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Histone H3 transcript stability in alfalfa

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Abstract

The stability of histone H3 transcripts in alfalfa for replication-dependent and -independent gene variants was measured by northern analysis under conditions of inhibition of transcription and/or translation. Replication-dependent histone H3.1 transcripts were about three-fold less stable than the equally polyadenylated mRNA for replacement variant H3.2 histone. In actively growing suspension cultures treated with dactinomycin half-lives of 2 and 7 h were observed for H3.1 and H3.2 mRNAs, respectively. mRNA stabilities were also measured indirectly by histone protein synthesis. The translation inhibitor cycloheximide strongly increased mRNA levels for both histone H3 variants. The dependence of histone mRNA turnover on translation in animals also appears to exist in plants. The combination of inhibition of transcription and translation by dactinomycin and cycloheximide was used in an indirect assessment of H3 mRNA stability throughout the cell cycle in partially synchronized and cycle-arrested cultures. Destabilization of replication-dependent histone H3.1 mRNA was detected in non-S phase cells.

Introduction

In alfalfa two-thirds of histone H3 protein exists as variant form H3.1 [44], a replication-dependent histone variant [17] for which ca. 50 gene copies exist per haploid genome [34, 35, 49]. In contrast, one-third of histone H3 protein is the constitutively expressed [17] replacement histone variant H3.2 [45], produced from three gene copies only [34, 35]. Histone H3 variant protein synthesis rates appear even more disproportionate. In asynchronous suspension cultures of alfalfa A2 cells histone H3.2 is synthesized at a rate at least twice as high as observed for the replication-dependent H3.1 variant [45]. The rapid turnover of a highly acetylated fraction of the newly synthesized H3.2 protein leads to the observed steady-state protein variant ratio [45]. The contrast between the number of histone H3 variant gene copies and the level of histone H3 variant protein synthesis suggests more levels of gene expression control than the purely transcriptional regulation described to date for histone genes in plants [3, 7, 19, 22, 24].

Post-transcriptional control of cell cycle-regulated histone genes in animals strictly depends on the primary and secondary structure of newly synthesized mRNA. The presence of a T-

hyphenated 3′ stem-loop structure and a closely associated downstream sequence is required for proper transcript processing, mediated by U7 snRNP (small nuclear ribonucleoprotein) complexes. Poly(A)$^-$ mRNA transport to and translation in the cytoplasm also require a complete 3′ UTR (untranslated region) [39]. Translation and the 3′ stem-loop structure are required for mRNA degradation [12, 31]. Inhibition of translation stabilizes histone mRNAs [1, 41]. The half-life of histone mRNA, during S phase almost one hour, is reduced four-fold upon the natural completion of replication or when DNA synthesis is inhibited. The mechanism underlying this feedback control is unknown but excess histone protein may play a role [16, 28, 32].

Polyadenylated histone mRNA for mammalian replacement histone H3.3 is not subject to this control [15, 47]. In yeast, production of polyadenylated histone mRNA in the cell cycle is primarily regulated by transcription. There are no experimental data to suggest differential control at the processing level [28]. There exists, however, evidence that yeast histone mRNAs are subject to modulation of stability. The clearest example is dosage compensation: doubling the copy number of the H2A-H2B genes HTA1-HTB1 resulted in a two-fold decrease in the H2B mRNA half-life [29]. Also, it has been shown that mRNA concentrations from constitutively transcribed histone genes displayed periodic fluctuation during the cell cycle [30]. The 3′ coding and untranslated regions of yeast histone mRNAs are necessary for this cell cycle regulation [30, 52].

In plants all histone mRNAs are polyadenylated, whether transcribed from cell cycle regulated [5, 6, 34, 51] or constitutive [7, 8, 34] histone genes. Experimental evidence to date has suggested that plant histone gene expression is primarily or exclusively controlled by transcriptional regulation [3, 43]. Here we report the observation that the polyadenylated mRNA for replication-dependent variant H3.1 is much less stable than the similarly polyadenylated mRNA for replacement histone H3.2. This difference can partly account for the relative synthesis rates of the histone H3 variant proteins [45]. Inhibition of histone mRNA decay by the translation inhibitor cycloheximide supports the notion that histone mRNA degradation in plants is linked to translation, as in animals. Furthermore, variation in histone H3 mRNA stabilities in partially synchronized and cell-cycle arrested cultures suggests that a mechanism of feedback control exists.

Material and methods

Alfalfa plant cell cultures

Alfalfa (*Medicago varia*) A2 cells were cultured as described before [45]. Exponential growth was maintained by dilution of dense suspension cultures (2.0 to 2.5 ml of settled cells from 10 ml culture after a settling time of 5 min; this is also referred to as '20–25%' culture) at 3 day intervals into MS medium to a density of 8–10%. Cells blocked in G1 phase were obtained when growing cells were collected by centrifugation at 500 × *g*, resuspended in phosphate-free MS medium and cultured for four more days [33]. Replication in cell cultures was blocked by mimosine addition for 24 h to 0.5 mM from a filter-sterilized stock solution of 10 mM in water (pH adjusted with KOH to 5.8) [26, 46]. A2 suspension culture, abundant in S phase cells, was obtained by treatment with hydroxyurea as described before [19] at a concentration of 10 mM [9]. Colchicine (0.05% w/v) was used to enrich for mitotic cells as described [9, 14].

Radioactive cell labeling

Relative rates of DNA and RNA synthesis were assessed by cell labeling with [methyl-^{3}H]-thymidine (20 Ci/mmol, Dupont-New England Nuclear) and [5,6-^{3}H]-uridine (38.5 Ci/mmol, Dupont-New England Nuclear), respectively, at 1 μCi per ml culture for 30 and 60 min. Label incorporation was measured as cpm incorporated into a 5% trichloroacetic acid-insoluble pellet or into a 0.5 M perchloric acid hydrolysate (70 min 70 °C) supernatant of homogenized cells.

Histone H3 protein synthesis rates were mea-

sured in exponentially growing A2 suspension cultures by labeled for 30 min with 150 μCi *L*-[4,5-^{3}H(N)]-lysine (94 Ci/mmol, Dupont-New England Nuclear) per 50 ml culture.

Northern analysis of histone H3 mRNA

Total RNA was prepared from A2 suspension cells using TRIzol (Gibco-BRL) exactly as described [20]. Equal amounts of total RNA (20 μg), as determined as UV absorbance and confirmed by standard agarose gel electrophoresis in Tris-borate-EDTA (TBE) with ethidium bromide staining of ribosomal RNAs, were electrophoresed in duplicate in formaldehyde agarose gels, transferred in 20 × SSC to positively charged nylon membrane (Boehringer), and equal loadings were confirmed by methylene blue staining of the membrane [37]. Hybridization was performed on parallel blots with histone H3 variant-specific dioxigenin-labeled probes as prescribed by Boehringer for the Genius system with detection by CSPD chemiluminescence (Tropix). Histone H3.1-specific probe was prepared from a 520 bp head-to-tail tetramer [18] of the *Cla* I-*Hin*f I fragment of H3.1 genomic clone ALH3-1.1 [51]. H3.2-specific probe was prepared from a 820 bp head-to-tail tetramer [18] of the *Bsm* I-*Mun* I fragment of H3.2 cDNA clone pH3c-11 [51]. The hybridization signal was quantitated by densitometry on a range of film exposures to assure linearity.

Histone protein synthesis

Histones were extracted from lysine-labeled cells at various times after addition of dactinomycin or cordycepin and fractionated by reversed-phase HPLC into histone H2B, H2A with H4, and H3 pools as described before [45]. The specific radioactivity of histone H3 variants was measured as cpm per absorbance at 214 nm. Histone H3 was electrophoresed on acetic acid-urea-Triton X-100 (AUT) gels. All histones were analyzed on SDS gels. Gels were stained by Coomassie Brilliant Blue, quantitated by densitometry, fluorographed and quantitated as described before [44]. Label incorporation in all histone species was expressed in arbitrary units as fluorographic film absorbance per Coomassie absorbance with all densitometric measurements within linear exposure ranges.

Determination of histone mRNA half-life stability

Turnover of histone H3 mRNA was measured as decreasing mRNA amounts in northern or protein synthesis analyses over time upon inhibition of gene transcription by dactinomycin or cordycepin. Dactinomycin (actinomycin D in mannitol, Sigma) was dissolved in water to 1 mg/ml, filter-sterilized and used at 10 μg/ml unless otherwise specified. Cordycepin was dissolved at 50 mg/ml 95% ethanol, diluted to 12.5 mg/ml with water, filter-sterilized and used at 125 μg/ml unless otherwise specified. mRNA half-life estimates were calculated by exponential decay analysis of densitometry measurements from chemiluminescent northern and fluorography registrations on Kodak XAR film using SigmaPlot (Jandel Scientific).

The stability of histone H3 mRNA was established indirectly by the protein synthesis inhibitor cycloheximide as developed for CHO cells [15] by comparing in northern analyses the mRNA concentrations in untreated cells *vs.* cells incubated for 2 h with 10 μg/ml cycloheximide. The cycloheximide stock solution of 5 mg/ml in water was filter-sterilized. To assess the effect of cycloheximide on histone gene transcription histone RNA levels were also measured after 2 h in 10 μg/ml dactinomycin in the absence and presence of cycloheximide [15, 41].

Results

Stability of histone H3 mRNAs in asynchronously growing cultures

The stability of mRNA species can be measured when gene transcription is blocked. This is most

commonly done by measurement of steady-state mRNA levels following complete inhibition of transcription by inhibitors like dactinomycin [13]. Uridine incorporation, a crude measure for transcription, was inhibited in alfalfa A2 suspension cells by incubation for 2 h with low concentrations of dactinomycin (Fig. 1A). Histone protein synthesis, typically a fair but indirect measure for histone mRNA concentrations, was also inhibited as seen for replication-dependent histone H3.1 and for the other core histones (Fig. 1B).

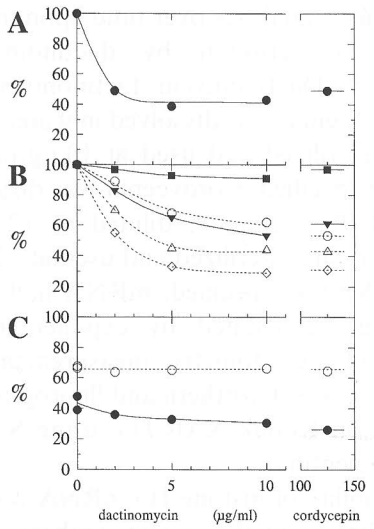

Fig. 1. Inhibition of histone synthesis by dactinomycin and cordycepin. Exponentially growing alfalfa A2 suspension cultures were pre-incubated with dactinomycin and cordycepin at the concentrations shown in μg/ml, followed by a 30 min incubation with radioactive tracers in the continued presence of the inhibitor. A. Incorporation of tritiated uridine, as per Materials and methods, into A2 cells relative to incorporation in the absence of inhibitors. B. Incorporation of tritiated lysine as a measure of the rate of histone synthesis, relative to the untreated control samples, was determined from the specific radioactivity of tritiated lysine incorporated in histone H3.1 (solid triangles) and H3.2 (solid squares) in the HPLC eluent and in AUT gels, and of histone H4 (open circles), H2A (open triangles) and H2B (open diamonds) in SDS gels, as described in Materials and methods. C. Relative proportion of histone H3.1 within total histone H3 steady-state protein (open circles), determined as absorbance in the HPLC eluent and as Coomassie density in AUT gels, and of newly synthesized histone H3.1 within total *de novo* synthesized histone H3 (solid circles), determined by liquid scintillation of the HPLC eluent and as fluorographic density in AUT gels, as described in Materials and methods.

Synthesis of the constitutively expressed replacement histone variant H3.2 [17, 45] which represents a minor fraction of total histone H3 but the majority of new histone H3 synthesis (Fig. 1C), was much less affected (Fig. 1B). This is reflected in the changing ratio of *de novo* histone H3 variant synthesis (Fig. 1C). This observation indicated that histone H3.2 mRNA might be more stable than the mRNAs of the S phase-dependent core histones.

Since mRNA stability may be affected by inhibitor treatment [13], additional and independent modes of transcriptional inhibition were evaluated, as done by others [10, 27]. Cordycepin, an adenosine analog that interferes with transcript processing and polyadenylation [10, 22, 27], inhibits uridine incorporation and histone synthesis in alfalfa effectively after a preincubation of 2 h (Fig. 1) at concentrations from 50 to 250 μg/ml. α-Amanitin, an inhibitor for RNA polymerases, failed to inhibit uridine incorporation and histone synthesis at concentrations up to 10^{-5} M with or without preincubation for 2 h (results not shown). Such failure has also been observed in tobacco [27].

Despite the fact that inhibition of uridine incorporation was incomplete at 10 μg/ml dactinomycin and at 125 μg/ml cordycepin (Fig. 1A), we proceeded to measure steady-state mRNA concentrations for both histone H3 variants by northern analysis in the knowledge that half-life values obtained under these maximally attainable levels of transcriptional inhibition would represent upper limit values. Inhibition of uridine incorporation increased within 1 h, with a slight lag for cordycepin (Fig. 2A). Decreasing mRNA concentrations for histone H3.1 and H3.2 were observed by northern analysis upon inhibition of transcription by dactinomycin (Fig. 3A) and cordycepin (Fig. 3B). Quantitation of these analyses (Fig. 2B and 2C, respectively) showed an exponential decay for both histone H3 mRNAs with half-lives of ca. 2 h for H3.1 and of 6 and 9 h for H3.2 (Table 1). Apparently the mRNA of replacement histone variant H3.2 is 3 to 4 times more stable than that of replication-dependent variant H3.1.

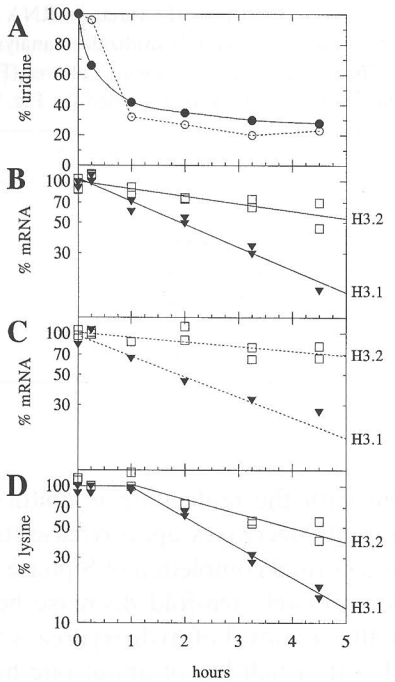

Fig. 2. Determination of histone H3 mRNA half-lives in growing cell cultures. Alfalfa A2 suspension cultures were incubated with 10 μg/ml dactinomycin (solid lines) or 125 μg/ml cordycepin (broken lines) for the indicated period of time. A. Incorporation of tritiated uridine during 30 min in dactinomycin (solid circles) or cordycepin (open circles), relative to incorporation in the absence of transcriptional inhibitors. The two middle panels show the level of mRNA for H3.1 (solid triangles) and H3.2 (open squares), determined by northern analysis, relative to mRNA levels in untreated cultures, in the presence of actinomycin (B) or cordycepin (C). D. Specific radioactivity of tritiated lysine, incorporated for 30 min in dactinomycin into histone H3.1 (solid triangles) and H3.2 (open squares), determined from liquid scintillation counting and absorbance in the HPLC eluent and from fluorography and Coomassie density in AUT gels, as described in Materials and methods, relative to values obtained in the absence of dactinomycin. The straight lines, representing exponential decay, were calculated for panels B and C using all data points and for panel D excluding data within the first 0.5 h.

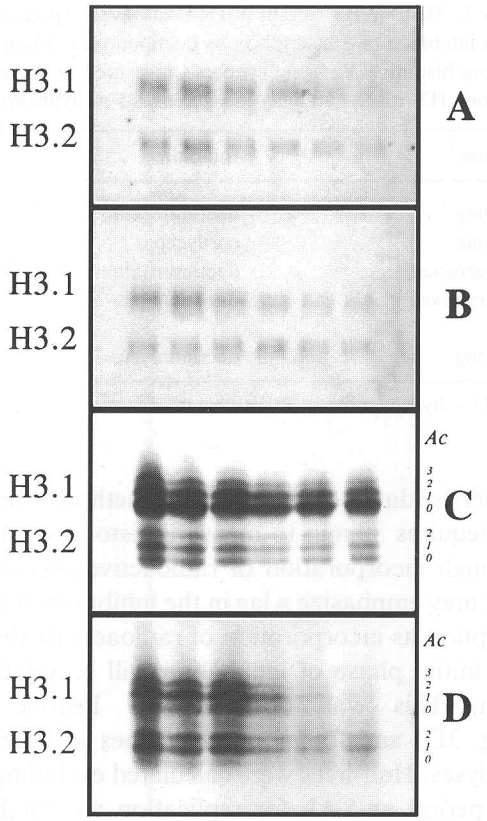

Fig. 3. Gel analysis of histone H3 mRNA and protein levels upon inhibition of transcription. A. Northern blot of histone H3 variant mRNA upon treatment of A2 cells with dactinomycin, as described for Fig. 2B. Lanes from left to right represent treatment for 0, 0.25, 1.0, 2.0, 3.25 and 4.5 h. To facilitate comparison of mRNAs, exposures of the same blot with different probes were offset to prevent overlap of H3.2 mRNA with the slightly shorter H3.1 mRNA. The chemiluminescent exposures with different probes were adjusted to obtain similar band densities. Consequently, the band intensities of H3.1 *vs.* H3.2 mRNA do not reflect mRNA amounts. B. Northern analysis, as in panel A except for transcriptional inhibition by cordycepin. Quantitation as in Fig. 2C. C. AUT gel Coomassie staining pattern of histone H3 protein prepared from the cultures identical to those shown in panel a, incubated with tritated lysine as described in Materials and methods and purified by HPLC. Protein loading levels were not adjusted between lanes. Detectable levels of acetylation are marked for histone H3 variant proteins. D. Fluorography of the AUT gel of panel C, with equivalent marking for acetylated, newly synthesized H3 variant proteins.

De novo protein synthesis provides a measure of mRNA available for translation and thus can assess indirectly mRNA decay, assuming that translational efficiency is equal and constant. Tritiated lysine incorporation into histones was measured by histone purification and fractionation through reversed phase HPLC chromatography with determination of specific radioactivity, AUT gel electrophoresis, gel staining (Fig. 3C) and fluorography (Fig. 3D). Synthesis of all core histones was determined using SDS gel electro-

Table 1. Histone H3 variant mRNA half-lives. Apparent half-lives with standard errors of histone H3 variant mRNA measured upon inhibition of transcription by dactinomycin (10 μg/ml) or cordycepin (125 μg/ml) by northern hynridization analysis and by *de novo* histone H3 protein synthesis measured by incorporation of tritiated lysine in exponentially growing cultures (Figs. 2, 3). Histone H3 mRNA stability measurements in hydroxyurea-arrested and -released cultures were as described for Fig. 5.

Culture[1]	Inhibitor	Analysis	$T_{1/2}$ H3.1	$T_{1/2}$ H3.2
growing	dactinomycin	northern	1.8 ± 0.2 h	5.6 ± 1.2 h
growing	cordycepin	northern	2.0 ± 0.3 h	9.1 ± 2.6 h
HU-arrested	dactinomycin	northern	1.5 ± 0.1 h	7.1 ± 1.1 h
HU-released	dactinomycin	northern	1.7 ± 0.2 h	
growing	dactinomycin	lysine label	1.4 ± 0.1 h	2.9 ± 0.5 h

[1] HU = hydroxyurea

phoresis (data not shown). This method of analysis requires 30 min to measure histone synthesis through incorporation of radioactive precursors and may emphasize a lag in the inhibition of transcription as incorporation of radioactivity during the initial phase of incubation will be relatively high. This was observed for histone H3 (Fig. 3D) and for all core histones in SDS gel analyses. Half-lives were calculated excluding the lag period as 1.4 h for replication variant H3.1 (Table 1), 1.5 h for histone H4 and ca. 1 h for histones H2A and H2B. The stability of the replacement H3.2 mRNA was two-fold higher than that of the H3.1 variant (Table 1). This indirect determination of histone H3.1 mRNA stability is virtually identical to the direct measurement by northern analysis. It may be slightly lower due to the fact that in the protein synthesis experiments a definite lag period could be identified, and thus excluded, from mRNA stability calculations (Fig. 2D). Even though the same factor probably applies to the H3.2 variant, the apparent H3.2 mRNA stability measured by *de novo* protein synthesis, is about two-fold lower than that measured directly (Table 1). The possible significance of this difference is discussed later.

Histone H3 protein synthesis and mRNA levels in arrested cell cycles

The high concentration of histone H3.1 mRNA in alfalfa A2 culture, abundant in S phase cells by treatment with the replication inhibitor hydroxyurea, rapidly decreases upon release from inhibition and normal completion of S phase [19, 23]. The approximately ten-fold decrease between 4 and 7 h after removal of hydroxyurea would correspond with a half-life of about one hour. The values obtained in our experiments are higher (Table 1). However, they were measured while transcription was inhibited [13]. Also, they are the average for a heterogeneous population of cells in various phases of the cell cycle and thus with varying rates of histone transcription and steady-state mRNA levels. Transcripts of the replication-variant H3.1 are barely detectable, if at all, in cells outside S phase [17, 19]. Thus, the rate of mRNA decay measured for this variant would reflect primarily its stability in actively replicating cells. In contrast, the replication-independent pattern of expression of the H3.2 genes [17] would tend to yield an average mRNA stability across all phases of the cell cycle.

To assess possible differences in H3 transcript stability throughout the cell cycle we employed a series of established protocols [9, 14, 19, 33, 46] that will arrest alfalfa cells at different stages of the cell cycle. Analysis of such stable cell populations that are expected to reflect distinct phases of the unperturbed cell cycle, were confirmed by measurements in cells following release from cell cycle arrest (not shown).

Early exponentially growing alfalfa A2 cultures contain a significant fraction of cells in S phase with the majority of the rest in G1 phase [9, 33].

As shown before [45], about one-third of *de novo* histone H3 protein synthesized is replication variant H3.1 (Fig. 4A). When progression through S phase is impeded by hydroxyurea, the fraction of cells in S phase increases and the rate of radioactive thymidine incorporation is elevated [9, 19]. A three-fold increase ($295 \pm 62\%$, $n = 6$) in H3.1 protein synthesis is seen (Fig. 4B), a direct reflection of the increase in H3.1 mRNA concentrations (Fig. 4D). Extended culture of cells in phosphate-free medium causes an arrest in G1 phase and the disappearance of S phase cells [33]. This is reflected in the reduction to $15 \pm 4\%$ ($n = 3$) in the synthesis of replication-dependent histone H3.1 protein (Fig. 4C). The response on synthesis rates of core histones H2A, H2B and H4, measured by fluorography of SDS gels (results not shown), mimicked the effects observed for replication-dependent histone H3.1 synthesis: a three-fold increase when the proportion of S phase cells was increased by hydroxyurea and a virtual cessation of synthesis upon phosphate starvation.

Under these different conditions of arrest in cell cycle progression, the steady-state concentrations of cell cycle-independent H3.2 mRNA decreased (Fig. 4D), with a similar two-fold reduction in protein synthesis rates (Fig. 4B, 4C) to $54 \pm 17\%$ ($n = 15$). A high synthesis rate is required for this replacement histone to maintain the integrity of transcribed chromatin in plants [45]. A reduction in this need in arrested cells is not unexpected. However, this effect supports the notion that feedback control on the translation of histone mRNA into protein exists.

Histone H3 mRNA stability in different stages of the cell cycle

Histone H3 northern analyses of these cell cycle-arrested cultures were performed upon dactinomycin inhibition of transcription. The relatively high concentration of replication-dependent H3.1 mRNA in cells, treated with hydroxyurea (Fig. 5A), decayed under these conditions with a half-life of 1.5 ± 0.1 h ($n = 5$). The same half-life

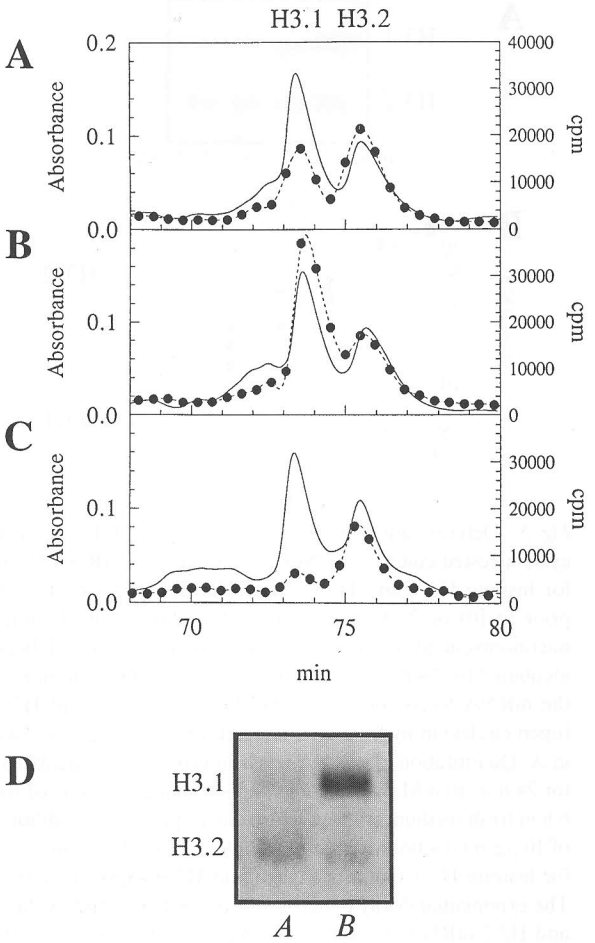

Fig. 4. Histone H3 variant mRNA and protein synthesis levels in cell cycle-arrested cultures. A. Alfalfa A2 cells were grown in exponential cultures and incubated for 30 min with tritiated lysine, as described in Materials and methods. Histone proteins were extracted and fractionated by reversed-phase HPLC. Histone H3 proteins eluted at a concentration of ca. 52% acetonitrile. In the experiments shown, histone H3.1 eluted at 73.5 min and histone H3.2 at 75.5 min. The absorbance at 214 nm (line) is shown with the radioactivity as cpm per 0.5 ml fraction (solid circles on broken line). Panel B shows the same analysis for histone H3 for a culture that was incubated for 24 h in 10 mM hydroxyurea. The absorbance pattern, with associated lysine incorporation, represents the same amount of total histone H3 present in A in order to facilitate comparison between different panels. Panel C shows the same analysis as in panel B for a culture, grown for 4 days in phosphate-free MS medium. D. Northern analysis of mRNA levels for histone H3.1 and H3.2 obtained from the cultures of A and B. Data presentation is as described in the legend of Fig. 3. The RNA sample for the culture in C was not available.

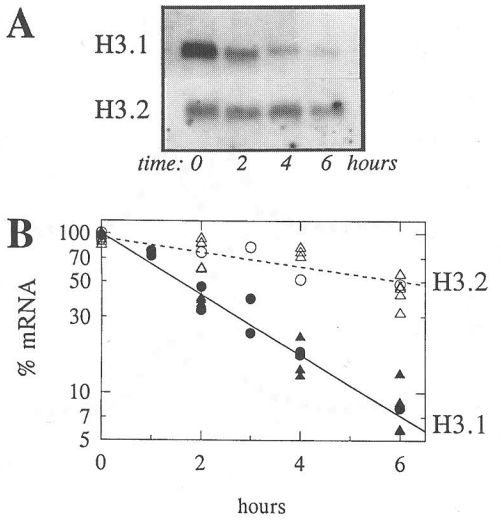

Fig. 5. Determination of histone H3 mRNA half-lives in cell cycle-arrested cultures. A. Northern analysis of mRNA levels for histone H3.1 and H3.2, shown as described for Fig. 3, prior to (0) or 2, 4 and 6 h after the addition of 10 μg/ml dactinomycin to an A2 suspension culture which had been incubated for 24 h in 10 mM hydroxyurea. B. Quantitation of the mRNA levels for histone H3.1 (solid circles) and H3.2 (open circles) in hydroxyurea-arrested cell cultures, as shown in A. Quantitation of mRNA levels in cell cultures, incubated for 24 h in 10 mM hydroxyurea and subsequently cultured for 6 h in fresh medium without hydroxyurea prior to the addition of 10 μg/ml dactinomycin and sampling for northern analysis, for histone H3.1 (solid triangles) and H3.2 (open triangles). The exponential decay patterns shown for H3.1 mRNA (line) and H3.2 mRNA (broken line) were calculated using all data shown.

(1.7 ± 0.2 h, $n = 2$) was measured when hydroxyurea cells were released from this block and H3 mRMA concentrations were measured during inhibition of transcription by dactinomycin, applied 6 h after release. Within experimental error the much slower decay rates for H3.2 mRNA under these conditions were also the same (Fig. 5B, Table 1). In these cultures, as in asynchronously growing ones, the mRNA stability of H3.1 is primarily measured in cells with high mRNA levels. The similarity between H3.1 stabilities in all these experiments (Table 1) is consistent with this interpretation. Cell cycle arrest by hydroxyurea, by itself, apparently has no effect.

In animal cells, histone mRNA turnover directly depends on translation [12, 38]. This observation has been used to measure indirectly

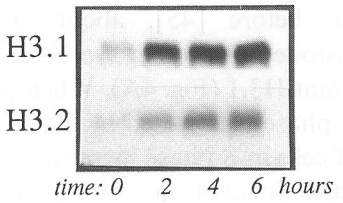

Fig. 6. Cycloheximide effect on steady-state levels of histone H3 mRNA. Total RNA was extracted from alfalfa A2 suspension cultures grown without (0) or after 2, 4 and 6 h with 10 μg/ml cycloheximide. Histone H3.1 and H3.2 mRNA levels were determined by northern analysis and shown as described for Fig. 3.

the stability of histone mRNAs [15]. Cycloheximide inhibition of translation causes the strongest increase in histone mRNA concentrations for conditions with the fastest turnover rates [1, 15, 25, 41]. Thus, stability of replication-dependent histone mRNAs can be assessed outside of S phase [15, 25, 40] but no absolute rates of mRMA decay can be obtained. In plant cells the same dependence of histone mRNA turnover on translation may exist. Treatment of wheat seedlings with cycloheximide raised histone H3 mRNA from undetectable to highly abundant levels [21]. Incubation of alfalfa A2 cells with cycloheximide strongly increased the mRNA concentration for both histone mRNAs (Fig. 6).

Since cycloheximide can also affect histone gene transcription [2, 11, 21], we compared the effects of cycloheximide with and without dactinomycin to detect major effects on transcription and to assess whether the mode of action of cycloheximide remains the same under different cell culture conditions. Increase in mRNA concentrations by cycloheximide with dactinomycin should largely be due to mRNA stabilization. The increase by cycloheximide alone could represent a combined effect of a higher rate of transcription and a lower rate of mRNA decay. The decrease by dactinomycin is a control for inhibition of transcription and should be consistent with the turnover rates measured in dactinomycin (Fig. 2, Fig. 5). The response of the two H3 mRNAs to cycloheximide was quite distinct (Fig. 7). Exposure of growing cultures to cycloheximide for 2 h increased the mRNA concentration for both H3

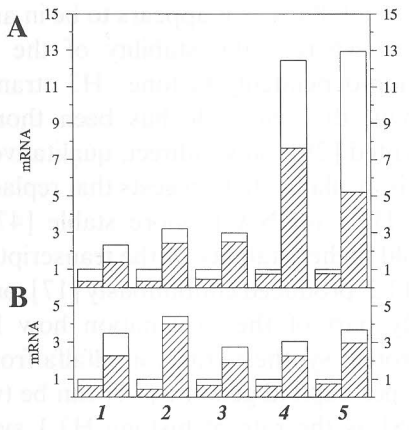

Fig. 7. Response of H3 mRNA levels to cycloheximide and dactinomycin. A. Histone H3.1 mRNA levels were analyzed by northern analysis, as described in Materials and methods, under various culture conditions and the level in cultures without addition of any drug are shown as the 1.0 standardized level in the left, open column for each set of paired columns. The left, filled column represents the amount of H3.1 mRNA observed 2 h after the addition of 10 μg/ml dactinomycin. The right, open column shows the standardized amount of H3.1 mRNA observed 2 h after addition of cycloheximide to 10 μg/ml and the filled column the amount 2 h after addition of 10 μg/ml cyclohaximide and 10 μg/ml dactinomycin. The Y-axis shows the numerical scale for comparison with the control amount of H3.1 mRNA without any additions. The conditions used to analyse the effect of cycloheximide were, from left to right: 1, exponentially growing cultures of alfalfa A2 cells; 2, cell cultures incubated for 24 h in 10 mM hydroxyurea; 3, cell cultures incubated for 3 days in phosphate-free MS medium; 4, cell cultures incubated for 24 h in 10 mM hydroxyurea and subsequently, without hydroxyurea, for 24 h in 0.05% colchicine; 5, cell cultures incubated for 24 h in 0.5 mM mimosine. B. Northern analysis of histone H3.2 mRNA, shown exactly as described for A.

variants about three-fold (Fig. 7, lane 1) with dactinomycin reductions as expected (Fig. 2). Cycloheximide addition had the same effect in cultures with a higher or a lower fraction of S phase cells, produced by hydroxyurea treatment (Fig. 7, lane 2) or phosphate starvation (Fig. 7, lane 3), respectively. This not necessarily implies that the effect of cycloheximide at all points in the cell cycle is invariant. In non-S phase cells, collected in mitosis by colchicine after cell cycle arrest with hydroxyurea (Fig. 7A, lane 4) or in replication block attained by prolonged incubation with mimosine [26, 46], as confirmed by cessation of thymidine incorporation in treated cultures and a reduction in the steady state level of the replication variant H3.1 mRNA (results not shown), the increase from the low level of replication-dependent H3.1 mRNA was much larger. This, and the relatively high inhibition by dactinomycin, suggest that in non-S phase cells the replication-dependent H3.1 genes are transcribed, in agreement with the dual promoter activity of plant histone genes [22], that their transcription is affected by cycloheximide treatment, and that the H3.1 mRNA produced is highly labile (Fig. 7A). Such effects were not observed for histone H3.2 (Fig. 7B), consistent with the much more constant level of replication-independent H3.2 mRNA throughout the cell cycle [17].

Discussion

Higher plants, like yeast, possess histone mRNAs with long, polyadenylated 3′UTR sequences that lack the stem-loop structure and downstream sequence elements, essential for the posttranscriptional regulation of replication variant histone mRNAs of animals. This, the analysis of promoter elements responsible for cell cycle regulation in plants and their relatively long cell cycle have suggested that in higher plants histone mRNA levels are controlled by transcription [3, 43]. In a previous study we have demonstrated that the promoter region of one of the H3.1 genes, ALH3-1.1, conferred meristem-specific expression on the β-glucurodinase (GUS) reporter gene [19]. While this construct showed elevated mRNA concentrations in S phase cells, however, the difference between the mRNA concentrations in S and post-S phase cells was only two- to three-fold, much lower than the 15-fold difference observed for the endogenous histone H3.1 transcripts [19]. This suggested that, in addition to the transcriptional component, other regulatory mechanisms might control histone H3 mRNA levels.

In the present study, using different experimental approaches, we present our findings about a differential stability of histone H3 gene variant

mRNAs in alfalfa. In our first series of experiments we observed that the constitutively expressed H3.2 mRNA is about three times more stable than the mRNA of replication variant H3.1 genes. First, the turnover of histone H3 mRNA was measured directly by northern analysis, following a pattern of simple exponential decay in asynchronously growing cells, treated with dactinomycin (Fig. 2B) and cordycepin (Fig. 2C). We selected two different types of inhibitors because it is known that inhibition of transcription by drugs may induce artificial stabilization of mRNA [13, 32, 42]. The similarity in mRNA stability observed with dactinomycin and cordycepin may suggest that the measured mRNA decay rates reflect reality (Table 1). In our alfalfa A2 suspension cultures with their very small cell clusters and direct accessibility of each cell to the growth medium, we did not see the hour lag phase in dactinomycin effect observed with tobacco cells [27]. Even though inhibition of uridine incorporation appeared incomplete (Fig. 3A), the simple exponential decay patterns (Fig. 2, Fig. 5) suggest that inhibition of histone gene transcription is effective very shortly after application, and that the half-life values obtained (Table 1) represent real mRNA turnover rather than upper limits of mRNA stability. As a different experimental approach, we measured mRNA decay indirectly as the decreasing rate of *de novo* histone protein synthesis when transcription was inhibited. For histone H3.1, the half-life values obtained were close to those seen by monitoring directly the pattern of histone H3.1 mRNA disappearance (Table 1). This not only confirmed the northern analyses, but also supports the validity of our assumption that translational efficiency would not change during drug treatment. In the case of the H3.2 variant, however, stability of the mRNA, measured directly by the rate of *de novo* protein synthesis, was two-fold less than that measured directly by northern analysis. This points to the possibility that a variable fraction of the total H3.2 mRNA is translatable. The implications of this idea are discussed below.

The higher stability of the replacement H3.2 mRNA *versus* replication-dependent H3.1 mRNA in alfalfa is as it appears to be in animals. However, whereas the stability of the animal replication-dependent histone H3 transcripts throughout the cell cycle has been thoroughly documented [28], only indirect, qualitative information is available that suggests that replacement histone H3.3 mRNA is more stable [47]. The three-fold higher stability of the transcript for histone H3.2, produced continuously [17], provides probably part of the explanation how histone H3.2 protein synthesis rates in alfalfa from only 3 genes per haploid genome [34] can be twice as high [45] as the rate of histone H3.1 synthesis from at least 50 genes [34, 49], even when their expression is limited to S phase [17].

An average half-life of 1.5 h for histone H3.1 mRNA decay (Table 1) cannot account for the rapid disappearance of H3.1 transcripts upon S phase completion [19, 23]. The rapid, about ten-fold decrease in the mRNA concentration even in partially synchronized callus suspension cultures would require a half-life of about 1 h. Since this appeared to be less than the rate of H3.1 decay in asynchronous cultures, we had to assess mRNA stability throughout the cell cycle. Because synchrony is often lost soon after removal of synchronizing agents, we used methods that would cause the arrest or slow-down of the cell cycle at distinct points. We used hydroxyurea to increase the fraction of cells in S phase. At low concentrations (10 mM) this drug causes a slow-down of cell cycle progression through S phase without effectively blocking DNA replication. The increase of the fraction of cells in S phase is reflected by an increased rate of radioactive thymidine incorporation (results not shown) [19], elevated levels of histone mRNA, for example for histone H3.1 (Fig. 4D), and increased rates of synthesis of histone proteins, H3.1 (Fig. 4B) and all other replication-dependent histones (results not shown). The interpretation of these observations is supported by flow cytometry analyses [9, 19]. At much higher concentrations (100 mM) these effects are not observed [4, 22], probably due to severe inhibition of cell metabolism, reflected in the known drop in cell viability [9]. The rate of H3.1 mRNA decay measured with dacti-

nomycin for cultures arrested by hydroxyurea (Fig. 5) was essentially the same as in asynchronous cultures (Fig. 2). This supports the argument that the alfalfa A2 cells treated with low concentrations of hydroxyurea are essentially the same as unperturbed S phase cells.

Since the H3.1 mRNA concentration is very low outside of S phase [17, 19], we used a different approach to evaluate the stability of the replication-dependent H3.1 transcripts in the absence of replication. Stabilization of histone mRNAs by cycloheximide in animals has been well documented, although the basis for the increase in mRNA levels has not been fully analyzed [2, 12]. The assumption is that in cycloheximide mRNAs with a high rate of turnover will increase more in steady-state level than less labile ones [15]. We observed that cycloheximide strongly increased the mRNA concentrations of both H3 variants in our plant cells (Fig. 6). Because transcriptional activation might also be induced by cycloheximide [2, 11, 21], we used a combination of cycloheximide and dactinomycin so that transcriptional and stability components that cause the rise in mRNA levels might be distinguished. In S phase cells obtained by hydroxyurea treatment [9], in cells cultures in phosphate-free medium [33] and in asynchronous cultures alike, similar increases, approximately three-fold, in histone transcript concentrations were observed with cycloheximide. The reduction in the response in dactinomycin showed that cycloheximide affects gene transcription (Fig. 7). In contrast to arrest at the G1/S boundary by a short application of mimosine, prolonged incubation of mammalian cells with this drug results in cells that pass through the transition point and slow down in S phase when DNA replication is blocked. This was reflected by a decreased rate of bromodeoxyuridine incorporation and a low level of histone H4 mRNA [46]. In our alfalfa callus suspension culture, A2 cells respond to a 24 h mimosine treatment in a very similar way (results not shown). In these cells, and in mitotic cells arrested by the metaphase inhibitor colchicine, the low concentrations of the replication-dependent H3.1 mRNA were increased more than tenfold by cycloheximide treatment (Fig. 7). The strong reduction in the response by dactinomycin indicates that cycloheximide caused transcriptional activation of the H3.1 genes in these cells in addition to mRNA stabilization (Fig. 7). Based on these results and assuming that cycloheximide has the same effect in all cell populations, the indirect cycloheximide method has demonstrated that differences in H3.1 mRNA stability exist under different culture conditions. In suspension cultures, the stability in the majority of cells in G1 or S phase appears to be similar. Upon inhibition of replication and during mitotic block, H3.1 mRNAs are less stable. If we accept that inhibition of DNA replication by mimosine [46] is analogous to the natural completion of S phase [28, 48], the observed changes may reflect processes that occur at the end of S phase or in transition to G2 phase. In this light, our results are in agreement with those of Harris and coworkers [15] who found that in CHO cells histone mRNA stability was the same in G1 and S and that it decreased strongly when cells entered G2 phase. Also, Morris and coworkers [25] observed in the HeLa cell cycle that H3 mRNA was less stable in late S phase, most stable in early S and that G2/M/G1 represented an intermediate stability condition. In our experiments, in asynchronous cultures, the increase by cycloheximide was the same as in hydroxyurea-treated cells (Fig. 7). This is in good agreement with the direct measurements of H3.1 mRNA stability in dactinomycin because the asynchronous cultures, used 24 h after subculture, contain a high percentage of A2 cells that are entering DNA replication, as determined by thymidine labeling (unpublished results). Our results would explain the observed difference of the average H3.1 mRNA stability in dactinomycin (Table 1) and in highly synchronous cells, released from hydroxyurea [19, 23].

As shown in Fig. 6, the level of H3.2 mRNA also becomes elevated upon cycloheximide treatment. To our knowledge, this is the first report of a replacement histone responding to such a treatment. The degree of response to cycloheximide may vary somewhat with growth conditions or culture treatment; hydroxyurea, for example, gave

a slightly stronger response (Fig. 7). Such differences were not detected in the direct measurements of H3.2 mRNA stability in dactinomycin (Table 1). This could be due to a feedback response when transcription is reduced, as suggested by the decrease in H3.2 mRNA concentrations in phosphate-free medium (Fig. 4). Double phosphate starvation synchronization of A2 cells showed that H3.2 mRNA concentrations varied with changes in DNA replication levels but with peak values later than the maximum in DNA synthesis and H3.1 mRNA [17]. H3.2 mRNA concentrations also increase in response to general stresses like treatment of the A2 plant cell wall with protoplasting enzymes, auxin shock and phosphate refeeding after deprivation [17]. While these responses likely are transcriptional, modulation of mRNA stability may also play a role. In a number of cases it has been documented that external conditions such as heat shock, hormones and growth factors affect mRNA stability [32]. Quantitatively, the three-fold increase in the H3.2 mRNA concentration under conditions of cycloheximide inhibition of translation appears out of proportion to the four-fold increase in H3.1 mRNA (Fig. 7) when one compares these values with the half-lifes of 2 h *versus* 7 h for H3.1 and H3.2 mRNA, respectively, obtained by measuring mRNA decay rates directly (Table 1).

The apparent instability of H3.2 mRNA, assessed indirectly by the translation inhibitor cycloheximide, is more in line with the indirect determination of a half-life of ca. 3 h, obtained by measuring the rate of *de novo* H3.2 protein synthesis (Table 1). The interpretation of this similarity is not completely clear. It appears consistent with our observation that only a fraction of H3.2 mRNA is involved in translation and that this mRNA fraction has a turnover rate faster than observed overall for histone H3.2 mRNA by northern analysis. This could not be detected by measuring mRNA decay in dactinomycin-blocked cells because this faster decay pattern is obscured by a more gradual one. In early logarithmic culture, a significant proportion of the cells is involved in DNA replication. In these cells all the replication-dependent histones are synthesized during the short period of S phase. It is possible that different histone mRNAs compete for translational machinery. In such a situation, the abundant replication-dependent mRNAs would limit translation of the lower amounts of replacement variant H3.2 mRNA, and thus, only a fraction of this mRNA would contribute to protein synthesis. In line with this view is our finding that in A2 cultures, treated with hydroxyurea and abundant in S phase cells, the rate of H3.2 histone protein synthesis is reduced (Fig. 4B). mRNA that is used in this translation into H3.2 protein appears to be less stable with a half-life of 3 h (Table 1) and a relatively higher increase in H3.2 mRNA levels when cycloheximide is used to block translation (Fig. 7B, column 2). The decreased stability of this H3.2 mRNA fraction may involve a feedback control, possibly similar to the mechanism observed in animal cells [28].

Our results have demonstrated that the changing concentrations of histone H3 mRNAs under various culture conditions of alfalfa [17] are regulated at multiple levels. These include transcription [19] and mRNA stability, each contributing to the overall concentration of histone H3 mRNA, and the rate of histone H3 variant protein synthesis (Fig. 4). Also, our results show that in plants turnover of histone mRNA may depend on translation as it does in other eukaryotic cells [12, 36]. Experiments are in progress to try to define the elements of mRNA stability and feedback control.

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