

# Developmental and Cell Cycle Regulation of Alfalfa *nucMs1*, a Plant Homolog of the Yeast Nsr1 and Mammalian Nucleolin

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We report here the isolation and characterization of the *nucMs1* alfalfa cDNA, whose predicted amino acid sequence structurally resembles the yeast Nsr1 protein and animal nucleolins. These proteins consist of an N-terminal acidic domain, centrally located RNA recognition motifs (RRMs), and a C-terminal glycine- and arginine-rich domain. In comparison with animal nucleolins that contain four RRMs, NucMs1 more closely resembles the yeast Nsr1 protein, which contains only two RRMs. A NucMs1 C-terminal peptide antibody specifically recognized a 95-kD nucleolar protein in alfalfa cells that changed its localization in a cell cycle-dependent manner. The *nucMs1* transcript and p95<sup>nucMs1</sup> protein levels correlated with cell proliferation, and *nucMs1* gene expression was found to be induced in the G<sub>1</sub> phase upon mitogenic stimulation of G<sub>0</sub>-arrested leaf cells. In situ hybridization analysis of different alfalfa organs during various developmental stages showed that *nucMs1* gene expression is highest in root meristematic cells, but it is also found in other meristematic cells of the plant body. *nucMs1* expression is tightly linked to cell proliferation but does not depend on a particular cell cycle phase. No *nucMs1* expression was observed in cells that had exited the cell cycle and were undergoing differentiation or polar growth, indicating that *nucMs1* may not be necessary for processes other than cell proliferation.

## INTRODUCTION

The nucleolus is the cell compartment for ribosome biogenesis and is composed mainly of chromatin, which consists of multiple rRNA genes and ribonucleoproteins (reviewed in Hadjiolov, 1985). Ribonucleoproteins are composed of pre-rRNAs and ribosomal as well as nonribosomal protein components. Several of the nonribosomal proteins have been identified in animals. Among these, nucleolin is considered

to play a key role in regulation of rDNA transcription (Bouche et al., 1987), preribosomal synthesis, and ribosomal assembly and maturation (Herrera and Olson, 1986; Bugler et al., 1987). Nucleolin also influences nucleolar chromatin structure through its interaction with DNA and histones (Olson and Thompson, 1983; Erard et al., 1988). Moreover, it has been suggested that nucleolin is involved in cytoplasmic–nucleolar transport of preribosomal particles (Borer et al., 1989). In animals, nucleolin is a large protein with a molecular mass of 90 to 110 kD. This protein is highly phosphorylated (Olson et al., 1974). Whereas in mitosis, nucleolin is phosphorylated by the cyclin-dependent kinase Cdc2 (Belenguer et al., 1990; Peter et al., 1990), during interphase it is found to be phosphorylated predominantly by casein kinase II (Caizergues-Ferrer et al., 1987; Belenguer et al., 1990).

To date, nucleolin cDNA sequences have been determined from hamster (Lapeyre et al., 1987), mouse (Bourbon et al., 1988), *Xenopus* (Caizergues-Ferrer et al., 1989), human (Srivastava et al., 1989), and chicken (Maridor and Nigg, 1990). All of the encoded protein sequences have a highly conserved tripartite structure. The N-terminal domain shows homology with the high mobility group of proteins and interacts with

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nucleolar chromatin (Erard et al., 1988). This is also the domain that contains the target sites for phosphorylation by Cdc2 kinase (Belenguer et al., 1990; Peter et al., 1990) and casein kinase II (Caizergues-Ferrer et al., 1987). It has been shown that nucleolin phosphorylation modulates chromatin condensation in conjunction with histone H1 (Kharrat et al., 1991) and is correlated with nucleolar transcriptional activity (Kang et al., 1974; Ballal et al., 1975). The N-terminal domain also contains bipartite nuclear localization sequence (NLS) motifs and recognizes simian virus 40-type monopartite NLS motifs (Xue et al., 1993). The central domain of nucleolin contains four RNA recognition motifs (RRMs), and the C terminus consists of glycine- and arginine-rich (GAR) repeats. The RRM motifs have been shown to specify the interaction with the external transcribed spacer region of the primary rRNA transcripts, whereas the GAR domain is involved in increasing the efficiency of binding but not of specificity (Ghisolfi et al., 1992).

The yeast *NSR1* gene was isolated on the basis of its ability to bind to simian virus 40-type NLS motifs (Lee et al., 1991). Deletion of *NSR1* impairs pre-rRNA processing and the production of mature 18S rRNA and leads to a severe growth defect (Kondo and Inouye, 1992; Lee et al., 1992). The encoded Nsr1 protein has striking structural homology with animal nucleolins in that it contains an acidic N terminus, centrally located RRM motifs, and a C-terminal GAR domain. Nsr1 may be the yeast equivalent of animal nucleolin (Xue et al., 1993). However, the Nsr1 protein is considerably smaller than the animal nucleolins. This is due mainly to the absence of two RRM motifs in the central part of the Nsr1 protein (Kondo and Inouye, 1992). Nsr1 also contains a bipartite NLS, and either the N-terminal region or the central RRM region is needed to localize Nsr1 to the nucleolus (Yan and Melese, 1993). Nsr1 binds to both ribosomal proteins and pre-rRNA and has been suggested to facilitate their interaction (Xue and Melese, 1994).

The goal of our study was to isolate nucleolin-like genes from plants. Here, we report the isolation of the *nucMs1* cDNA (for nucleolin of *Medicago sativa*) from alfalfa. The predicted NucMs1 protein sequence shows the characteristic tripartite structure known from animal nucleolins. Alignment of NucMs1 with animal nucleolins revealed the absence of ~180 amino acids containing two RRM motifs. The NucMs1 protein resembles the structure of the Nsr1 protein from yeast, which also lacks the central RRM motifs. A NucMs1 antibody recognized a 95-kD nuclear protein in fractionated alfalfa cell extracts. The p95<sup>nucMs1</sup> protein was found to be confined to the nucleolus during interphase, becoming distributed over the whole cell with the onset of mitosis. In situ hybridization and RNA gel blot analyses with alfalfa plants indicated that the *nucMs1* gene is not expressed in nondividing cells but is induced in the G<sub>1</sub> phase of the cell cycle at a similar time as a D-type cyclin gene. In proliferating cells, *nucMs1* transcript levels did not change in a cell cycle phase-specific manner but disappeared when cells had exited the cell cycle and were undergoing differentiation.

## RESULTS

### Cloning of Alfalfa *nucMs1*

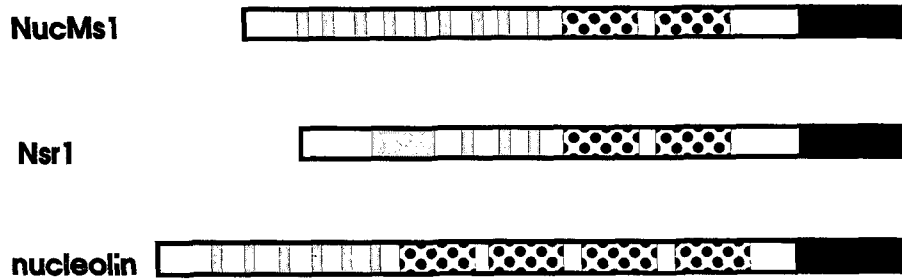
Two cDNA libraries prepared from suspension-cultured alfalfa cells were screened with radiolabeled oligonucleotides encoding the highly conserved GAR domain of mammalian nucleolin genes. Seven cDNA clones were isolated by using this procedure. The longest clone, denoted *nucMs1*, is 1.8 kb long. Sequence analysis indicated homology of the open reading frame with animal nucleolins but showed that the cDNA is truncated at the 5' end. RNA gel blot analysis revealed a length of 2.4 kb for the mature *nucMs1* mRNA (data not shown), indicating that ~600 nucleotides are missing from the full-length cDNA. The missing 5' region was obtained by polymerase chain reaction (PCR) amplification of reverse-transcribed RNA with synthetic oligonucleotides complementary to the 5' end of the *nucMs1* cDNA clone. Sequence analysis of the PCR product showed complete identity with *nucMs1* in the overlapping region, indicating that the 5' PCR product was derived from the same gene.

The original screening of the alfalfa cDNA libraries led to the isolation of seven cDNA clones. *nucMs1* was the longest. Restriction analysis and sequencing of all seven clones indicated that at least three classes of *nucMs* genes exist in alfalfa. Alignment of the predicted protein sequences resulted in scores ranging between 93 and 96% identity. Because alfalfa is an autotetraploid outcrossing species, it is possible that the different transcripts are encoded by different alleles. Alternatively, the different cDNAs may encode different members of a gene family that has evolved by recent duplications. DNA gel blot hybridization with *nucMs1* produced a number of bands, also indicating the presence of several closely related genes in the genome of alfalfa (data not shown).

### NucMs1 Has the Tripartite Structure of Yeast Nsr1 and Animal Nucleolins

The assembled full-length nucleotide sequence of *nucMs1* contains a single open reading frame of 2008 nucleotides, which is preceded by several in-frame stop codons and potentially encodes a protein of 636 amino acids.

The predicted NucMs1 protein has a tripartite structure and is depicted schematically in Figure 1. A 340-amino acid long, highly acidic N-terminal region is separated from a GAR C terminus by a central region of ~230 amino acids. The N terminus consists of nine repeats (Figure 1, gray boxes) that are composed of stretches of seven to 12 aspartic or glutamic acid residues that contain four to eight interspersed serine residues. The acidic stretches are delimited by 10- to 20-amino acid long, highly basic repeats that are characterized by a high content of lysine and proline residues. The central domain of



**Figure 1.** Schematic Depiction of the Structural Features of the Primary Sequences of Alfalfa NucMs1, Yeast Nsr1, and Mammalian Nucleolin Proteins. The gray boxes indicate the N-terminal acidic repeats that are delimited by stretches of mostly basic sequences. The RRM repeats are denoted by dotted boxes. The C-terminal GAR domains are shown as solid boxes. The *nucMs1* nucleotide and predicted amino acid sequences have the GenBank, EMBL, and DDBJ accession number X88845.

NucMs1 consists of two ~70–amino acid long repeats homologous with RRM, whereas the C-terminal part is highly glycine and arginine rich.

The NucMs1 protein has highest similarity with yeast Nsr1 and animal nucleolins. NucMs1 has a highly conserved tripartite structure and many important regulatory sequence elements; however, it also has some surprising differences (see alignment of alfalfa NucMs1, yeast Nsr1, and mammalian nucleolins in Figure 1). Both plant, yeast, and animal proteins contain several acidic repeats in their N-terminal regions. However, compared with the yeast Nsr1 and animal nucleolins, the plant sequence contains considerably more but shorter acidic repeats. The acidic repeats of the yeast Nsr1 and the plant NucMs1 proteins also contain considerably more serine residues per acidic repeat than do those found in the sequences of animal nucleolins.

Nucleolin becomes phosphorylated by Cdc2 kinase at the onset of mitosis (Belenguer et al., 1990; Peter et al., 1990). The S/TPXK motif that corresponds to the mitotic phosphoacceptor sites in the animal nucleolin protein can also be found three times in the N terminus of NucMs1. However, no such recognition motif was found in the yeast Nsr1 sequence (Lee et al., 1991).

The bipartite NLS in the chicken nucleolin protein KKK-KEMANKSAPEAKKKK was shown to be responsible for targeting nucleolin to the nucleus (Schmidt-Zachmann and Nigg, 1993). Several potential bipartite NLS sequences in the N-terminal regions of NucMs1, yeast Nsr1, and nucleolin conform to the consensus motif for nuclear targeting (Dingwall and Laskey, 1991).

Comparison of NucMs1 with animal nucleolins revealed major differences in the central RRM. Whereas animal nucleolins contain four RRM repeats, only two RRM are present in the NucMs1 sequence. Yeast Nsr1 also contains only two RRM and in this respect resembles more closely the plant NucMs1 protein. Comparison of the two RRM domains in the NucMs1 protein (29% identity) with those of the mammalian nucleo-

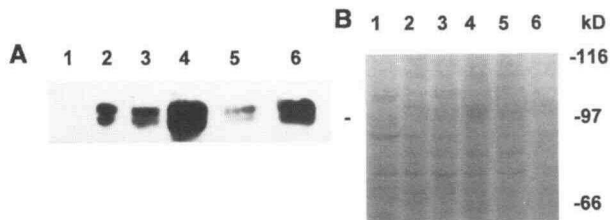
lins and the yeast Nsr1 yielded similar or slightly higher overall scores (30 to 41%).

The yeast Nsr1, the plant NucMs1, and the animal nucleolin proteins all contain a highly conserved C-terminal GAR domain, which appears to play a role in strengthening the binding of these proteins to RNA (Ghisolfi et al., 1992). Overall, the highly conserved structure of the NucMs1, the Nsr1, and nucleolins suggests that these proteins might perform homologous functions.

#### **p95<sup>nucMs1</sup> Shows Cell Cycle-Dependent Nucleolar Localization**

To identify and study the NucMs1 protein, antibodies were raised against a synthetic peptide encoding the C-terminal 12 amino acids of the predicted NucMs1 amino acid sequence. Immunoblotting crude extracts of proliferating cells with affinity-purified NucMs1 antibody produced a double band of 90 to 95 kD, representing different phosphorylated forms of NucMs1 (see below).

To investigate the intracellular distribution of p95<sup>nucMs1</sup>, cytosolic and nuclear fractions were prepared from logarithmically growing cells. In immunoblots, p95<sup>nucMs1</sup> was exclusively detected in extracts of the nuclear but not of the cytoplasmic fraction (Figure 2A, lanes 2 and 1, respectively). To test whether p95<sup>nucMs1</sup> is associated with any particular structure of the nucleus, consecutive extraction of the nuclear preparation with EDTA, 0.5 M NaCl, 1 M NaCl, and finally 7 M urea was performed. Whereas extraction with EDTA yielded some p95<sup>nucMs1</sup> protein to become dissociated from the nucleus (Figure 2A, lane 3), considerably more protein was extracted with 0.5 M NaCl (Figure 2A, lane 4). Increasing the salt concentration to 1 M NaCl only yielded a relatively small amount of p95<sup>nucMs1</sup> protein (Figure 2A, lane 5). However, when the nuclear preparation was further treated with 7 M urea, a considerable amount



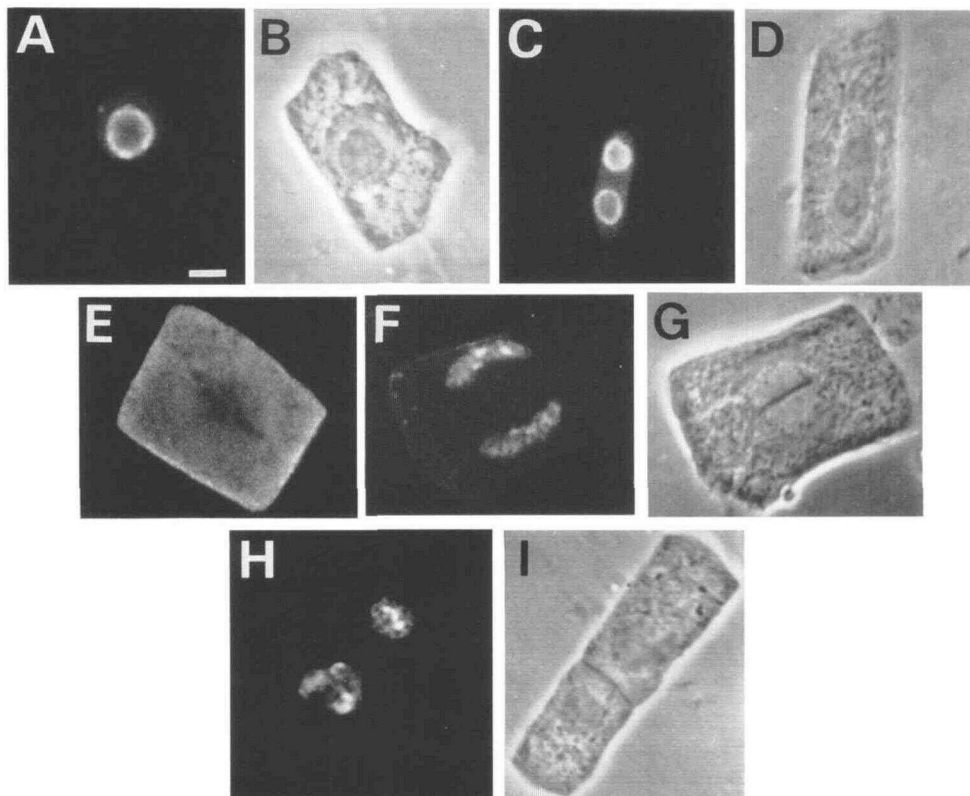
**Figure 2.** NucMs1 Is a Nuclear Protein Associated with Different Nuclear Structures.

(A) Immunoblots of cytoplasmic (lane 1) and nuclear (lane 2) fractions from exponentially growing alfalfa cells and of successive extracts from the nuclear fraction with EDTA (lane 3), 0.5 M NaCl (lane 4), 1 M NaCl (lane 5), and 7 M urea (lane 6). Twenty micrograms of protein was loaded per lane, blotted to nitrocellulose filters, and decorated with affinity-purified C-terminal NucMs1 antibody. Bar indicates the relative position of NucMs1 on the gel.

(B) Coomassie Brilliant Blue R 250–stained gel of the same samples as shown in (A). The numbers at right indicate the relative mobility of marker proteins.

of p95<sup>nucMs1</sup> protein was extracted (Figure 2A, lane 6). These results indicate that p95<sup>nucMs1</sup> is associated with different structures of the nucleus.

To study the cellular localization of p95<sup>nucMs1</sup>, alfalfa root tip cells were analyzed by indirect immunofluorescence microscopy with the C-terminal NucMs1 antibody. In interphase cells (Figures 3A and 3B), p95<sup>nucMs1</sup> was predominantly found in the nucleolus, although some staining was consistently found in the nucleus. In Figures 3C and 3D, which show an interphase cell with two nucleoli in a single nucleus, p95<sup>nucMs1</sup> is found predominantly in both nucleoli, although some protein is found in the nucleus (Figure 3C). During mitosis, a dramatic change in the cellular location of p95<sup>nucMs1</sup> was observed. In metaphase cells, p95<sup>nucMs1</sup> is evenly distributed throughout the cytoplasm (Figure 3E). At this stage, p95<sup>nucMs1</sup> could never be colocalized with chromosomes but is concentrated in the periphery of the cell cortex. In telophase cells, when cell plate formation became visible (Figures 3F and 3G), p95<sup>nucMs1</sup> was found to be associated with chromosomes in small aggregates.



**Figure 3.** Cell Cycle–Dependent Intracellular Localization of NucMs1.

Alfalfa root tips were decorated with the C-terminal NucMs1 antibody and analyzed by indirect immunofluorescence microscopy.

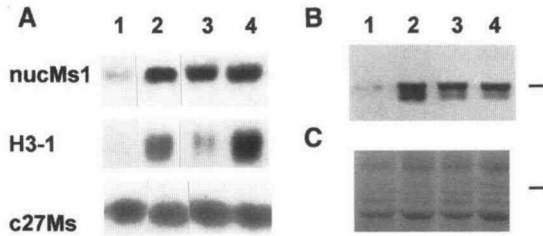
(A) to (D) Interphase cell containing a single nucleolus [(A) and (B)] or two nucleoli [(C) and (D)]. Immunofluorescence [(A) and (C)] and transmission [(B) and (D)] light microscopy were used.

(E) Immunofluorescence microscopy of a metaphase cell.

(F) and (G) Telophase cell. Immunofluorescence (F) and transmission (G) light microscopy were used.

(H) and (I) Mitotic cell in late cytokinesis. Immunofluorescence (H) and transmission (I) light microscopy were used.

Bar in (A) = 5  $\mu$ m for (A) to (I).



**Figure 4.** Cell Proliferation-Dependent Expression of the *nucMs1* Gene.

(A) Transcript analysis of stationary phase cells (lane 1), logarithmically dividing cells (lane 2), cells arrested by aphidicolin in the G<sub>1</sub>-to-S transition (lane 3), and cells after release from the aphidicolin block in the S phase (lane 4). An RNA gel blot containing 20  $\mu$ g of total RNA per lane was analyzed with radiolabeled fragments of the *nucMs1*, histone H3-1, and *c27Ms* genes.

(B) NucMs1 protein levels of stationary phase cells (lane 1), logarithmically dividing cells (lane 2), aphidicolin-arrested G<sub>1</sub>-to-S transition cells (lane 3), and S phase cells (lane 4). The immunoblot contains 20  $\mu$ g of total protein per lane and was probed with an affinity-purified C-terminal NucMs1 antibody.

(C) A Coomassie Brilliant Blue R 250-stained gel of the same samples as shown in (B).

Bars in (B) and (C) indicate the relative position of the NucMs1 protein on the gel.

Although p95<sup>*nucMs1*</sup> is mainly located on the chromosomes at this stage, a small fraction of p95<sup>*nucMs1*</sup> was consistently observed in the cytoplasm. In late cytokinesis, after fusion of the new cross wall with the old cell walls (Figures 3H and 3I), p95<sup>*nucMs1*</sup> is found exclusively in aggregated form on the decondensing chromosomes in the reconstituting nucleoli (Figure 3H).

#### Cell Proliferation-Dependent Expression of the *nucMs1* Gene

Animal nucleolin is known to be expressed at high levels in proliferating cells. To study the expression of the *nucMs1* gene, stationary alfalfa cells were compared with logarithmically growing cells. In a transcript analysis of stationary phase cells, barely any mRNA of the *nucMs1* gene or the S phase-specific histone H3-1 gene was detected (Figure 4A, lane 1). mRNA levels of *c27Ms* (Pay et al., 1992), an alfalfa gene that is constitutively expressed during the cell cycle (Meskiene et al., 1995), were equally high in stationary and dividing cells (Figure 4A, lanes 1 and 2, respectively). Immunoblots of crude extracts of these stationary phase cells with affinity-purified NucMs1 antibody showed traces of the p95<sup>*nucMs1*</sup> protein (Figure 4B, lane 1). In contrast, severalfold higher *nucMs1* transcript and p95<sup>*nucMs1*</sup> protein levels were detected in actively dividing cells (Figures 4A and 4B, lanes 2, respectively). In cells that were arrested at the G<sub>1</sub>-to-S transition of the cell cycle

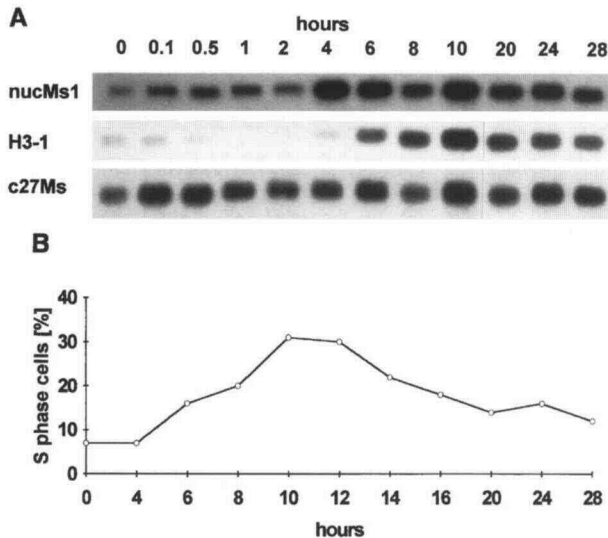
with the DNA polymerase inhibitor aphidicolin, *nucMs1* transcript and p95<sup>*nucMs1*</sup> protein levels were as high as in asynchronously dividing cells (Figures 4A and 4B, lanes 3 and 2, respectively). In contrast, histone H3-1 transcript levels were much lower in aphidicolin-arrested cells than in proliferating cells (Figure 4A, lanes 3 and 2, respectively). After release of the aphidicolin block by washing out the drug, cells entered synchronously into S phase and histone H3-1 transcript levels increased (Figure 4A, lane 4). However, *nucMs1* mRNA and p95<sup>*nucMs1*</sup> protein levels did not change considerably under these conditions (Figures 4A and 4B, lanes 4, respectively).

The presence of a 90-kD NucMs1 band in proliferating cells but not in stationary phase cells might indicate the presence of degradation products. Animal nucleolin has autolytic activity, and it has been suggested that its function might be regulated by proteolytic degradation. Although we cannot exclude this possibility, the 95-kD NucMs1 band was predominant at the G<sub>1</sub>-to-S transition and during S phase of dividing cells. Because nucleolin is also modified during different steps of the cell cycle by phosphorylation (Caizergues-Ferrer et al., 1987; Belenguer et al., 1990; Peter et al., 1990), we think these electrophoretic variations could reflect different phosphorylated NucMs1 isoforms. Preliminary evidence confirms this notion, showing correlating changes of electrophoretic mobility and phosphorylation of the NucMs1 protein during entry into mitosis (L. Bögge, unpublished results).

#### During Reentry into the Cell Cycle, the *nucMs1* Gene Is Induced in the G<sub>1</sub> Phase of the Cell Cycle

The cell proliferation-dependent expression of the *nucMs1* gene was further investigated in cells that were allowed to resume cell division after phosphate starvation. As determined by flow cytometric analysis, >90% of the cells were arrested in G<sub>1</sub> phase after growth in phosphate-free medium. The addition of phosphate initiated the reentry into the cell cycle, and after 6 hr, cells entered S phase (Figure 5B). A peak in the mitotic index was detected after 28 hr. *nucMs1* gene expression was induced 4 hr after readdition of phosphate, whereas transcript levels of the histone H3-1 gene only increased 2 hr later (Figure 5A). The increase of histone H3-1 transcript levels 6 hr after the readdition of phosphate correlated with the onset of DNA replication (Figure 5B). These results show that *nucMs1* gene expression is induced before S phase, that is, in the G<sub>1</sub> phase of the cell cycle.

To study the reentry into the cell cycle in a different system, fully differentiated leaf pieces that are naturally arrested with a 2C DNA content (Meskiene et al., 1995) were incubated in a medium containing mitogenic concentrations of auxin and cytokinin. Under these conditions, leaf cells reenter the cell cycle in G<sub>1</sub> phase and begin DNA synthesis after ~3 days (Meskiene et al., 1995). When expression of the *nucMs1* gene was compared with that of the histone H3-1 gene over a time course of 6 days (Figure 6), *nucMs1* gene expression was induced 4 hr after mitogen stimulation (Figure 6, lane 4). In



**Figure 5.** The *nucMs1* Gene Is Induced in the G<sub>1</sub> Phase of the Cell Cycle.

(A) RNA gel blot analysis of phosphate-starved G<sub>1</sub>-arrested alfalfa cells and different time points after readdition of phosphate. Poly(A)<sup>+</sup> RNA was isolated from 100 μg of total RNA and hybridized with radiolabeled fragments of the *nucMs1*, histone H3-1, and *c27Ms* genes. (B) Percentage of S phase cells as determined by flow cytometry at various time points after refeeding of phosphate.

comparison, histone H3-1 gene expression was not observed before 3 days (Figure 6, lane 7) and correlated with the onset of DNA synthesis (data not shown). *nucMs1* gene expression showed maximal levels 24 hr after mitogen stimulation (Figure 6, lane 6). When cells entered S phase and histone H3-1 mRNA became detectable, *nucMs1* transcript levels decreased again (Figure 6, lane 7).

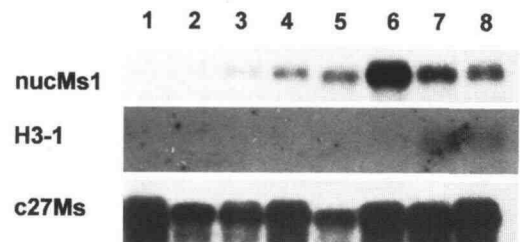
Recently, we isolated an alfalfa cyclin gene homologous with mammalian D-type cyclins (Dahl et al., 1995). Like their mammalian counterparts, alfalfa cyclin gene expression was induced in the G<sub>1</sub> phase of the cell cycle, well before the onset of DNA synthesis. Interestingly, the *nucMs1* gene was expressed at exactly the same time, indicating that these genes could be regulated by the same upstream factors. In summary, the experiments with leaf and suspension-cultured cells demonstrate that the *nucMs1* gene is not expressed under nondividing conditions but becomes induced in the G<sub>1</sub> phase when cells reenter the cell cycle.

#### In Situ Hybridization of Alfalfa Organs Reveals Proliferation- but Not Growth-Dependent Expression of the *nucMs1* Gene during Root Development

To consider *nucMs1* gene expression in a developmental context, transcript levels of the *nucMs1* gene were determined by

RNA gel blot and in situ hybridization analysis of different organs. The transcript pattern of the *nucMs1* gene on RNA gel blots was compared with that of the histone H3-1 gene (Figure 7). Histone H3-1 gene expression was found predominantly in flower bud and vegetative meristem (Figure 7, lanes Fb and Vm, respectively), strictly correlating with the state of mitotic activity of the organs. Although *nucMs1* transcript was abundant in flower bud and vegetative meristem (Figure 7, lanes Fb and Vm, respectively), considerably higher expression levels were observed in roots (Figure 7, lane R). Although the basis for this preferential expression of the *nucMs1* gene in roots is unclear, it may be that the different *nucMs* genes isolated during the screen of the cDNA library are expressed in an organ- or tissue-specific manner. Experiments to determine such a regulation are under way.

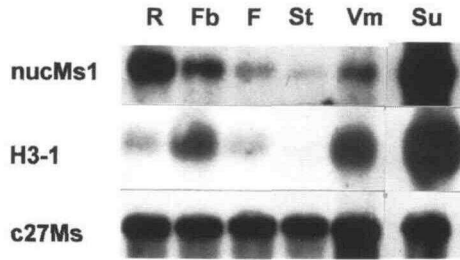
In agreement with RNA gel blot analysis, in situ hybridization also detected highest *nucMs1* transcript levels in roots (Figure 8A). Similar to the strictly S phase-specific expression pattern of the histone H3-1 gene in the root tip (Figure 8B), *nucMs1* gene expression was exclusively observed in the meristematic region (Figure 8A). However, in contrast to the spotty cell-specific pattern obtained with the histone H3-1 antisense probe (Figure 8B), *nucMs1* transcript levels did not appear to vary in the proliferating cells (Figure 8A), indicating that the *nucMs1* gene is constitutively expressed in dividing cells and does not change in different cell cycle phases. No expression of the *nucMs1* gene was observed in the quiescent center, which typically consists of nondividing cells (Figure 8A). Although *nucMs1* gene expression was seen in the root cap initials (Figure 8A), no transcript could be detected in the root cap, a tissue that consists of cells that have exited the cell cycle and undergo differentiation. No *nucMs1* expression was observed in the elongation zone (Figure 8A, upper part of the section), a region of the root in which cells stop dividing and grow in a polarized manner. These results show that



**Figure 6.** *nucMs1* Gene Induction Is an Early Response in Mitogen-Stimulated Leaf Cells.

RNA gel blot analysis of leaf pieces before (lane 1) and at 0.5 hr (lane 2), 1 hr (lane 3), 4 hr (lane 4), 8 hr (lane 5), 24 hr (lane 6), 3 days (lane 7), and 6 days (lane 8) after stimulation by auxin and cytokinin. Poly(A)<sup>+</sup> RNA was isolated from 100 μg of total RNA and hybridized with radiolabeled fragments of the *nucMs1*, histone H3-1, and *c27Ms* genes.





**Figure 7.** The *nucMs1* Gene Is Most Highly Expressed in Roots.

RNA gel blot analysis of root (R), flower bud (Fb), mature flower (F), stem (St), vegetative meristem (Vm), and suspension-cultured alfalfa cells (Su). Poly(A)<sup>+</sup> RNA was isolated from 100 µg of total RNA and hybridized with radiolabeled fragments of the *nucMs1*, histone H3-1, and *c27Ms* genes.

*nucMs1* gene expression correlates with cell proliferation but not with growth or differentiation in the absence of cell division.

#### Stage-Specific Expression of *nucMs1* during Leaf Development

An adult alfalfa plant always contains leaves at different stages of development. In young immature alfalfa leaves, mitoses can be seen throughout the leaf blade, whereas mature leaves show no mitotic activity. When longitudinal sections of immature leaves were analyzed by in situ hybridization with antisense probes of the *nucMs1* and histone H3-1 genes, transcripts of both genes were observed in cells of all leaf tissues (Figures 8C and 8D, respectively), correlating with the high mitotic activity in the organ. Mature leaves contain cells that have exited the cell cycle and have differentiated to photosynthetically active tissues. When these tissues were analyzed, no expression of either the *nucMs1* (Figure 8E) or the histone H3-1 gene (Figure 8F) was observed. These results are consistent with a cell proliferation-dependent regulation of the *nucMs1* gene.

#### *nucMs1* Gene Expression Is a Marker for Proliferation Events during Flower Development

Flower development is initiated by a reprogramming of the vegetative meristem into a floral meristem. One of the earliest detectable events is the mitotic activation of the quiescent central zone of the apical dome (Steeves and Sussex, 1989). It is only at a later time that organ primordia are formed. To investigate the expression of the *nucMs1* gene in the context of the changes in cell division activities during alfalfa flower development, longitudinal sections of flower buds in different stages of development were hybridized with antisense probes of the *nucMs1* and histone H3-1 genes (Figures 8G to 8J). In very young flower buds, as depicted in Figure 8G, *nucMs1* transcripts were found predominantly in cells of the apical dome

of the floral meristem but also in the bracts and the flower primordia. In longitudinal sections of older flower buds, when sepals had already formed and petal, stamen, and carpel primordia could be distinguished, expression of the *nucMs1* gene was confined to the newly formed floral organs (Figure 8I). The sites of *nucMs1* expression again correlated with the areas of major cell cycle activity, as indicated by the presence of histone H3-1 transcripts in these regions (Figure 8J).

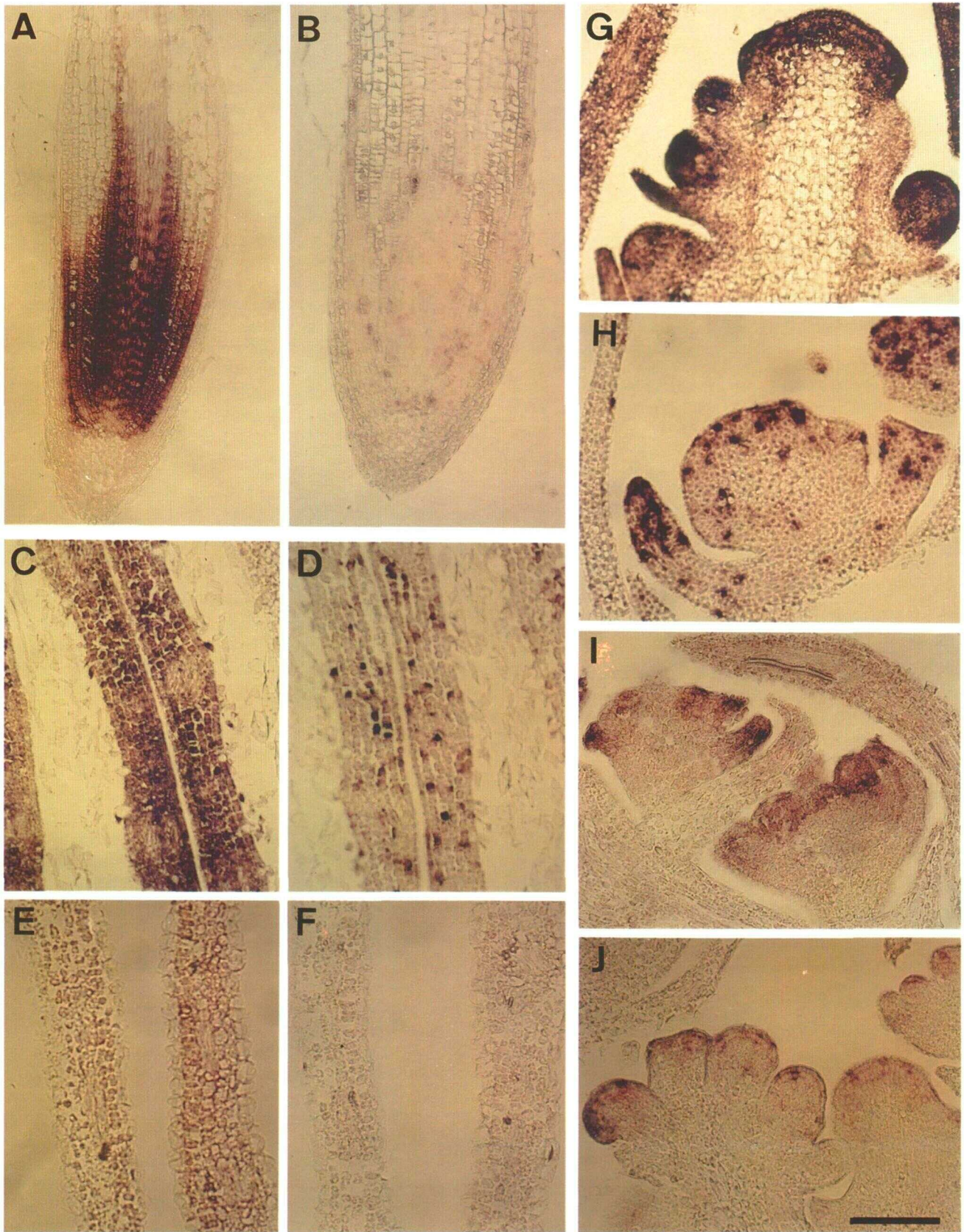
All experiments were controlled by in situ hybridizations of longitudinal sections of all organs and developmental stages with digoxigenin-labeled sense probes of the *nucMs1* and the histone H3-1 genes. Under the conditions used, no staining was obtained in any of the experiments (data not shown). Taken together, the patterns of *nucMs1* and histone H3-1 gene expression in all organs and developmental stages strictly correlated with a proliferative state of the cells. However, in contrast to the cell cycle S phase-specific expression of the histone H3-1 gene, the *nucMs1* gene was expressed in all phases of the cell cycle.

#### DISCUSSION

The nucleolus contains a variety of proteins that appear to be involved in rDNA transcription, ribosomal assembly, and maturation. Several nucleolar proteins that have been studied in detail, such as B23, fibrillarin, and nucleolin, revealed characteristic motifs, which appear to represent functional protein domains. Among these, nucleolin and fibrillarin have RRRMs also found in nucleoplasmic proteins. These two proteins also contain GAR domains that seem to function in binding single-stranded nucleic acids. Several nucleolar proteins that bind to proteins containing NLS motifs have N-terminal acidic repeat motifs that contain serine residues and can be phosphorylated by casein kinase II. Although animal and yeast nucleolar proteins have been studied in great detail, such studies are still lacking in plants. In this report, the isolation and characterization of the alfalfa *nucMs1* cDNA and NucMs1 protein are reported. Structural and expression analyses of the *nucMs1* gene suggest that NucMs1 is a plant homolog of the yeast Nsr1 and mammalian nucleolins. First, the predicted NucMs1 protein sequence has highest similarity with yeast Nsr1 and animal nucleolins and shows all the prominent structural features of these proteins, such as an acidic N-terminal region with alternating acidic and basic repeats, RRRMs in the central domain, and a C-terminal GAR domain (Lapeyre et al., 1987; Bourbon et al., 1988; Srivastava et al., 1989; Maridor et al., 1990). Second, NucMs1 is a nucleolar protein showing a cell cycle-dependent localization. Third, *nucMs1* expression strongly correlates with the proliferative state of the cell. By these criteria, NucMs1 can be classified as an Nsr1/nucleolin homolog from higher plants.

A major difference between NucMs1 and animal nucleolins has been observed in the central part of the predicted proteins. In all animal nucleolins, four RRRMs are found (Lapeyre





**Figure 8.** In Situ Hybridization of *nucMs1* and Histone H3-1 Genes in Root Tips and during Different Stages of Leaf and Flower Development.



et al., 1987; Bourbon et al., 1988; Srivastava et al., 1989; Maridor and Nigg, 1990). In comparison, the alfalfa *NucMs1* sequence contains only two RRM and highly resembles the structure of the yeast *Nsr1* protein, which also contains only two RRMs. The lack of two RRMs might have interesting functional implications. In vitro studies with animal nucleolin have revealed proportionality between number of binding sites and binding capacity for rRNA (Bugler et al., 1987). Therefore, twice the amount of *NucMs1* molecules would be required to bind to the same length of rRNA as nucleolins from animals.

The N-terminal region of *NucMs1*, yeast *Nsr1*, and animal nucleolins is composed of alternating basic and acidic repeats and shows limited similarity with the DNA binding regions of histones and high mobility group proteins. Compared with animal nucleolins and the yeast *Nsr1*, the N-terminal region of *NucMs1* has many more acidic residues. Because several of these acidic residues fit the consensus sequence of casein kinase II, which can phosphorylate animal nucleolins, these residues might be important for the regulation of the interaction of nucleolins with nucleic acids. Apart from the capacity to bind to rRNA, nucleolin can also interact with DNA (Olson and Thompson, 1983; Sapp et al., 1986; Erard et al., 1988) and was recently shown to bind to double-stranded matrix attachment regions (Dickinson and Khowi-Shigematsu, 1994). These authors also showed that nucleolin was present in soluble and bound fractions in nuclei of human erythroleukemia cells. Extraction of alfalfa nuclei also indicated association of *NucMs1* with different nuclear structures. These results were confirmed by immunofluorescence microscopy studies that consistently showed a certain amount of *NucMs1* in the extranucleolar region of the nucleus in interphase cells. Several bipartite NLS consensus motifs are also present in the N-terminal domains of the yeast *Nsr1*, animal nucleolins, and the plant *NucMs1* sequence. Nucleolin has been shown to shuttle between nucleus and cytoplasm, possibly serving as a transporter for ribosomal proteins into the nucleolus (Borer et al., 1989). Therefore, phosphorylation events can be expected to influence the ability of nucleolin to associate with nucleic acids and function as a cytoplasmic–nuclear transporter.

In animals, nucleolin is assumed to be required for the transcription, processing, and assembly of rRNA into ribosomal particles (Bouche et al., 1984). Deletion analysis of the yeast *NSR1* gene is consistent with this idea and shows a severe growth defect associated with the accumulation of 35S pre-rRNA. Although nucleolin expression has been found to correlate strongly with cell proliferation (Bugler et al., 1982), only one study was performed on nucleolin gene expression when cells reenter the cell cycle, showing that nucleolin protein accumulates after growth factor stimulation of G<sub>0</sub>-arrested cells (Bouche et al., 1987). Both nucleolin and rRNA synthesis precede DNA synthesis under these conditions. The analysis of *nucMs1* expression during resumption of cell division of phosphate-starved alfalfa cells revealed a similar regulation in that *nucMs1* transcription was induced before onset of DNA synthesis.

The totipotent capacity of plant cells to resume cell division and organogenesis from a differentiated state is well known. Mature nondividing alfalfa leaf cells that are photosynthetically active can be stimulated to reverse their differentiation state and resume cell division and organogenesis under in vitro conditions. Apart from nutrients, the presence of the plant hormones auxin and cytokinin is absolutely required for the induction of cell proliferation as well as for organogenesis. Our studies showed that, in comparison with cyclins, which are induced at specific stages during the reentry of leaf cells into the cell cycle, *nucMs1* gene expression is induced at the same time as that of *cycMs4*. The induction of the *nucMs1* and the *cycMs4* genes occurs in the G<sub>1</sub> phase before the onset of DNA synthesis. In situ studies of alfalfa roots, leaves, and flower buds showed a strict correlation of *nucMs1* and *cycMs4* gene expression with a proliferative state. As was seen most clearly in roots, *nucMs1* and *cycMs4* gene expression is tightly linked to cell division, and cells that have exited the cell cycle and are undergoing differentiation or polarized growth do not contain any *nucMs1* or *cycMs4* transcript.

Previous studies with different cell cycle marker genes (Dahl et al., 1995; Meskiene et al., 1995) have indicated that root cells exit the cell cycle in the G<sub>1</sub> phase, which in animals is

**Figure 8.** (continued).

Longitudinal sections (10  $\mu$ m thick) of alfalfa root tips, leaves, and flowers were hybridized with digoxigenin-labeled antisense and sense fragments of *nucMs1* and histone H3-1.

- (A) Bright-field microscopy of a root tip that was hybridized with a *nucMs1* antisense fragment.
  - (B) Bright-field microscopy of a root tip that was hybridized with a histone H3-1 antisense fragment.
  - (C) Bright-field microscopy of young leaves that were hybridized with a *nucMs1* antisense fragment.
  - (D) Bright-field microscopy of young leaves that were hybridized with a histone H3-1 antisense fragment.
  - (E) Bright-field microscopy of mature leaves that were hybridized with a *nucMs1* antisense fragment.
  - (F) Bright-field microscopy of mature leaves that were hybridized with a histone H3-1 antisense fragment.
  - (G) Bright-field microscopy of a floral meristem that was hybridized with a *nucMs1* antisense fragment.
  - (H) Bright-field microscopy of a floral meristem that was hybridized with a histone H3-1 antisense fragment.
  - (I) Bright-field microscopy of a flower bud that was hybridized with a *nucMs1* antisense fragment.
  - (J) Bright-field microscopy of a flower bud that was hybridized with a histone H3-1 antisense fragment.
- Bar in (J) = 150  $\mu$ m for (A) to (J).

usually denoted as G<sub>0</sub>. The strict proliferation dependency of *nucMs1* synthesis raises an important issue regarding the relationship of growth control and cell proliferation. Although it is clear that dividing cells require a high translational output and therefore high numbers of ribosomes, growing or differentiating cells still require protein synthesis. If NucMs1 is involved in ribosomal synthesis, it would be expected that *nucMs1* expression might still be required during growth or differentiation. However, we could not detect *nucMs1* expression in elongating root cells or in differentiating cells. Furthermore, when we examined suspension-cultured cells in stationary phase that still grew but did not divide, *nucMs1* transcript and NucMs1 protein also disappeared. These observations do not indicate a household role of NucMs1 and favor the idea that NucMs1 might play a role in cell proliferation. Considering the similar kinetics of the expression of *cycMs4*, a G<sub>1</sub> cyclin, and the *nucMs1* gene, and the evidence that G<sub>1</sub> cyclins are the prime candidates to convey mitogenic signals to the cell cycle machinery, it is tempting to speculate that NucMs1 might be involved in coupling such signals to the translational machinery.

## METHODS

### Screening of cDNA Libraries

Alfalfa (*Medicago sativa*) cDNA libraries (Hirt et al., 1992) were hybridized with the radiolabeled oligonucleotide GGIGGIAA<sup>7</sup>AGGIGGIGGI, which is homologous with the highly conserved C terminus of animal nucleolins. A recombinant plasmid, denoted *nucMs1* (for nucleolin of *M. sativa*), with an insert length of 1800 bp, was sequenced with the T7 polymerase sequencing kit, according to the manufacturer's recommendations (Pharmacia, Uppsala, Sweden). Because the cDNAs contained coding information for alfalfa nucleolins but were all truncated at their 5' ends, two oligonucleotides (CAGGCTTGCATCTTCATCTTCATCGG and TATGAATCCAGCTTCTTTGTGGGTGCACTCAC) were synthesized and used for the isolation of the missing 5' end with the 5' AmpliFINDER RACE kit, according to the manufacturer's instructions (Clontech, Palo Alto, CA).

### Antibody Preparation, Purification, and Protein Blotting

A 12-amino acid long peptide corresponding to the predicted C terminus of NucMs1 was synthesized. The peptide was coupled with an additional cysteine residue to trypsin inhibitor protein as a carrier and used for immunization of rabbits. Immune sera were affinity purified on the peptide coupled to CNBr-activated Sepharose CL 4B (Harlow and Lane, 1988). For protein blotting, samples containing 50 µg of protein were separated on 10% SDS-polyacrylamide gels and subsequently transferred to polyvinylidene difluoride (Millipore, Milford, MA) membranes in 50 mM Tris base, 50 mM boric acid buffer, pH 8.3, in a liquid electroblotting system (Hoefer, San Francisco, CA) at constant 30 V over night, with cooling. After Ponceau staining, the filters were blocked in 5% milk powder, 0.05% Tween 20 in PBS buffer for 2 hr. The affinity-purified first antibody was applied at a concentration of 1 to 2 µg/mL in blocking buffer. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody, and

the reaction was visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as substrate.

### Cell Fractionation

The separation of cytoplasm and nuclei and the purification of nuclei were achieved from protoplasts isolated from suspension-cultured cells, as described by Hadlaczy et al. (1983). Nuclear proteins were subsequently extracted with either 10 mM EDTA, 0.5 M NaCl, 1 M NaCl, or 7 M urea in a buffer of 25 mM Tris-HCl, pH 7.5, 0.5 mM phenyl methylsulfonyl fluoride, 1 mM DTT, and 10 µg/mL leupeptin. After the addition of SDS sample buffer, the fractions, containing a total of 10 µg of protein, were separated by SDS-PAGE and immunoblotted with the nucleolin antibody.

### Immunofluorescence Detection

Alfalfa seedling roots were fixed and stained as described by Traas et al. (1992). Immunofluorescence photographs were taken using a Bio-Rad MRC 600 confocal scanning laser microscope fitted on a Nikon microscope with a ×60 apochromatic Nikon objective (model NA 1.40). An argon ion laser provided the spot source of excitation light, and synchronized moving mirrors generated the sweep pattern. Eight sweeps of each photograph were collected and averaged. Images were recorded using Bio-Rad software. Photographs were taken from a high-resolution flat screen monochrome monitor.

### Isolation of RNA and RNA Gel Blotting

Total RNA was extracted from cultured cells and plant organs of alfalfa, as described by Cathala et al. (1983). Twenty micrograms of total RNA was loaded in each slot of a formaldehyde-agarose gel and transferred to Hybond N filters (Amersham). Hybridization was performed at 42°C in 50% formamide, according to standard protocols, followed by washing steps of increasing stringency.

### Cell Culture, Synchronization, and Mitogen Activation of Leaves

These analyses were performed as described by Meskiene et al. (1995).

### In Situ Hybridization

For preparation of hybridization probes, a 500-bp HindIII fragment of the 3' coding region of *nucMs1* was cloned into the pBluescript SK+ (Stratagene) vector. Digoxigenin labeling of sense and antisense probes by in vitro transcription, tissue preparation, and in situ hybridization were as described by Fobert et al. (1994).

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