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Differential Expression of Histone H3 Gene Variants during Cell Cycle and Somatic Embryogenesis in Alfalfa

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ABSTRACT

Northern analysis has revealed substantial differences in mRNA accumulation of the two histone H3 gene variants represented by pH3c-1 and pH3c-11 cDNA clones. Both in partially synchronized cell suspension cultures and in protoplast-derived cells from alfalfa, *Medicago varia*, the maximal level of the histone H3-1 gene transcript coincided with the peak in [^{3}H]thymidine incorporation. Histone H3-11 mRNA was detectable in cells throughout the period of the cell cycle studied. Various stress factors such as medium replacement, enzyme digestion of the cell wall, osmotic shock, and auxin treatment considerably increased the level of the histone H3-11 transcript. In alfalfa (*Medicago sativa*), the presence of H3-11 mRNA in unorganized tissues of microcallus suspension and in somatic embryos induced by auxin treatment supports the idea that this H3 variant exists in a continously active state of transcription. During embryo development, the early globular stage embryos showed increased accumulation of histone H3-11 mRNA in comparison with the later stages. The highest level of the histone H3-1 transcript was detectable 1 day after treatment of callus tissues with 2,4-dichlorophenoxyacetic acid. Somatic embryos contained appreciable levels of histone H3-1 transcripts at all stages of somatic embryo development. These observations suggest that the histone H3-1 gene belongs to the class of replication-dependent histone genes. The histone H3-11 gene showed characteristics of a constitutively expressed replacement-type histone gene, with a specific characteristic that external factors can influence the level of gene transcription.

Isolation and molecular characterization of various classes of histone genes have significantly advanced our present understanding of the structural and regulatory role of histones as fundamental components of the nucleosomal structure in eukaryotic chromatin. The regulation of histone gene expression has mostly been studied in lower eukaryotes and animal cell systems (13, 16, 20). The complexity of expression patterns is evident from the detection of various histone types, each with their own characteristic pattern of regulation. Despite the fact that a number of histone genes from higher plants have been sequenced, as yet only few studies of their expression have been reported (11, 15, 18, 27).

Waterborg *et al.* (24) have shown by acid-urea-PAGE with a transverse gradient of Triton X-100 that there are two different histone H3 protein variants in alfalfa. They differ in their steady-state levels of acetylation. Protein sequence analysis (22) has shown that the H3.1 and H3.2 histone protein variants appear to be the products of genes that correspond with the cDNAs pH3c-1 and pH3c-11, respectively, which were determined by Wu *et al.* (28). Previously, we detected significant variation in the level of total histone H3 transcripts in different tissues of alfalfa with a particularly high level of mRNA in somatic embryos (27). In the present work, we extend these studies using gene-variant-specific 3′-end probes to analyze differential expression of the H3 histone gene variants during the cell cycle and during embryo development. Northern analysis has allowed us to discriminate between a completely and a partially cell cycle-dependent pattern of mRNA expression. This study of two histone H3 cDNA clones suggests that each represents a distinct member of the family of housekeeping histone genes in plants with its own function and a distinct mechanism for regulation of gene expression.

MATERIALS AND METHODS

Tissue Culture Systems

A fast growing cell suspension culture was established from primary callus tissues from *in vitro* grown plants of alfalfa, *Medicago varia* cv Rambler (A_2). Cultures containing single cells and small multicellular colonies were maintained in Murashige-Skoog medium (14) supplemented with 1 mg/L 2,4-D and 0.2 mg/L KIN[1]. The cell suspensions were subcultured twice/week. For studies of somatic embryogenesis, we used a so-called MCS (7). They were established by the use of callus tissues from petioles of plants of RA3 genotype *Medicago sativa* L. described first by Stuart and Strickland (21). The MCS consists of dedifferentiated cells grown as small clusters in liquid culture based on Schenk-Hildebrandt medium (19) with 15 μM NAA and 10 μM KIN. Somatic embryogenesis is induced by treatment of MCS with 100 μM 2,4-D in the presence of 10 μM KIN for 1 h. Subsequently, the MCS was washed twice and cultured in hormone-free Schenk-Hildebrandt liquid medium supplemented with 30 mM proline and 10 mM $(NH_4)_2SO_4$. Somatic embryos were formed after 3 weeks and could be fractionated according to size and developmental stage by sieving through various pore size meshes and manual selection. The pore sizes were: globular

[1] Abbreviations: KIN, kinetin; MCS, microcallus suspension; NAA, naphthaleneacetic acid; SSC, standard sodium citrate (8.75 g/L NaCl and 4.41 g/L sodium citrate [pH 7.0]; bp, base pair.

stage embryos, 300 to 500 μm; heart-shaped embryos, 500 to 800 μm; and torpedo-shaped embryos, >800 μm. Leaf protoplasts from *M. varia* cv Rambler (A_2) were isolated and cultured as described by Deák *et al.* (6).

Synchronization of Cell Suspension Cultures by Double Phosphate Starvation Method

A synchronization protocol based on the method described by Amino *et al.* (1) was used. Cells were transferred 2 d after subculture through washes to phosphate-free medium at a low cell density (3×10^5 cell/mL) and cultured for 3 d. At this time, KH_2PO_4 was added to a final concentration of 0.2 mM. After 1 d, the cells were centrifuged, washed, and transferred to phosphate-free medium. Four days later, KH_2PO_4 was readded to a final concentration of 0.675 mM.

Determination of the Mitotic Index and Incorporation of [^{3}H]Thymidine

Samples from cell suspensions and protoplast cultures were treated with colchicine (0.5 mg/L) for 3 h. After Carnoy I fixation, cells were stained with carbol fuchsin (10), and the mitotic index was determined by analysis of 1000 cells.

Labeling of cells with [^{3}H]thymidine (Radiochemical Center, Amersham, U.K.; 777 GBq/μmol) was carried out by incubation of the cells at 20 or 37 KBq/mL [^{3}H]thymidine for 3 h at 25°C on a rotary shaker. Cells were collected by centrifugation for 3 min at 100*g* in Eppendorf tubes and lysed in 1 mL cold 7% (w/v) TCA. Lysates were centrifuged, and the pellets were washed with 7% (w/v) TCA, with 80% (v/v) ethanol and finally 0.2 N perchloric acid. Pellets were resuspended in hot 0.5 N perchloric acid for 30 min. After centrifugation, the radioactivity was measured in the supernatant using a toluol-based scintillation cocktail. All sampling was performed in triplicate.

Preparation of Total RNA and Northern Hybridization

Cellular RNA was isolated according to the method described by Cathala *et al.* (3), which is based on extraction in the presence of quanidium thiocyanate and selective precipitation with lithium chloride.

Total RNA (10–24 μg) was electrophorased in a formaldehyde-agarose gel (1.4%, w/v) and transferred to a Hybond-N membrane (Amersham, Inc.) according to the manufacturer's instructions. The *Eco*RV-*Pst*I fragment of pH3c-1 and the *Pvu*II-*Hin*dIII fragment of pH3c-11 (28) were isolated and labeled by the random-priming method described by Feinberg and Vogelstein (8). Sequence analysis of the cDNA insert Msc27 used as an internal control in Northern analysis did not reveal significant homology to any known gene according to the databank search.

Northern blots were hybridized overnight at 42°C in a solution consisting of 50% formamide, 3 × SSC, and 0.1% SDS; 0.25% nonfat milk powder and 3 to 6×10^6 cpm/mL [^{32}p]dATP labeled probe DNA. After hybridization, the filters were washed at 65°C twice with 3 × SSC and 0.1% SDS and once with 0.1 SSC and 0.1% SDS. Autoradiography was performed at −70°C for 12 to 36 h.

RESULTS

Probe Specificity

Nucleotide sequences of previously analyzed histone H3 cDNAs of alfalfa were compared (28), and it appeared possible to generate hybridization probes specific for the two gene variants from the distinct 3′-untranslated transcript regions. In the present Northern analysis, we used the 200-bp *Eco*RV-*Pst*I fragment from cDNA pH3c-1 and the 360-bp *Pvu*II-*Hin*dIII fragment of cDNA pH3c-11. These fragments each contain a short sequence from the coding region, so it became necessary to prove the specificity of the probes. The labeled 3′-end fragments hybridized quite specifically only to their own cDNA insert under the hybridization conditions used (Fig. 1). Based on quantification by scintillation counting, self-hybridization was at least two orders of magnitude higher than cross-hybridization.

Changes in the Levels of Histone H3-Variant mRNA in Partially Synchronized Alfalfa Cells

Fast-growing suspension cultures with predominantly cycling cells as well as leaf mesophyll protoplast-derived cells can provide partially synchronous cell populations. When cells were grown in liquid cultures and exposed twice to phosphate starvation, the second addition of phosphate could induce synchrony during subsequent growth shown by peaks in [^{3}H]thymidine incorporation and mitotic index values (Fig. 2). Total cellular RNA preparations from these A_2 cultures were hybridized with 3′-end probes that were specific for the two histone H3 gene variants. H3–1 transcripts could be detected in abundance only within a narrow time range. The maximal level of mRNA from this gene coincided with the peak of [^{3}H]thymidine incorporation.

Northern analysis with the H3–11 probe revealed a significant level of H3–11 transcript throughout the period of the cell cycle studied. Quantitative analysis showed two maxima in hybridization intensity. The first one was seen shortly after adding phosphate to the culture medium and the second one was observed 15 to 21 h later. The highest transcript level of the H3–11 gene was reached later than the maximum in H3–1 transcript accumulation.

The results of the hybridization with another cDNA (Msc27) used as an internal control to quantify the amount of RNA loaded in each gel lane are shown in Figure 2.

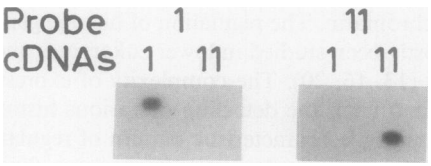

Figure 1. Analysis of the specificity of the probes used in Northern hybridization. Histone H3 cDNA inserts were prepared from H3c-1 (1) and pH3c-11 (11) plasmids by digestion with *Pst*I. In each lane, 30 ng of insert DNA was fractionated by electrophoresis, transferred to nitrocellulose, and hybridized with the 3′-end sequences from both cDNAs as ^{32}P-labeled probes. Hybridization conditions were identical with those used in Northern analysis.

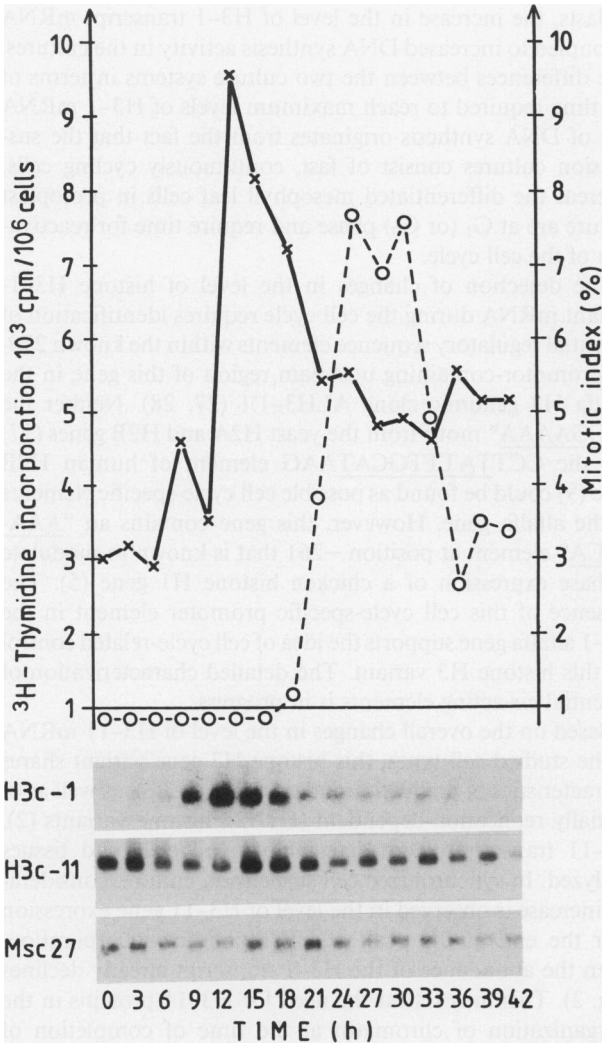

Figure 2. mRNA levels of H3 histone gene variants in a partially synchronized alfalfa cell suspension culture. After readdition of phosphate to the culture medium, [^{3}H]thymidine incorporation (X) and the mitotic activity index (O) were monitored by sampling at 3-h intervals. Total cellular RNA (15 μg) isolated from each sample were hybridized with three probes: H3c-1 and H3c-11 represent the 3′-end gene-specific fragments from histone H3 cDNAs; Msc27 is a selected cDNA that is used as a reference probe.

As an alternative approach, we analyzed the expression of these histone H3 variants in protoplast-derived cell populations. It is generally accepted that freshly isolated protoplasts are in G_1 phase (26). After resynthesis of the cell wall, the mitotic cycle is reactivated, mainly by addition of synthetic auxins like 2,4-D in the presence of cytokinins like zeatin riboside.

A peak of [^{3}H]thymidine incorporation on day 2 of a culture of alfalfa protoplasts (Fig. 3) indicates a detectable degree of synchrony when cells enter S phase. The pattern of expression of the two histone H3 variants is quite different. Accumulation of H3–1 transcript is restricted to days 2 through 4 when the [^{3}H]thymidine incorporation is also increased. Whereas H3–1 mRNA is not detectable in the leaf tissues used as a source to prepare protoplasts, transcripts of the H3–11 gene are present, although to a slight extent. Interestingly, the abundance of this mRNA is increased by the protoplast isolation procedure. Subsequently, during continued culture, the level of H3–11 gene transcript gradually decreased. Taken together these two systems have shown that mRNA accumulation of H3–1 and H3–11 histone H3 genes are under significantly different control.

Expression of Histone H3 Gene Variants after Induction of Somatic Embryogenesis by Auxin Treatment in Dedifferentiated Callus Tissues

Previously, we detected a high level of mRNA of histone H3 at all developmental stages of alfalfa somatic embryogenesis (27). Considering the existence of histone H3 gene variants with different expression patterns as shown above, we have reanalyzed the levels of H3 gene transcripts during induction of somatic embryogenesis. The tissue culture system used for these studies is based on alfalfa MCS. In this case, dedifferentiated cells are grown as actively proliferating multicellular clusters up to 3 mm in diameter in liquid cultures supplemented with the weak auxin NAA. Treatment of MCS with a strong auxin (100 μM 2,4-D) for 1 h is sufficient to trigger embryogenic development through initiation of organized growth of MCS. Figure 4 shows the results of Northern

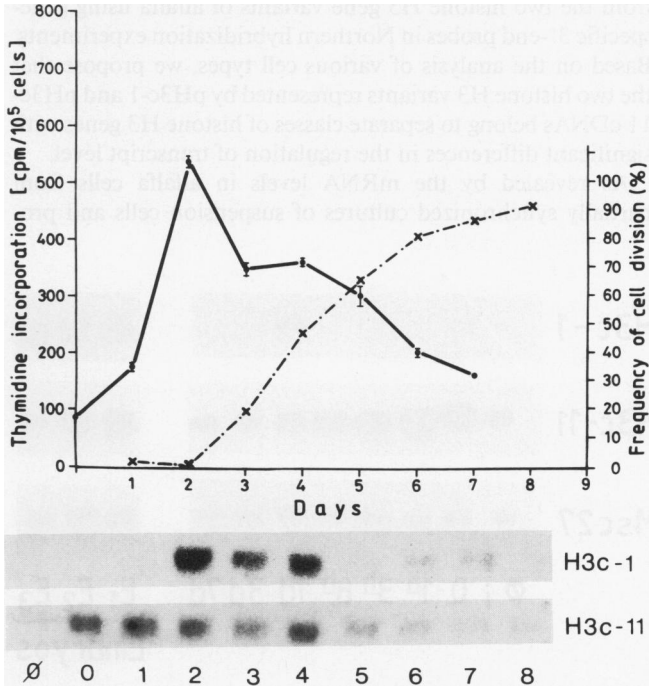

Figure 3. The time course of [^{3}H]thymidine incorporation (——), frequency of cells undergoing cell division (–·–·–·––), and level of transcript of two histone H3 gene variants during culture of alfalfa mesophyll protoplasts. Total cellular RNA was isolated from leaf tissues (ø), from protoplasts at the end of protoplasting enzyme digestion (O) and from cultured, protoplast-derived cells at sampling intervals of 1 d. H3c-1 and H3c-11 represent the signal obtained with 3′-end gene-specific hybridization probes from histone H3 cDNAs. Each lane contains 10 μg of RNA.

analysis of various RNA samples isolated from uninduced MCS or from 2,4-D-treated tissues which were subsequently cultured in hormone-free medium and from different stages of embryo development.

The amount of H3–1 transcript is low in MCS with the exception of a sharp increase 1 d after 2,4-D treatment. In contrast to MCS, differentiated embryos contained significant levels of H3–1 mRNA. The transcript level of the H3–11 gene is constantly high. Importantly, the 2,4-D treatment itself acts as an inducer for activation of H3–11 expression. Comparison of the various stages of embryo development revealed a considerable degree of stage specificity. In early-stage globular embryos, the level of H3–11 mRNA is much higher than at later stages. These differences suggest differential gene expression during the process of somatic embryogenesis.

DISCUSSION

Two distinct variants of histone H3 have been identified previously in alfalfa on the basis of electrophoretic mobility in an acid-urea-polyacrylamide gel system with a transverse gradient of Triton X-100 (23, 24). The existence of these histone H3 variants was clearly shown by nucleotide sequence comparison of cDNA clones isolated from alfalfa (28). Different classes of histone H3 genes with specific patterns of expression are also known in other eukaryotic organisms (2, 20). In the present study, we compared the levels of mRNA from the two histone H3 gene variants of alfalfa using gene-specific 3′-end probes in Northern hybridization experiments. Based on the analysis of various cell types, we propose that the two histone H3 variants represented by pH3c-1 and pH3c-11 cDNAs belong to separate classes of histone H3 genes with significant differences in the regulation of transcript level.

As revealed by the mRNA levels in alfalfa cells from partially synchronized cultures of suspension cells and protoplasts, the increase in the level of H3–1 transcript mRNA is coupled to increased DNA synthesis activity in the cultures. The differences between the two culture systems in terms of the time required to reach maximum levels of H3–1 mRNA and of DNA synthesis originates from the fact that the suspension cultures consist of fast, continuously cycling cells, whereas the differentiated mesophyll leaf cells in protoplast culture are at G_1 (or G_0) phase and require time for reactivation of the cell cycle.

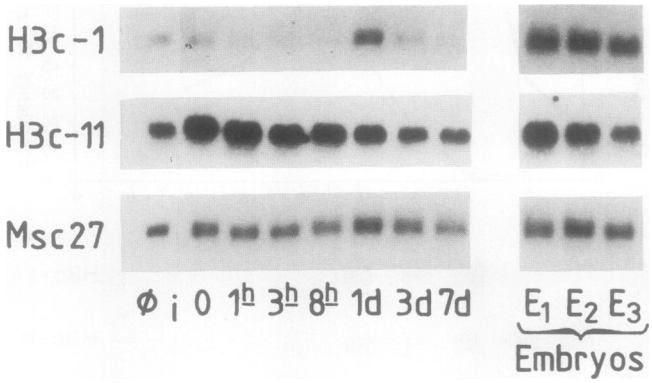

Figure 4. Amounts of histone H3 variants RNA in alfalfa MCS and in somatic embryos. Total cellular RNA was isolated from MCS grown under noninductive condition in the presence of 15 μM NAA and 10 μM KIN (ø), from MCS treated with 100 μM 2,4-D in the presence of 10 μM KIN for 1 h (O), and from MCS grown in hormone-free liquid medium for different periods of time shown as hours and days after induction (i). Stages of embryo development are referred to as globular (E_1), heart (E_2), and torpedo (E_3), based on the shape of the embryos separated for RNA extraction. Amounts of RNA are 24 μg/slot. Probes used for Northern analysis are the same as in Figure 2.

The detection of changes in the level of histone H3–1-variant mRNA during the cell cycle requires identification of potential regulatory sequence elements within the known 279-bp promotor-containing upstream region of this gene in the alfalfa H3 genomic clone ALH3–1.1 (27, 28). Neither the "GCGAAAA" motif from the yeast H2A and H2B genes (17) nor the CCTTATTTGCATAAG element of human H2B gene (9) could be found as possible cell cycle-specific elements in the alfalfa gene. However, this gene contains an "AAACACA" element at position −261 that is known to modulate S-phase expression of a chicken histone H1 gene (5). The presence of this cell cycle-specific promoter element in the H3–1 alfalfa gene supports the idea of cell cycle-related control for this histone H3 variant. The detailed characterization of potential *cis*-acting elements is in progress.

Based on the overall changes in the level of H3–11 mRNA in the studied cell types, this histone H3 gene variant shares characteristics of replication-independent (H3.3), as well as of partially replication-dependent (H2A2), histone variants (2). H3–11 transcripts were detectable in all cells and tissues analyzed. In synchronized cell suspension culture, considerable increase is observed in the level of H3–11 gene expression near the end of the peak of [^{3}H]thymidine incorporation, when the abundance of the H3–1 transcript already declines (Fig. 2). This may reflect the need for H3–11 proteins in the reorganization of chromatin at the time of completion of DNA duplication.

Increased amounts of H3–11 mRNA were observed in the phosphate-starved cells within the first 3 h after readdition of phosphate (Fig. 2), in freshly prepared mesophyll protoplasts (Fig. 3), in auxin-shocked MCS (Fig. 4), and at early stages of embryo development (Fig. 4). In all the above cases, the studied cells undergo significant metabolic changes linked to alterations in the pattern of gene expression. Formation of chromatin in a transcriptionally active state in cells stressed by external stimuli and during the developmental switch toward embryogenesis can be postulated as a prerequisite for a proper cellular response. Our observations about the presence of H3–11 transcripts in these cultured alfalfa cells might be correlated with the observation that the steady-state level of acetylation of the protein produced by the H3–11 gene, the histone H3.2-variant protein, was much higher than in the other H3 protein variant (24). Also, salt stress increased the multiacetylated forms of the two histone H3-variant proteins (23). In view of the possible role of histone acetylation in the formation of transcriptionally active chromatin (4) or the signal function of acetylation that modulates histone-protein and histone-DNA interactions (12), we think that the H3–11 gene product could be involved in changing chromatin structure and in the reprogramming of gene expression.

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