THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1993 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 268, No. 7, Issue of March 5, pp. 4912-4917, 1993
Printed in U.S.A.

Histone Synthesis and Turnover in Alfalfa

FAST LOSS OF HIGHLY ACETYLATED REPLACEMENT HISTONE VARIANT H3.2*

(Received for publication, September 4, 1992)

Jakob H. Waterborg‡

From the Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri, Kansas City, Missouri 64110-2499

Histone synthesis in alfalfa tissue culture cells was studied by labeling with tritiated lysine, purification of histone proteins by reversed-phase high pressure liquid chromatography, and fluorography of acid/urea/Triton X-100 polyacrylamide gels. Minor histone variant H3.2 was synthesized twice as fast as major variant H3.1. The predicted difference in histone H3 variant turnover was examined during continued growth. More than 50% of newly synthesized histone H3.2 and 20% of new H3.1 were lost from chromatin over a period of 100 h. This produced a ratio between the stable remaining portions of each new histone H3 variant protein identical to that of the steady-state histone H3 variants. The labile portion of new histone H3.2 (half-life of 20 h) was rapidly lost specifically from transcriptionally active chromatin as judged by the acetylation level of nearly 1.5 acetylated lysines/histone molecule, a level 50% higher than the acetylation in histone H3.2 overall and three times that of histone H3.1. These results and the constitutive level of H3.2 gene expression identify histone H3.2 of alfalfa as a functional replacement histone variant. The extent of its preferential assembly into active chromatin nucleosomes and the rapid rate of its subsequent loss indicate significant dissolution of plant nucleosomes during gene transcription.

Histone genes represent the archetypical example of genes expressed during a limited phase of the cell cycle with histone protein synthesis coordinately dependent on DNA replication (1). In contrast, some histone genes are expressed in a constitutive or tissue-specific manner, even in nonproliferating cells (2, 3). A major function of these latter histone genes appears to be repair of chromatin structure through the assembly into nucleosomes on DNA sequences that have reduced nucleosomal densities as a result of losses incurred during gene transcription (3–6). Over time and in the absence of DNA replication they replace prior existing nucleosomes within the chromatin structure. This has given this type of constitutively expressed histone variant the functional name of *replacement* histone. Histone variant H3.3 in mammals and birds represents the most well studied example of such a replacement histone (2–4). Its primary protein sequence is highly conserved, only slightly different from that of replication-dependent H3 histones, and it is produced continuously from poly(A)$^+$ mRNA (2–4, 7).

Recently a minor histone H3 gene in alfalfa has been recognized as the first histone H3 gene in plants with an apparent constitutive pattern of expression (8, 9). It exists in approximately four gene copies/genome,[1] which produce one third of the steady-state histone H3 protein in a variant form named H3.2 (10). The other two-thirds is produced by some 40 genes (11) in a replication-dependent pattern of expression (9). This major histone variant, H3.1, differs from the H3.2 form in only 4 residues (8, 12). Both gene classes appear to be transcribed into poly(A)$^+$ mRNA transcripts in all plants (8, 9, 13–15). Considering the relative time required to traverse the S phase and the other phases of the alfalfa cell cycle, the discrepancy between the number of histone H3 variant genes and amounts of steady-state protein suggests the presence of additional levels of regulation that may include differences in gene transcription rates, stability of mRNAs, efficiency of translation, and turnover of protein.

A protein identical to histone H3.2 of alfalfa has also been found in all mono- and dicotyledonous plants analyzed (15–17). The nonmeristematic expression of the histone H3.2 of *Arabidopsis thaliana* (15) supports the idea that this protein will be found to be constitutively expressed in all plants. To stress the similarities in pattern of expression and primary protein sequence with the constitutively expressed class of animal histone H3.3 replacement variants, it has been called histone H3.3-like (15) or histone H3.III (16). This paper presents experiments that show that histone H3.2 of alfalfa is also functionally equivalent to the class of replacement H3.3 histones in animal cells.

Replacement histone H3.3 in chicken has been found to be enriched and more highly acetylated in transcriptionally active chromatin of nonproliferating cells (18) apparently as a result of preferential assembly of replacement nucleosomes into transcribed chromatin (19). The high level of histone acetylation is caused by its presence in a dynamically highly acetylated chromatin environment, a condition that appears to be required for gene transcription (20–22). This preferential localization implies that the newly assembled replacement nucleosomes also become subject to the increased rate of nucleosome dissolution typical for transcribed chromatin (5, 6, 23, 24). It predicts (i) that the rate of replacement histone turnover is higher than that of the replication-dependent histone variant, and (ii) that the fraction of replacement histone subject to this increased rate of turnover will be more highly acetylated, a reflection of the chromatin environment from which it is lost.

The current study of alfalfa histone H3 variant synthesis

* This work was supported by National Science Foundation Grant DCB-9118999. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Division of Cell Biology and Biophysics, School of Biological Sciences, Rm. 414 BSB, University of Missouri-Kansas City, 5100 Rockhill Rd., Kansas City, MO 64110-2499. Tel.: 816-235-2591; Fax: 816-235-5158.

[1] A. J. Robertson and J. H. Waterborg, unpublished results.

was initiated to test both predictions. The observation that more than 50% of newly synthesized histone H3.2, characterized by a very high level of acetylation, was rapidly lost from chromatin, supports the identification of histone H3.2 of alfalfa as a functional replacement histone. The observed magnitude of turnover of alfalfa H3.2 with a very short half-life of less than 1 day suggests that nucleosome dissolution during transcription in this plant chromatin occurs much more readily than in transcribed chromatin of animal cells.

MATERIALS AND METHODS

Alfalfa (*Medicago varia*). cv Rambler suspension cultures named A2 (25) were labeled with 2.25 mCi of L-[4,5-^{3}H]lysine (86 Ci/mmol, Du Pont-New England Nuclear)/140-ml culture at a density of 30% for 2 h during log phase growth at 25 °C. Cells were collected by centrifugation for 4 min at 800 × *g*, washed once with 160 ml of fresh medium, and diluted into 300 ml of fresh MS medium (Sigma). One aliquot of 25 ml was taken immediately, and cells (1.6-ml cell pellet) were collected by centrifugation at 800 × *g* and stored at −20 °C. Subsequent samples were taken between 3 and 334 h. These were equivalent to the initial sample and contained a fraction of 25/300 of the pulse-labeled cells. The actual amount of cells in these samples increased from the initial value (1.6-ml cell pellet) over the course of the experiment because of continued cell proliferation to a 58-ml cell pellet (see Fig. 3). Fresh medium was added to maintain a cell density of less than 20% to assure continued growth. Histones were extracted from whole cells, and histone H3 variants were purified and separated by reversed-phase HPLC[2] on a Zorbax Protein-Plus column developed at 1 ml/min with a gradient from 20 or 30 to 60% acetonitrile in water with 0.1% trifluoroacetic acid as described before (12, 25). Elution of histones was monitored and quantitated by absorbance at 214 nm and by liquid scintillation counting. Histone identity was confirmed by acid/urea/Triton X-100 (AUT) gel electrophoresis and fluorography as described before (26). Fractions with histone variants H3.1 and H3.2 were pooled, as were fractions with histone H2B and fractions containing both histone H4 and H2A. These pools were adjusted to 2 mM 2-mercaptoethanol and lyophilized until dry, prior to AUT-gel electrophoresis, Coomassie staining, quantitative fluorography and densitometry, as described (12).

RESULTS

Histone synthesis can be measured by incorporation of radioactive lysine into newly synthesized protein and determination of the specific radioactivity of each purified histone species, taking into account differences in lysine content. Alfalfa histones were prepared from a suspension culture of A2 cells incubated for 2 h with tritiated lysine and fractionated by reversed-phase HPLC as described before (26) (Fig. 1*A*). Label incorporation was measured by liquid scintillation counting of the HPLC eluent (Fig. 1*B*) and by fluorography of pooled histone fractions fractionated by AUT-gel electrophoresis (results not shown). The highest specific radioactivities were observed in the three highly lysine-rich histone H1 forms of alfalfa (10) which nearly coelute during reversed-phase HPLC (26) and in lysine-rich histone H2B. Histone H4, an arginine-rich core histone with a relatively low content of lysine (25), was labeled to the lowest specific radioactivity of all histones. Remarkably, the two histone H3 variants of alfalfa which are identical in lysine content and nearly identical in primary protein sequence (12) differed from each other in specific labeling by a factor of 1.8 (Fig. 1*B*). This shows that the lower abundance variant H3.2 is synthesized at a rate almost twice that of the high abundance H3.1 form and implies that the rate of turnover of at least a fraction of newly synthesized histone H3.2 must be higher than the turnover of new variant H3.1 protein.

Turnover of histones is very slow with half-lives in whole animals ranging from 100 to more than 300 days and has been difficult to quantitate because of significant rates of cell death and DNA turnover (for discussion, see Ref. 2). The approach to label histones in proliferating cells in culture and to follow the decay of histone-specific radioactivity after induction of terminal cell differentiation and cessation of cell proliferation and histone synthesis has been flawed by the continued synthesis of replication-independent histone variants (2, 27, 28). Under these experimental conditions core histone half-lives range from 14 to 140 days with slower turnover for the central core histones H3 and H4, which are less likely to be exchanged (29), and significant differences in turnover rates of replication-dependent and -independent histone variants (27). In this study we chose to follow the decrease in histone-specific radioactivity under conditions of continued growth. This approach avoids the flaws of these earlier experiments. However, the time period during which specific radioactivities can be measured experimentally is limited and determined strongly by the initial specific radioactivities attainable.

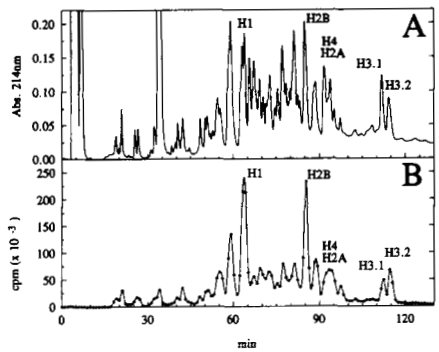

FIG. 1. **Fractionation of [^{3}H]lysine pulse-labeled alfalfa histones.** *Panel A*, absorbance elution profile during reversed-phase HPLC chromatography of histones extracted from a 0.75-ml cell pellet of alfalfa A2 cells, labeled in 15 ml of medium for 2 h with 0.08 mCi of tritiated lysine. *Panel B*, pattern of incorporated radioactivity determined by liquid scintillation counting of 0.1 ml of the 1-ml fractions. The rest of each fraction was used for AUT-gel electrophoresis and fluorography to confirm the identity of the marked labeled histone forms as described before (10, 12).

Alfalfa A2 suspension cultures were incubated for 2 h with a very high amount of tritiated lysine, and equivalent aliquots of cells were taken immediately after pulse labeling and after periods ranging from 3 h up to 334 h (15 days). Histones were prepared, fractionated by reversed-phase HPLC, and the specific radioactivity of histone H3.1 and H3.2 variants was determined as the ratio of radioactivity (Fig. 2*B*) and absorbance at 214 nm (Fig. 2*A*). As seen before, minor histone variant H3.2 was labeled almost twice as much as H3.1 (Fig. 2*B*). Samples obtained during continued growth clearly showed a drop in histone-specific radioactivity. Example radioactivity elution profiles for equal amounts of histone protein (Fig. 2*A*) are shown for samples taken after 13 h (Fig. 2*C*), 71 h (Fig. 2*D*), and 238 h (Fig. 2*E*) of continued growth in the absence of radioactive lysine. A decrease in the specific radioactivity of both histone variants is obvious with a clearly sharper drop for histone H3.2 than for H3.1 protein. When these measurements were corrected for the amount of cells obtained from equivalent aliquots of pulse-labeled cells (Fig. 3), assuming that increases in cell mass equal increases in histone protein, a measure for the specific activity of labeling is obtained that is corrected for continued histone protein synthesis (Fig. 4*A*).

The specific radioactivity of histone H3.2 decreased over a period of 100 h from approximately 200 to 80 (arbitrary units) to a level of 42.6 ± 3.8% (n = 5), whereas the label in histone

[2] The abbreviations used are: HPLC, high pressure liquid chromatography; AUT, acid/urea/Triton X-100.

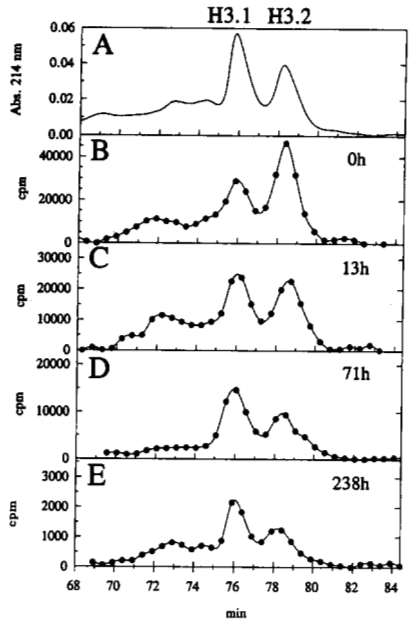

FIG. 2. **Fractionation of histone H3 variant proteins of alfalfa.** Absorbance elution profile during reversed-phase HPLC chromatography of histones extracted from a 1.4-ml cell pellet of A2 cells (*panel A*) and of similar amounts of histone labeled *in vivo* with tritiated lysine (see "Materials and Methods") after 0 (*panel B*), 13 (*panel C*), 71 (*panel D*), or 238 h (*panel E*) of continued growth. Histone variant H3.1 elutes prior to histone H3.2 at approximately 51–53% acetonitrile in the solvent. The radioactivity in cpm/0.5-ml fraction is given.

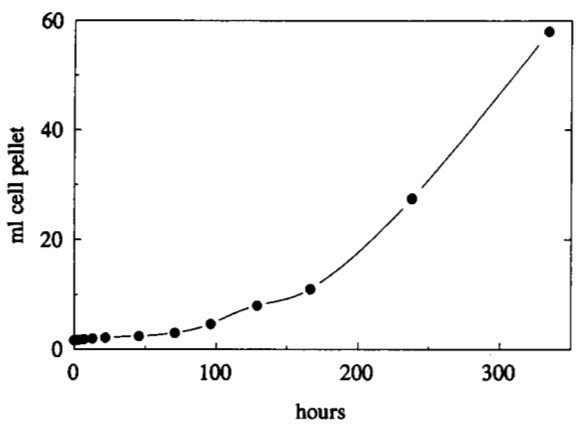

FIG. 3. **Growth curve of alfalfa A2 suspension culture.** Increase in cell mass collected by centrifugation from equivalent initial aliquots of lysine-labeled A2 suspension cell cultures during 15 days of continued near-logarithmic growth from 25 to 900 ml of culture medium is shown.

H3.1 dropped approximately 20% to 78.5 ± 3.8% ($n = 5$). The rate of loss of label appeared equivalent. The residual label in both histones did not change significantly over the next 10 days of continued logarithmic growth in suspension culture (Fig. 4*A*) or over a period of 1 month of slower growth as callus cultures on agar (results not shown). This showed that the residual fraction of both histone variants is stable with turnover rates that cannot be estimated within the time frame of the experimental protocol.

The combination of a high rate of histone synthesis of variant H3.2 and a more extensive turnover than H3.1 apparently produces the observed steady-state distribution of histone H3 variant protein with 57.7 ± 2.2% ($n = 8$) of histone H3 protein in major variant H3.1 protein. Measurement of

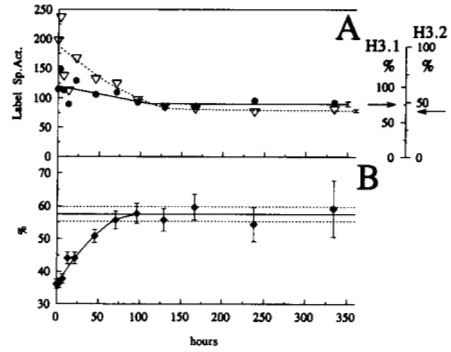

FIG. 4. **Turnover of newly synthesized histone H3 protein.** *Panel A*, the specific radioactivity (*Label Sp. Act.*) of histone variants H3.1 and H3.2 synthesized in the presence of tritiated lysine was determined from radioactivity (cpm) and amount of protein (214 nm absorbance) measured during reversed-phase chromatography (Fig. 2) and adjusted for the increase in cell mass (Fig. 3). Standard deviation errors of this calculation for samples between 100 and 350 h for histone H3.1 (*circles on continuous line*) and H3.2 (*triangles on broken line*) are shown. The relative loss of lysine label from each variant is shown on the *right*. *Panel B*, the relative distribution of label between H3.1 and H3.2 is presented as the percentage of label (*diamonds* with standard deviation bars) present in histone H3.1. For comparison a *line* (with error limits as *broken lines*) is shown for the percentage of variant H3.1 that is part of total histone H3 protein.

the fraction of label incorporated into newly synthesized H3.1 protein revealed that over the period of 100 h of growth with measurable histone H3 turnover the fraction of labeled H3.1 increases from 35% to become identical to the fraction of steady-state H3.1 protein (Fig. 4*B*).

Quantitative densitometry of Coomassie-stained AUT gels with pooled histone H3 variant preparations (Fig. 5*A*) confirmed the high level of steady-state histone acetylation of variant H3.2 (10, 12, 17, 26) measured in this study as 1.02 ± 0.04 ($n = 4$). The acetylation level for variant H3.1, 0.56 ± 0.04 ($n = 4$), is much lower (Fig. 6*B*). When new histone H3 was analyzed in the same way by fluorography, the acetylation patterns for new and preexisting histone H3.1 were similar (Fig. 5). However, the level of acetylation of new H3.2 was dramatically higher with prominent amounts of mono-, di-, and triacetylated forms (Fig. 5*B*) and 1.18 ± 0.03 acetylated lysine residues/newly synthesized H3.2 (Fig. 6*B*). During continued growth this level rapidly dropped to below that of steady-state H3.2 (Fig. 5*E*) to 0.85 ± 0.03 ($n = 5$) acetylated lysines/remaining new but stable H3.2 (Fig. 6*B*). The shift in the relative amounts of acetylated forms of new histone H3.2 during this period is presented in Fig. 6*A*, with the steady-state distributions for both histone variants presented on the *right*. Over the same period that histone acetylation in new H3.2 changed, new H3.1 remained on average at the same level of acetylation as preexisting H3.2 protein (Fig. 6*B*).

Data scatter in histone-specific radioactivity measurements for new histones H3.1 and H3.2 (Fig. 4*A*) appears to be caused by inherent inaccuracy in determinations of the amounts of individual histone H3 variants (Fig. 2*A*) and the radioactivity associated with them (Fig. 2, *B–E*). The error level decreased significantly when calculations depended on radioactivity measurements alone with the accuracy of liquid scintillation counting as primary source of error (Fig. 4*B*). When the amount of labeled histone H3.2 was standardized on the label in histone H3.1 (Fig. 7*A*) without correction for the small but detectable loss of new histone H3.1 (Fig. 4*A*), a new measure for histone H3.2 turnover was obtained. The fact that the fraction of stable new histone H3.2, 43 ± 4% ($n = 5$) (Fig. 7*A* on the *right*), was identical to that obtained directly (Fig.

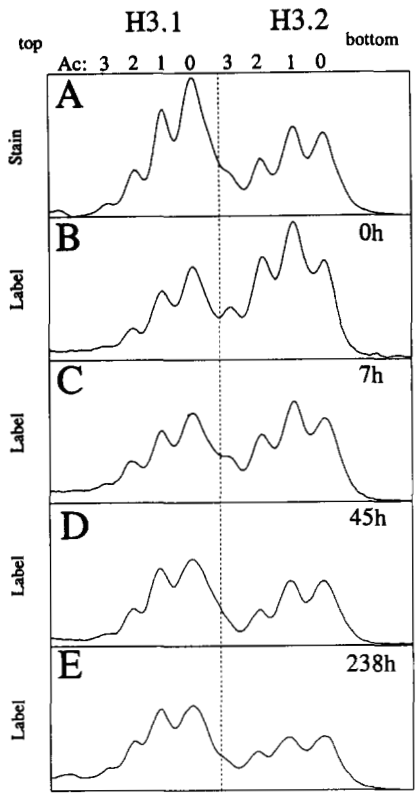

FIG. 5. **Acetylation of histone H3 variants.** Histone H3 variant proteins, pulse-labeled *in vivo* and purified by HPLC, were lyophilized and electrophoresed on AUT gels (*top* and *bottom* are indicated). The pattern of Coomassie-stained histone H3 (*Stain*) (*panel A*) and associated radioactivity (*Label*) in properly exposed fluorographs (*panels B–E*) were measured by densitometry and quantitated by determining overlapping patterns of Gaussian curves for each band. Examples are shown for the radioactivity patterns of histone H3 after 0 (*panel B*), 7 (*panel C*), 45 (*panel D*), and 238 h (*panel E*) of continued growth. The level of non- through triacetylation for both histone variants is indicated. The overlap between highly acetylated forms of histone H3.2 and low acetylated forms of histone H3.1 prevents calculation of the absolute levels of acetylation for each variant (12).

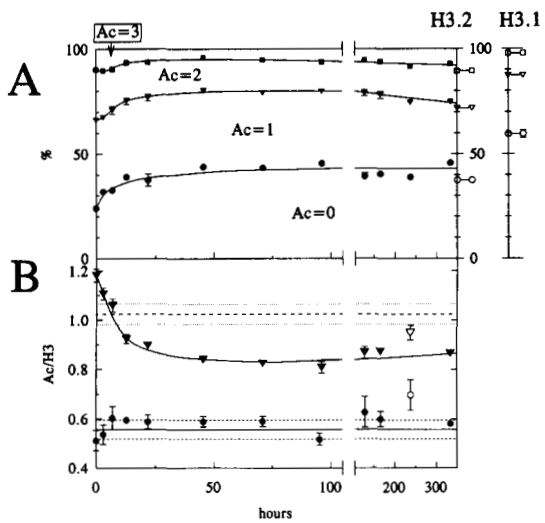

FIG. 6. **Quantitative analysis of histone H3 variant acetylation.** *Panel A*, quantitative densitometry of AUT-gel fluorographs gave the cumulative relative distribution for nonacetylated (*Ac = 0*; *circles*), monoacetylated (*Ac = 1*; *triangles*), diacetylated (*Ac = 2*; *squares*), and triacetylated (*Ac = 3*) newly synthesized histone H3.2 after lysine labeling and during continued growth. Standard deviation errors of the measurement are shown for all symbols or fall within the size of the symbol. For comparison the cumulative relative distribution for steady-state acetylation of histone variant H3.2 obtained by densitometry of Coomassie-stained AUT gels is shown by *open symbols* on the *right* figure axis and for variant H3.1 to the *right* of the figure. *Panel B*, the value of acetylated lysine residues/histone H3 molecule (*Ac/H3*) was calculated from these densitometry analyses for new histone H3.1 (*circles*) and H3.2 (*triangles*) and for steady-state histone H3.1 (*line* with standard deviation limits shown by two *broken lines*) and H3.2 (*broken line* with standard deviation limits shown by two *dotted lines*). The data represented by *open symbols* were excluded from further data analysis.

4*A*) appears to validate this method which allowed determination of an apparent half-life of approximately 20 h for the labile fraction of new H3.2 (Fig. 7*A*, *inset*).

This approach was also applied to estimate the turnover of newly synthesized histones H2B, H2A, and H4 for which the amount of protein could not be determined from reversed-phase HPLC elution profiles (Fig. 1*A*) preventing calculation of histone-specific radioactivities. Approximately 75% of new histone H2B was lost over a period of 15 days with a half-life of 2 days (Fig. 7*B*), but the remaining new histone H2B was not necessarily stable. Significant changes in the level of acetylation of newly synthesized major histone variant H2B.1 were not observed when analyzed by AUT-gel fluorography (results not shown). Determination of the contribution of new histone H4 and new H2A to the pattern of loss of label from the HPLC fractions containing both histones (Fig. 7*C*) also required AUT-gel analysis and fluorography. The *broken line* in Fig. 7*C* shows the contribution of lysine-rich histone H2A with a half-life estimated at 30 h (Fig. 7*C*, *inset*). Over the course of the experiment, loss of new H2A histone, primarily major H2A.3 and minor H2A.1 (10), was like that of new histone H2B. The amount of label in new histone H4, relative to histone H3.1, may have increased slightly during the first few days of continued growth (Fig. 7*C*). This suggests that newly synthesized histone H4 is at least as stable as histone H3.1 protein and may indeed be completely stable. AUT-gel fluorography did not reveal changes in the steady-state level of acetylation of new histone H4 (results not shown).

DISCUSSION

High rates of transcription of chromatin by RNA polymerases can cause extensive loss of nucleosomes from DNA as clearly demonstrated by transcription of ribosomal genes. Even moderate rates of transcription have been shown to reduce the nucleosomal density on active gene sequences (24, 30, 31). Considering the requirements of the transcription process and the size of RNA polymerase complexes, it remains amazing and poorly understood how DNA can be transcribed while maintaining a nucleosomal configuration (23, 32, 33). Experimental evidence suggests with varying degrees of confidence that nucleosomes dissolve or jump around RNA polymerase complexes, that nucleosomes split and transiently unfold, and that transient dissociation of H2A-H2B dimers may allow DNA transcription without dissociation of any H3-H4 tetramer structures (5, 23, 32–35).

Loss of nucleosomes requires repair of the chromatin structure, either during DNA replication when most histone synthesis occurs or outside of S phase. The latter option is the only one available in nonproliferating cells. Histone H3.3 of animals has been recognized as a constitutively produced histone variant that participates in the assembly of nucleosomes outside of S phase and has thus been named a replacement histone (4). No other core histone is known to have sequence variants with equivalent function. Histones H2A and H2B appear to exchange rather readily between nucleo-

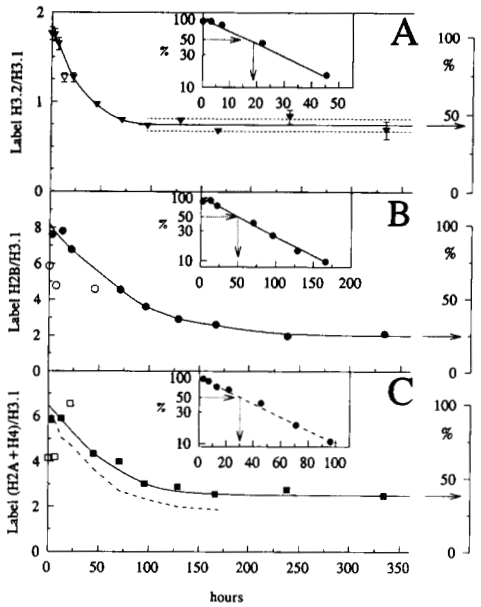

FIG. 7. **Quantitative analysis of turnover of newly synthesized core histones.** Specific radioactive labeling of histones H3.2 (*panel A, triangles*), H2B.1 (*panel B, circles*), H2A.3 with H2A.1 and H4 (*panel C, squares*) was calculated from the radioactivity observed in reversed-phase HPLC peaks that coincided with identified peaks of absorbing protein (Fig. 1) *relative* to the radioactivity associated with histone H3.1. This reduced the error for H3.2 determinations to the error bars shown or to an error range within the size of the symbols. The *broken lines* show the level of standard deviation error for the combined calculated values obtained between 95 and 350 h of continued growth. For the other histones a similar degree of accuracy has been assumed, although not enough data were collected to substantiate this idea or to determine the stability of the fraction of residually labeled histone. For each histone species AUT-gel fluorography showed that at least 90% of the radioactivity was associated with the histone bands (results not shown). *Open symbols* were excluded from the calculations of the apparent decay in radioactivity (*lines* in all *panels*), half-life calculations (*insets*), and for calculation of the residual amount of new histone remaining at the end of the experiment (shown on the *right* of each *panel*). AUT-gel fluorography was used to determine the amount of labeled new H2A variants (*panel C, broken line*) relative to labeled histone H4. Half-life determinations for histone H3.2 (*inset* in *panel A*), histone H2B.1 (*inset* in *panel B*), and histone H2A.3 with H2A.1 (*inset* in *panel C*) assumed that residual amounts of new histone were completely stable and apply only to the labile fraction of each new histone form.

somes during transcription and replication (5, 29, 33) and may participate as dimers in the formation of replacement nucleosomes. The relative rates of turnover of these histones (2, 27, 28) support this idea. In this study, equivalent turnover of H2A and H2B histones was also seen (Fig. 7). Remarkably, histone H4 has been shown to be more stable than even replication-dependent H3 histones, here also confirmed for alfalfa (Fig. 7). These observations suggest that replacement histone H3.3 provides an essential starting point to assemble a nucleosome in the absence of DNA replication. H3.3 may form the nucleosomal kernel of the H3-H4 tetramer by binding histone H4 from previously displaced nucleosomes and complete the octamer by binding two mobile H2A-H2B dimers. This specific function of histone H3.3 may explain why this histone variant in both animals and plants is even more highly conserved in primary protein sequence than replication-dependent forms of histone H3 (7, 15–17, 36).

The connection between chromatin transcription and the need to repair chromatin by assembly of replacement nucleosomes was experimentally confirmed by the observation that replacement histone H3.3 of chicken in nonproliferating cells preferentially accumulated in transcriptionally active chromatin domains (18, 19). As a consequence the level of acetylation of these histone H3.3 variant molecules was found to be higher than seen for histone in bulk chromatin. The preferential localization of replacement histone into transcriptionally active and labile chromatin domains predicts a higher rate of loss and thus turnover for these histone molecules than for replication-dependent histone variants or for replacement variant histone molecules assembled into nucleosomes during S phase. The rate of turnover of highly acetylated chicken histone H3.3 relative to lower acetylated histone H3 forms has never been experimentally determined, but the 2.5-fold faster turnover of acetylated relative to nonacetylated histone H4 in Friend cells provides support for this prediction (28).

All characteristics that identify chicken histone H3.3 as a replacement histone have been shown to exist in alfalfa histone H3.2, and by extension in all histone H3.III variants of plants: constitutive gene transcription (9, 15), high steady-state levels of acetylation (16, 17), and the existence of a fraction of new histone H3.2 that exhibits increased turnover. Reduction of the average acetylation level of new histone H3.2 from 1.18 to 0.85 acetylated lysines/molecule (Fig. 6) when almost 60% of the new protein is lost (Fig. 4) suggests that the histone H3.2 molecules lost had an average level of acetylation of 1.45 (± 0.08).

This level of acetylation is much higher than the steady-state acetylation of 0.5 for the major histone H3.1 variant of alfalfa. Comparison of the alfalfa results with the differences in histone H3.3 acetylation of transcriptionally active *versus* bulk chromatin preparations of chicken (18, 19) revealed that the differences in alfalfa were much more prominent. The differences in histone turnover rates are also very large when the half-life of the highly labile, highly acetylated fraction of new histone H3.2 (< 1 day) is contrasted with the stability over 15 days for the remaining new H3.2 and for the majority of the new H3.1 molecules (Figs. 4 and 7*A*). The 60% labile fraction of histone H3.2 is much larger than the presumed fraction of transcriptionally active and competent chromatin in alfalfa. The same is true for the abundance of the highly acetylated histone H3.2 in alfalfa and for the equivalent histone H3.III histones in all other plants (16, 17). This is much higher than the levels of replacement histone H3.3 in animal cells, even after a long term absence of cell proliferation (2, 37, 38). These combined observations suggest that the rate of nucleosome dissolution and thus the need for assembly of replacement nucleosomes in plant chromatin is much larger than observed to date in animal systems.

The sequences of plant histones differ only slightly from their equally conserved animal counterparts. Very few single residue changes have been found in histones H3 and H4. Although the terminal domains of H2A, H2B, and H1 histones in plants are typically longer than in animals (12, 25, 36, 39, 40), histone octamers and nucleosomes formed with plant histones appear as stable as their animal equivalents (41–43). In addition, alfalfa histone H4, most of new histone H3.1, and 40% of new H3.2 appear completely stable (Figs. 4 and 7). These data support the notion that the stability of animal and plant chromatin is essentially the same. Therefore the higher rate of plant nucleosome dissolution during transcription may well depend on differences in animal and plant RNA polymerases, transcription-associated proteins, or their interaction with the plant histones. The validity of this idea must be evaluated experimentally by transcription *in vivo* and *in*

vitro of defined reconstituted chromatins.

Acknowledgment—I gratefully acknowledge W. Morgan for a critical reading of the manuscript and for frequent advice and discussions.

REFERENCES

1. Stein, G., Stein, J., Chrysogelos, S., Dell'Orco, R., Hickey, E., Kroeger, P., Morris, T., Nick, H., Pauli, U., Van Wijnen, A., Weber, L., and Zambetti, G. (1989) in *Histones and Other Basic Nuclear Proteins* (Hnilica, L. S., Stein, G. S., and Stein, J. L., eds) pp. 269–317, CRC Press, Boca Raton FL
2. Wu, R. S., Panusz, H. T., Hatch, C. L., and Bonner, W. M. (1986) *CRC Crit. Rev. Biochem.* **20,** 201–263
3. Marzluff, W. F. (1989) in *Histones and Other Basic Nuclear Proteins* (Hnilica, L. S., Stein, G. S., and Stein, J. L., eds) pp. 319–329, CRC Press, Boca Raton FL
4. Zweidler, A. (1984) in *Histone Genes: Structure, Organization and Function* (Stein, G., Stein, J., and Marzluff, W. F., eds) pp. 339–371, John Wiley and Sons, New York
5. Jackson, V. (1990) *Biochemistry* **29,** 719–731
6. Pfaffle, P., Gerlach, V., Bunzel, L., and Jackson, V. (1990) *J. Biol. Chem.* **265,** 16830–16840
7. Wells, J. R. E., Coles, L. S., and Robins, A. J. (1989) in *Histones and Other Basic Nuclear Proteins* (Hnilica, L. S., Stein, G. S., and Stein, J. L., eds) pp. 253–267, CRC Press, Boca Raton FL
8. Wu, S.-C., Gyorgyey, J., and Dudits, D. (1989) *Nucleic Acids Res.* **17,** 3057–3063
9. Kapros, T., Bögre, L., Nemeth, K., Bako, L., Gyorgyey, J., Wu, S. C., and Dudits, D. (1992) *Plant Physiol.* **98,** 621–625
10. Waterborg, J. H., Winicov, I., and Harrington, R. E. (1987) *Arch. Biochem. Biophys.* **256,** 167–178
11. Wu, S.-C., Bögre, L., Vincze, E, Kiss, G. B., and Dudits, D. (1988) *Plant Mol. Biol.* **11,** 641–650
12. Waterborg, J. H. (1990) *J. Biol. Chem.* **265,** 17157–17161
13. Chaubet, N., Chaboute, M.-E., Clement, B., Ehling, M., Philipps, G., and Gigot, C. (1988) *Nucleic Acids Res.* **16,** 1295–1304
14. Chaboute, M. E., Chaubet, N., Clement, B., Gigot, C., and Philipps, G. (1988) *Gene (Amst.)* **71,** 217–223
15. Chaubet, N., Clement, B., and Gigot, C. (1992) *J. Mol. Biol.* **225,** 569–574
16. Waterborg, J. H. (1991) *Plant Physiol.* **96,** 453–458
17. Waterborg, J. H. (1992) *Plant Mol. Biol.* **18,** 181–187
18. Ridsdale, J. A., and Davie, J. R. (1987) *Nucleic Acids Res.* **15,** 1081–1095
19. Hendzel, M. J., and Davie, J. R. (1990) *Biochem. J.* **271,** 67–73
20. Pfeffer, U., Ferrari, N., Tosetti, F., and Vidali, G. (1988) *Exp. Cell Res.* **178,** 25–30
21. Zhang, D.-E., and Nelson, D. A. (1988) *Biochem. J.* **250,** 233–240
22. Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1988) *EMBO J.* **7,** 1395–1402
23. Kornberg, R. D., and Lorch, Y. (1991) *Cell* **67,** 833–836
24. Bedoyan, J., Gupta, R., Thoma, F., and Smerdon, M. J. (1992) *J. Biol. Chem.* **267,** 5996–6005
25. Waterborg, J. H. (1992) *Biochemistry* **31,** 6211–6219
26. Waterborg, J. H., Harrington, R. E., and Winicov, I. (1990) *Biochim. Biophys. Acta* **1049,** 324–330
27. Grove, G. W., and Zweidler, A. (1984) *Biochemistry* **23,** 4436–4443
28. Tsvetkov, S., Ivanova, E., and Djondjurov, L. (1989) *Exp. Cell Res.* **180,** 94–105
29. Louters, L., and Chalkley, R. (1985) *Biochemistry* **24,** 3080–3085
30. Widmer, R. M., Lucchini, R., Lezzi, M., Sogo, J., Edstrom, J.-E., and Koller, T. (1984) *EMBO J.* **3,** 1635–1641
31. Björkroth, B., Ericsson, C., Lamb, M. M., and Daneholt, B. (1988) *Chromosoma (Berl.)* **96,** 333–340
32. Thoma, F. (1991) *Trends Genet.* **7,** 175–177
33. Van Holde, K., Lohr, D. E., and Robert, C. (1992) *J. Biol. Chem.* **267,** 2837–2840
34. Walker, J., Chen, T. A., Sterner, R., Berger, M., Winston, F., and Allfrey, V. G. (1990) *J. Biol. Chem.* **265,** 5736–5746
35. Lee, M.-S., and Garrard, W. T. (1991) *EMBO J.* **10,** 607–615
36. Wells, D., and Brown, D. (1991) *Nucleic Acids Res.* **19,** 2173–2188
37. Urban, M. K., Franklin, S. G., and Zweidler, A. (1979) *Biochemistry* **18,** 3952–3960
38. Wunsch, A. M., and Lough, J. (1990) *Cell Diff. Dev.* **30,** 19–25
39. Spiker, S. (1982) *J. Biol. Chem.* **257,** 14250–14255
40. Lindsey, G. G., Orgeig, S., Thompson, P., Davies, N., and Maeder, D. L. (1991) *J. Mol. Biol.* **218,** 805–813
41. Bavykin, S. G., Undritsov, I. M., Usachenko, S. I., Strokov, A. A., Bogdanov, Y. F., and Mirzabekov, A. D. (1988) *FEBS Lett.* **228,** 149–152
42. Hofmann, D., Zentgraf, H., and Kahl, G. (1989) *FEBS Lett.* **256,** 123–127
43. Moehs, C. P., and Spiker, S. (1991) in *3rd International Congress of Plant Molecular Biology*, University of Tucson, AZ (Halleck, R. B., ed) Abstr. 330