

# Synthesis of Rat Myosin Light Chains in Heterokaryons Formed between Undifferentiated Rat Myoblasts and Chick Skeletal Myocytes

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**ABSTRACT** The control of gene expression during terminal myogenesis was explored in heterokaryons between differentiated and undifferentiated myogenic cells by analyzing the formation of species specific myosin light chains of chick and rat skeletal muscle. Dividing L6 rat myoblasts served as the biochemically undifferentiated parent. The differentiated parental cells were mononucleated muscle cells (myocytes) that were obtained from primary cultures of embryonic chick thigh muscle by blocking myotube formation with EGTA and later incubating the postmitotic cells in cytochalasin B. Heterokaryons were isolated by the selective rescue of fusion products between cells previously treated with lethal doses of different cell poisons. 95–99% pure populations of heterokaryons formed between undifferentiated rat myoblasts and differentiated chick myocytes were obtained. The cells were labeled with [<sup>35</sup>S]methionine, and whole cell extracts were analyzed on two-dimensional polyacrylamide gels. These heterokaryons synthesize the light chain of chick myosin and both embryonic and adult light chains of rat skeletal myosin. Control homokaryons formed by fusing undifferentiated cells to themselves did not synthesize skeletal myosin light chains. Control heterokaryons formed between undifferentiated rat myoblasts and chick fibroblasts also failed to synthesize myosin light chains. These results indicate that differentiated chick muscle cells provide some factor that induces L6 myoblasts to synthesize rat myosin light chains. This system provides a model for investigating the processes by which differentiated cell functions are induced.

Cells expressing different differentiated programs mutually suppress specialized functions when hybridized (1). This observation suggests that during the developmental program cells may produce molecules that inhibit alternate pathways. Previous experiments have not addressed the issue of whether differentiated cells contain molecules that can induce differentiation in cells from earlier stages of the same developmental pathway. The behavior of heterokaryons formed by fusing a differentiated cell to its undifferentiated precursor would resolve this question. We describe here the application of this approach to the study of the control of skeletal myosin light chain synthesis during terminal differentiation.

Skeletal myosin has a molecular weight of ~450,000 and is composed of two 200,000 mol wt heavy chains associated with several different light chains ranging in molecular weight from 15,000 to 30,000 (2). Although both fast and slow light chains

can be expressed in cultured muscle cells (3), the principal forms synthesized in short-term cultures are the fast light chains. Because the fast light chain 1 (LC1) is the most prominent form on two-dimensional polyacrylamide gels, LC1 was selected as the protein marker for the expression of differentiated functions. Chick and rat myoblasts were chosen for the present experiments because the skeletal myosin light chains specific to each species can be resolved on two-dimensional polyacrylamide gels, permitting the identification of differentiated markers from each parental genome.

The established line of L6 rat myoblasts (4) was chosen to be the undifferentiated parent because of the ease of maintaining dividing cultures in a biochemically undifferentiated state. These cells are determined for myogenesis, and are capable of differentiating if they are permitted to reach confluency. Differentiated L6 cells synthesize only the embryonic form of

skeletal myosin LC1 (5–7). Embryonic chick skeletal muscle cells served as the differentiated parent. Cultures of freshly isolated chick embryonic myoblasts are morphologically and biochemically undifferentiated. After dividing several times they become postmitotic and spontaneously fuse to form multinucleated myotubes. Myotube formation itself is not a prerequisite for the expression of differentiated functions because muscle-specific products are synthesized in mononucleated cells that have been prevented from fusing by low calcium concentration (8–11), cytochalasin B (12), or other agents (13, 14).

Mononucleated cells were used to avoid gene dosage effects that could occur if multinucleated myotubes were fused to undifferentiated mononucleated myoblasts. Mononucleated differentiated muscle cells are referred to as myocytes to distinguish them from both multinucleated myotubes and undifferentiated myoblasts.

Classic hybridization approaches in which fused cells must divide and produce hybrid colonies were inappropriate for these experiments. Because the difference between these parental cells is only one of several cell divisions, the expression of differentiated functions in hybrid clones isolated many cell generations after fusion would probably not reflect the initial molecular interactions. Furthermore, because differentiated myocytes are postmitotic, restricting the analysis to only those clones capable of cell division would clearly bias results against the expression of the differentiated functions of the postmitotic cells. We thus performed our analysis on heterokaryons, the initial fusion products before cell division has begun. The results of these experiments demonstrate that undifferentiated rat myoblasts are stimulated to synthesize both embryonic and adult rat skeletal myosin light chains when fused to chick myocytes. This suggests but does not fully establish the presence of positive inductive regulatory molecules in the terminally differentiated chick cells that govern the expression of this protein.

## MATERIALS AND METHODS

### Cell Lines

The established line of L6 rat myoblasts (4) was cultivated in medium composed of four parts Dulbecco's modified Eagle's medium to one part medium 199 supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Although this line will grow in lower concentrations of fetal bovine serum or horse serum, high concentrations of FBS were chosen to ensure that the cells remained in an undifferentiated rapidly proliferating state. Cells were subcultivated three times weekly before confluency was reached. The subclone of L6 is the same as the one used in the studies of Whalen et al (5, 6). It is near diploid and has a modal number of 41 chromosomes (15).

### Primary Embryonic Chick Cultures

Skeletal myoblasts were obtained from the thigh muscle of 12-d embryonic chickens. Thigh muscles were dissected, minced, then aspirated vigorously in serum-free medium. Debris was removed by serial filtration through cotton gauze and lens paper. Cell number was determined in a Coulter counter (Coulter Electronics Inc., Lehigh Valley, Pa.), with typical yields of  $15\text{--}20 \times 10^6$  cells per embryonic thigh. Comparison hemocytometer counts with trypan blue indicated that ~50% of the particles (cells plus debris) detected with the Coulter counter represented viable cells. Myoblasts were cultivated in 15-cm plastic petri dishes (Integrid dishes; Falcon Plastics; Div. Becton, Dickinson & Co., Oxnard, Calif.) that had been coated by pretreatment with a 1 percent pigskin gelatin solution (Sigma Chemical Co., St. Louis, Mo.). Cells were preplated at  $9 \times 10^7$  cells/dish for twenty min to remove contaminating fibroblasts, and the myoblasts remaining in solution in each dish were distributed among four additional dishes. Cultures were maintained in medium consisting of four parts Dulbecco's modified Eagle's medium to one part medium 199, 5% horse serum, 1% chick embryo extract, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. 1.75 mM EGTA was added 24 h

later to prevent myotube formation, and  $10^{-5}$  M cytosine arabinoside was added the following day to further reduce fibroblast contamination (16).

Myosin synthesis was considerably reduced in our EGTA-blocked cultures compared to normal differentiated cultures, as has been reported by other laboratories (8, 17). In addition, many myocytes detached from the dish after several days treatment with EGTA. For these reasons, the medium was changed on the 3rd of culture to one containing 2  $\mu$ g/ml cytochalasin B as the agent preventing spontaneous fusion to form myotubes (12). Cells were initially blocked with EGTA because many myoblasts were still dividing at the time the first cells began to differentiate. Adding cytochalasin B at that time would have resulted in many myocytes becoming binucleate, because of the inhibition of cytokinesis by cytochalasin B (18). Myosin light chain synthesis approached control levels under this regimen. Chick myocytes were used to form heterokaryons after 6–8 d in culture. Cells were fed fresh medium the day before an experiment. After the culture had been trypsinized, the cells were subjected to an additional 30-min differential adhesion in the absence of cytochalasin. Because the rounded, cytochalasin B-treated myocytes reattach very slowly compared to fibroblasts, this final step resulted in a substantial enrichment for differentiated myocytes.

### Biochemical Selection for Heterokaryons

The general procedure we have developed for the biochemical selection of heterokaryons has been described in detail elsewhere (19). The specific values for doses and cell number given below are representative, but were adjusted according to the needs of the individual experiment. Cells were treated for 30 min at 0°C in Hanks' balanced salt solution (BSS) containing either iodoacetamide or diethylpyrocarbonate (Sigma Chemical Co.). Typical doses of the irreversible inhibitors used to kill parental cells were 4.5 mM iodoacetamide or 0.004% diethylpyrocarbonate for chick myocytes and 5.5 mM iodoacetamide or 0.0055% diethylpyrocarbonate for L6 rat myoblasts. Because the aqueous half-life of diethylpyrocarbonate is on the order of 15 min at 0°C, all stock dilutions of diethylpyrocarbonate were made in absolute ethanol. The final dilution into Hanks' BSS coincided with the start of the treatment time. After the 30-min treatment, cells were pelleted and washed.  $10^7$  cells that had been treated with iodoacetamide were mixed with  $10^7$  diethylpyrocarbonate-treated cells, the mixture was pelleted, the supernate discarded, and then the cells were centrifuged a final time to remove traces of saline from the walls of the test tube. The pellet was then aspirated to dryness and resuspended in polyethylene glycol 1000 (PEG) (20), which was diluted with medium containing 15% dimethylsulfoxide (21). Under these conditions, 35% PEG (vol/vol) fused 10–15% of the cells with essentially no toxic effects. The pellet of  $2 \times 10^7$  cells was resuspended in 0.2 ml of 35% PEG for 45 s, then diluted with 1 ml of complete medium. The cells were then plated at  $2 \times 10^5$  cells/cm<sup>2</sup> in 15% FBS. Cells were plated in high serum to prevent heterokaryons or control cells from differentiating as a response to culture conditions rather than to the interactions occurring as a result of cytoplasmic mixing.

The following protocol was adopted to reduce the toxicity of dying parental cells. The cells were treated and fused on day 0 and initially plated at low density in bacteriological-grade petri dishes. Heterokaryons take 24–36 h to recover from the trauma of biochemical selection before they are able to reattach. Bacterial dishes were used to ensure that the cells remained in suspension during the 24–48 h before final plating (see below). On day 1 aliquots of the floating cells were transferred to tissue-culture microwells, then fixed, stained and counted the next day (day 2). Experiments in which >95% of the nuclei were in heterokaryons were then processed as follows. Based upon the specific rescue obtained, the surface area necessary to give a surviving heterokaryon density of 60,000 cells/cm<sup>2</sup> was calculated. The remaining cells were then centrifuged onto a Ficoll-sodium diatrizoate cushion (lymphocyte sedimentation medium, density 1.08; Litton Bionetics). Most of the dead cells were unable to exclude the Ficoll and therefore pelleted, whereas most of the viable heterokaryons remained at the Ficoll-medium interface. The interface cells were then harvested, washed, and plated in the calculated surface area in gelatinized Falcon microtiter II dishes. The cells were washed and fed [<sup>35</sup>S]methionine containing medium on the afternoon of day 3. After labeling overnight, the cells were harvested the morning of day 4. In some cases cells were labeled at earlier or later time-points. Using this approach, 19 of 20 experiments showed the induction of rat myosin light chains. Homokaryon controls were also performed using this procedure.

In all experiments, aliquots of chick myocytes or L6 rat myoblasts were plated on bacterial dishes for the first 2 d, then replated at equivalent densities in microtiter wells and labeled along with the heterokaryons. These controls verified the differentiated or undifferentiated status of the parental cells.

### Cell Labeling and Two-Dimensional Gels

Methionine free Eagle's minimal essential medium containing 10% undialyzed FBS was conditioned by a 24-h exposure to confluent dishes of chick fibroblasts.

Conditioning produced a twofold increase in subsequent [ $^{35}$ S]methionine incorporation, possibly by reducing the cold methionine contributed by the serum. The use of dialyzed serum produced inferior results. Heterokaryons were washed once with methionine-free medium and then labeled overnight in conditioned methionine(-) medium containing 300  $\mu$ Ci/ml of [ $^{35}$ S]methionine. The next morning cells were washed twice in Hanks' BSS, rinsed very quickly with distilled water to remove salts, and extracted directly into lysis buffer (22).

Two-dimensional gels were run according to O'Farrel (22). pH 4–6 ampholytes (Bio-Rad Laboratories, Richmond, Calif.) were used for isoelectric focusing and 12.5% polyacrylamide gels separated proteins by molecular weight. The thickness of the second dimension was reduced to 0.6 mm to increase the sensitivity of autoradiographic detection of [ $^{35}$ S]methionine without resorting to fluorography.  $1-3 \times 10^5$  TCA-precipitable counts were loaded for each sample. Dried gels were exposed to Kodak XR-5 medical x-ray film for  $9-12 \times 10^6$  cpm  $\times$  d.

Myosin heavy chains aggregate and do not form discrete spots on two-dimensional gels. The migration of chick and rat skeletal myosin light chains on two-dimensional gels has been well characterized (3, 5, 7). The location of these light chains in cell extracts was confirmed by their absence from undifferentiated cells, their appearance after differentiation, their comigration with the myosin light chains of myofibrils prepared from chick and rat thigh muscle, and their copurification with the myosin heavy chains after treatment with anti-myosin heavy chain antibodies.

### Partial Purification of Actomyosin

Actomyosin from heterokaryons was purified by a modification of the method of Luzzati and Drugeon (23). Labeled heterokaryons were washed twice in cold Hanks' BSS, once in cold low-salt buffer (30 mM KCl, 10 mM Tris, pH 7.0, 14 mM 2-mercaptoethanol) and then lysed in cold low-salt buffer containing 0.5% Nonidet P-40 (NP-40) and 30  $\mu$ g of cold carrier chick actomyosin. The extract was centrifuged for 10 min at 4°C in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), the supernate discarded, and the pellet extracted for 3 h in high-salt buffer (600 mM KCl, 10 mM Tris, pH 7.0, 14 mM 2-mercaptoethanol). Insoluble material was removed by a second 10-min centrifugation, and the dissolved actomyosin was diluted 1:20 with distilled water and allowed to precipitate overnight. After a final 10-min centrifugation, the pellet was extracted into lysis buffer and analyzed on two-dimensional gels.

### Immune Purification of Myosin

The myosin synthesized in heterokaryons was purified using *Staphylococcus aureus* to precipitate immune complexes formed between antimyosin antibodies and heterokaryon cell extracts. Rabbit antibodies prepared against the heavy chain of fetal bovine skeletal muscle were obtained as a gift from Dr. J.-C. Lelong, Paris. Formaldehyde-inactivated *Staphylococcus aureus*, strain Cowan I, was prepared according to Kessler (24). Labeled heterokaryons were extracted with magnesium salt buffer MSB (1 mM MgCl<sub>2</sub>, 0.25 M KCl, 50 mM Tris, pH 7.4, 1% NP-40), and the nuclei removed by centrifugation. 25  $\mu$ l of antiserum was added to an extract from  $10^5$  heterokaryons in 100  $\mu$ l of MSB and incubated at 0°C for 5 h. 250  $\mu$ l of a 10% solution in MSB of inactivated bacteria was added, incubated for 15 min at 0°C, then pelleted in a Beckman microfuge. The pellet was washed twice in washing buffer (0.15 M NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% sodium lauryl sulfate, 5 mM EDTA, 5 mM nonradioactive methionine) then extracted for 15 min at room temperature into O'Farrel's lysis buffer (22) and processed for two-dimensional gels.

## RESULTS

### Isolation of Heterokaryons

Heterokaryons were obtained by selection with irreversible biochemical inhibitors. In this system, each parental population is treated with a lethal dose of a different irreversible agent. After the unreacted inhibitor is washed away, the two populations are mixed and fused with PEG. Because a different lesion is produced in each population, complementation (rescue) can occur in heterokaryons, whereas parental cells and homokaryons die. Labeling experiments have demonstrated that only authentic heterokaryons are rescued (19). Fig. 1 shows the typical appearance of biochemically isolated heterokaryons between chick myocytes and rat myoblasts. The differential staining properties of the chick (light) and rat (dark) nuclei provide clear confirmation that the selective

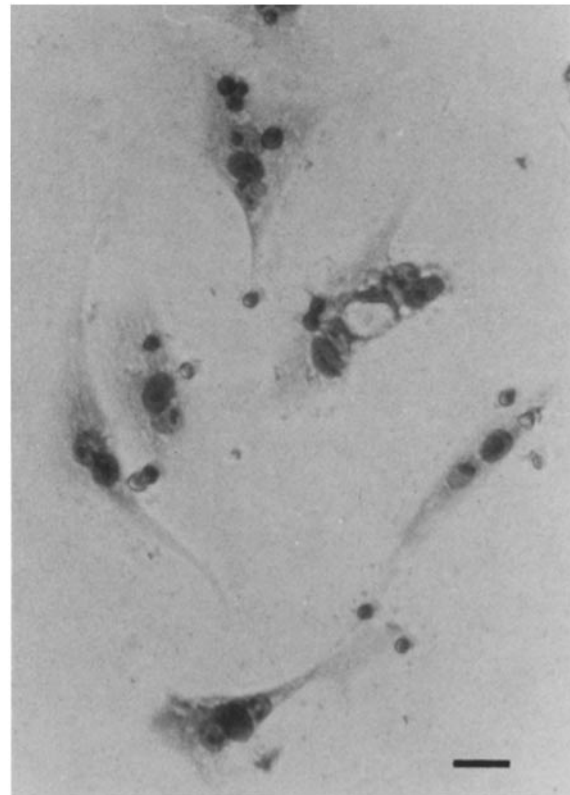


FIGURE 1 Heterokaryons between chick myocytes and rat myoblasts. In Giemsa-stained heterokaryons, the nuclei of chick myocytes stain more lightly than the L6 rat nuclei, so the heterokaryon nature of the binucleated cells is evident. These cells were from a 98% pure population of heterokaryons formed by fusing iodoacetamide-treated chick myocytes to diethylpyrocarbonate-treated rat myoblasts. Cells were fixed 3 d after fusion. Bar, 20  $\mu$ m.

technique isolates heterokaryons and not homokaryons. It is nonetheless possible to isolate homokaryons if one treats different aliquots of the same cell type with different selective agents and performs self  $\times$  self fusions. Rat  $\times$  rat and chick  $\times$  chick monokaryons made in this manner did not show differential nuclear staining.

### Myosin Light-chain Synthesis in Myocyte-Myoblast Heterokaryons

Populations in which >95 percent of the cells were heterokaryons between chick myocytes and rat myoblasts were labeled overnight with [ $^{35}$ S]methionine 4 d after heterokaryon formation, and total cell extracts were analyzed on two-dimensional gels. Fig. 2 demonstrates that these myoblast heterokaryons synthesized at least three types of skeletal myosin LC1: chick fast, rat adult fast, and rat embryonic. Two independent tests confirmed that the proteins synthesized in the heterokaryons were myosin light chains. Fig. 2B shows that the proteins behave like myosin light chains in that they copurify with actomyosin upon repeated cycles of high ionic strength extraction and low ionic strength precipitation. The three light-chain spots are also highly enriched after their immune purification with an antibody directed against the myosin heavy chain (Fig. 2D).

The same pattern of light chain synthesis was observed if the selective treatments were inverted, i.e., if chick myocytes treated with iodoacetamide were fused to rat myoblasts treated with

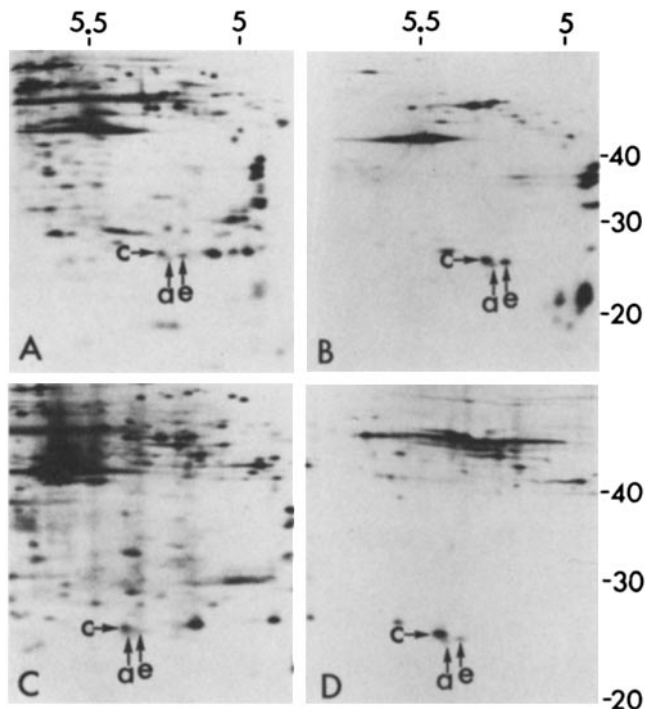


FIGURE 2 Light-chain synthesis in myocyte-myoblast heterokaryons. The two-dimensional gels of heterokaryons from two different experiments are shown. The skeletal myosin in light chains are identified as c (chick fast LC1), a (adult rat fast LC1), and e (embryonic rat LC1). (A) Total cytoplasmic extract, (B) actomyosin from the experiment in A purified by high salt extraction and low salt precipitation, (C) total cytoplasmic extract, (D) anti-myosin precipitate from the experiment in C. Five times as much radioactivity was loaded on the gels of total extracts as on the gels of purified material. The approximate pH and molecular weight ( $\times 10^{-3}$ ) is indicated on the horizontal and vertical axes, respectively.

diethylpyrocarbonate (Fig. 3A) or if chick myocytes treated with diethylpyrocarbonate were fused to rat myoblasts treated with iodoacetamide (Fig. 3B). The induction of rat myosin is thus not dependent upon the specific biochemical treatment combination employed. This induction was reproducible, and was observed in 19 of 20 experiments.

L6 myoblast self  $\times$  self homokaryons were constructed to verify that the biochemical selection procedure itself did not induce myogenic differentiation. Fig. 3C shows that L6 homokaryon cell extracts exhibited no radioactivity in the light-chain region of the gels. In every experiment, aliquots of untreated and unfused L6 rat myoblasts were plated, labeled, and analyzed to verify the extent of their undifferentiated state. This control was performed to exclude the possibility that areas of local confluence in the proliferating L6 stock populations might have caused a small percentage of L6 cells to differentiate. Indeed, 5% of the experiments were discarded because the synthesis of small but detectable amounts of rat embryonic myosin light chain was observed in these untreated L6 cells. Adult rat light chains were never observed in these controls.

Chick myocyte homokaryons (Fig. 3D) did not show radioactivity corresponding to the location of rat myosin light chains, and they continued to synthesize chick skeletal light chains. As an additional control, L6 homokaryons were cocultivated with chick myocyte homokaryons (Fig. 3E). Extracts of these cells showed the synthesis of only chick myosin light chains, demonstrating that diffusible factors from the chick cells were not

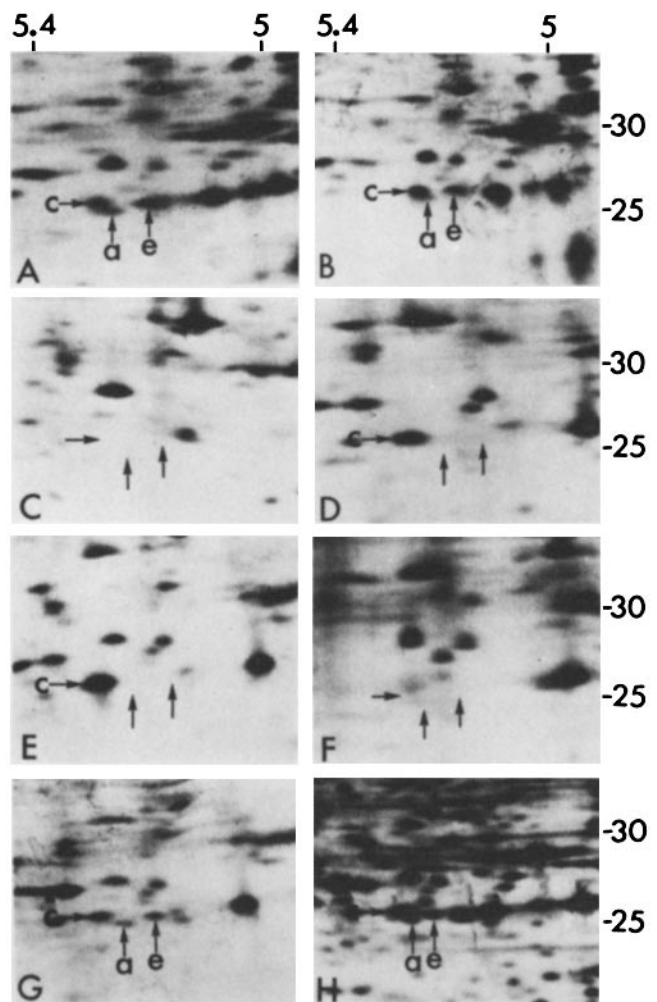


FIGURE 3 Two-dimensional polyacrylamide gels of heterokaryon and control cell extracts. Only the region of the gels containing the myosin light chains is shown. The light chains are labeled as in Fig. 2. Unlabeled arrows point to areas on the gel where relevant light chains would appear if synthesis were occurring. (A) Heterokaryons between iodoacetamide-treated chick myocytes and diethylpyrocarbonate-treated rat myoblasts, (B) heterokaryons between diethylpyrocarbonate-treated chick myocytes and iodoacetamide-treated rat myoblasts, (C) rat myoblast homokaryons, (D) chick myocyte homokaryons, (E) the myoblast and myocyte homokaryons of B and C cocultivated for three days before labeling, (F) heterokaryons between rat myoblasts and chick fibroblasts, (G and H) heterokaryons between chick myocytes and rat myoblasts cultivated in  $10^{-5}$  M cytosine arabinoside (G) or  $16 \mu\text{M}$  5-bromodeoxyuridine (H). The approximate pH and molecular weight ( $\times 10^{-3}$ ) is indicated on the horizontal and vertical axes, respectively.

able to induce rat myosin synthesis under the culture conditions of these experiments. Thus, the synthesis of adult and embryonic rat skeletal myosin light chains was observed only when the chick myocytes were fused with L6 cells.

Because of the delay imposed by the biochemical selection procedure, the earliest time examined was 2 d after heterokaryon formation. Although both embryonic and adult rat LC1 were synthesized at that time, the relative proportion of adult light chain increased over the next few days.

Heterokaryons between L6 rat myoblasts and chick fibroblasts were constructed to determine if nonspecific interactions in avian-mammalian heterokaryons were responsible for the appearance of differentiated rat proteins. Fig. 3F shows that

these heterokaryons did not synthesize skeletal myosin light chains.

### Lack of Requirement for DNA Synthesis before Rat Myosin Induction

Whether a specific "quantal" cycle of DNA synthesis is necessary before the expression of terminal myogenesis is an unresolved question (25–30). Several approaches were adopted to determine if DNA synthesis was required for the synthesis of rat myosin in heterokaryons between chick myocytes and rat myoblasts. Inhibitors of DNA synthesis and inhibitors of differentiation dependent on DNA synthesis failed to prevent the induction of the rat myosin light chains. Cytosine arabinoside ( $10^{-5}$  M) reduces DNA synthesis in L6 rat myoblasts by >90% within 8 h of its addition to the culture medium (30). 5-Bromodeoxyuridine (BUdR) in concentrations >3  $\mu$ M inhibits the differentiation of L6 rat myoblasts if it is present during DNA synthesis (30, 31). Heterokaryons exposed to either  $10^{-5}$  M cytosine arabinoside (Fig. 3G) or 16  $\mu$ M BUdR (Fig. 3H) for the first 4 d after PEG-induced fusion still showed the induction of both forms of rat myosin LC1. Finally, heterokaryons plated in the presence of [ $^3$ H]thymidine did not incorporate label during the first 6 d after fusion. Table I demonstrates that only 1% of the heterokaryon nuclei incorporated [ $^3$ H]thymidine during this period.

### Possibility of Gene Dosage Effects

Although differentiated functions are suppressed in 1:1 hybrids, a variety of studies has shown that if the hybrid cell contains twice as many chromosomes from the differentiated parent as from the undifferentiated one it can continue to express differentiated functions (32–40). In some gene dosage combinations the undifferentiated cell can be activated to produce differentiated products (34–37, 40). The majority of heterokaryons between chick myocytes and rat myoblasts in this study are binucleates; however, ~20–30% of the cells are trinucleated or tetranucleated (Table II). Under the counter hypothesis that undifferentiated myoblasts suppress differentiated functions, myosin synthesis might nonetheless continue in those heterokaryons containing more chick than rat nuclei. The identification of possible gene dosage effects in these heterokaryons will be accomplished once antibodies specific for rat skeletal myosin have been obtained.

Table II also shows that the number of binucleates decreases and the number of multinucleated cells increases during the first 3 d after the cells are plated in tissue-culture dishes. This suggests that binucleated heterokaryons are functionally dif-

TABLE I  
[ $^3$ H]Thymidine Incorporation in Heterokaryons and Control Cells\*

Cells	Total nuclei counted	[ $^3$ H]Thymidine labeled nuclei	Percent labeled nuclei
Chick myocyte $\times$ L6	1,031	14	1
L6 myoblasts	1,241	1,015	82
Chick myocytes	1,003	336	33‡

\* Biochemically selected heterokaryons and untreated control cells were plated in 5  $\mu$ Ci/ml of [ $^3$ H]thymidine, then fixed and processed for autoradiography on the 6th d of the experiment.

‡ Because myocytes are postmitotic, this labeling is primarily a result of fibroblast contamination.

TABLE II  
Size distribution of Multinucleated Cells in Myocyte-Myoblast Heterokaryons

Time after heterokaryon formation*	No. nuclei/cell‡				
	1	2	3	4	5 or more
d					
3	3	79	13	3	4
6	2	51	19	9	19

\* Heterokaryons were transferred from bacterial to tissue-culture grade petri dishes on the evening of day 2, so day 6 represents the behavior of the cells after ~3.5 d of attached culture.

‡ Numbers represent percent of total cells. About 250 cells were counted for each determination.

ferentiated and are fusing to form multinucleated myotubes. However, we cannot yet determine whether rat products are contributing to this process or whether it results from chick molecules present on the heterokaryon cell membrane.

### DISCUSSION

Previous somatic cell hybridization experiments between cells of different histotypic origins have led to the concept that differentiated functions are usually suppressed in hybrids between differentiated and "undifferentiated" (fibroblastoid) cells. The present experiments were designed in part to test the hypothesis that alternate regulatory controls might be exhibited if the "undifferentiated" parent was a precursor cell within the same developmental lineage.

These experiments demonstrate that heterokaryons between chick myocytes and undifferentiated rat myoblasts synthesize both chick and rat skeletal myosin light chains. The phenomenon is independent of the specific drug combination used in the selective system, because inverted treatments give the same results. L6 homokaryons do not show the induction of myosin light chains as a result of the biochemical selection procedure, nor do chick homokaryons show the appearance of new proteins in the light chain region of the gel. Thus this phenomenon is not likely to be an artifact resulting from the selection technique. Simple cocultivation of chick myocytes and L6 cells is not sufficient to induce rat myosin synthesis under the culture conditions of these experiments.

These observations imply that the chick cell is providing a factor that either initiates the rat program of differentiation or at least the synthesis of rat myosin light chains. Although this factor might operate by directly inducing terminal myogenesis, a variety of secondary inductive mechanisms are possible. For example, an inhibition of cell division in the L6 nucleus because of the presence of the chick myocyte cytoplasm or nucleus, the provision by the chick membrane of a receptor that responds to the culture environment with a signal to differentiate, or the presence of a chick enzyme that alters the cells metabolism in a critical fashion all could potentially stimulate L6 to express differentiated functions. Such secondary mechanisms must nonetheless be specific for myogenic cells, because chick fibroblasts do not induce the synthesis of skeletal myosin light chains when fused to L6 cells.

Differentiated L6 myotubes synthesize only the embryonic form of skeletal myosin LC1 (5–7). The appearance of adult rat light chain in these heterokaryons suggests either that the chick cells contain factors that release the block of adult light-chain synthesis in L6 myotubes or that the chick cells dilute an L6 suppressive factor.

The lack of DNA synthesis in the heterokaryon indicates that the expression of at least some differentiated rat proteins is not dependent upon a specific cycle of DNA synthesis during which "reprogramming" might occur. Similar conclusions were obtained by Nadal-Ginard (30) using a highly aneuploid subclone of L6. He showed that populations in which >90% of the cells were capable of forming clones could also differentiate and form myotubes in the absence of additional DNA synthesis. This demonstrated that G<sub>1</sub> cells had the option to differentiate or divide. Our results, using an entirely different approach and a nearly diploid line of L6 myoblasts, confirm the conclusion that DNA synthesis is not a prerequisite for the expression of differentiated functions in L6 rat myoblasts.

These experiments differ in several fundamental aspects from previous somatic cell hybridization studies. The use of purified populations of heterokaryons before the onset of cell division eliminates the possibility of artifacts resulting from the loss of particular chromosomes or bias resulting from the selection of only those heterokaryons capable of division to form hybrid clones. The analysis of tens of thousands of independently fused cells ensures that the observed result is representative of the phenotypic interactions that are occurring. The use of diploid (or near-diploid) cells reduces the possibility that the observations reflect abnormal cell interactions. Finally, the isolation of heterokaryons has permitted the use of cells from defined developmental stages that differ from each other by only a few cell divisions.

We have shown that differentiated chick myocytes are able to induce the synthesis of both fetal and adult rat skeletal myosin light chains when fused to undifferentiated L6 rat myoblasts. This suggests that the control of terminal myogenesis may be under positive inductive regulation.

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