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 protein levels correlated with cell proliferation, and nucMs1 gene expression was found to be induced in the G1 phase upon mitogenic stimulation of Go-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 (Lapayre 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...
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 RRMs 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 RRMs, 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 RRMs in the central part of the Nsrl protein (Kondo and Inouye, 1992). Nsr1

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 differ-
NucMsl consists of two ~70-aa repeats homologous with RRM repeats, whereas the C-terminal part is highly glycine and arginine rich.

The NucMsl protein has highest similarity with yeast Nsr1 and animal nucleolins. NucMsl has a highly conserved tripartite structure and many important regulatory sequence elements; however, it also has some surprising differences (see alignment of alfalfa NucMsl, yeast Nsr1, and mammalian nucleolin 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 NucMsl 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 NucMsl. 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 NucMsl, yeast Nsr1, and nucleolin conform to the consensus motif for nuclear targeting (Dingwall and Laskey, 1991).

Comparison of NucMsl with animal nucleolins revealed major differences in the central RRMs. Whereas animal nucleolins contain four RRM repeats, only two RRMs are present in the NucMsl sequence. Yeast Nsr1 also contains only two RRMs and in this respect resembles more closely the plant NucMsl protein. Comparison of the two RRM domains in the NucMsl protein (29% identity) with those of the mammalian nucleolins and the yeast Nsr1 yielded similar or slightly higher overall scores (30 to 41%).

The yeast Nsr1, the plant NucMsl, 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 NucMsl, the Nsr1, and nucleolins suggests that these proteins might perform homologous functions.

**p95^NucMsl Shows Cell Cycle-Dependent Nucleolar Localization**

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

To investigate the intracellular distribution of p95^NucMsl, cytosolic and nuclear fractions were prepared from logarithmically growing cells. Immunoblots, p95^NucMsl 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^NucMsl 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^NucMsl 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^NucMsl protein (Figure 2A, lane 5). However, when the nuclear preparation was further treated with 7 M urea, a considerable amount...
Figure 2. NucMsl 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 NucMsl antibody. Bar indicates the relative position of NucMsl 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>nucMsl</sup> protein was extracted (Figure 2A, lane 6). These results indicate that p95<sup>nucMsl</sup> is associated with different structures of the nucleus.

To study the cellular localization of p95<sup>nucMsl</sup>, alfalfa root tip cells were analyzed by indirect immunofluorescence microscopy with the C-terminal NucMsl antibody. In interphase cells (Figures 3A and 3B), p95<sup>nucMsl</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>nucMsl</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>nucMsl</sup> was observed. In metaphase cells, p95<sup>nucMsl</sup> is evenly distributed throughout the cytoplasm (Figure 3E). At this stage, p95<sup>nucMsl</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>nucMsl</sup> was found to be associated with chromosomes in small aggregates.
Although p95\textsubscript{nuclMst} is mainly located on the chromosomes at this stage, a small fraction of p95\textsubscript{nuclMst} 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\textsubscript{nuclMst} is found exclusively in aggregated form on the decondensing chromosomes in the reconstituting nucleoli (Figure 3H).

**Cell Proliferation-Dependent Expression of the nucMst\textsubscript{1} Gene**

Animal nucleolin is known to be expressed at high levels in proliferating cells. To study the expression of the nucMst\textsubscript{1} gene, stationary alfalfa cells were compared with logarithmically growing cells. In a transcript analysis of stationary phase cells, barely any mRNA of the nucMst\textsubscript{1} 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 NucMst\textsubscript{1} antibody showed traces of the p95\textsubscript{nuclMst} protein (Figure 4B, lane 1). In contrast, severalfold higher nucMst\textsubscript{1} transcript and p95\textsubscript{nuclMst} protein levels were detected in actively 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, nucMst\textsubscript{1} mRNA and p95\textsubscript{nuclMst} protein levels did not change considerably under these conditions (Figures 4A and 4B, lanes 4, respectively).

The presence of a 90-kD NucMst\textsubscript{1} band in proliferating cells but not in stationary phase cells might indicate the presence of degradation products. Animal nucleolin has autoproteolytic activity, and it has been suggested that its function might be regulated by proteolytic degradation. Although we cannot exclude this possibility, the 95-kD NucMst\textsubscript{1} band was predominant at the G\textsubscript{1}-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 NucMst\textsubscript{1} isoforms. Preliminary evidence confirms this notion, showing correlating changes of electrophoretic mobility and phosphorylation of the NucMst\textsubscript{1} protein during entry into mitosis (L. Bögre, unpublished results).

**During Reentry into the Cell Cycle, the nucMst\textsubscript{1} Gene Is Induced in the G\textsubscript{1} Phase of the Cell Cycle**

The cell proliferation-dependent expression of the nucMst\textsubscript{1} 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\textsubscript{1} 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. nucMst\textsubscript{1} 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 nucMst\textsubscript{1} gene expression is induced before S phase, that is, in the G\textsubscript{1} 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\textsubscript{1} phase and begin DNA synthesis after ~3 days (Meskiene et al., 1995). When expression of the nucMst\textsubscript{1} gene was compared with that of the histone H3-1 gene over a time course of 6 days (Figure 6), nucMst\textsubscript{1} gene expression was induced 4 hr after mitogen stimulation (Figure 6, lane 4). In
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 G1 phase of the cell cycle, well before the onset of DNA synthesis. Interestingly, the nucMsl 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 nucMsl gene is not expressed under nondividing conditions but becomes induced in the G1 phase when cells reenter the cell cycle.

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

To consider nucMsl gene expression in a developmental context, transcript levels of the nucMsl gene were determined by RNA gel blot and in situ hybridization analysis of different organs. The transcript pattern of the nucMsl 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 nucMsl 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 nucMsl 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 nucMsl 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), nucMsl 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), nucMsl transcript levels did not appear to vary in the proliferating cells (Figure 8A), indicating that the nucMsl gene is constitutively expressed in dividing cells and does not change in different cell cycle phases. No expression of the nucMsl gene was observed in the quiescent center, which typically consists of nondividing cells (Figure 8A). Although nucMsl 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 nucMsl 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 nucMsl expression is not limited to the root tip but extends throughout the root meristem, indicating that it is a key player in maintaining cell division in the root.
nucMsl very young flower buds, as depicted in Figure 8G, and histone H3-1 genes (Figures 8G to 8J). In nucMsl of the stem (St), vegetative meristem (Vm), and suspension-cultured alfalfa scripts were found predominantly in cells of the apical dome stages of development were hybridized with antisense probes development, longitudinal sections of flower buds in different stages of development. In young immature alfalfa leaves, mitoses can be seen throughout the leaf blade, whereas mature leaves show almost no mitoses. Whole leaves were analyzed by in situ hybridization with antisense probes of nucMsl, 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)+ RNA was isolated from 100 μg of total RNA and hybridized with radiolabeled fragments of the nucMs1, histone H3-1, and c27Ms genes.

Both nucMs1 and histone H3-1 gene expression correlated with cell proliferation but is only at a later time that organ primordia are formed. To investigate the expression of the nucMsl 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 sepal 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 the proliferative state of the cells. By these criteria, NucMs1 can be classified as an Nsr1/nucleolin homolog from higher plants. 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 RRMs are found (Lapeyre...
Figure 8. In Situ Hybridization of nucMsl 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 NucMs7 sequence contains only two RRMs 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 NucMs7 molecules would be required to bind to the same length of rRNA as nucleolins from animals.

The N-terminal region of NucMs7, 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 NucMs7 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 nucleoli also indicated association of NucMs7 with different nuclear structures. These results were confirmed by immunofluorescence microscopy studies that consistently showed a certain amount of NucMs7 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 NucMs7 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 G0-arrested cells (Bouche et al., 1987). Both nucleolin and rRNA synthesis precede DNA synthesis under these conditions. The analysis of nucMs7 expression during resumption of cell division of phosphate-starved alfalfa cells revealed a similar regulation in that nucMs7 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, nucMs7 gene expression is induced at the same time as that of cycMs4. The induction of the nucMs7 and the cycMs4 genes occurs in the G1 phase before the onset of DNA synthesis. In situ studies of alfalfa roots, leaves, and flower buds showed a strict correlation of nucMs7 and cycMs4 gene expression with a proliferative state. As was seen most clearly in roots, nucMs7 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 nucMs7 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 G1 phase, which in animals is

Figure 8. (continued).

Longitudinal sections (10 μm thick) of alfalfa root tips, leaves, and flowers were hybridized with digoxigenin-labeled antisense and sense fragments of nucMs7 and histone H3-1.

(A) Bright-field microscopy of a root tip that was hybridized with a nucMs7 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 nucMs7 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 nucMs7 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 nucMs7 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 nucMs7 antisense fragment.
(J) Bright-field microscopy of a flower bud that was hybridized with a histone H3-1 antisense fragment.

Bar in (J) = 150 μm for (A) to (J).
usually denoted as G0. The strict proliferation dependency of nucMs7 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 cells still require protein synthesis. If NucMs7 is involved in ribosomal synthesis, it would be expected that nucMs7 expression might still be required during growth or differentiation. However, we could not detect nucMs7 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, nucMs7 transcript and NucMs7 protein also disappeared. These observations do not indicate a household role of NucMs7 and favor the idea that NucMs7 might play a role in cell proliferation. Considering the similar kinetics of the expression of cycMs4, a G1 cyclin, and the nucMs7 gene, and the evidence that G1 cyclins are the prime candidates to convey mitogenic signals to the cell cycle machinery, it is tempting to speculate that NucMs7 might be involved in coupling such signals to the translational machinery. The reaction was visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indoly 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 Hadlaczky 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).
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REFERENCES


Fobert, P.R., Coen, E.S., Murphy, G.J.P., and Doonan, J.H. (1994). Patterns of cell division revealed by transcriptional regulation of genes during the cell cycle in plants. EMBO J. 13, 616–624.


Kondo, K., and Inouye, M. (1992). Yeast NSR1 protein that has structural similarity to mammalian nucleolin is involved in pre-ribosomal RNA processing. J. Biol. Chem. 267, 16252–16258.


