70707 (M. sativa CD2 kinase mRNA).