

# MYOSIN SYNTHESIS IN EMBRYONIC CHICKEN FIBROBLASTS

RONALD B. YOUNG, WERNER G. BERGEN, and PETER B. BLAUWIEKEL

From the Departments of Food Science and Human Nutrition, Biomechanics, and Animal Husbandry, Michigan State University, East Lansing, Michigan 48824

## ABSTRACT

The rate of constitutive myosin synthesis was measured in cultures of replicating embryonic chicken skin fibroblasts by pulse labeling with [ $^3\text{H}$ ]leucine. These cells synthesized the 200,000-dalton heavy chain of myosin (MHC) at a rate of  $3.2 \times 10^3$  molecules/cell/min. Additionally, an independent estimate of the MHC synthesis rate needed to maintain a constant level of constitutive MHC/cell was calculated from total protein content, percentage MHC, fibroblast doubling time, and MHC half-life. This calculated rate of  $\sim 2.9 \times 10^3$  molecules/cell/min was in close agreement with the measured rate. By comparison, the synthesis rate of myofibrillar MHC in fully activated muscle cell cultures was  $\sim 2.9 \times 10^4$  molecules/nucleus/min.

**KEY WORDS** fibroblasts · constitutive myosin · synthesis rate · muscle cells

The intracellular location, content, and functional properties of the constitutive contractile proteins in nonmuscle cells have been studied widely in recent years. Thin filaments, for example, have been observed ultrastructurally in nonmuscle cells by electron microscopy (19, 20, 21, 25), by antibody staining with antiactin (13) and antitropomyosin (12, 16), and by binding of fluorescently labeled heavy meromyosin to actin filaments (23, 24). Analysis of actin content in nonmuscle cells by a variety of preparative and biochemical techniques is discussed by Pollard and Weihing (21). Less is known about constitutive myosin heavy chain (MHC) metabolism in nonmuscle cells. Constitutive myosin has been estimated to compose  $\sim 0.5$ – $3\%$  of the total protein in a variety of nonmuscle cells (1, 3, 9, 17, 18), and both constitutive MHC and actin in nonmuscle cells are the products of different genes than the myofibrillar myosin and actin found in muscle fibers (2–4, 6–8, 10, 22, 27, 28, 30). The purpose of the present research was to quantitatively meas-

ure the extent of activation of the constitutive MHC gene in replicating fibroblasts relative to that of the myofibrillar MHC gene in muscle cells.

## MATERIALS AND METHODS

### *Preparation of Fibroblast*

#### *Cell Cultures*

Skin that had been removed from the legs and undersides of 12-d embryonic chickens was placed into 10 ml of buffered saline solution (BSS), consisting of 137 mM NaCl, 2.7 mM KCl, 1.0 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgCl}_2$ , 0.15 mM  $\text{NaH}_2\text{PO}_4$ , 6.0 mM  $\text{NaHCO}_3$ , 5.5 mM glucose, pH 7.4. Trypsin was added to a concentration of 0.05%, and the suspension was incubated at  $37^\circ\text{C}$  for 5–10 min with repeated aspiration to aid fibroblast dissociation. After the suspension had become somewhat milky in appearance, the cell suspension was centrifuged at 700 g for 5 min at room temperature, and the cell pellet was resuspended in complete culture medium (Eagle's Minimum Essential Medium plus 5% chicken embryo extract and 10% horse serum). Cells were counted with a hemocytometer and plated at a density of  $\sim 5 \times 10^6$  cells/15-cm diameter Falcon tissue culture dish (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.). Fibroblasts were incubated at  $37^\circ\text{C}$  in a 95% air-5%  $\text{CO}_2$  atmosphere in 15 ml of complete

medium, and medium was replenished every 24 h. When each fibroblast culture became nearly confluent, it was subcultured by trypsinization into three additional 15-cm culture dishes. Approximately 3 d were required for the fibroblasts to approach confluence under these conditions, and all cells had been subcultured three to four times before each experiment. Doubling times ranged from 15 to 19 h, and, as shown in Fig. 1, the total protein content of these cultures closely paralleled the cell number.

## Preparation of Muscle

### Cell Cultures

Muscle cell cultures were isolated from 12-d chick embryos (29, 31) and plated in 15-cm Falcon tissue culture dishes (coated with 0.25 mg of collagen) at an initial cell density of  $2 \times 10^6$ /dish. Under these conditions, myogenic cells began to fuse after ~30–35 h. Cellular fusion increased rapidly for the next 24–36 h such that 70–80% of the nuclei were within myotubes by 70–80 h. Replication of nonmuscle cells was inhibited after 48 h by the presence of  $10^{-7}$  M fluorodeoxyuridine. Muscle cultures were analyzed at 5 d of age.

### Nuclei Counts

Cultures to be counted were rinsed two times with BSS at 37°C, fixed in absolute methanol for 5 min, and stained with Giemsa stain for 20 min at room temperature. At least 1,000 nuclei were counted in randomly chosen fields at a final magnification of 320, and the total number of nuclei per dish was calculated.

### MHC Synthesis Rate

The rate of MHC synthesis in fibroblasts was determined by pulse labeling with 10  $\mu$ Ci [ $^3$ H]leucine/ml in

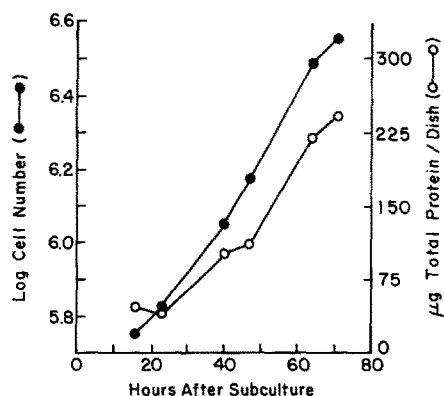


FIGURE 1 Proliferation rate and total protein accumulation rate in replicating fibroblasts. Number of cells and total protein content in 6-cm dishes were determined as described in Materials and Methods. The experiments reported here utilized cells between 40 and 60 h after subculture.

complete culture medium at 37°C for 4 h. At the end of the labeling period, the dishes were rinsed once with cold BSS and twice with cold 0.25 M KCl, 0.02 M Tris, pH 7.4, and the cells were scraped from the surface with a plastic spatula into 1.0 ml of 0.25 M KCl, 0.02 M Tris, pH 7.4. Cells were then homogenized with 20 strokes of a 7-ml Dounce-type glass homogenizer (Wheaton Scientific Div., Wheaton Industries, Millville, N. J.; tightly fitting A pestle), and the homogenate was centrifuged at 12,000 g for 10 min. Approximately 20  $\mu$ g of chicken thigh muscle myosin prepared according to Serayderian et al. (26) was added to the supernate to ensure easy visualization of MHC after electrophoresis. Skeletal muscle myosin was used as carrier in these experiments because of the relative ease with which large quantities can be prepared compared to fibroblast myosin and because skeletal muscle and fibroblast MHC have identical electrophoretic mobilities (Fig. 2D). TCA was added to a final concentration of 10%, and insoluble protein was collected by centrifugation and rinsed two additional times with cold 5% TCA. Residual TCA was then extracted by rinsing three times with ether. The pellet was dissolved in 0.075 ml of 1.0% SDS, 0.05 M Tris-HCl, pH 7.1, 0.5% 2-mercaptoethanol, 20% glycerol, and 0.01% pyronin Y by heating at 100°C for 10 min. Electrophoresis was carried out at 0.5 mA per gel on 10% polyacrylamide gels cross-linked with 0.1% *N,N'*methylene-bisacrylamide in 0.2 M Tris-glycine buffer (pH 8.8) and 0.1% SDS. Gels were stained with 0.1% Coomassie Blue in an  $H_2O$ :methanol:acetic acid mixture (43:50:7 by volume) and destained electrophoretically in a  $H_2O$ :methanol:acetic acid mixture (87.5:5:7.5 by volume). Destained gels were frozen in dry ice, and 0.5-mm slices were taken through the region of the gels containing the 200,000-dalton subunit of myosin. Slices were dissolved in 0.2 ml of 30%  $H_2O_2$  by heating at 50°C for 3 h.

To convert MHC dpm into number of MHC actually synthesized, the specific radioactivity of the [ $^3$ H]leucine used for pulse labeling in the cell culture medium was measured. The assumptions were made that intracellular [ $^3$ H]leucine specific radioactivity equilibrated rapidly with the extracellular pool and that the intracellular and extracellular [ $^3$ H]leucine-specific radioactivities were equal. That these are valid approximations is strongly reinforced by Fig. 3 and the data of McKee et al. (15). To determine [ $^3$ H]leucine specific radioactivity, TCA was added to radioactive cell culture medium to a final concentration of 10%. Samples were placed at 2°C for at least 2 h to permit the insoluble protein to aggregate, and the TCA-insoluble material was removed by low-speed centrifugation. The supernate was extracted three times with 3 vol of ether to remove TCA, HCl was added to 0.01 N, and each sample was loaded onto a small Dowex-50 column (Dow Corning Corp., Midland, Mich.) equilibrated with 0.01 N HCl. Columns were washed with water:methanol (1:1) until all non-amino-acid radioactivity was eluted, and the amino acids were

eluted from the column with 30%  $\text{NH}_4\text{OH}$ . Eluted samples were then dried in a Buchler Rotary Evapo-Mix (Buchler Instruments Div., Searle Diagnostics Inc., Fort Lee, N. J.) and resuspended in 0.20 ml of 0.01 N HCl. One aliquot of this material was analyzed on Ionex-25 SA-Na thin-layer plates (Brinkmann Instruments, Inc., Westbury, N. Y.) to determine  $^3\text{H}$ leucine dpm/ $\mu\text{l}$ . A second aliquot of this mixture was analyzed on a Technicon amino acid analyzer (Technicon Instruments Corp., Tarrytown, N. Y.) to determine nmol leucine/ $\mu\text{l}$ .  $^3\text{H}$ leucine specific radioactivity (dpm/nmol) was then calculated. MHC dpm were converted to number of MHC by assuming that each MHC contains 160 leucine residues (11), a value that is quite constant in divergent types of myosin.

### *MHC Half-Life*

Proliferating fibroblasts were incubated in the presence of complete culture medium containing 1  $\mu\text{Ci}$   $^3\text{H}$ leucine/ml for 16 h to ensure complete prelabeling of protein with  $^3\text{H}$ leucine. Cultures were then grown in complete medium without  $^3\text{H}$ leucine for the remainder of the chase experiment. At the end of the labeling period and at 12-h intervals thereafter for 36 h, duplicate 10-cm plates were rinsed once each with BSS and isolation buffer (0.25 M KCl, 0.02 M Tris-HCl, pH 7.4) to remove residual radioactivity. Cells were then scraped, homogenized, and prepared for electrophoretic analysis of radioactivity in MHC as described under "MHC Synthesis Rate." To standardize and summarize data from separate experiments, the radioactivity found in MHC at each of the times indicated was calculated as a percentage of the initial value, and the logarithm of these values was plotted as a function of time after initiation of the chase period. The amount of time necessary for the radioactivity to decrease to 50% of its initial value was used as the half-life of MHC.

### *Purification of Fibroblast Myosin*

Myosin was purified from fibroblasts by a procedure similar to that of Ostlund and Pastan (18). Rapidly dividing fibroblasts that had been pre-labeled with 1  $\mu\text{Ci}$   $^3\text{H}$ leucine/ml for 16 h were rinsed three times with cold BSS and scraped into the residual BSS adhering to the dishes. These cells were collected by centrifugation at 1,000 g for 1 min and stored at  $-20^\circ\text{C}$  for up to 3 mo. Cells containing 50–100 mg of protein were thawed, the cell suspension was made 0.6 M KCl, 2 mM dithiothreitol (DTT), and 15 mM Tris-HCl, pH 7.2, and cells were homogenized 20 times with a Dounce homogenizer. This cell homogenate was made 5 mM in ATP and  $\text{MgCl}_2$  and then centrifuged at 150,000 g for 2 h. The supernate was then chromatographed over a  $0.9 \times 54$  cm column of Bio-Gel A-15m (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 0.6 M KCl, 2 mM DTT, 0.5 mM ATP, 0.5 mM  $\text{MgCl}_2$  and 15 mM Tris-HCl, pH 7.2. Myosin was located by measuring the

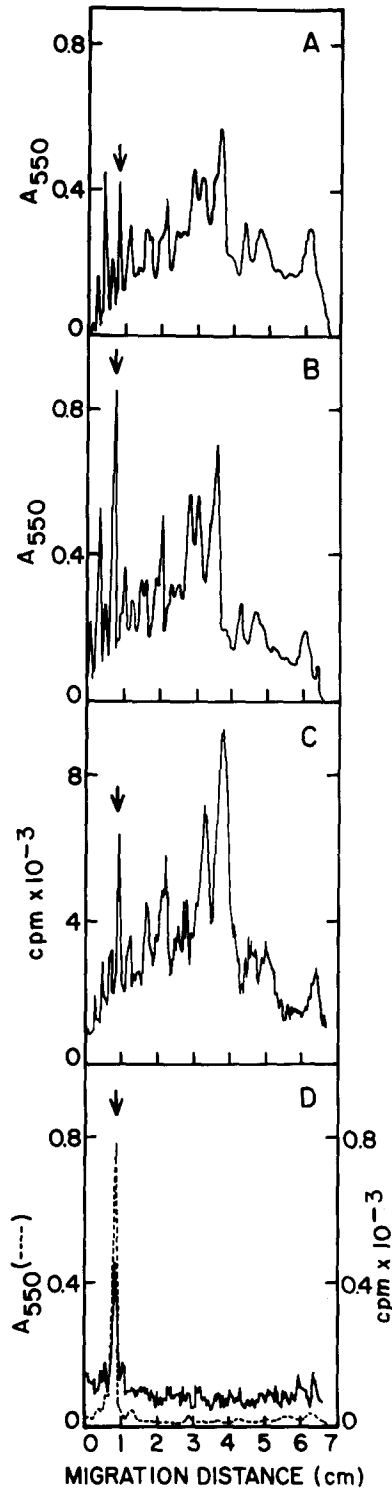
ATPase activity in an aliquot of each 2-ml fraction. ATPase assays were as described elsewhere (1) in 0.6 M KCl, 2 mM ATP, 2 mM EDTA, 2 mM DTT, and 15 mM Tris-HCl, pH 7.2. The fractions exhibiting ATPase activity were pooled, and solid  $(\text{NH}_4)_2\text{SO}_4$  was added to 60%. The protein was collected by centrifugation at 15,000 g for 15 min, dissolved in 0.25 M NaCl, 15 mM Tris-HCl, pH 7.2, and dialyzed for 24 h at  $2^\circ\text{C}$  against two changes of the same buffer. Aliquots of this protein solution were mixed with an equal volume of 1.0% SDS, 0.5% 2-mercaptoethanol, 20% glycerol, 0.01% pyronin Y, and 0.05 M Tris-HCl, pH 7.1, and electrophoresis was carried out as already described. The fractions exhibiting ATPase activity contained a high proportion of MHC (c.f. Fig. 2D), and those fractions devoid of ATPase activity did not contain detectable quantities of 200,000-dalton polypeptide.

### *Measurement of Total Protein and MHC Content of Fibroblasts*

Fibroblast cultures were rinsed four times with BSS at  $2^\circ\text{C}$  to remove residual protein adhering to the cells from the culture medium. The cells were then scraped from the surface of the culture dish with a plastic spatula into 1.0 ml of 0.25 M KCl, 0.01 M  $\text{MgCl}_2$ , 0.01 M Tris, pH 7.4, and homogenized 20 times with a Dounce homogenizer. Duplicate dishes were simultaneously stained with Giemsa stain so that the number of cells could also be determined. Aliquots of the cell homogenates were removed for total protein analysis by the method of Lowry et al. (14). The remainder of the cell homogenate was then centrifuged at 12,000 g for 15 min to sediment nuclei, mitochondria, and other cellular debris. Aliquots of the 12,000 g supernate were then electrophoresed on SDS-polyacrylamide gels as already described. This procedure results in >90% recovery of MHC from fibroblasts as evidenced by additional experiments in which the 12,000 g pellet was re-extracted with the same buffer containing 0.6 M NaCl. The results reported in Table I are based on experiments in which only one extraction was carried out as described above. The percentage of MHC in the 12,000 g supernate was estimated from gel scans at 550 nm of the Coomassie Blue-stained gels. Gels were scanned in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a Gilford gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a Hewlett-Packard 3380S integrator (Hewlett-Packard Co., Palo Alto, Calif.). The percentage of MHC was then calculated, and this percentage of MHC was multiplied by the total protein content/cell to obtain MHC content/cell.

## RESULTS

The resolution of MHC in gel scans of fibroblast cell extracts is shown in Fig. 2A. When skeletal muscle myosin was added to these extracts to aid in positive MHC identification, the height of only



one of the peaks was significantly enhanced (Fig. 2B). [<sup>3</sup>H]leucine incorporated into MHC during pulse labeling can be resolved by the gel slicing procedure described in Materials and Methods from bands of both slightly higher and lower molecular weights (Fig. 2C), and the fact that skeletal muscle MHC and fibroblast MHC have identical electrophoretic mobilities is shown in Fig. 2D.

In order for kinetics of [<sup>3</sup>H]leucine incorporation into MHC to accurately reflect MHC synthesis, equilibration of extracellular [<sup>3</sup>H]leucine with the intracellular leucine pool must occur rapidly. A manifestation of constant, maximum intracellular specific radioactivity would be a linear rate of [<sup>3</sup>H]leucine incorporation into MHC during a pulse label. Fibroblast and muscle cultures were therefore labeled for 5, 10, 20, 30, 60, 120, 180, and 240 min with 10 µCi/ml [<sup>3</sup>H]leucine. MHCs were isolated on SDS-polyacrylamide gels, and [<sup>3</sup>H]leucine incorporation was examined as a function of time (Fig. 3). Linear extrapolation of [<sup>3</sup>H]leucine incorporation to 0 dpm illustrates that equilibration of extracellular [<sup>3</sup>H]leucine with the intracellular pool must have been nearly instantaneous. Thus, direct comparison of constant-length pulse-label data is a valid method for comparing rates of MHC synthesis. Only the first 60 min of the experiments are shown in Fig. 3, so the behavior in the first 30 min is more readily observed; incorporation was linear for the remainder of the 4-h pulse.

The constitutive myosin found in nonmuscle cells, such as fibroblasts, and the myofibrillar myosin found in muscle fibers are the products of

FIGURE 2 Distribution of total fibroblast protein and MHC bands on SDS-polyacrylamide gels. The small arrow in each panel indicates the band identified as MHC. (A) Scan at 550 nm of a Coomassie Blue-stained SDS-polyacrylamide gel containing fibroblast cytoplasmic protein. (B) A duplicate sample similar to that shown in panel A, except that ~10 µg of skeletal muscle myosin was added before electrophoresis. (C) The distribution of radioactivity in 0.5-mm gel slices taken throughout the length of the gel shown in panel B. (D) Coelectrophoresis of skeletal muscle MHC and fibroblast MHC. The dashed line is the A<sub>550</sub> tracing of ~10 µg of chicken thigh muscle myosin, and the solid line represents the radioactivity in [<sup>3</sup>H]leucine-labeled fibroblast myosin prepared as detailed in Materials and Methods.

different structural genes (2, 3, 8, 22). Consequently, the magnitude of expression of these two distinct gene products was measured in their respective cell types (Table I). Proliferating fibroblasts synthesize  $\sim 3.2 \times 10^3$  constitutive MHC/cell/min, whereas fully activated multinucleated muscle fibers synthesize  $\sim 2.9 \times 10^4$  myofibrillar MHC/nucleus/min. These latter data from embryonic chicken muscle cultures are essentially iden-

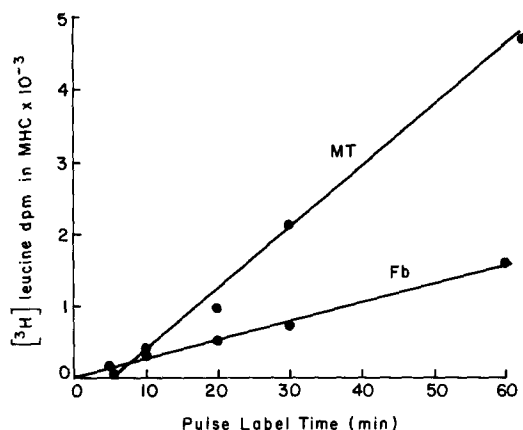


FIGURE 3 Initiation of linear MHC synthesis in proliferating fibroblasts (*Fb*) and 5-d muscle cultures (*MT*). Cells were labeled with  $10 \mu\text{Ci}$   $[^3\text{H}]$ leucine/ml for the times indicated, and incorporation into MHC was measured as outlined in Materials and Methods. The amount of time required for initiation of linear synthesis of  $[^3\text{H}]$ leucine-labeled MHC is very short, implying that the intracellular  $[^3\text{H}]$ leucine specific radioactivity rapidly reaches a constant, maximum level. The linear regression equations are: Fibroblasts,  $y = 26.1x + 22.2$  ( $x = -0.85$  min when  $y = 0$ ); muscle cells,  $y = 85.3x - 467$  ( $x = 5.47$  min when  $y = 0$ ).

tical to one other report in which embryonic Japanese quail muscle cultures were used (5).

The constitutive MHC synthesis rate in fibroblasts, which was  $\sim 10\%$  of the maximum myofibrillar MHC synthesis rate by myotubes (Table I), was perhaps higher than anticipated based on the difference in the ability of these two cell types to accumulate their respective cell-specific myosin. Therefore, an attempt was made, using independently obtained data, to calculate the MHC synthesis rate of replicating fibroblasts. In a simplified view of fibroblast MHC metabolism, only two basic needs must be met in order for MHC content to remain constant. First, there is continuous degradation of all proteins in cells; consequently, fibroblasts need to synthesize enough MHC to replace those molecules destroyed by proteolysis. Secondly, fibroblasts are continuously replicating, and the remaining MHC synthesis must keep MHC content/cell constant during the dilution caused by replication. The doubling time (or generation time) of fibroblasts was  $\sim 18$  h, hence the quantity of MHC in each culture must likewise double every 18 h in order for the average MHC content/cell to remain constant. Table II shows that  $\sim 2.29 \times 10^3$  MHC must be synthesized in each cell per minute to offset the dilution in MHC caused by cell replication. Additionally, the MHC synthesis rate needed to replace those molecules degraded by proteolysis can be calculated. The equation that describes changes in MHC content can be expressed as:

$$dc/dt = T - CD,$$

where  $dc/dt$  is the rate of change in the average

TABLE I  
MHC Synthesis Rate in Proliferating Fibroblasts and Multinucleated Myotubes\*

Cell type	No. of nuclei/culture ( $\times 10^6$ )	$[^3\text{H}]$ leucine incorporated into MHC $\ddagger$ (dpm $\times 10^4$ )	No. of MHC molecules synthesized $\S$ ( $\times 10^3$ )	No. of MHC molecules synthesized/nucleus/min $^{\parallel}$ ( $\times 10^3$ )
Proliferating fibroblasts	$2.08 \pm 0.2$	$2.10 \pm 0.2$	1.58	3.17
5-d muscle cultures	$10.10 \pm 0.9$	$28.08 \pm 0.07$	70.4	29.0

\* Mean  $\pm$  SEM of six experiments for fibroblasts and five experiments for muscle cultures. Each experiment consisted of quadruplicate 15-cm culture dishes.

$\ddagger$   $[^3\text{H}]$ leucine dpm incorporated into MHC during a 4-h pulse label. Counting efficiency was 30–40% and was calculated for each gel slice using an external standard.

$\S$  No. of MHC molecules synthesized during a 4-h pulse label. This value was calculated using a mol wt of  $2.0 \times 10^6$  daltons for MHC, 160 leucine residues/MHC and a  $[^3\text{H}]$ leucine specific radioactivity of  $5 \times 10^4$  dpm/nmol for fibroblast pulse labels and  $1.5 \times 10^4$  dpm/nmol for muscle cell pulse labels.

$^{\parallel}$  Calculated by dividing the data in column three by 240 min (amount of time elapsed during the pulse label) and by the data in column one. In the 5-d muscle cultures, the  $\sim 20\%$  mononucleated cells were assumed to contribute negligible amounts of MHC and were excluded from the calculation.

TABLE II  
*Estimation of Fibroblast MHC Synthesis Rate Required to Maintain a Constant MHC Level*

Total protein per cell*	MHC per cell‡	No. of MHC per cell§	Doubling time <sup>¶</sup>	MHC half-life <sup>¶¶</sup>	MHC synthesis requirement to offset dilution during replication**	MHC synthesis requirement to offset degradation‡‡	Total MHC synthesis requirement§§
pg	pg		h	h	MHC/cell/min	MHC/cell/min	MHC/cell/min
76 ± 3	0.82	2.47 × 10 <sup>6</sup>	18	51	2.29 × 10 <sup>3</sup>	0.56 × 10 <sup>3</sup>	2.85 × 10 <sup>3</sup>

\* Calculated from Fig. 1.

‡ Calculated by assuming that 1.08% of fibroblast total protein is MHC (determined as described in Materials and Methods).

§ Calculated by assuming MHC mol wt is 2 × 10<sup>5</sup>.

¶ Calculated from Fig. 1.

¶¶ Calculated from Fig. 4.

$$** \text{ Replication MHC/cell/min} = \frac{2.47 \times 10^6 \text{ MHC}}{\text{cell}} \times \frac{1}{18 \text{ h}} \times \frac{1 \text{ h}}{60 \text{ min}} = 2.29 \times 10^3.$$

$$‡‡ \text{ Degradation MHC/cell/min} = \frac{2.47 \times 10^6 \text{ MHC}}{\text{cell}} \times \frac{\ln 2}{51 \text{ h}} \times \frac{1 \text{ h}}{60 \text{ min}} = 5.60 \times 10^2.$$

§§ Calculated as the sum of MHC synthesis needed to offset the dilution caused by replication (column 6) and MHC synthesis needed to offset the loss caused by degradation (column 7).

number of MHC/cell as a function of time,  $T$  is the MHC translation rate in molecules/cell/min,  $C$  is the MHC concentration in molecules/cell, and  $D$  is the first-order MHC degradation constant. Since the assumption has been made that MHC/cell is constant,  $dc/dt = 0$  and, therefore,  $T = CD$ . Furthermore,  $D$  is related to the half-life of MHC (in minutes) by the relationship,  $D = \ln 2/t^{1/2}$ , and the following equation describes the MHC translation rate needed to meet the demands caused by MHC turnover:

$$T = \frac{C \cdot \ln 2}{t^{1/2}}.$$

The measured MHC half-life was found to be ~51 h (Fig. 4), in reasonable agreement with the fibroblast MHC half-life of ~2.7 days previously reported by Rubinstein et al. (22). Thus, the rate of MHC synthesis required to offset degradation was calculated to be 560 MHC/cell/min (Table II). The sum of these independently calculated synthesis requirements (Table II) is similar to the experimentally measured synthesis rate shown in Table I.

#### DISCUSSION

Synthesis of ~2.9–3.2 × 10<sup>3</sup> constitutive MHC/cell/min by fibroblasts is apparently needed in order for each cell to maintain a constant myosin level. This range was established by two independent methods. By comparison, the synthesis rate of myofibrillar MHC (which is the product of a

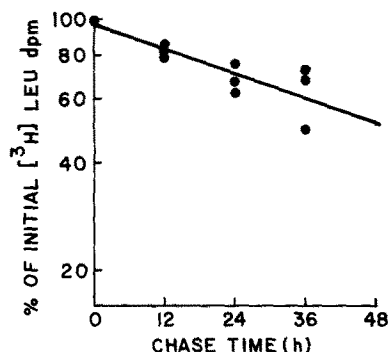


FIGURE 4 Half-life of MHC in replicating fibroblasts as determined by following the rate of disappearance of [<sup>3</sup>H]leucine. The quantity of radioactivity in MHC was measured at each time point, and the data from each experiment were normalized as detailed in Materials and Methods. Each point is the mean of duplicate measurements, and the line best fitting these data corresponds to a half-life of 51 h.

different gene than fibroblast constitutive myosin) in muscle cultures is ~2.9 × 10<sup>4</sup> MHC/nucleus/min. Understanding the mechanism regulating the differential expression of these two MHC genes so that one of them directs production of 10-fold more protein than the other is a key question in contractile protein metabolism. The fibroblasts used in the present experiments were undergoing rapid replication. If the constitutive contractile proteins are indeed involved in a variety of cellular processes (including cell cleavage, maintenance of shape, chromosomal movements, endo- and exo-

cytosis, and pseudopodia formation), rapidly proliferating fibroblasts would be expected to require substantial quantities of myosin.

In these experiments, it was assumed that all of the material migrating with the mol wt of 200,000 daltons during SDS-polyacrylamide gel electrophoresis was MHC. The pulse-labeling experiments and MHC half-life measurements were analyzed by this criterion. There are several large peptides in cells, and it was possible for some of these proteins to have molecular weights similar to that of MHC. The resolution of electrophoretic analysis used in this work (Fig. 2) was sufficient to resolve closely migrating bands, and no evidence of band broadening or double peaks was ever observed. Additionally, as mentioned in Materials and Methods, myosin was localized during gel filtration chromatography by measurement of its ATPase activity. The fractions not exhibiting ATPase activity were also essentially devoid of a 200,000-dalton peptide when the protein in these fractions was analyzed by SDS-polyacrylamide gel electrophoresis. Another argument against contamination of MHC from other peptides can be made from the data of Ostlund and Pastan (18). Two plasmacytoma cell lines contained no protein band with mol wt of 200,000 daltons and no K<sup>+</sup>-activated ATPase activity. This, therefore, is one case where the absence of a 200,000-dalton peptide is accompanied by the absence of any detectable myosin ATPase activity. Clearly, this is a weak argument; however, these results collectively suggest that contamination of MHC with other peptides is not a problem.

When the total MHC synthetic needs in fibroblasts were broken down into the two components of turnover and replication (Table II), an interesting fact is observed. Approximately 80% of the total MHC synthesis by these logarithmically growing cells is needed simply to prevent dilution during replication. The remaining 20% is necessary to replace MHC destroyed by turnover. In view of these results, it would be interesting to examine regulation of MHC synthesis in confluent cultures where cell number is constant and where synthesis and degradation are presumably identical. Because cell number would be constant, and because MHC requirements would therefore be diminished, MHC content in confluent cells could be modulated either by a drastically diminished synthesis rate, by accelerated turnover in the presence of a constant synthesis rate, or most likely by a combination of these factors. In this

regard, it is interesting to note that Rubinstein et al. (22) have shown that myofibrillar myosin and actin synthesis and degradation are apparently highly coordinated in muscle cells, and it seems reasonable that constitutive myosin and actin metabolism would likewise be closely coordinated. In fact, constitutive myosin prelabeled in replicating precursor myogenic cells (which are nonmuscle cells for all practical purposes) can be chased into differentiated myotubes, but this constitutive myosin continues to be degraded at approximately the same rate as in the precursor cells (22). Additionally, this constitutive myosin was degraded faster than the myofibrillar myosin elaborated in the newly formed multinucleated myotubes, further attesting to the basic biochemical differences between constitutive and myofibrillar contractile proteins and their mechanisms of regulation.

In conclusion, the rate of constitutive MHC synthesis in replicating embryonic chicken skin fibroblasts is  $\sim 2.9\text{--}3.2 \times 10^3$  MHC/cell/min. This synthesis rate seems sufficiently high to meet the contractile needs of logarithmically proliferating fibroblasts, but it should be re-emphasized that these measurements were made under a very specific set of conditions for a specific cell type. Individual cell types likely modulate MHC synthesis in response to the unique set of conditions with which they are faced.

The authors gratefully acknowledge the assistance of Anne Tuomari and Dianne Young.

Michigan Agricultural Experiment Station Article No. 8120. Research for this work was supported by the Muscular Dystrophy Association, the Michigan Agricultural Experiment Station Project Nos. 1241 and 1265, and Biomedical Research Support Grants from the College of Agriculture and Natural Resources and the College of Osteopathic Medicine.

Received for publication 10 June 1977, and in revised form 29 November 1978.

## REFERENCES

1. ADELSTEIN, R. S., M. A. CONTI, G. S. JOHNSON, I. PASTAN, and T. D. POLLARD. 1972. Isolation and characterization of myosin from cloned mouse fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3693-3697.
2. BURRIDGE, K., and D. BRAY. 1975. Purification and structural analysis of myosins from brain and other non-muscle tissues. *J. Mol. Biol.* **99**: 1-14.
3. CHI, J. C. H., N. RUBINSTEIN, K. STRAHS, and H. HOLTZER. 1975. Synthesis of myosin heavy and light chains in muscle cultures. *J. Cell Biol.* **67**:523-537.
4. COHEN, R., M. PACIFICI, N. RUBINSTEIN, J. BIEHL, and H. HOLTZER. 1977. Effect of a tumour promoter on myogenesis. *Nature (Lond.)*. **266**:538-540.
5. EMERSON, C. P., and S. K. BECKNER. 1975. Activation of myosin

- synthesis in fusing and mononucleated myoblasts. *J. Mol. Biol.* **93**: 431-447.
6. FELLINI, S. A., and H. HOLTZER. 1976. The localization of skeletal light meromyosin in cells of myogenic cultures. *Differentiation*. **6**:71-74.
  7. GRUENSTEIN, E., and A. RICH. 1975. Non-identity of muscle and non-muscle actins. *Biochem. Biophys. Res. Commun.* **64**:472-477.
  8. HOLTZER, H. 1970. Myogenesis. In *Cell Differentiation*. O. Schjeide and J. Je Willis, editors. Van Nostrand Reinhold Co., New York. 476-503.
  9. JOHN, H. A., M. PATRINO-GEORGIOULAS, and K. W. JONES. 1977. Detection of myosin heavy chain mRNA during myogenesis in tissue culture by *in vitro* and *in situ* hybridization. *Cell*. **12**:501-508.
  10. HOLTZER, H., J. M. MARSHALL, and H. FINCK. 1957. An analysis of myogenesis by the use of fluorescent antimyosin. *J. Cell Biol.* **3**:705-723.
  11. KIRSHENBAUM, D. M. 1977. A compilation of amino acid analyses of proteins. XI. Residues per thousand residues. *Anal. Biochem.* **79**:470-501.
  12. LAZARIDES, E. 1975. Tropomyosin antibody: The specific localization of tropomyosin in non-muscle cells. *J. Cell Biol.* **65**:549-561.
  13. LAZARIDES, E., and K. WEBER. 1974. Actin antibody: The specific visualization of actin filaments in non-muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2268-2272.
  14. LOWRY, O., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
  15. MCKEE, E. E., D. E. RANNELS, and H. E. MORGAN. 1977. Compartmentation of the intracellular pool of free phenylalanine and the specific activity of phe-tRNA in perfused rat heart. *Fed. Proc.* **36**:1980.
  16. MASAKI, T. 1975. Tropomyosin-like protein in chick embryo fibroblasts. *J. Biochem.* **77**:901-904.
  17. OSTLUND, R. E., and I. PASTAN. 1974. Myosin in cultured fibroblasts. *J. Biol. Chem.* **249**:3903-3907.
  18. OSTLUND, R. E., and I. PASTAN. 1976. The purification and quantitation of myosin from cultured cells. *Biochim. Biophys. Acta.* **453**:37-47.
  19. PERRY, M. M., H. A. JOHN, and N. S. T. THOMAS. 1971. Actin-like filaments in the cleavage furrow of the newt egg. *Exp. Cell Res.* **65**: 249-253.
  20. POLLARD, T. E., and S. ITO. 1970. Cytoplasmic filaments of *Amoeba proteus*. I. The role of filaments in consistency changes and movement. *J. Cell Biol.* **46**:267-289.
  21. POLLARD, T. D., and R. WEIHING. 1974. Actin and myosin and cell movement. *CRC Crit. Rev. Biochem.* **2**:1-65.
  22. RUBINSTEIN, N., J. CHI, and H. HOLTZER. 1976. Coordinated synthesis and degradation of actin and myosin in a variety of myogenic and non-myogenic cells. *Exp. Cell Res.* **97**:387-393.
  23. SANGER, J. W. 1975. Changing patterns of actin localization during cell division. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1913-1916.
  24. SANGER, J. W. 1975. Intracellular localization of actin with fluorescently labeled heavy meromyosin. *Cell Tiss. Res.* **161**:431-444.
  25. SCHROEDER, T. E. 1973. Actin in dividing cells: Contractile ring filaments bind heavy meromyosin. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1688-1692.
  26. SERAYDERIAN, K., E. J. BRISKEY, and W. F. H. M. MOMMAERTS. 1967. The modification of actomyosin by  $\alpha$ -actinin. I. A survey of experimental conditions. *Biochim. Biophys. Acta.* **133**:399-411.
  27. STORIT, R. V., D. M. COEN, and A. RICH. 1976. Tissue-specific forms of actin in the developing chick. *Cell*. **8**:521-527.
  28. STORIT, R. V., and A. RICH. 1976. Chick cytoplasmic actin and muscle actin have different structural genes. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2346-2350.
  29. TEPPERMAN, K., G. MORRIS, F. ESSIEU, and S. M. HEYWOOD. 1975. A mechanical dissociation method for preparation of muscle cell cultures. *J. Cell Physiol.* **86**:561-566.
  30. WHALEN, R. G., G. S. BUTLER-BROWNE, and F. GROS. 1976. Protein synthesis and actin heterogeneity in calf muscle cells in culture. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 2018-2022.
  31. YOUNG, R. B., D. E. GOLL, and M. H. STROMER. 1975. Isolation of myosin-synthesizing polysomes from cultures of embryonic chicken myoblasts before fusion. *Dev. Biol.* **47**:123-135.