Changes in the H-1 Histone Complement during Myogenesis. I. Establishment by Differential Coupling of H-1 Species Synthesis to DNA Replication

EDWARD WINTER,* DANIEL LEVY,* and JOEL S. GORDON*‡
*The Cellular and Developmental Biology Program and ‡Department of Anatomical Sciences, State
University of New York at Stony Brook, Stony Brook, New York 11794. Dr. Levy's present address is
Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts
02138. Dr. Gordon's present address is Basic Research, Johnson and Johnson, Skillman, New Jersey
08558.

ABSTRACT   Proportions of the four major chicken H-1 histones (referred to as H-1's a–d)
change during in vitro skeletal myogenesis. As myoblasts fuse and differentiate into myotubes,
the relative amount of H-1 c increases dramatically. The change occurs primarily because
synthesis of the H-1 species is coupled to DNA synthesis to different extents. H-1 c synthesis
is least tightly coupled to DNA replication in precursor myoblasts and in differentiated
myotubes. Thus H-1 c synthesis predominates after dividing myoblasts fuse into postmitotic
myotubes. This results in the replacement of pre-existing H-1 and therefore increases the
relative amount of H-1 c. Differences in the stability of the H-1's are also involved in changing
H-1 proportions. The results show that changes in H-1 proportions during myogenesis are a
consequence of withdrawal from the cell cycle. The data provides a general mechanistic
explanation of how tissue-specific H-1 proportions are established.

Histones are the major structural proteins of eucaryotic chromatin. Two molecules each of histones H2A, H2B, H3, and H4 make up the nucleosomal core. Approximately 140 base pairs of DNA are wrapped in two turns around the nucleosome which is responsible for the repeating 'beads on a string' morphology of chromatin (for reviews of nucleosomal structure see references 1 and 2). The H-1 histones bind to the regions of DNA which link adjacent nucleosomes (3–6), and the outer portion of the nucleosomal particle (7, 8). The H-1's have a central role in organizing the repeating nucleosomal array into a helix having a diameter of ~300 Å (9–14).

Heterogeneity within each of the histone classes has been observed. H-1 heterogeneity is especially widespread. This heterogeneity reflects the existence of a limited number of primary amino acid sequence variants which are subject to numerous postsynthetic modifications (for review see reference 15). The H-1 sequence variants are present in tissue- (16, 17, and reviewed in reference 18) and developmental- (19–21) specific proportions. Changes in H-1 proportions have also been observed after hormone induction (22–24), after viral transformation (25), and after the occurrence of changes

in cellular growth rates (26–28). During the differentiation of cells characterized by quiescent nuclei, such as nucleated erythrocytes (29) and spermatocytes (reviewed in 30), the H-1's are substantially replaced by cell-specific H-1–like proteins. These correlations of changes in the H-1 complement with changes in nuclear activity have led to the suggestion that H-1 variants have different functions (31).

To understand the functional and structural significance of H-1 variants, it is important to elucidate how their proportions are established during cytodifferentiation. Most histone synthesis is coupled to DNA replication in both higher and lower eucaryotic somatic cells (for review see reference 32). However, several studies have shown that the coupling of H-1 synthesis may not be as stringent as the coupling of nucleosomal histone synthesis (33–38). H-1 synthesis which occurs in the absence of DNA replication may play a role in altering H-1 proportions. Zweidler has proposed that histone variants expressed in somatic cells are of two general types: replication variants whose synthesis is tightly coupled to DNA replication, and replacement variants which are expressed regardless of DNA synthetic activity (39). According to this hypothesis,

changes in DNA synthetic rates would alter the balance of replication- and replacement-variant synthesis and thus alter histone-variant proportions. This idea is supported by the observation that H-1⁰, an H-1 variant found in high relative amounts in nondividing mammalian tissues, continues to be synthesized at high levels in the absence of DNA replication (28, 40–42).

In vitro myogenesis is an ideal system for studying how H-1 proportions are regulated during cytodifferentiation. Relatively homogeneous populations of rapidly dividing myoblasts synchronously fuse into nondividing myotubes with the concomitant expression of muscle-specific genes. Thus the role that coupling of H-1-variant synthesis to DNA replication plays in establishing H-1-variant proportions can be evaluated in nondividing and dividing cells of the same developmental lineage.

This report shows that proportions of the four major chicken H-1's (H-1's *a–d*) change during myogenesis. The relative amount of H-1 *c* increases dramatically upon fusion. The change is primarily due to differential coupling of H-1 species synthesis to DNA replication. H-1 *c* synthesis is least tightly coupled in both myoblasts and myotubes. The predominant synthesis of H-1 *c* in postmitotic cells results in its increase relative to the other H-1's in the nondividing myotube. Differences in the stabilities of the four H-1's are also involved in changing the H-1 proportions. The results show that the changes in H-1 proportions during myogenesis are a consequence of withdrawal from the cell cycle.

MATERIALS AND METHODS

Cell Culture: Myoblasts were prepared by mechanical dissociation (43) of the thigh musculature dissected from 11–12-d-old White Leghorn chicken embryos (utility grade, SPAFAS Inc., Norwich, CT). Unless stated otherwise, cells were inoculated into 850-cm³ gelatin-coated plastic roller bottles at 2–4 × 10⁸ cells in 60–70 ml of Earle's minimum essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 15% horse serum and 4% embryo extract prepared from 12-d-old chicken embryos (44). The bottles were placed on a roller bottle apparatus at 37°C after equilibrating the media with 92% air, 8% CO₂. The roller apparatus was initially set at 1 rpm for 8–12 h to allow cells to settle and adhere to the substratum. The rate was then increased to 4–5 rpm. A comparison of myogenic differentiation in roller bottles and in standard 100-cm gelatin-coated tissue culture dishes reveals no difference in H-1 synthesis and turnover, growth rate, fusion, or accumulation of acetylcholine receptor and creatine phosphokinase activity. A more detailed characterization and description of the technique for growing chicken myogenic cells in roller bottles will be described elsewhere.

Myoblasts were harvested 16–24 h after preparation of the cultures, before fusion was evident. Dividing cells in the myotube cultures were selectively killed with cytosine arabinoside (Ara C).[1] Upon the definitive appearance of fused cells at day 3, the media was replaced twice at 24-h intervals with 20 ml of fresh growth media containing 10⁻⁵ M Ara C. This results in the elimination of the vast majority of the mononucleated cells as judged by phase-contrast microscopy. Subsequent analysis of myotubes was carried out within 24 h after the addition of fresh growth media without Ara C.

Proteins and DNA were isotopically labeled by incubating cells for 4 h in 10 ml of labeling media consisting of Earle's minimum essential medium without lysine, leucine, and methionine (Gibco Laboratories), 15% dialyzed horse serum (Gibco Laboratories), and 4% dialyzed embryo extract containing 100 μCi of [³H]lysine (4,5,³H[N], 75 Ci/mM) and/or 2 μCi of [¹⁴C]thymidine (methyl-¹⁴C, 50 mCi/mM) (New England Nuclear, Boston, MA). Incorporation of [³H]lysine into the H-1's, and into the High Mobility Group (HMG) proteins 1, 2A, 2B, 14, and 17 was linear for up to 8 h. Control experiments showed that cells incubated for up to 24 h in this media survive and differentiate on schedule when returned to normal media.

H-1 turnover rates were determined after labeling cells with [³H]lysine for 4 h. This was followed by three 15-min washes with growth media supplemented with 1 mM unlabeled L-lysine and incubation for increasing times in growth media supplemented with 1 mM L-lysine.

Extraction and Electrophoresis of Histones: Cells were harvested with a rubber policeman into 15 ml of phosphate-buffered saline (PBS) and sedimented for 6 min at 800 g. In preparation for extracting the H-1's, the pellet was immediately resuspended in 1.5 vol of 30 mM MgCl₂, 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1.0 mM dithiothreital, and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was sonically disrupted with a Branson sonifier outfitted with a microprobe. H-1's were extracted from the sonicate with 5% perchloric acid (PCA) by the technique of Johns (45).

Total histones were extracted from partially purified nuclei. The cells were lysed by resuspension in 30 mM MgCl₂, 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1.0 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100, and a homogenate of the cell suspension was generated at 4°C with 35 up and down strokes with a motorized teflon-glass homogenizer. Nuclei were sedimented by centrifugation at 1,600 g. Histones were extracted from these crude nuclear preparations at 4°C with 0.4 N H₂SO₄ by the technique of Panyim et al. (46).

SDS PAGE of histones was carried out using the discontinuous buffer system of Laemmli (47). Total histones were resolved on 15% polyacrylamide gels electrophoresed at 140 V for 4–5 h. The H-1's were resolved on 17% polyacrylamide gels electrophoresed at 140 V for 7–8 h. Acetic acid-urea-polyacrylamide gels were run as described by Panyim and Chalkey (48). 30 μg of H-1 in 8 μl of loading buffer (8 M urea, 4% β-mercaptoethanol, 22% sucrose, and 1.3 N acetic acid) was applied to wells of a 30-cm-long slab gel which had been pre-electrophoresed overnight at 100 V in the opposite direction from which it was to be run. The H-1's were resolved by electrophoresis at 250 V for 72 h.

The gels were stained with 0.25% Coomassie Blue in 50% methanol and 10% acetic acid overnight and destained in 50% methanol and 10% acetic acid. Photographic transparencies of the gels were prepared with Polaroid type 46-L continuous tone film. Fluorograms of the gels were made using preflashed X-ray film as described by Laskey and Mills (49). The relative staining intensity of protein bands was quantitated by integrating the area under peaks with an electronic digitizer (Numonics Corp., Lansdale, PA, model 1224), generated from tracings of the transparencies using a microdensitometer (Joyce Loebl, Gateshead, England). Fluorograms were quantitated similarly. The relative specific activities of protein bands were calculated by dividing the area under the fluorogram peaks by the area under the stained gel peaks. Multiple fluorographic and photographic exposures were quantitated to insure operation within the linear response range of the films. In selected experiments, radioactivity in H-1 protein bands was directly measured by scintillation spectrometry and the relative amounts of radioactivity compared with that determined by fluorography. Gel slices were oxidized using a model 306 Tri-Carb sample oxidizer (Packard Instrument Co., Downers Grove, IL), and the radioactivity was determined. Relative incorporation of radioactivity obtained by the two methods within a given experiment were identical.

Immunological Identification of the H-1's: Confirmation of the identity of putative H-1's was obtained by Western blot analysis. Proteins resolved on SDS polyacrylamide gels were electrophoretically transferred to nitrocellulose filters at a constant 10 V/cm (0.8–1.4 amps) in 20 mM Tris-HCl, 150 mM glycine, and 20% methanol for 12–16 h. Approximately half of the protein was eluted from the gel under these conditions, allowing the gel to be stained for comparison with the autoradiogram. Proteins resolved on acid-urea gels were transferred for 4–5 h at 10 V/cm. (1.0–1.5 amps) using 7% acetic acid as the transfer solution. The nitrocellulose replicas were incubated in 3% bovine serum albumin (BSA) (Pentax, fraction V), 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, for 1 h at 40°C to eliminate nonspecific binding. Filters were then incubated with 10 μg/ml of affinity-purified rabbit anti-chicken H-1 antibody (a gift from Dr. Manuel Perucho, see reference 50 for details) in 3% BSA, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, and 0.05% Nonidet P-40 for 2 h. This was followed by a 2-h incubation with 0.5–1.0 μCi/ml [¹²⁵I]-Protein A (8 μCi/μg) (New England Nuclear) in the same buffer. The filters were rinsed with water followed by three 15-min washes in 1.0 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, and 0.4% Sarkosyl (wt/vol), and then rinsed with water, air dried, and exposed to X-ray film.

Miscellaneous Assays and Procedures: DNA was quantitated in triplicate on aliquots of a whole cell sonicate by the microfluorometric assay of Kissane and Robins (51) as modified by Santoianna and Ayala (52), using salmon sperm DNA as a standard. Incorporation of [¹⁴C]thymidine into DNA was quantitated by measuring radioactivity incorporated into TCA-precipitable material recovered by filtration.

RESULTS

H-1 Proportions Change during Myogenesis

The relative amounts of the major H-1 species found in myoblasts and myotubes were compared by analyzing 5%

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[1] *Abbreviations used in this paper:* Ara C, cytosine arabinoside; HMG, High Mobility Group; PCA, perchloric acid.

PCA extracts of whole cells on acid-urea-polyacrylamide gels (Fig. 1). The same four species (referred to as *a–d* in order of increasing mobility) were found in both myoblasts and myotubes. They were present, however, in different proportions. Myotubes showed increased relative amounts of H-1*c*. To eliminate the possibility that the change is due to differential degradation or extraction during sample preparation, a mixing experiment was done. The H-1's were extracted from a mixture of cells consisting of unlabeled cells at one developmental stage in 10-fold excess (protein:protein) over radioactively labeled cells at the other stage. The resulting H-1 staining pattern was characteristic of the unlabeled cell type, whereas the fluorographic pattern was characteristic of the radiolabeled cell type (Fig. 2). Thus, the difference in H-1 proportions between myoblasts and myotubes pre-exists extraction. We have previously shown that the four major H-1 species in chick muscle are not derived from the phosphorylation of a smaller number of H-1 species (53), and in the following paper we demonstrate that they are different primary amino acid sequence variants.

The protein bands referred to as H-1's *a–d* co-migrate with authentic H-1's (isolated from purified chicken thymus chromatin) on acid-urea-polyacrylamide gels where mobility is primarily a function of charge. The putative H-1's also co-migrate with authentic H-1 on SDS polyacrylamide gels where mobility is primarily a function of molecular weight. On SDS gels only three H-1 bands are observed. H-1's *b* and *d* co-migrate most rapidly, followed by H-1*c* and H-1*a* (53). The increase in the level of H-1*c* in myotubes can also be observed in this gel system (see Fig. 3). These proteins were unequivocally identified as authentic H-1's with an affinity-purified rabbit anti-H-1 antibody. Upon Western blot analysis, the three bands resolved on SDS gels bound the antibody (Fig. 3). Furthermore, only those bands that co-migrated with authentic H-1's bound the antibody when total cell extracts were analyzed. When resolved on acid-urea gels, all four H-1 bands bound the antibody (data not shown). When total extracts from myoblasts and myotubes were compared, the increase in the relative amount of H-1*c* was seen in the antibody-detected bands (Fig. 3).

FIGURE 2 Mixing experiment in which an excess of unlabeled myoblasts was combined with radiolabeled myotubes. The H-1's were extracted with 5% PCA and displayed on an acid-urea-polyacrylamide gel. The stained gel (A), fluorographic exposure (B), and corresponding optical density scans are shown for comparison.
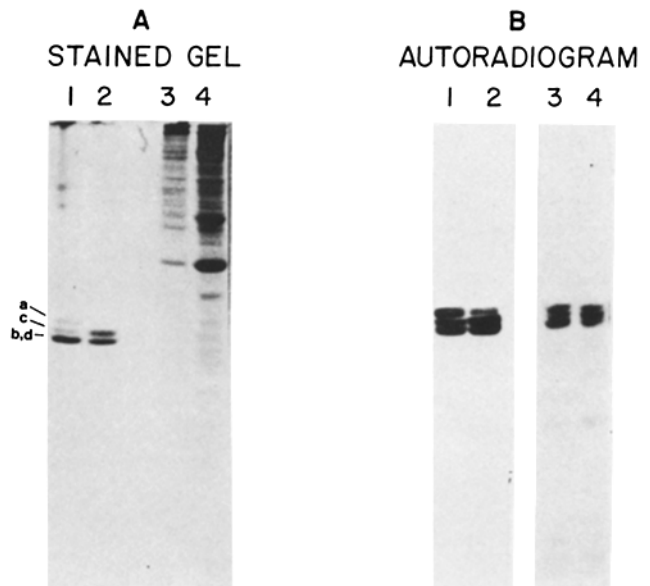
FIGURE 3 Immunological identification of H-1's by Western blot analysis. H-1's extracted using 5% PCA from myoblasts (lane *1*) and from myotubes (lane *2*), and total cell extracts from myoblasts (lane *3*) and from myotubes (lane *4*) were resolved on SDS polyacrylamide gels. A shows the gel stained with Coomassie Blue and B shows the autoradiogram of the antibody-detected bands. In B, lanes *1* and *2* are from a 1-d exposure, and lanes *3* and *4* are from a 1-wk exposure of the autoradiogram.
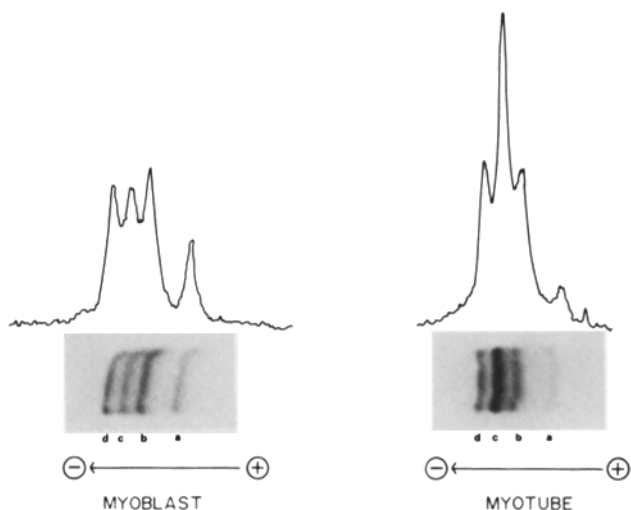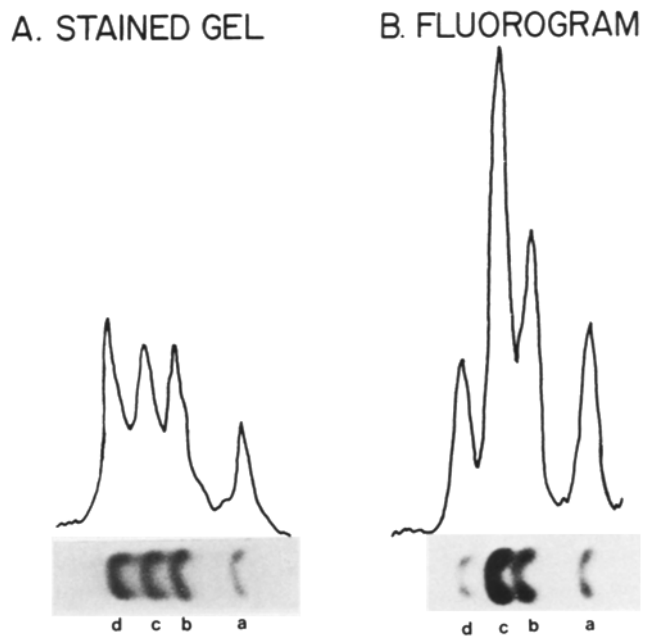
FIGURE 1 Comparison of myoblast and myotube H-1's resolved on an acid-urea-polyacrylamide gel. The corresponding optical density scans are shown for comparison. The H-1's are from right (H-1*a*) to left (H-1*d*). The arrow indicates the direction of electrophoresis.

H-1 Synthesis Continues in the Absence of DNA Synthesis

Numerous studies have shown that most histone synthesis is restricted to the S phase of the cell cycle. More recent work suggests that there are exceptions to this phenomenon. Continued H-1 synthesis after myotube formation and withdrawal

from the cell cycle could be involved in changing the H-1 proportions. Thus, the degree to which H-1 synthesis and DNA replication are coupled during myogenesis was investigated.

Myoblast and myotube cultures were incubated with [³H]-lysine and [¹⁴C]thymidine for 4 h. Incorporation of [¹⁴C]-thymidine per microgram of DNA in myotubes was 5 ± 2% of that found in myoblast cultures. To compare the synthesis of total histones, we extracted them from isolated nuclei, resolved them by SDS PAGE, and determined the relative incorporation of [³H]lysine by fluorography (Fig. 4). Incorporation of [³H]lysine into myotube core histones (relative specific activities) decreased to 7 ± 3% of that found in myoblast cultures. In contrast, incorporation of label into myotube H-1's continued at 26 ± 3% of the rate found in myoblasts. Thus H-1 synthesis is less tightly coupled to DNA replication than core histone synthesis in differentiated myotubes.

The uncoupling of H-1 synthesis from DNA replication could be a phenomenon specific to differentiated myotubes or it could also occur in precursor myoblasts. To determine if myoblasts can synthesize H-1's in the absence of DNA replication, we treated cultures with increasing concentrations of Ara C to inhibit DNA synthesis, and the incorporation of [³H]lysine into electrophoretically separated H-1's and core histones was monitored by fluorography (Fig. 5a). At 50 µM Ara C, DNA synthesis was maximally inhibited to 5 ± 2% of that found in untreated control cultures. At this Ara C concentration, [³H]lysine incorporation into the core histones was maximally inhibited to 7 ± 3% of that found in untreated myoblasts. The decrease in DNA synthesis was paralleled by a similar decrease in core histone synthesis at all Ara C concentrations (Fig. 5b). This is not the case with H-1 synthesis. H-1 synthesis was maximally inhibited to only 24 ± 3% of the rate found in untreated cultures at Ara C concentrations that inhibited 95% of the DNA synthesis. H-1 synthesis was inhibited less than DNA or core histone synthesis at all Ara C concentrations (Fig. 5b).

To eliminate the possibility that the [³H]lysine is incorporated into nonhistone contaminants co-migrating with the H-1's, we extracted them with 5% PCA. H-1's prepared from myogenic cells in this manner are >80% pure, and are easily resolved from other protein species under the electrophoretic

conditions used (reference 53 and unpublished results). Incorporation of [³H]lysine into myotube H-1's prepared in this manner was 21 ± 3%, and in myoblasts treated with 50 µM, Ara C was 24 ± 2% of that found in untreated myoblasts
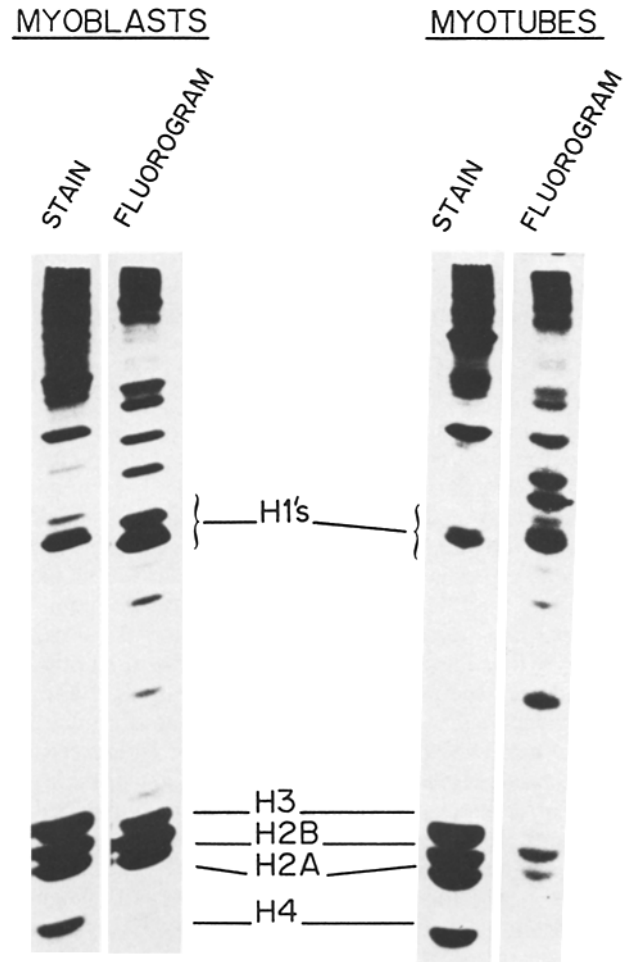

FIGURE 4 Comparison of total histone synthesis in myoblasts and in myotubes. Histones were extracted from myoblast and myotube nuclei from cultures incubated identically with [³H]lysine. After resolution on an SDS polyacrylamide gel, the stained gel was subjected to fluorography.
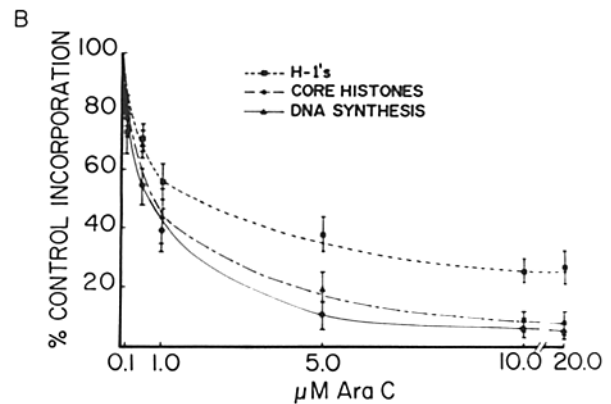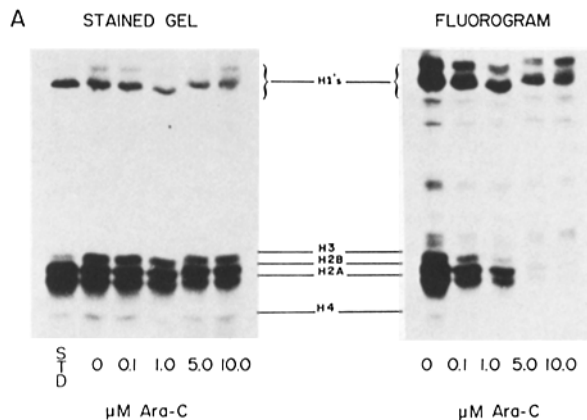

FIGURE 5 Effect of Ara C on histone synthesis in myoblasts. A shows the Coomassie Blue–stained gel and corresponding fluorogram of total histones extracted from myoblasts treated with increasing concentrations of Ara C as indicated. B shows the relative labeling rates of DNA, the core histones, and the H-1's quantitated from three independent experiments similar to that shown in A.
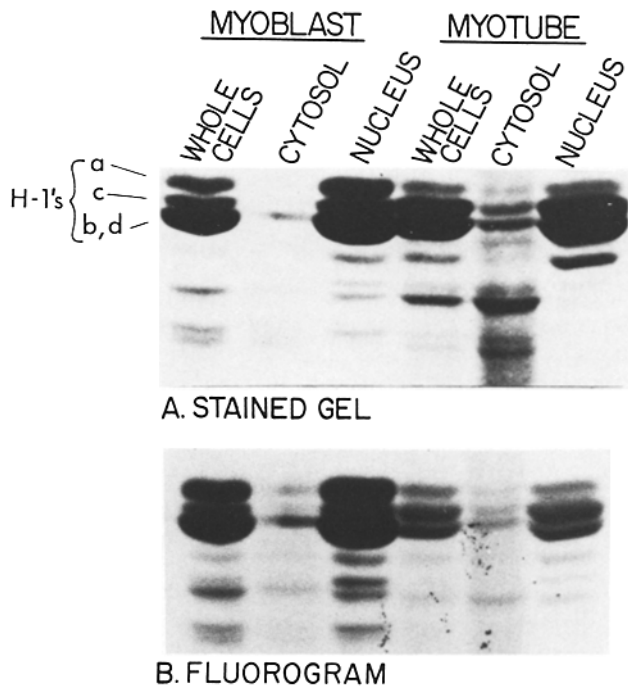
A. STAINED GEL

B. FLUOROGRAM

FIGURE 6 Cellular localization of H-1's synthesized in myoblasts and myotubes. Cells were incubated for 30 min with [³H]lysine and separated into nuclear and cystosolic fractions. The H-1's were extracted, and then displayed on SDS polyacrylamide gels which were subsequently subjected to fluorography. Three times the cell volume was analyzed in the cytosolic fractions compared to the nuclear fractions.
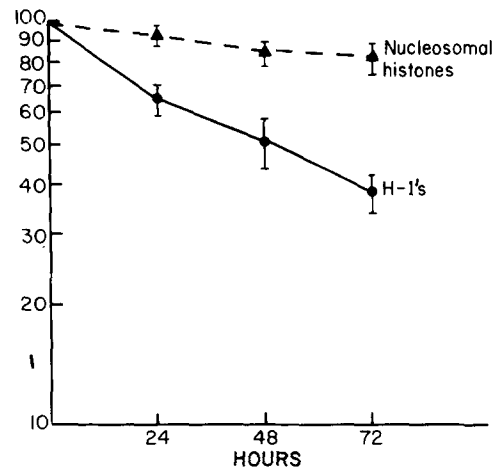

FIGURE 7 Turnover of H-1's and nucleosomal histones synthesized in myoblasts. Myoblasts were labeled with [³H]lysine and cells were harvested at 24-h intervals after the isotope was removed. Histones were extracted from isolated nuclei, displayed on SDS polyacrylamide gels, and the relative amount of H-1 and nucleosomal histone radioactivity remaining at the indicated times was analyzed by fluorography. Each point is the average of four independent determinations.
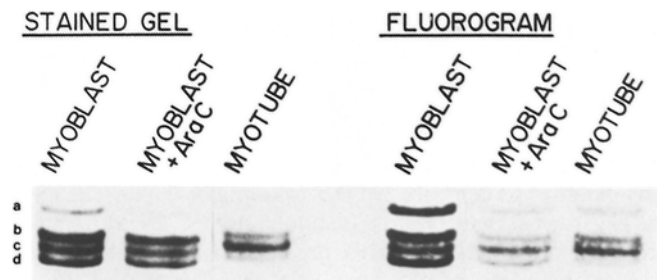

FIGURE 8 H-1 species synthesis in myoblasts, in myoblasts treated with Ara C, and in myotubes analyzed on an acid-urea-polyacrylamide gel. H-1a is the upper, slowest-migrating electrophoretic band followed by H-1's b, c, and d.

(data not shown). Thus H-1 synthesis in myogenic cells appears to consist of two components, one which is coupled to DNA replication, and one, representing ~25% of the H-1 synthesis which is uncoupled. This latter component is maintained in nondividing myotubes.

The H-1 synthesized in the absence of DNA synthesis could either be directly incorporated into nuclei or remain part of a rapidly turning over cytoplasmic pool. These two possibilities were distinguished by comparing the amount of newly synthesized H-1 in the cytoplasmic and nuclear fractions of myoblasts and myotubes after a 30-min incubation in [³H]-lysine (Fig. 6). More than 95% of the labeled and unlabeled H-1 was found in the nuclear fraction in all cases. Similar results were obtained with Ara C–treated myoblasts (data not shown). Since the specific activity of the H-1's extracted from all fractions was indistinguishable, a cytoplasmic H-1 pool of significant proportions is unlikely. Thus, most if not all newly synthesized H-1 is rapidly incorporated into nuclei in the presence or absence of DNA replication.

Since the H-1's are synthesized in the absence of DNA replication and incorporated into nuclei, it is likely that they turn over in myogenic cells more rapidly than the core histones whose synthesis is tightly coupled to DNA replication. This was confirmed by incubating myoblasts with [³H]lysine for 4 h and allowing differentiation to proceed in media supplemented with excess unlabeled lysine. The H-1's were isolated at various times after labeling and the decrease in H-1 radioactivity was monitored by fluorography. H-1's synthesized in myoblasts turned over during myogenesis with a half-life of 48 h (Fig. 7). Core histone turnover is barely detectable during the 4-d period measured. The turnover rate of H-1 synthesized in myotubes was also determined. H-1's synthe-

sized in myotubes turned over with a half-life of 72 h (data not shown). Thus H-1's synthesized in the presence or absence of DNA replication turn over more rapidly than the nucleosomal histones. Results discussed later suggest a likely explanation for the difference between the apparent turnover rates of H-1's synthesized in myoblasts and myotubes.

Synthesis of the H-1 Species Is Coupled to DNA Replication to Different Extents

To determine if H-1 species synthesis is coupled to DNA replication to different extents, we examined the relative labeling rates of the individual H-1 species. Fig. 8 shows a fluorogram of an acid-urea-polyacrylamide gel used to resolve the H-1's from untreated myoblasts, Ara C–treated myoblasts, and myotubes incubated identically with [³H]lysine. Both the nonproliferative myotubes and myoblasts treated with Ara C show an increased relative labeling of H-1c compared to untreated myoblasts. In Ara C–treated myoblasts, labeling of H-1a was 17%, H-1b 18%, and H-1d 15% of that found in untreated myoblasts. In contrast, H-1c labeling in Ara C–treated myoblasts continued at 65% of the rate found in untreated myoblasts. In myotubes, the labeling of H-1a, H-1b, and H-1d was 14, 15, and 13%, respectively, of that found

in myoblasts. H-1c labeling in myotubes was 58% of that found in myoblasts. These results show that H-1 species synthesis is coupled to DNA replication to different extents with H-1c synthesis being least tightly coupled. Thus, the increase in the relative amount of H-1c during myogenesis results, at least in part, because its synthesis is least tightly coupled to DNA replication.

The H-1 Species Have Different Stabilities

Differences in stabilities of the individual H-1 species during myogenesis could also contribute to the changes in H-1 proportions. To test this possibility, we examined the relative stabilities of the H-1's resolved on acid-urea-polyacrylamide gels. Fluorographic analysis showed that the H-1's have different stabilities during myogenesis with H-1c being the most stable, followed by H-1d, H-1b, and H-1a (Fig. 9). The absolute turnover rates of the individual H-1's can be calculated from their relative turnover rates (Fig. 9a) and the absolute turnover rate for total H-1's (Fig. 7). The half-lives of H-1a, H-1b, H-1c, and H-1d are 19, 48, 80 and 78 h, respectively. The differences in H-1 stabilities explain why total H-1 synthesized in myoblasts apparently turn over more rapidly than total H-1's synthesized in myotubes since the latter synthesize relatively more of the most stable H-1c species. Thus differential degradation of the H-1's is also involved in determining the unique H-1 proportions present in the differentiated myotube.

DISCUSSION

The relative proportions of the four major H-1's change during in vitro myogenesis. As myoblasts fuse and differentiate into myotubes, the relative amounts of H-1's a, b, and d decrease while the relative amount of H-1c more than doubles. The change in H-1 proportions is not the result of Ara C treatment of myogenic cells during the preparation of myotube cultures (see Materials and Methods) since it is also observed when myotubes are prepared in the absence of the drug (data not shown). In a previous study, changes were not detected in H-1 proportions of nuclei isolated from 8-, 11-, and 18-day-old chicken thigh tissue undergoing myogenesis

(53). Single, undifferentiated myoblasts were not separated from multinucleated muscle fibers in that study. The H-1 proportions measured in vivo are most likely average H-1 proportions from cells at various stages of myogenic differentiation. Because of the high cytoplasm to nuclei volume ratio of myotubes, and a possible selective isolation of myoblast nuclei, the myoblast H-1 contribution may have obscured that of the myotubes. We have examined this by comparing the H-1 proportions extracted from relatively homogeneous myotubes (as judged by phase-contrast microscopy) prepared from thigh musculature with single cell preparations. The myotubes and single cells were separated by filtration after trypsinizing dissected thigh musculature. The H-1 proportions from in vivo and in vitro myotube preparations are similar to each other and different from the pattern common to in vivo and in vitro myoblasts. Thus, the changes in H-1 proportions occurring during in vitro myogenesis reflect the changes occurring in vivo.

Our results show that H-1's are synthesized in the absence of DNA synthesis, accumulate in the nucleus, and replace pre-existing H-1's throughout in vitro myogenesis. The selective uncoupling of H-1c synthesis from DNA replication and the relative stability of H-1c provides the explanation for H-1c accumulation in myotubes. As myoblasts withdraw from the cell cycle, H-1c synthesis, which is least tightly coupled to DNA replication, predominates. Concurrently the H-1's are turning over at the same rate as in myoblasts with H-1c being the most stable. The combined result is that H-1c accumulates in the differentiated muscle fiber.

Chicken myogenesis is also accompanied by a change in proportions of another class of structural chromosomal proteins, the high molecular weight HMG proteins (reference 53 and unpublished results). In this case, the increase in the proportion of HMG 1 appears to be regulated through a mechanism different from that observed with the H-1's, since its synthesis, as that of all of the HMGs in chicken muscle cells, is not directly coupled to DNA replication (unpublished results).

The relative amount of H-1c also increases in cultured chick embryo fibroblasts as the rate of cell division decreases following density-dependent inhibition of cell growth (refer-
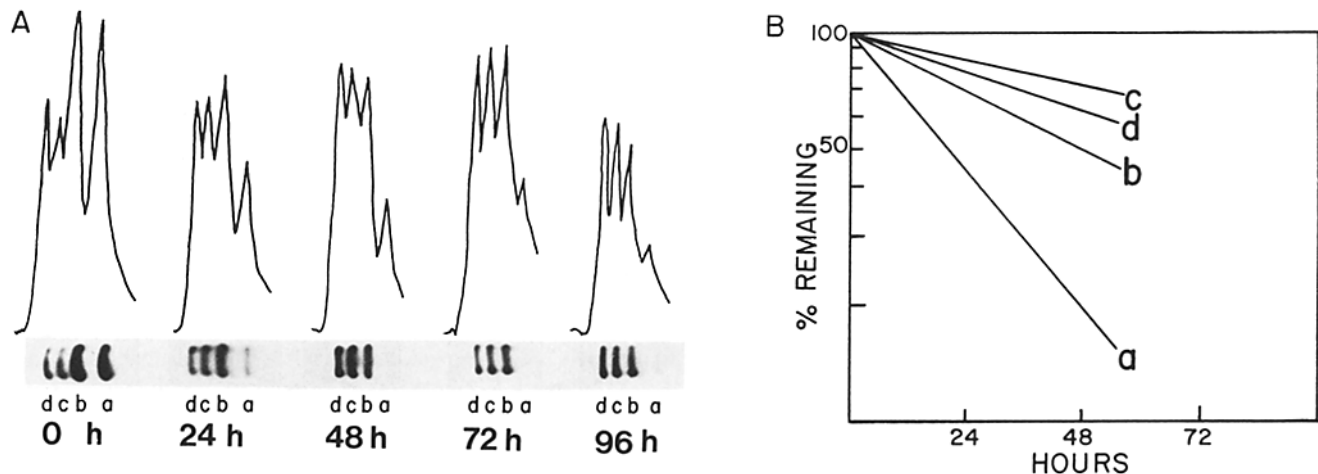

FIGURE 9   Stabilities of the H-1 species. Myoblasts were labeled with [³H]lysine and cells were harvested at increasing times after the radioactive media was removed and replaced with media containing excess unlabeled lysine. A shows different fluorographic exposures of the H-1's resolved on acid-urea-polyacrylamide gels and the corresponding optical density scans. B shows the turnover rates of the individual H-1's calculated from three independent determinations of the relative turnover rates of the individual H-1's and from the turnover rate of total H-1's (see Fig. 6).

ence 54 and unpublished results). As in myogenic cells, H-1 synthesis can continue in the absence of DNA synthesis at confluency or following Ara C treatment. Furthermore, H-1 c synthesis is less tightly coupled to DNA replication than synthesis of H-1's a, b, or d (unpublished results). In addition, a survey of the H-1 composition of different chicken tissues has revealed that those tissues undergoing a low rate of cell division have high relative amounts of H-1 c (55). These results suggest that the selective uncoupling of H-1 c synthesis from DNA replication is not restricted to myogenic and fibroblastic cells, but is a widespread phenomenon responsible for establishing H-1 proportions in many chicken cell types.

Individual H-1 species also accumulate in nondividing cells of other organisms. In mammals, a specific H-1 species, H-1^0, accumulates in nondividing cells. We have shown that the relative amount of an H-1 species tentatively identified as H-1^0 increases substantially during differentiation of the rat E63 myogenic cell line (unpublished results). Synthesis of this species is less tightly coupled to DNA replication than synthesis of the other H-1's in Ara C–treated myoblasts and myotubes (unpublished results). It has also been demonstrated that H-1^0 synthesis is uncoupled from DNA synthesis in hydroxyurea-treated Friend erythroleukemia cells (40), in mouse fibroblasts (28), in mouse neuroblastoma cells (41), and in Chinese hamster ovary cells (42) in tissue culture. In addition, the synthesis of another H-1 variant found in increased proportions in nondividing mouse tissues is also preferentially uncoupled from DNA replication in mouse fibroblasts (28). These results suggest that differential coupling of H-1 species synthesis to DNA replication is a widespread phenomenon involved in establishing tissue-specific H-1 proportions.

Our results support Zweidler's hypothesis that H-1 variants can be separated into replication variants whose expression is tightly coupled to ongoing DNA synthesis, and into replacement variants which are expressed regardless of DNA synthetic activity (39). It meets the prediction of this hypothesis that changes in the rate of DNA synthesis would alter the balance of replacement- and replication-variant histone synthesis and thus alter the histone-variant proportions. Wu and Bonner found that a small percentage of nucleosomal histone synthesis occurs in the absence of DNA replication and is limited to specific subspecies (56); this could reflect a similar mechanism involving the nucleosomal histones.

Recently, Smith et al. completed a survey of different avian tissues and cell types in an attempt to identify H-1^0 (54). Their results suggested that avian cells lack an H-1^0 homologue. The similarity between the regulation of H-1 c synthesis in chickens and H-1^0 synthesis in mammals suggests that they may be functionally homologous.

The biological significance of the selective synthesis of specific H-1 species in nonproliferative cells remains to be determined. It is possible, on one hand, that H-1 c in chickens and H-1^0 in mammals impart a unique structural configuration on chromatin required for its function after withdrawal from the cell cycle. Alternatively, the H-1's in which synthesis is uncoupled from DNA replication may not differ functionally from the other H-1's. H-1 turnover may be necessary for the maintenance or functioning of chromosomes. If so, the continued synthesis of a specific H-1 species may reflect a gene or set of genes which has evolved to carry out this function. An appealing possibility extending from the known participation of the H-1's in organizing nucleosomes into

higher orders of chromatin structure is that H-1 turnover would be involved in allowing higher orders of chromatin structure to unfold in nondividing cells. It has recently been demonstrated that the removal of H-1 is a necessary prerequisite for transcriptional activation of the developmentally regulated Xenopus 5S RNA genes in vitro (57). It is possible that the turnover of H-1 in nondividing cells allows transcriptional factors to interact with specific DNA sequences, thus allowing changes in gene expression to occur in nonproliferating cells. The finding of selective uncoupling of specific H-1 species synthesis from DNA replication, leading to the selective accumulation in nondividing cells, raises a number of interesting possibilities. An understanding of the regulation of H-1-variant synthesis in myogenic cells should provide further insight into these questions. The following paper is an initial step in that direction.

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