

Changes in the H-1 Histone Complement during Myogenesis. II. Regulation by Differential Coupling of H-1 Variant mRNA Accumulation to DNA Replication

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ABSTRACT We have shown that changes in proportions of the four chicken H-1's during *in vitro* myogenesis are primarily the result of differential coupling of their synthesis to DNA replication (see the previous paper). We show here that the four major chicken H-1's are encoded by distinct mRNAs which specify primary amino acid sequence variants. Accumulation of the H-1-variant mRNAs is coupled to DNA replication to different extents. The level of mRNA encoding H-1c (the H-1 variant that increases relative to the other H-1's in nondividing muscle cells) is completely uncoupled. In contrast, the level of mRNAs encoding H-1's *a*, *b*, and *d* (which have levels that decrease in nondividing muscle cells) are more tightly coupled. Polyadenylation is not involved in uncoupling H-1c mRNA accumulation from DNA replication.

The H-1 histones are small, basic proteins that have been implicated in organizing nucleosomes into higher orders of chromatin structure. In all species examined, the H-1's are highly heterogeneous. Where examined in detail, the heterogeneity reflects numerous postsynthetic modifications of a limited number of amino acid sequence variants. The sequence variants show tissue specific distributions (1, 2). In addition, changes in H-1-variant proportions have been correlated with changes in developmental (3-5), differentiative (6, 7), and physiologic states (8-12). Though little is known about the biological consequences of these changes, their widespread correlation with changes in nuclear activity have led to suggestions that the H-1 variants have different functions. An understanding of how changes in the H-1 complement of a cell are regulated may provide insight into their role in regulating the functional state of chromatin.

We have shown that a dramatic change in H-1 proportions during *in vitro* chick myogenesis is the result of differential coupling of their synthesis to DNA replication (13). As myoblasts withdraw from the cell cycle, fuse, and differentiate into myotubes, the amount of H-1c increases relative to the amount of H-1's *a*, *b*, and *d*. As a family, synthesis of the

H-1's is less tightly coupled to DNA replication than is nucleosomal histone synthesis. In addition, there are differences in the degree to which synthesis of the individual H-1's is coupled, with H-1c synthesis being substantially less tightly coupled than synthesis of H-1's *a*, *b*, and *d*. The continued synthesis of the H-1's in the absence of DNA synthesis occurs in conjunction with a turnover of nuclear H-1. As myogenic cells stop dividing and differentiate into myotubes, H-1c synthesis predominates, thus increasing the relative amount of H-1c in the terminally differentiated myotube.

H-1c is present in high relative amounts in many nonproliferating chicken tissues (14), suggesting that uncoupling of its synthesis from DNA replication is not limited to the myogenic lineage. In addition, there are differences in the degree to which H-1-variant synthesis is coupled to DNA replication in other organisms (15-18, and unpublished results). Thus differential coupling of H-1-variant synthesis to DNA replication appears to be a widespread mechanism for establishing tissue-specific H-1 proportions. It follows that chicken myogenesis *in vitro* provides a useful system for eliciting how differential coupling is regulated, and how this in turn establishes tissue-specific H-1 proportions.

Previous amino acid composition analysis suggested that the major chicken H-1's are primary amino acid sequence variants (1, 2). We now confirm that the four chicken H-1's are primary sequences variants by partial peptide analysis and *in vitro* translation. It can be shown that H-1 mRNA levels are less tightly coupled to DNA synthesis than the levels of mRNA encoding the nucleosomal histone H-4. Moreover, there are differences in the degree to which H-1-variant mRNA levels are coupled to DNA synthesis, with H-1c mRNA levels being completely uncoupled. Thus, changes in H-1 proportions during myogenesis are regulated at the level of messenger RNA accumulation.

MATERIALS AND METHODS

Cell Culture: Myogenic cells were prepared by mechanical dissociation of the thigh musculature (19) dissected from 11-12-d-old White Leghorn chickens (SPAFAS Inc., Norwich, CT). Cells were grown in 850-cm³ gelatin-coated plastic roller bottles in Dulbecco's modified Eagle's medium supplemented with 15% horse serum and 4% embryo extract as previously described (20). Myoblasts were harvested at 16-24 h after preparation of the cultures, prior to fusion. After day 3, and upon definitive appearance of fused cells, the myotubes were freed of dividing cells with two 24-h treatments with 10⁻⁵ M cytosine arabinoside (Ara C).¹ The myotubes were harvested within 24 h after the replacement with fresh media without Ara C.

Extraction and Electrophoresis of H-1's: H-1's were extracted by virtue of their solubility in 5% perchloric acid (PCA) using a previously described modification (13) of the technique of Johns (21). H-1's and H-1 proteolytic fragments were resolved on 17% polyacrylamide gels containing SDS, electrophoresed at 140 V for 7-8 h. Acid-urea-polyacrylamide slab gel electrophoresis was carried out by a modification (13) of the method of Panyim and Chalkey (22). H-1's dissolved in 6-10 μ l of loading buffer (8 M urea, 4% β -mercaptoethanol, 22% sucrose, and 1.3 N acetic acid) were applied to 30-cm-long pre-electrophoresed slab gels and electrophoresed at 250 V for 72 h. Proteins were stained with 0.25% Coomassie Blue in 50% methanol and 10% acetic acid overnight and destained in 50% methanol and 10% acetic acid. Silver staining of proteins was carried out by the technique of Switzer et al. (23), as modified by Wray et al. (24). Photographic transparencies of the gels were prepared using Polaroid type 46-L continuous tone film.

Partial Proteolytic Analysis of H-1's: A modification of the technique for digestion of proteins in gel slices described by Cleveland (25) was used to compare proteolytic fragments of the four H-1's. The modification allows a wide variety of proteases to be used since the digestion buffer can be varied to optimize enzymatic activity. Gel slices containing the four H-1's were prepared from a preparative acid-urea-polyacrylamide gel on which 0.5 mg of chicken thymus H-1's were resolved. After electrophoresis, the H-1's were localized by Coomassie Blue staining of strips cut from the edges of the gel. The H-1-containing region of the gel was excised and cut into 0.25-cm-wide strips running parallel to the direction of electrophoresis. The strips were washed three times in 100 mM Tris-HCl, pH 8.0, lyophilized, and stored in sealed tubes. Lyophilization before protease treatment was found to increase the efficiency of digestion, presumably by increasing the accessibility of the H-1's to the proteases. Digestions with *Staphylococcus aureus* V-8 protease (Miles Laboratories Inc., Elkhart, IN), trypsin (TPCK-treated from bovine pancreas, Worthington Biochemical Corp., Freehold, NJ), and elastase (from porcine pancreas, Worthington Biochemical Corp.), were carried out by rehydrating the gel slices in 100 mM Tris, pH 8.0, containing increasing concentrations of the appropriate enzyme. Collagenase (CLS III, Worthington Biochemical Corp.) digestions were in 100 mM Tris-HCl, pH 8.0, and 3 mM CaCl₂. After incubating the slices for 30 min at 37°C, digestions were terminated by washing three times in ice cold H₂O. The gel slices were lyophilized, rehydrated in loading buffer for 20 min, and placed on the surface of an SDS polyacrylamide gel so that resolution was 90° relative to the first dimension. Proteolytic fragments were visualized by silver staining. Multiple concentrations of each enzyme ranging from 1-25 μ g/ml were tested. Examples presented in the text were chosen to show the maximum number of unique proteolytic fragments observed.

RNA Extraction: RNA was sequentially extracted with guanidine-HCl and phenol as previously described (26). Cycling myoblasts, myoblasts pre-treated for 2 h with 10⁻⁵ M Ara C to inhibit DNA synthesis, or myotubes were each harvested directly into ice cold 7.3 M guanidine-HCl, 20 mM KOAc, 5

mM EDTA, and 1.0 mM dithiothreitol using a rubber policeman. The suspension was homogenized with a polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY) and one half-volume of ice cold 95% ethanol was added. After storage for 2 h at -20°C, the RNA-containing precipitate was collected by centrifugation at 4000 \times g for 30 min at -10°C. The precipitate was suspended in 10 ml of 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 50 mM Tris-HCl, pH 9.0, and further extracted with an equal volume of water-saturated phenol:chloroform:isoamyl alcohol (50:50:1). The recovered aqueous phase was re-extracted with the above phenol:chloroform:isoamyl alcohol mixture two more times before being brought to 66 mM ammonium acetate and 70% ethanol. After overnight storage at -20°C, the precipitated RNA was collected by centrifugation. The RNA was washed three times by resuspension and centrifugation in 66 mM ammonium acetate in 70% ethanol, lyophilized, dissolved in H₂O, and stored at -80°C.

Hybridization Analysis: Relative levels of electrophoretically resolved H-1 and H-4 mRNAs were determined by hybridization (Northern) analysis using H-1 cDNA (described by Cole, L. S., and J. R. E. Wells, manuscript submitted for publication) and H-4 DNA (a derivative of lambda CHO-1, described in reference 27) cloned in plasmid pBR 322. Plasmid DNAs were radioactively labeled to an approximate specific activity of 10⁸ cpm/ μ g by nick translation (reagents obtained from Bethesda Research Laboratories, Gaithersburg, MD).

50 μ g of total RNA was electrophoresed for 400 volt hours on 1.8% agarose gels containing 2.2 M formaldehyde in 20 mM 3-[N-morpholino]propanesulfonic acid, 5 mM NaOAc, 0.1 mM EDTA, pH 7.0. The gels were washed for 10 min in H₂O at 65°C and subsequently stained for 30 min in 1 μ g/ml ethidium bromide to verify that equal amounts of RNA were contained in each lane. The RNA was transferred to nitrocellulose filters in 20 \times standard saline citrate (SSC) (1 \times = 150 mM NaCl, 15 mM sodium citrate) for 12 h as described by Thomas (28) except that the gel was soaked in 20 \times SSC for 1 h prior to transfer. After the filters were dried at 80°C for 3 h under vacuum, they were incubated overnight at 37°C in 20 ml of 50% formamide, 5 \times SSC, 5 \times Denhardt's solution, 0.1% SDS, and 25 mM NaH₂PO₄, pH 6.5, containing 100 μ g/ml heat-denatured *Escherichia coli* DNA. This was followed by a 48-h incubation at 37°C in 10 ml of the above solution containing 2-3 \times 10⁷ cpm ³²P-labeled DNA. After hybridization, the nitrocellulose filters were washed successively with 5 \times SSC, 0.1% SDS at room temperature for 10 min; 5 \times SSC, 0.1% SDS at 60°C for 30 min; 2 \times SSC, 0.1% SDS at 60°C for 30 min; and finally with 1 \times SSC, 0.1% SDS at 60°C for 30 min. The filters were dried and exposed to preflashed X-ray film at -80°C using an intensifying screen (Cormex Lightning-Plus, DuPont Co., Wilmington, DE) as described by Laskey and Mills (29). Levels of hybridization were determined by scintillation spectrometry of excised radioactive bands which were localized by superimposing the autoradiogram onto the nitrocellulose filter and were corrected for nonspecific hybridization.

In Vitro Translations: Total RNA was translated in a nuclease-treated wheat germ *in vitro* translation system prepared without lysine (Bethesda Research Laboratories Inc., 8107 SB) supplemented with 200 μ Ci/ml ³H-l-lysine (4,5-³H[N], 90 Ci/mM) (New England Nuclear, Boston, MA). Total translational activity was monitored by scintillation spectrometry of 5% TCA-precipitable material which had been incubated at 90°C for 30 min to hydrolyze aminoacyl-tRNAs. Protein synthesis behaved as a linear function of RNA concentration up to 140 μ g/ml of total RNA. Translations were for 3 h at 25°C during which time incorporation was linear with respect to time. Acetylation by the *in vitro* translation system was inhibited by the addition of oxaloacetate and citrate synthase (type II from porcine heart, Sigma Chemical Co., St. Louis, MO), as described by Palmiter (30).

H-1 translation products were extracted from the wheat germ reaction mixture with 5% PCA. Aliquots of the reaction mixtures containing equivalent levels of protein radioactivity (1.5-2.0 ml) were mixed with 20 μ g of chicken thymus H-1. H-1's were subsequently extracted using 5% PCA and analyzed by SDS or acid-urea-polyacrylamide gel electrophoresis. The gels were prepared for fluorography and exposed to preflashed X-ray film (29). H-1 translation products were quantitated by integrating the area under densitometric peaks of the fluorogram with an electronic digitizer (Numonics Corp., Lansdale, PA, model I224). Densitometric scans were generated with a microdensitometer (Joyce Loebl, Gateshead, England). Differential loss of H-1 during extraction and sample preparation was monitored by quantitating recovery of the added H-1 by integrating areas under densitometric peaks of the stained gel.

RESULTS

The Four H-1's Have Different Amino Acid Sequences

To determine whether the four chicken H-1's are amino

¹ Abbreviations used in this paper: Ara C, cytosine arabinoside; PCA, perchloric acid; SSC, standard saline citrate.

acid sequence variants, we compared the peptide fragments generated by digestion of the H-1's with V-8 protease, elastase, trypsin, and type III collagenase. The four H-1 species were first resolved by acid-urea-polyacrylamide gel electrophoresis. The region of the gel containing the H-1's was excised and cut into strips along the direction of migration. These slices were then incubated with one of the proteases and were subsequently placed on the surface of an SDS polyacrylamide gel such that electrophoresis in the second dimension was 90° relative to the first dimension.

Proteolytic fragments of the four H-1's generated by V-8 protease, trypsin, elastase, and collagenase are shown in Fig. 1. A number of proteolytic fragments common to all four H-1's were generated with each of the enzymes tested. In addition, fragments can be identified which are unique to the individual H-1's. Of the 15 major H-1 *a* proteolytic fragments generated with the four enzymes, six had unique molecular weights (V-8 protease and elastase each gave two unique fragments, and collagenase and trypsin each gave one). Of the 14 major H-1 *c* proteolytic fragments, two were unique (one unique fragment generated by V-8 protease and one by collagenase). H-1 *b* and *d* peptides generated with V-8 protease, trypsin, and elastase were identical. Two proteolytic fragments unique to H-1 *b* were observed when collagenase was used. The data indicates that the amino acid sequence of H-1 *a* is different from that of H-1 *c*, which are both different from that of H-1's *b* and *d*. The data also suggests that H-1's *b* and *d* have unique amino acid sequences.

That all four H-1's are unique sequence variants is strongly supported by in vitro translation analysis. The major 5% PCA-soluble translation products directed by total myoblast RNA co-migrated with authentic H-1 on SDS polyacrylamide gels (Fig. 2). As with in vivo-synthesized H-1's, only three bands are observed on SDS gels due to the failure to resolve H-1's *b* and *d*. Identity as H-1's was further established by recovery with an affinity-purified anti-H-1 antibody (data not shown).

When the 5% PCA-soluble translation products were analyzed by acid-urea-polyacrylamide gel electrophoresis, multiple bands were observed (Fig. 3A). Careful examination re-

veals four pairs of overlapping doublets. The upper member of each doublet co-migrates with H-1 synthesized in vivo. Histones synthesized in vivo are subject to acetylation (for review see reference 31). Wheat germ in vitro translation reaction mixtures have been shown to contain acetylating activity (30). If acetylation of the H-1's synthesized in vitro is incomplete, the upper member of each doublet could represent the acetylated H-1, while the lower member could be unmodified. To test this possibility, we inhibited acetylation by adding oxaloacetate and citrate synthase to the translation reaction (30). Under these conditions only four translation products which co-migrate with the lower member of each pair of doublets were observed (Fig. 3B). Thus the lower members of each pair of doublets are the unacetylated pre-

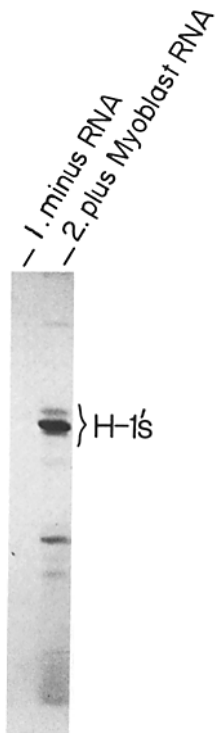


FIGURE 2 H-1 in vitro translation products directed by total myoblast RNA. 20 μ g of total myoblast RNA was translated in a 0.2-ml wheat germ cell-free translation reaction supplemented with [3 H]lysine. The 5% PCA extract of the reaction mixture with and without mRNA was displayed on an SDS polyacrylamide gel and analyzed by fluorography.

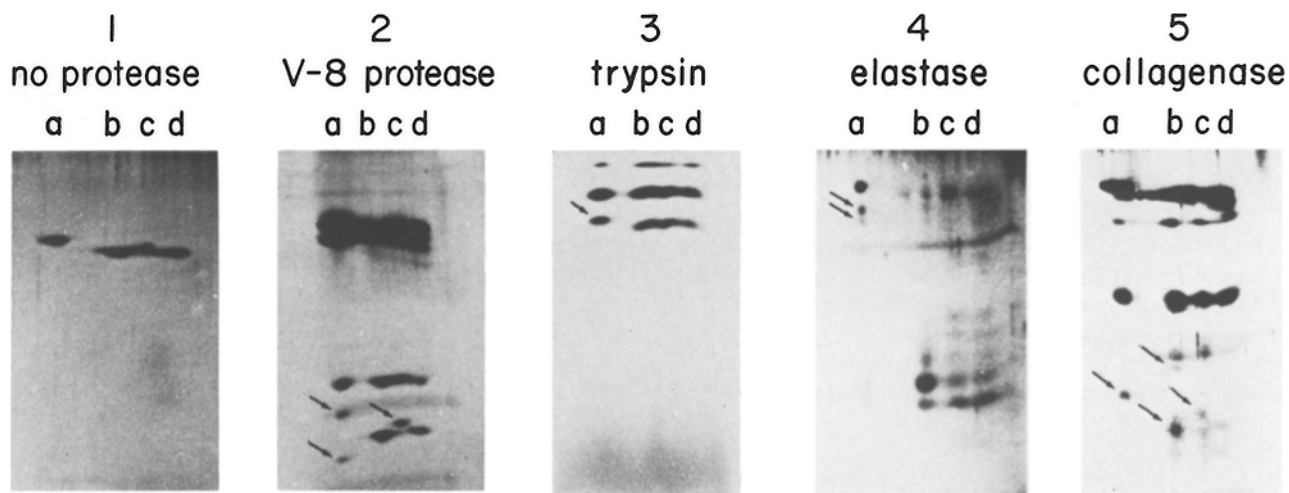


FIGURE 1 Partial proteolytic analysis of the four chicken H-1's. Gel slices containing the four electrophoretically resolved H-1's were incubated in buffer containing the indicated protease. The proteolytic fragments were subsequently electrophoresed 90° relative to the first dimension and silver stained as described in Materials and Methods. The examples shown represent enzyme concentrations that yielded the maximum number of fragments unique to the individual H-1's (indicated by arrows). The gels were electrophoresed for different lengths of time.

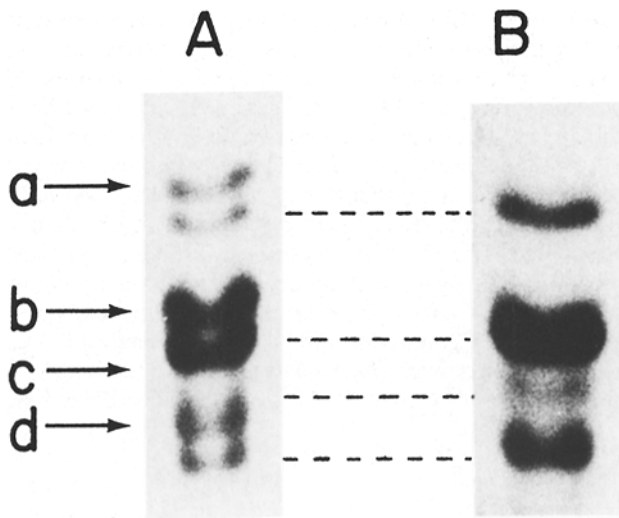


FIGURE 3 Effect of depleting acetyl CoA pools on H-1 *in vitro* translation products. Myoblast mRNA was translated in the absence (A) or presence (B) of oxaloacetate and citrate synthase. The 5% PCA extracts were resolved on an acid-urea-polyacrylamide gel. The arrows indicate the positions where the *in vivo*-synthesized H-1's migrate.

cursor forms of the H-1's. Coupled with the partial peptide analysis, these results strongly suggests that the four H-1's are primary sequence variants.

H-1 mRNA Is Present in Nondividing Myogenic Cells

Previous work showed that H-1 synthesis is less tightly coupled to DNA synthesis than nucleosomal histone synthesis throughout myogenesis (13). H-1 synthesis is only partially inhibited in myoblasts in which DNA synthesis has been inhibited with Ara C, or after cells withdraw from the cell cycle upon fusion into myotubes. In contrast, core histone synthesis is tightly coupled to DNA synthesis. To determine if synthesis is regulated at the level of messenger RNA accumulation, we assayed the relative amounts of H-1 mRNA by hybridization using the Northern transfer technique.

Hybridization analysis shows that the level of H-1 mRNA is less tightly coupled to DNA synthesis than the level of mRNA encoding the nucleosomal histone H-4. H-1 and H-4 mRNAs were present at decreased levels in Ara C-treated myoblasts and in nondividing myotubes when compared with untreated myoblasts (Fig. 4). However, the decrease in H-1 mRNA levels was substantially less dramatic than the decrease in H-4 mRNA levels. Quantitative analysis reveals that the amount of H-1 mRNA present in Ara C-treated myoblasts was $18 \pm 3\%$ and in myotubes was $13 \pm 2\%$ of that found in untreated dividing myoblasts. In contrast, the amount of H-4 mRNA present in Ara C-treated myoblasts was $5 \pm 2\%$ and in myotubes was $3 \pm 2\%$ of that found in untreated myoblasts.

The Level of Translatable RNA Encoding The Four H-1 Species Is Coupled to DNA Replication to Different Extents

To determine if the levels of mRNA encoding each of the variants is differentially coupled to DNA synthesis, we assayed

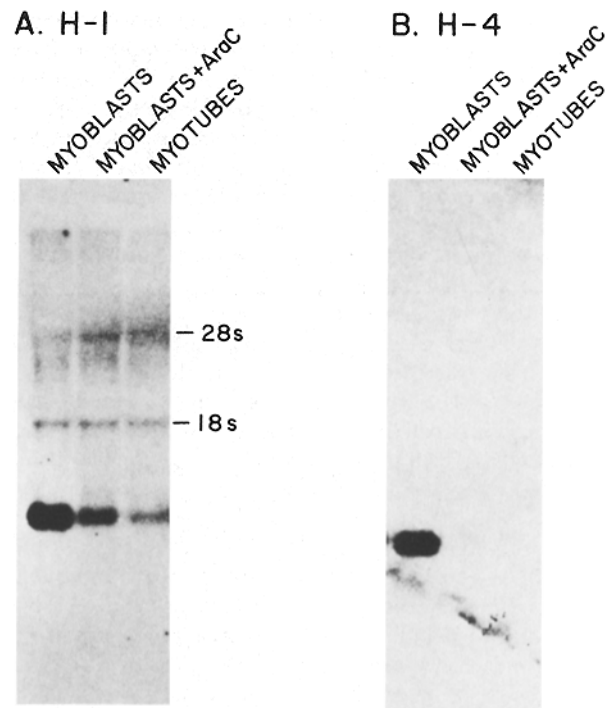


FIGURE 4 Hybridization (Northern blot) analysis of H-1 and H-4 mRNA levels in myogenic cells. Equal amounts of total RNA extracted from myoblasts, from Ara C-treated myoblasts, and myotubes was electrophoretically resolved on an agarose gel. The RNA was transferred to nitrocellulose filters and hybridized with radioactively labeled H-1, or H-4, DNA.

changes in the amounts of translatable mRNA encoding the four H-1's. The H-1 *in vitro* translation products directed by RNA from untreated myoblasts, Ara C-treated myoblasts, and myotubes are shown in Fig. 5. H-1 *c* translatable mRNA was preferentially retained in the cells which are not synthesizing DNA as compared to dividing myoblasts. In Ara C-treated myoblasts and in myotubes, the level of translatable H-1 *a*, *b*, and *d* mRNA decreased to $<18\%$ of the level found in untreated myoblasts. In contrast, the level of translatable H-1 *c* mRNA was indistinguishable in myoblasts, in Ara C-treated myoblasts, and in myotubes. The results show that the level of H-1 *c* mRNA is uncoupled from DNA synthesis. It follows that the uncoupling of H-1 *c* synthesis previously observed in myogenic cells occurs at the level of mRNA accumulation.

H-1 Messages Are Not Polyadenylated

In most cases, histone messages lack the 3' polyadenylate tracts typical of eucaryotic mRNA. However, several notable exceptions have been described. In several cell types in which polyadenylated histone messages have been observed, histone synthesis is uncoupled from DNA synthesis (32-34). These correlations between polyadenylated histone mRNA and uncoupled histone synthesis have led to suggestions that polyadenylation is involved in the process of uncoupling histone synthesis from DNA replication (32-35). We therefore asked if the H-1 mRNAs which accumulate in nondividing myogenic cells are polyadenylated. RNA from untreated myoblasts, Ara C-treated myoblasts, and myotubes was separated into poly (A)⁺ and poly (A)⁻ fractions by poly(U)-Sepharose chromatography (35). The H-1 mRNA content of each was

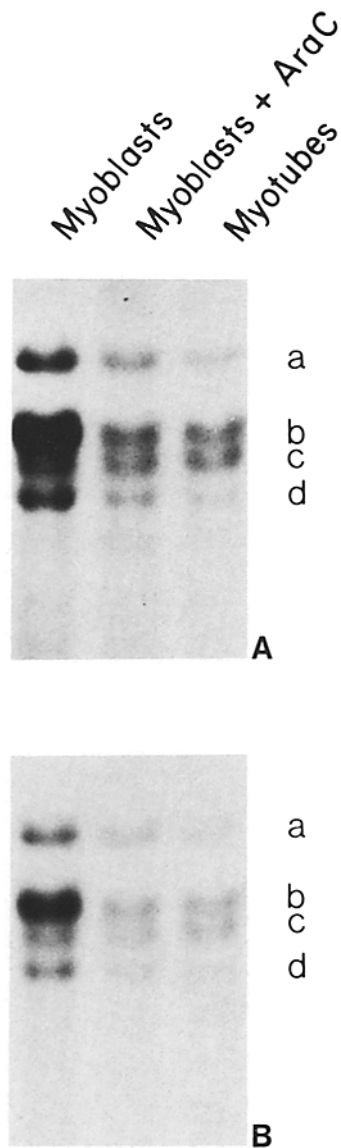


FIGURE 5 Comparison of translatable H-1 mRNA levels in myogenic cells. Total RNA extracted from myoblasts, Ara C-treated myoblasts, and myotubes was translated in a cell-free wheat germ *in vitro* translation system supplemented with oxaloacetate and citrate synthase. The H-1's were extracted from aliquots of the reactions that contained equivalent levels of protein radioactivity with 5% PCA, and were subsequently resolved by acid-urea-polyacrylamide gel electrophoresis followed by fluorography. Two different exposures of the same gel are shown for comparison. (A) 3-wk and (B) 1-wk exposure.

then analyzed by hybridization and *in vitro* translation analysis. The vast majority of total translation products were directed by the poly (A)⁺ fraction (Fig. 6A). However, after PCA extraction, H-1 translation products were detected only in the poly (A)⁻ fractions (Fig. 6B). Each variant was found in the same relative proportions as after translation of total RNA. Furthermore, hybridization analysis showed that H-1 mRNA is found exclusively in the poly (A)⁻ fraction in all cases (data not shown). Thus, H-1 mRNA is not polyadenylated in dividing and nondividing myogenic cells.

DISCUSSION

This study shows that the four chicken H-1's are encoded by

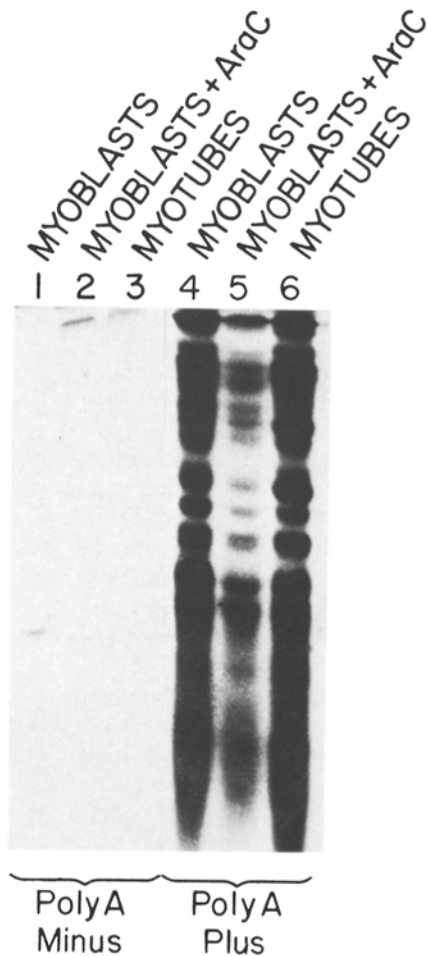
different messenger RNAs whose expression is coupled to DNA synthesis to varying extents. Accumulation of H-1c mRNA is completely uncoupled from DNA synthesis. This can account for the increase in the relative amount of H-1c after dividing myoblasts fuse into nondividing myotubes (see previous paper).

Peptide mapping and cell-free translation analysis both indicate that the four major chicken H-1's are amino acid sequence variants. A comparison of the peptides generated after partial digestion with four different proteases clearly distinguishes H-1a from H-1c and both from H-1's b and d. Only minor differences between H-1's b and d are observed with one of the enzymes. *In vitro* translation analysis of RNA extracted from myogenic cells supports the conclusion that the four chicken H-1's are primary amino acid sequence variants. When acetylation is inhibited, myogenic RNA directs the synthesis of four H-1's. We can not eliminate the possibility that the wheat germ translation system carries out a novel covalent modification which gives the appearance of four primary translation products. However, the observation that the relative amounts of the four H-1 translation products vary depending on the source of RNA makes this unlikely. Thus analysis by two independent techniques indicates that the four major chicken H-1's are primary amino acid sequence variants.

Analysis of the protease digestion products can define some of the differences between the H-1's. After V-8 protease digestion, H-1a and H-1c yield different numbers of peptides, some of which have unique molecular weights. The unique H-1a and H-1c fragments generated by V-8 protease, which has a specificity for glutamic and aspartic acid residues, suggests that these proteins have different numbers and locations of these amino acids. One of the two major trypsin digestion products of H-1a has a larger apparent molecular weight than that characteristic of the others. This digestion product is relatively resistant to proteolysis following extended trypsin digestions (data not shown). Since it is known from trypsin digestions of H-1's from other organisms that the central globular region is relatively resistant (36), this result suggests that the H-1a central domain is larger than that of H-1's b, c, and d. The differences distinguishing H-1b and H-1d are more difficult to define since they are observed only with commercial collagenase which usually contains contaminating proteases of undefined specificity (37, 38). Taken as a whole, the data indicate that the structure of H-1a (the least stable H-1 species) is the most highly diverged. H-1c (which preferentially accumulates during myogenesis) is also clearly different from the others. The data suggest that H-1's b and d are very similar. That the H-1's whose proportions change most dramatically during myogenesis are also the most unique (as determined by partial proteolytic mapping) suggests that fundamental changes in chromatin structure may be taking place in these cells. Further understanding of the differences between the four proteins awaits the cloning and sequencing of the genes which encode them.

In the previous paper we demonstrated that synthesis of the H-1's is less tightly coupled to DNA synthesis than nucleosomal histone synthesis in both myoblasts and myotubes. This study shows that the difference in coupling is regulated at the level of mRNA accumulation. It is likely that the changes in H-1 mRNA levels determined by hybridization with the cloned H-1 cDNA underestimate the amount of total H-1 message in the nondividing cell types. Preliminary studies

A. Total Translation Products



B. H-1 Translation Products

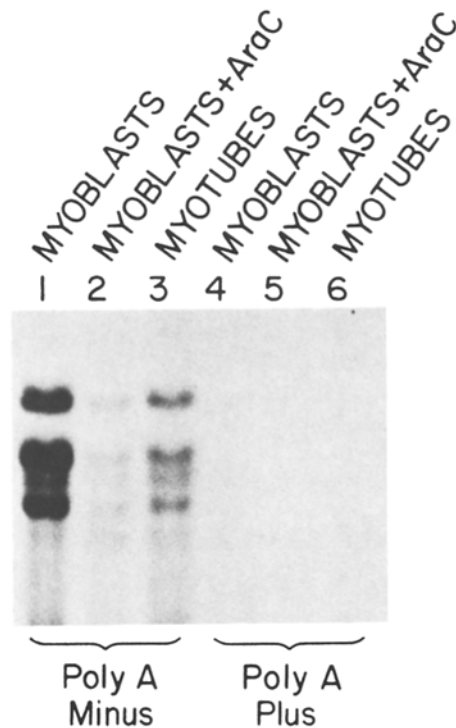


FIGURE 6 Analysis of the H-1 mRNA content in poly-A⁺ and poly-A⁻ fractions from myogenic cells. RNA extracted from myoblasts, from Ara C-treated myoblasts, and from myotubes was separated into poly-A⁺ and poly-A⁻ fractions using poly(U)-Sephacrose. Each fraction was translated in vitro in the presence of oxaloacetate and citrate synthase, and an aliquot of the total translation products was resolved by SDS PAGE followed by fluorography (A). The single band observed in lane 1 co-migrates with H2B, the most lysine-rich core histone. The H-1's were then extracted from the remaining translation reaction with 5% PCA and resolved by acid-urea-polyacrylamide gel electrophoresis followed by fluorography (B).

suggest that the cloned H-1 cDNA used in this study preferentially hybridizes to H-1*a* mRNA (data not shown). H-1*a* synthesis is more tightly coupled to DNA replication than synthesis of H-1*b*, *c*, or *d*. Thus the changes in hybridizable H-1 mRNA may preferentially reflect changes in the RNA whose level is most tightly coupled to DNA synthesis. However, even when this cloned DNA is used, the results show that H-1 mRNA levels are less tightly coupled to DNA synthesis than nucleosomal H-4 mRNA levels.

In the previous paper we also demonstrated that differential coupling of H-1-variant synthesis to DNA replication is responsible for changing H-1 proportions after dividing myoblasts differentiate into nondividing myotubes. The results described in this paper demonstrate that differential coupling is regulated at the level of mRNA accumulation.

Based on work in other systems, it is likely that most core histone and core histone mRNA synthesis is restricted to the late G-1/S phase of the cell cycle and that the mRNAs encoding the core histones are rapidly degraded after S phase. The results which we have presented suggest that H-1 mRNA metabolism is different, and that different H-1 mRNAs can

be independently regulated. For example, individual H-1 mRNAs may be transcribed and/or stabilized at other stages of the cell cycle and may not be subject to the same S phase controls as core histone mRNAs. However, since the levels of H-1 mRNAs do not increase during myogenesis, we do not know whether any H-1 mRNAs continue to be synthesized during myogenesis or whether they are simply not destabilized (or a combination of both). The underlying molecular mechanisms which result in the differential regulation of H-1 variant mRNA levels remains to be determined. It has been proposed that the lack of polyadenylation is involved in coupling histone synthesis to DNA replication (32-35). Our results show that H-1 mRNAs in both dividing and nondividing myogenic cells are not polyadenylated. Thus the polyadenylation of H-1 mRNA is not involved in uncoupling H-1*c* mRNA levels from DNA synthesis. It should be pointed out that while the relative amount of translatable H-1*c* mRNA increases during myogenesis, the in vivo and in vitro labeling patterns for H-1*c* (relative to H-1's *a*, *b*, and *d*) are not identical. H-1*c* mRNA is either preferentially translated in vivo or inefficiently translated in vitro. These results suggest

that H-1c mRNA may have other properties which play a role in regulating its expression. An understanding of the mechanism by which the cell distinguishes between the H-1 mRNAs may be particularly revealing.

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