Analysis of Muscle Protein Expression in Polyethylene Glycol-induced Chicken:Rat Myoblast Heterokaryons

STEPHEN F. KONIECZNY, JEANNE BENTLEY LAWRENCE, and JOHN R. COLEMAN
Division of Biology and Medicine, Brown University, Providence, Rhode Island, 02912. Dr. Konieczny's present address is Department of Biology, University of Virginia, Charlottesville, Virginia 22901. Dr. Lawrence's present address is Department of Anatomy, University of Massachusetts Medical Center, Worcester, Massachusetts 01605.

ABSTRACT Heterokaryons derived from polyethylene glycol-mediated fusion of myoblasts at different stages of development were used to investigate the transition of cells in the skeletal muscle lineage from the determined to the differentiated state. Heterokaryons were analyzed by immunofluorescence, using rabbit antibodies against the skeletal muscle isoforms of chicken creatine kinase and myosin, and a mouse monoclonal antibody that cross-reacts with chicken and rat skeletal muscle myosin. When cytochalasin B-treated rat L8(E63) myocytes (Konieczny S.F., J. McKay, and J. R. Coleman, 1982, *Dev. Biol.*, 91:11–26) served as the differentiated parental component and chicken limb myoblasts from stage 23–26 or 10–12-d embryos were used as the determined, undifferentiated parental cell, heterokaryons exhibited a progressive extinction of rat skeletal muscle myosin during a 4–6-d culture period, and no precocious expression of chicken differentiated gene products was detected. In the reciprocal experiment, 85–97% of rat myoblast × chicken myocyte heterokaryons ceased expression of chicken skeletal muscle myosin and the M subunit of chicken creatine kinase within 7 d of culture. Extinction was not observed in heterokaryons produced by fusion of differentiated chicken and differentiated rat myocytes and thus is not due to species incompatibility or to the polyethylene glycol treatment itself. The results suggest that, when confronted in a common cytoplasm, the regulatory factors that maintain myoblasts in a proliferating, undifferentiated state are dominant over those that govern expression of differentiated gene products.

The development of specific cell types within an embryo is characterized by the processes of determination and differentiation, which occur in sequential order. Determination can be defined as the process whereby a cell lineage becomes restricted to a particular developmental pathway, whereas differentiation can be thought of as the expression of an array of specific gene products characteristic of a given cell type. Although simple working definitions can be assigned to these processes, little is known about the specific molecular mechanisms that control an individual cell state.

Recently, we have begun to study these mechanisms by means of somatic cell hybridization, using in vitro muscle development as the principal model system. Myogenesis is advantageous for these types of investigations because myoblasts are easy to obtain from a variety of organisms, they can be selectively purified by a number of procedures, and they will differentiate in vitro under specific culture conditions. In addition to exhibiting the distinct morphology of the multinucleated muscle fiber, differentiated muscle cells also elaborate a variety of muscle-specific products such as myosin, actin, tropomyosin, the M subunit of creatine kinase, acetylcholine receptors, etc., making this developmental system unusually well suited to a number of experimental approaches (reviewed in references 1–3).

The emergence of specific cell types in the developing chicken limb bud has been studied extensively at the morphological and biochemical levels, both in vivo and in vitro (4). Limb buds are first identifiable at ~60 h of embryonic development as small outgrowths of mesenchyme derived from both somitic and somatopleural areas adjacent to the

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limb, covered by body wall ectoderm (5). By 96 h, cells of the limb mesoblast become developmentally restricted to the chondroblast, myoblast, or fibroblast lineages, and, as development progresses, differentiated chondrocytes become evident in the core region of the limb and differentiated muscle fibers begin to form in the peripheral regions (6–11). Thus, the chicken limb bud provides a model system in which a number of cell types can be analyzed at various stages of determination and differentiation.

Somatic cell hybridization has been used extensively to study gene expression in eucaryotic cells (reviewed in references 12 and 13). Most of these investigations have involved the analysis of proliferating hybrid cells, the mitotic progeny of the heterokaryons formed by cell fusion. Although proliferating cell hybrids can provide large amounts of material for quantitative biochemical analysis, gene linkage studies, and investigations on the stability of the determined state, they are generally not suitable for analysis of the regulatory processes governing the transition from the determined to the differentiated state. Hybrid clones that have been isolated and expanded are many cell divisions removed from the time of heterokaryon formation and have often lost substantial numbers of chromosomes, and the expansion process itself can select against cells that enter a nonproliferating, differentiated state. In addition, because of the need for extensive proliferation, one or both of the parental cell types are usually from a permanent cell line, often of undefined developmental history and potential.

The use of heterokaryons can circumvent some of the problems associated with proliferating hybrid cells. First, because both parental nuclei are maintained within a common cytoplasm, no chromosome loss occurs. Second, dosage effects can be analyzed by studying heterokaryons produced by different parental cell input ratios. Finally, individual heterokaryons and homokaryons can be scored for the presence or absence of specific gene products by immunofluorescence, permitting identification of those heterokaryons that express or cease to express a particular antigen.

In this study we report the analysis of heterokaryons produced by fusing differentiated rat myocytes to either (*a*) stage 23–26 chicken limb bud cells, (*b*) 10–12-d chicken embryo leg myoblasts, or (*c*) differentiated chicken leg myocytes. Heterokaryons were analyzed for the production of skeletal muscle myosin and the M subunit of creatine kinase (M-CK)[1] using specific antibodies. In heterokaryons resulting from fusion of undifferentiated chicken cells and differentiated rat myocytes, no precocious expression of chicken muscle myosin or M-CK was detected and rat myosin was slowly extinguished. Reciprocally, extinction of chicken muscle protein expression was observed after fusion of chicken myocytes with rat myoblasts. In control experiments, heterokaryons produced by fusing rat myocytes with chicken myocytes maintained expression of muscle proteins, which demonstrates that extinction is not a result of somatic cell fusion per se. These results strengthen the view that extinction of differentiated characteristics in heterokaryons may reflect intracellular regulatory processes that operate during cell differentiation.

[1] *Abbreviations used in this paper:* CB, cytochalasin B; DAPI, 4′-6-diamidino-2-phenylindole; DME, Dulbecco's modified Eagle's medium; HS, horse serum; M-CK, M subunit of creatine kinase; PEG, polyethylene glycol.

MATERIALS AND METHODS

Chicken Embryo Cells

LIMB BUDS: Four to six stage 23–26 chicken embryos (14) were removed from the egg and placed into cold PBS. After removing all extra-embryonic membranes, wing and leg buds were excised and placed into 5 ml of 1% pancreatin, 2% trypsin in Puck's saline A at 37°C. After 30 min, the limb buds were transferred into cold PBS and triturated gently 10 times with a pasteur pipette. This removed most of the ectoderm and the remainder was peeled away using watchmaker's forceps. The cleaned mesoderm was transferred into 0.05% trypsin for 10 min at 37°C and gently triturated until all large fragments had been dissociated. Complete medium was added, and the suspension was filtered through 20-μm pore size nylon and counted in a hemocytometer. Cell yield averaged 3–4 $\times$ 10^6 cells/embryo. Cells were plated at a density of 2 $\times$ 10^6/60-mm gelatinized dish in Dulbecco's modified Eagle's medium (DME) containing 10% horse serum (HS), 2.5% embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Cultures were incubated at 37°C in 5% CO_2 in air. Cells to be used for heterokaryon formation were harvested the following day by trypsinization.

LEG MYOBLASTS: Myoblasts were isolated from 10–12-d chicken embryo leg muscle without trypsinization, as described previously (15, 16). Leg muscle was removed, minced with scissors, suspended in complete medium, and triturated 30 times with a 10-ml pipette during a 10-min incubation at 37°C. After filtration through a double layer of lens paper and 20-μm pore size nylon, the cell suspension was incubated in nongelatinized tissue culture plates to permit selective attachment of fibroblasts. After 20 min, the cell suspension was removed, counted, and plated in complete medium at a density of 6 $\times$ 10^5 cells/60-mm gelatinized dish. Cultures were either harvested after 1 d for myoblast heterokaryon experiments or were used for production of mononucleate myocytes as described below.

DIFFERENTIATED MONONUCLEATE MYOCYTES: Pure populations of differentiated mononucleate myocytes were obtained as reported previously (16), except that 0.1 μCi/ml [^{3}H]thymidine (SA = 6.7 μCi/mM) was added at the time of plating. Cultures were fed fusion-inhibiting medium containing [^{3}H]thymidine at 24 h, and were re-fed at 72 h with the same medium lacking [^{3}H]thymidine. Fusion-inhibiting medium consisted of Ca^{++}-, Mg^{++}-free Eagle's minimum essential medium containing Chelex-100-treated HS (10%) and EE (2.5%), supplemented with 25 μM $CaCl_2$ (17). Cultures were used for heterokaryon experiments at 5 d, by which time they were devoid of contaminating fibroblasts and consisted of ~98% differentiated, mononucleate myocytes (16), of which at least 97% were [^{3}H]thymidine labeled.

Myogenic Rat Cell Lines

L6J1 AND E63: L6J1 is a subclone derived from rat L6 myoblasts (18, 19) and E63 is a subclone of the rat L8 line (20). Detailed growth and differentiation characteristics of L6J1 and E63 have been described (16, 20–22). Cell lines were maintained in DME containing 10% HS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were subcultured before confluency using 0.05% trypsin in Puck's saline A. Both lines are phenotypically stable under these conditions, exhibit massive fusion after confluency, and yield 94–97% myogenic colonies in clonal culture. Routine monitoring of cultures by DNA fluorochromasia (23) revealed no mycoplasma contamination.

DIFFERENTIATED E63 MYOCYTES: Cytochalasin B (CB) was used to inhibit fusion of rat myoblasts and to obtain differentiated, mononucleate myocytes (16, 24). Briefly, E63 cells were plated at an initial density of 1.5 $\times$ 10^5 cells/100-mm nongelatinized dish in DME and 10% HS, and were fed on days 2 and 4. On day 5, when cultures were confluent but exhibited minimal fusion, they were fed DME and 10% HS containing 2.5 μg/ml CB. During the next 48 h, a population of spherical, loosely adherent cells appeared that consisted primarily of differentiated, mononucleate myocytes. After isolation by the selective detachment procedures described previously (16), the spherical cells were pooled, replated at 7 $\times$ 10^5 cells/100-mm dish in medium lacking CB, and harvested the next day for heterokaryon experiments. In some experiments, 0.1 μCi/ml [^{3}H]thymidine was added with the medium changes on days 4 and 5, resulting in ~90% nuclear labeling of myocytes.

Polyethylene Glycol-induced Cell Fusion

Before fusion with polyethylene glycol (PEG), plates of each parental cell type were fixed for determination of the percentage of differentiated and/or [^{3}H]thymidine-labeled cells present. The remaining parental cell cultures were harvested using 0.05% trypsin and a mixed cell suspension was prepared. Aliquots of unmixed and of mixed parental cells were plated at a density of 10^5 cells/60-mm dish to serve as non-PEG-treated controls. The mixed cell sus-

pension, containing 2–3 × 10^6 cells of each parental type, was then centrifuged at 250 *g* for 5 min, the medium was removed, and the pellet was resuspended in 0.5 ml of 50% PEG 1500 in DME lacking HS (25). After 90 s, the mixture was diluted gently with 10 ml DME and pelleted, and the cells were resuspended in 5 ml DME and 10% HS. After incubation at 37°C for 30 min, the cells were pelleted again, resuspended in complete medium, and plated at a density of 3 × 10^5 cells/60-mm gelatinized dish. This protocol generally yielded 10–15% multinucleate cells, ~25% of which were heterokaryons.

At 24 h after plating, samples of control and PEG-fused cultures were fixed and the remaining plates were fed complete medium containing 1.75 mM EGTA to inhibit spontaneous fusion of parental cells and heterokaryons. Rat myoblasts do not fuse under these conditions (16). Some chicken myoblast fusion can occur in high-density cultures in 1.75 mM EGTA, but rarely were rat:chick heterokaryons observed in high-density control cultures not treated with PEG. As a further precaution against spontaneous fusion, only low-density cultures were analyzed except where indicated otherwise. Cultures were fixed at 24-h intervals, with an additional medium change at 72 h. In some experiments, [^{3}H]leucine at a concentration of 10 μCi/ml (SA = 40 Ci/mM) was added for 1.5 h before fixation.

Cell Fixation and Antibody Staining

Cultures were rinsed in PBS, fixed for 20 min with 3% *p*-formaldehyde, and then rinsed and stored at 4°C in PBS until completion of the experiment (4–7 d). Cells that were to be stained with antibodies were lysed with 0.05% Nonidet P-40 for 30 min. After rinsing in PBS, primary antibody was added for 20 min and plates were again rinsed in PBS. If the primary antibody was of rabbit origin, rhodamine-conjugated goat anti-rabbit IgG was added for 20 min. If the mouse monoclonal antibody XM1b was used, a rabbit anti-mouse IgG secondary antibody was added for 20 min, plates were rinsed in PBS, and rhodamine-conjugated goat anti-rabbit IgG was added for another 20 min. After a final PBS rinse, plates were coated with Kodak emulsion NTB-2 (Eastman Kodak Co., Rochester, NY) and stored at 4°C for 1–2 wk. Autoradiograms were developed with Kodak D-19 (Eastman Kodak Co.) and nuclei were counterstained for 10 min with the DNA fluorochrome 4′-6-diamidino-2-phenylindole (DAPI) (26).

The three antibodies used in these experiments have been characterized previously and are specific to skeletal muscle isoforms of myosin and creatine kinase (16). Antibodies to chicken myosin and M-CK were raised in rabbits and are species specific, reacting with chicken but not with rat muscle proteins. Anti-chicken myosin exhibits prominent A-band localization in cross-striated chicken muscle fibers in culture and a fibrillar staining pattern in mononucleate myocytes. It does not stain rat muscle fibers, nor do fibroblasts or myoblasts of either rat or chicken origin react with this reagent. Rabbit anti-chicken M-CK shows the same species and cell-type specificity, but differs from antimyosin in that it exhibits both cytoplasmic and M-line staining in differentiated chicken muscle cells. XM1b is a mouse monoclonal antibody that was raised against adult rat skeletal muscle myosin. It is not species specific, it reacts with both rat and chick skeletal muscle myosins, and it exhibits A-band localization in cultures containing rat or chick muscle fibers. XM1b stains mononucleate myocytes from both species, but only background fluorescence is detected in myoblasts or in fibroblasts. Rhodamine and DAPI fluorescence were visualized using a Zeiss Standard 16 microscope and the results were recorded photographically on Kodak Ektachrome 160 film (Eastman Kodak Co.).

Heterokaryon Identification

Heterokaryons were identified by differential nuclear morphology and visualized by DAPI-DNA fluorescence and by the presence or absence of silver grains over the nuclei. All heterokaryons were scored with respect to nuclear ratio and their ability to stain with antibodies against skeletal muscle myosin or M-CK. Cells were scored as negative if their immunofluorescence intensity did not significantly exceed the background staining found in unfused myoblasts in the same preparations. This level of background was comparable to that seen in controls run with nonimmune sera. From antibody dilution assays, we estimate that the staining technique used will detect a minimum of 10% of the amount of antigen present in the differentiated myocytes used in these experiments.

Materials

Fertile white leghorn chicken eggs were purchased from Spafas (Norwich, CT). Falcon or Corning tissue culture plates from Fisher Scientific Co. (Medford, MA) were used throughout, and all medium components were from Gibco Laboratories (Grand Island, NY) unless otherwise noted. [^{3}H]Thymidine and [^{3}H]leucine were purchased from New England Nuclear (Boston, MA), rhodamine-labeled IgG from Miles Laboratories Inc. (Elkhart, IN), rabbit antibodies to chicken skeletal muscle myosin from Antibodies Inc. (Davis, CA), DAPI from Boehringer Mannheim Biochemicals (Indianapolis, IN), pancreatin and CB from Sigma Chemical Co. (St. Louis, MO), Chelex-100 from Bio-Rad Laboratories (Rockville Centre, NY), and PEG from BDH Chemicals Ltd. (Poole, England). All other reagents were from Sigma Chemical Co. or Fisher Scientific Co.

RESULTS

The following experiments were performed to determine whether differentiated rat myocytes could activate precocious expression of chicken muscle-specific products, such as myosin and M-CK, after PEG-mediated fusion to undifferentiated chicken myoblasts. Rat E63 myocytes that had been isolated by the CB procedure served as the differentiated parent and are referred to hereafter as CB-E63 cells. We have demonstrated previously that these cells are terminally differentiated myocytes: they contain high levels of M-CK, express skeletal muscle myosin as determined by immunofluorescence with the monoclonal antibody XM1b, and have withdrawn from the cell cycle in the G_1 phase (16).

Heterokaryon 1: Rat CB-E63 Myocytes × Chicken Limb Bud Mesodermal Cells

Differentiated rat myocytes were fused by means of PEG to mesodermally derived cells from stage 23–26 chicken embryo limb buds. By this stage, limb bud cells are committed to muscle, cartilage, and connective tissue lineages (6–11). Although no firm estimates of the relative numbers of cells in each lineage exist, clonal assays (27) are consistent with the view that up to 45% may be in the myoblast compartment at this time, and ~1% already have begun to express skeletal muscle myosin or M-CK (Table I*A*). Before PEG fusion, 77% of the rat CB-E63 cells were myosin positive. Therefore, precocious expression of chicken products would be expected

TABLE I

Heterokaryon 1: Differentiated Rat CB-E63 Myocytes × Stage 23–26 Chicken Limb Bud Cells

(A) Parental cells:

Days postfusion	Cell type	Percent fluorescent with anti-myosin
0	Rat CB-E63 myocytes	77% (1,602)
0	Stage 23–26 chicken limb bud cells	1% (3,016)

(B) Heterokaryons:

Days postfusion	Percent fluorescent with anti-chicken myosin or M-CK*		Percent fluorescent with XM1b‡	
	1:1§	Total	1:1	Total
1	1% (132)¶	1% (234)	77% (73)	71% (143)
2	2% (104)	1% (193)	43% (89)	41% (173)
3	3% (112)	2% (189)	37% (75)	31% (176)
4	2% (95)	1% (174)	21% (71)	21% (136)

* Heterokaryons were stained with rabbit antibodies specific for chicken skeletal muscle myosin or for M-CK. The data were pooled, as no significant differences were observed between them.

‡ XM1b: mouse monoclonal antibody which stains chicken and rat myosin.

§ Nuclear ratios: 1:1: heterokaryons containing one rat and one chicken nucleus only. Total: all heterokaryons scored, regardless of nuclear ratio.

¶ Numbers in parentheses refer to the total number of cells scored.

All tables follow this convention unless otherwise stated.

in a maximum of 77% of the heterokaryons if activation occurred regardless of developmental lineage, and 35% (0.77 × 0.45) if it were restricted to the myoblast compartment. In actuality, the percentage was much lower.

Heterokaryons were analyzed by immunofluorescence at daily intervals for 4 d after PEG fusion and were categorized according to nuclear ratio (Table I*B*). Few heterokaryons were found with more than three nuclei. Regardless of the nuclear ratio, only 1% of heterokaryons were positive for chicken myosin or M-CK at 24 h and a maximum of 2–3% were found to be positive on subsequent days. These values do not differ significantly from the fraction of chicken cells already differentiated at the time of fusion (Table I*A*). A few cultures were also analyzed at 5 and 6 d postfusion to ensure that heterokaryons had sufficient time to express chicken differentiated gene products. Again, no detectable activation of chicken myosin or M-CK was found (Fig. 1, *A–C*). These results suggest that exposure to the internal environment of a CB-E63 myocyte does not elicit expression of characteristic muscle products by the chicken genome.

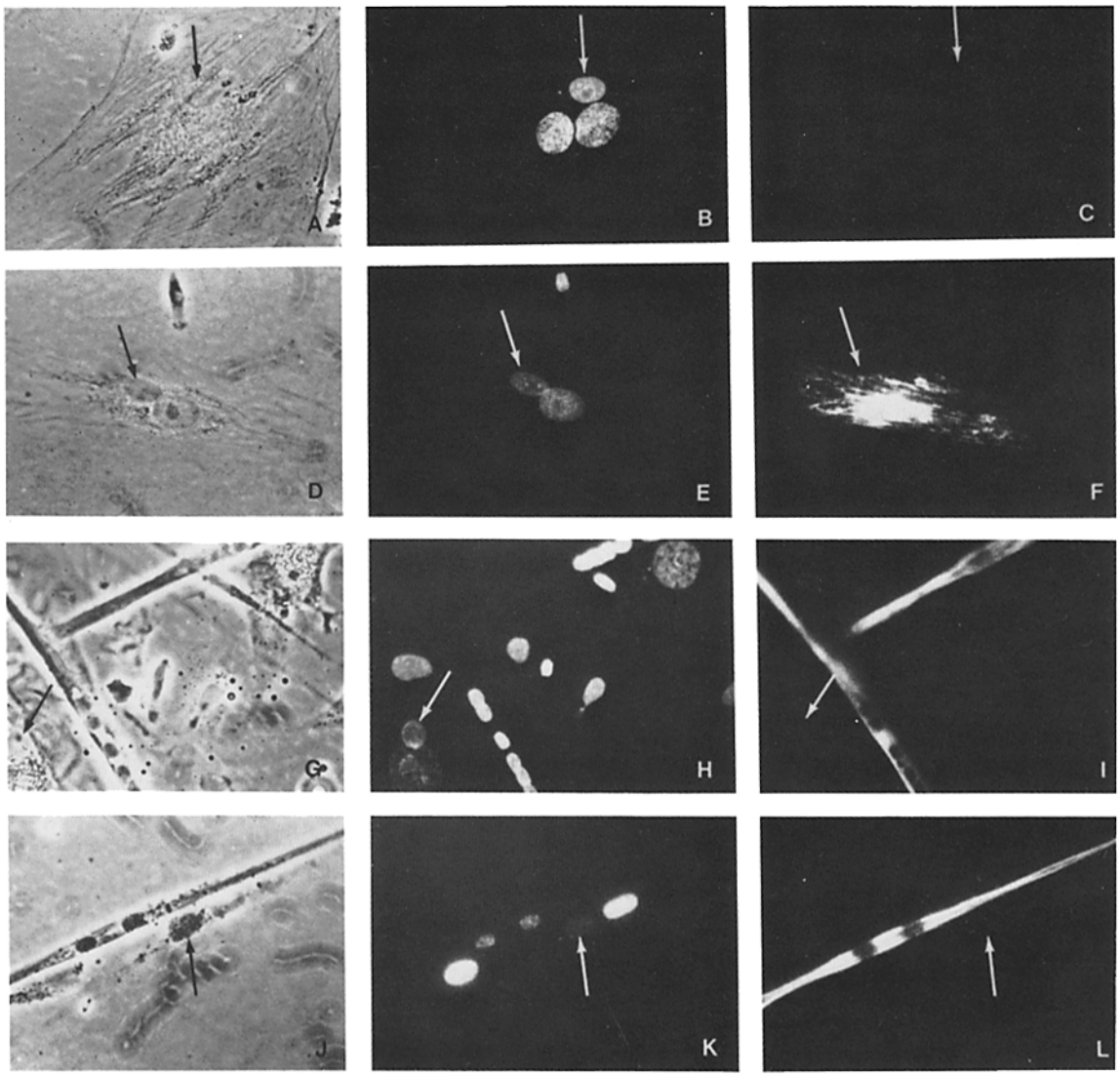

FIGURE 1 Representative chicken:rat heterokaryons from three different experiments. *Left*, phase contrast; *center*, DAPI fluorescence; *right*, rhodamine immunofluorescence. Arrows in all micrographs point to the single chicken nucleus present in each heterokaryon. (*A–C*) Trinucleated heterokaryon derived from 2 rat [^{3}H]thymidine-labeled CB-E63 myocytes and 1 Stage 24 chicken embryo limb bud cell, fixed 24 h postfusion and stained with anti-chicken M-creatine kinase. No rhodamine immunofluorescence is observed. (*D–F*) Binucleated heterokaryon produced from a [^{3}H]thymidine-labeled rat CB-E63 myocyte and a Stage 24 chicken embryo limb bud cell, fixed 24 h postfusion and stained with the monoclonal antibody XM1b. The heterokaryon exhibits positive fluorescence, indicating that myosin extinction has not yet occurred. (*G–I*) Culture containing a binucleated heterokaryon produced from a [^{3}H]thymidine-labeled rat CB-E63 myocyte and an 11-d-old chicken embryo leg myoblast, stained with anti-chicken myosin at 96 h postfusion. Following PEG treatment, the culture was plated at high cell density and some spontaneous fusion of chicken myoblasts has occurred. In this example, the heterokaryon and surrounding mononucleated cells from both rat and chicken parental cells are negative, while the chicken myofibers are positive. (*J–L*) Binucleated heterokaryon produced from a [^{3}H]thymidine-labeled, differentiated chicken myocyte and a cycling rat L6J1 myoblast, stained with anti-chicken myosin 96 h postfusion. The large number of silver grains over the chicken nuclei accounts for the weak DAPI fluorescence. Extinction of myosin is observed in the heterokaryon while expression is demonstrated in the binucleated chicken homokaryon. The focal plane in all phase contrast micrographs is on the cells, rather than on the silver grains. × 170.

Since no precocious activation of chicken skeletal muscle proteins was detected in these heterokaryons, analyses were performed to determine whether rat skeletal muscle myosin continued to be expressed. Heterokaryons were stained with the monoclonal antibody XM1b, which cross-reacts with both chicken and rat skeletal muscle myosins, and analyzed as described above. 1 d after PEG fusion, 71–77% of the heterokaryons were XM1b positive (Table I*B*; Fig. 1, *D–F*), a value that matches closely the fraction of myosin-positive CB-E63 cells in the prefusion population (Table I*A*). Furthermore, because only 1% of these heterokaryons contain chicken myosin, virtually all of the XM1b staining must be due to the presence of rat myosin. By 2 d after fusion, however, the fraction of heterokaryons containing rat myosin had dropped to 41–43%, and at 4 d only 21% remained positive with XM1b (Table I*B*). These results suggest that differentiated characteristics already expressed by CB-E63 myocytes are gradually extinguished in the heterokaryons.

The observed extinction of rat myosin and the absence of activation of chicken muscle proteins could reflect regulatory interactions influencing gene expression, or they could have a more trivial explanation, such as reduced cell viability after PEG fusion. To examine this, [^{3}H]leucine was added to 4-d post-fusion cultures and the plates were fixed after 1.5 h and processed for immunofluorescence and autoradiography. 80 heterokaryons were examined. All exhibited grain densities comparable to those observed over unfused parental cells, regardless of antibody staining characteristics. These results demonstrate that the heterokaryons analyzed in these experiments were viable, metabolically active cells.

Heterokaryon 2: Rat CB-E63 Myocytes × Chicken Embryo Leg Myoblasts

Although the embryonic limb bud contains cells that are determined to form muscle, the vast majority of these will continue to proliferate in vivo for at least another 10 d before differentiating. Thus, it is possible that the absence of activation of chicken muscle-specific genes in the experiments described above reflects the relatively primitive developmental state of the limb bud cells. To address this point, heterokaryon experiments were performed using 10–12 d embryonic leg myoblasts, up to 70% of which can differentiate within several cell cycles (S. F. Konieczny and J. R. Coleman, unpublished results; 28).

Chicken leg myoblasts were fused to differentiated rat CB-E63 myocytes and heterokaryons were analyzed for the expression of chicken muscle-specific myosin and chicken M-CK as described above. In this experiment, 68% of the rat CB-E63 cells and 9% of the chicken cells were differentiated at the time of fusion (Table II*A*). If myocyte × myocyte heterokaryons continue to express muscle proteins, and if activation rather than extinction occurs in myoblast × myocyte heterokaryons, a maximum of 71% differentiated heterokaryons would be expected ([0.68 × 0.09] + [0.68 × 0.91] + [0.32 × 0.09] = 0.71). As seen in Table II*B*, the fraction of 1:1 heterokaryons positive for chicken muscle proteins ranged from 2% at 2 d to 7% at 4 d postfusion (Fig. 1, *G–I*). When the data from heterokaryons were pooled to include all nuclear ratios, these values ranged from 5 to 9%. Since 9% of the cells in the parental chicken myoblast population were myosin positive, we conclude that no precocious expression of chicken muscle proteins occurred in this experiment. The range of values observed for 1:1 heterokaryons could reflect experimental variation or a transient loss of chicken muscle proteins after cell fusion. These alternatives are addressed in the next section (*Heterokaryon 3*).

TABLE II

Heterokaryon 2: Differentiated Rat CB-E63 Myocytes × 10–12-D Chicken Leg Myoblasts

(A) Parental cells:

Days postfusion	Cell type	Percent fluorescent with antimyosin
0	Rat CB-E63 myocytes	68% (477)
0	10–12-d chicken leg myoblasts	9% (684)

(B) Heterokaryons:

Days post-fusion	Percent fluorescent with anti-chicken myosin or M-CK		Percent fluorescent with XM1b	
	1:1	Total	1:1	Total
1	3% (156)	7% (321)	35% (31)	45% (88)
2	2% (165)	5% (332)	29% (55)	44% (124)
3	4% (182)	9% (309)	16% (45)	22% (86)
4	7% (110)	9% (207)	8% (26)	11% (53)

Heterokaryons were also stained with the antibody XM1b to determine whether rat myosin continued to be expressed. A progressive decrease in the percentage of myosin-positive heterokaryons was observed, and by 4 d postfusion, only 8–11% stained with this reagent (Table II*B*). Thus, extinction of rat skeletal muscle myosin expression occurred in these experiments as well as in heterokaryons involving limb bud cells. The results do not exclude the possibility that rat myosin expression is activated in rat myoblast × chick myocyte heterokaryons, since the observed values of 8–11% are slightly higher than the 6% expected if expression continued only in myocyte × myocyte heterokaryons (0.68 × 0.09 = 0.06). They are in fact the values one would expect if such activation occurred ([0.68 × 0.09] + [0.32 × 0.09] = 0.09). This was investigated in the experiment that follows.

Heterokaryon 3: Rat CB-E63 Myocytes × Chicken Embryo Leg Myocytes

The extinction of rat myosin expression observed in the experiments described above was an unexpected result because Carlsson et al. (29) had demonstrated previously that co-cultured chicken and rat myoblasts can fuse spontaneously, forming heterokaryon muscle fibers that synthesize both chicken and rat myosin. Our experiments differed from theirs in that the chicken myoblasts had not yet undergone the transition to spontaneous fusion competence, and heterokaryon formation was mediated by PEG. Our results suggested that when presumptive myoblasts are PEG fused with mononucleate myocytes, the phenotype of the presumptive myoblast is dominant in the resulting heterokaryons. The following experiment was performed to address the possibility that extinction was caused by the PEG fusion procedure per se, rather than by normal cellular regulatory control mechanisms.

Differentiated, mononucleate chicken myocytes were obtained by culture in Ca^{++}-, Mg^{++}-depleted medium (16) and fused by means of PEG with rat CB-E63 cells. As in the previous experiments, PEG-treated and control cultures were

TABLE III

Heterokaryon 3: Differentiated Rat CB-E63 Myocytes × Differentiated, Fusion-inhibited Chicken Myocytes

(A) Parental cells:

Days postfusion	Cell type	Percent fluorescent with antimyosin
0	Rat CB-E63 myocytes	62% (610)
0	Fusion-inhibited chicken myocytes	98% (208)

(B) Heterokaryons:

	Percent fluorescent with anti-chicken myosin or XM1b*	
Days postfusion	1:1	Total
1	63% (38)	77% (127)
2	62% (39)	64% (149)
3	58% (24)	58% (81)
4	67% (21)	72% (57)

* Heterokaryons were stained with antibodies specific for chicken skeletal muscle myosin or with XM1b, which stains both chicken and rat myosin. The data were pooled, as no significant differences were observed between them.

maintained in 1.75 mM EGTA to prevent spontaneous fusion, and heterokaryons were examined by immunofluorescence for continued expression of skeletal muscle myosin. At the time of fusion, 98% of the chicken cells and 62% of the rat CB-E63 cells consisted of differentiated myocytes (Table III*A*). If myocyte × myocyte heterokaryons remain differentiated and myocyte × myoblast heterokaryons undergo extinction, 61% of the fusion products would be expected to remain positive for myosin (0.98 × 0.62 = 0.61). As seen in Table III*B*, 58–67% of 1:1 heterokaryons and 58–77% of the total heterokaryon population remained positive for chicken or rat skeletal muscle myosin over the 4-d period. We have also found that when two differentiated chicken myocytes are PEG fused, 97–99% of the homokaryons continue to express myosin for at least 4 d (30). These results demonstrate that the PEG fusion procedure itself does not lead to extinction of the myogenic phenotype and they suggest that the extinction of muscle protein expression observed in heterokaryons 1 and 2 is due to regulatory factors contributed by the undifferentiated parental cell.

Finally, precocious expression of rat muscle proteins in rat myoblast × chick myocyte fusion products (31; and see *Heterokaryon 2* above) occurred, if at all, in only a minority of heterokaryons in this experiment. Although the 67–72% of myosin-positive heterokaryons present at 4 d postfusion exceeds the 61% expected from myocyte × myocyte fusions alone, it falls far below the 98% value that would result if all rat CB-E63 × chicken myocyte heterokaryons expressed myosin ([0.62 × 0.98] + [0.38 × 0.98] = 0.98).

Heterokaryon 4: Rat L6J1 Myoblasts × Chicken Embryo Leg Myocytes

While this investigation was in progress, Wright (31) reported the activation of rat muscle-specific myosin synthesis in mass populations of heterokaryons derived from cycling rat L6 myoblasts and differentiated chicken myocytes. In our experiments, using the reciprocal cross (rat myocytes × chicken myoblasts), extinction rather than activation was observed. Because of this difference in results, an experiment similar to Wright's was performed using rat L6J1 cycling myoblasts as the undifferentiated parental cell and fusion-inhibited chicken myocytes as the differentiated partner.

In this experiment, 97% of the chicken cells were myosin positive, whereas none of the rat L6J1 cells were differentiated before PEG fusion (Table IV*A*). As in our previous experiments, the percentage of cells that stained with muscle-specific antibodies decreased with time. Whereas 58% of the 1:1 heterokaryons were positive with anti-chicken myosin at 24 h postfusion, only 11% remained positive after 7 d (Table IV*B*; Fig. 1, *J–L*). Cultures stained with anti-chicken M-CK exhibited 14% positive heterodikaryons at 24 h and 3% at 7 d (Table IV*B*). Thus, the large majority of heterokaryons exhibit extinction of both chicken myosin and M-CK, with M-CK being lost more rapidly, as is also the case in heterokaryons derived from melanoma cells and chicken myocytes (30). Cultures stained with monoclonal antimyosin XM1b yielded results indistinguishable from those obtained with anti-chicken myosin (data not shown). The discrepancies between these results and those reported by Wright (31) will be addressed in the Discussion.

DISCUSSION

The experiments reported above demonstrate that differentiated rat myocytes are unable to elicit precocious synthesis of chicken muscle-specific products when fused to determined, undifferentiated chicken myoblasts. Furthermore, the differentiated phenotype of the rat myocyte is extinguished in such heterokaryons, as reflected by the progressive disappearance of skeletal muscle myosin.

Several observations argue against the possibility that extinction of muscle protein expression is due to nonspecific effects of PEG-induced cell fusion, such as cytotoxicity or disruption of cell organization, or to the interspecies nature of the heterokaryons. First, the number of heterokaryons found per unit area remained nearly constant during the 4-d postfusion period. This suggests that they are viable cells, because Doetschman and Jewett (32) have demonstrated that myoblasts detach from the substratum within 45–60 min after cell death. Second, incorporation of [^{3}H]leucine demonstrated directly that 4-d heterokaryons remain metabolically active and continue to engage in protein synthesis. Finally, rat myocyte × chick myocyte heterokaryons remained myosin positive throughout the duration of the experiment; thus, extinction is due neither to PEG fusion per se nor to species incompatibility. Taken together, these results suggest that extinction of muscle properties in heterokaryons is a conse-

TABLE IV

Heterokaryon 4: Undifferentiated Rat L6J1 Myoblasts × Differentiated, Fusion-inhibited Chicken Myocytes

(A) Parental cells:

Days postfusion	Cell type	Percent fluorescent with antimyosin
0	Rat L6J1 myoblasts	0% (600)
0	Fusion-inhibited chicken myocytes	97% (730)

(B) Heterokaryons:

	Percent fluorescent with anti-chicken myosin		Percent fluorescent with anti-chicken M-CK	
Days postfusion	1:1	Total	1:1	Total
1	58% (123)	69% (267)	14% (126)	27% (223)
4	18% (91)	25% (173)	9% (78)	14% (118)
7	11% (38)	15% (53)	3% (31)	5% (43)

quence of regulatory mechanisms active in the parental cells.

Fusion of cells in different phases of the cell cycle can lead to regulatory interactions that may contribute to the extinction of specific gene expression in heterokaryons (33–36). The myocytes used in our experiments are in an arrested or prolonged G_1 or G_0 state (16), whereas the presumptive myoblasts were from exponentially growing cultures in which 30–50% of the cells are in G_1 at any given time (H. U. Lee and J. R. Coleman, manuscript in preparation; 16, 26). Since 30% or more of the heterokaryons should have been derived from cells in G_0 or G_1, the extinction observed cannot be accounted for solely by regulatory interactions resulting from heterophasic fusions.

An assumption underlying this investigation is that PEG fusion of cells in suspension is a random process and is not selective with respect to developmental lineage or stage of differentiation. Two lines of evidence support this assumption. First, similar fusion frequencies, 10–15%, are obtained when populations greatly enriched for myoblasts, fibroblasts, or chondroblasts are treated with PEG under equivalent conditions (S. F. Konieczny, unpublished observations). Second, heterokaryons 3 and 4 both involve cells derived solely from the myogenic lineage, and both exhibit patterns of extinction consistent with fusion occurring as a random event.

Several other investigators have reported the extinction of differentiated properties within 1–5 d postfusion in heterokaryons involving cells from a variety of species and developmental pathways. These include phagocytic activity in mouse macrophage × mouse melanoma heterokaryons (37), dihydroxyphenylalanine oxidase activity in chicken fibroblast × mouse melanoma heterokaryons (38), expression of skeletal muscle myosin and M-CK in chicken myocyte × mouse melanoma heterokaryons (30), tyrosine aminotransferase inducibility in rat hepatoma × rat liver epithelial cell heterokaryons (39), and albumin production in rat hepatoma × mouse fibroblast heterokaryons (40). Thus, extinction in heterokaryons is a widely encountered phenomenon, perhaps more closely related to the state of differentiation than to developmental cell lineage or species origin of the parental cells.

With regard to the work presented here and elsewhere (30), it is interesting to note that the rates of extinction for M-CK and for skeletal muscle myosin differ (see *Heterokaryon 4*). This may reflect different mRNA and/or protein half-lives for these muscle products. Experiments from our laboratory suggest that M-CK has a half-life of ~14 h in cultured muscle, whereas the half-life of skeletal muscle myosin has been estimated to be 48–72 h (41, 42). These values correlate well with the rates of extinction of these proteins in heterokaryons and are consistent with the view that extinction involves inhibition of expression of previously active muscle protein genes rather than increased rates of protein degradation.

Although activation of differentiated gene products was not detected in this investigation, several previous reports have suggested that specific gene activation can occur in heterokaryons. Linder et al. (43, 44) demonstrated activation of chicken globin genes in heterokaryons produced by fusing transcriptionally inactive adult chicken erythrocytes with proliferating cells from several mammalian cell lines, including rat L6 myoblasts. A similar report by Bruno et al. (45) demonstrated activation of globin synthesis in reconstituted cells produced by fusing chicken erythroyctes to enucleated chicken or mouse fibroblasts. Such studies differ fundamentally from our work on myoblast heterokaryons in that they involve re-expression of previously active genes rather than precocious expression.

Heterokaryons produced by fusing rat myocytes with chicken myoblasts (Tables I and II) did not exhibit precocious expression of chicken skeletal muscle myosin or M-CK, as determined by immunoflourescence. In contrast, Carlsson et al. (29) demonstrated that co-cultured chicken and rat myoblasts can spontaneously form heterokaryon muscle fibers that express both chicken and rat myosin. Spontaneous fusion involves myoblasts that have undergone the transition to differentiation competence, whereas our experiments used, as one of the parental cells, proliferating myoblasts that had not yet undergone this change in physiological state. Whether or not the transition to differentiation competence involves a requisite series of cell divisions (H. U. Lee and J. R. Coleman, manuscript in preparation; 28, 46–49), our results with avian myoblasts suggest that it is not circumvented by exposure to the internal environment of a rat myocyte.

In investigations similar to ours, Wright (31) reported activation of rat myosin light-chain synthesis in heterokaryons between rat L6 myoblasts and chicken myocytes, and Blau et al. (50) demonstrated synthesis of human myosin light chains and M-CK in heterokaryons derived from mouse myotubes and human amniocytes. These experiments differ from ours in several fundamental ways. First, both Wright and Blau et al. used electrophoretic techniques to detect newly synthesized proteins in mass populations of cells, whereas our investigations used immunofluorescence to detect specific muscle gene products in individual cells. Although in our efforts to repeat Wright's experiment (Table IV) the majority of heterokaryons extinguished chicken M-CK and chicken myosin, 3–15% remained positive for at least 7 d post-fusion. It is possible that synthesis of rat muscle-specific products is initiated in this minority population and would be detected by two-dimensional gel electrophoresis. Unfortunately, antibodies specific to rat myosin or M-CK are not yet available to test this hypothesis.

Second, Wright and Blau et al. used a variety of inhibitors before and after fusion to purify selectively the heterokaryon population. The repercussions of such treatments on the regulatory molecules involved in the expression of differentiated gene products are unknown.

Third, the large majority of heterokaryons in our experiments were the products of 1:1 and 2:1 input ratios of the parental cell types. Few heterokaryons containing more than three nuclei were found in this investigation, but we have reported elsewhere (30) that extinction of M-CK in chicken myocyte-mouse melanoma heterokaryons is dosage dependent. In the study by Wright, 19% of the cells contained five or more nuclei, and 28% contained four or more. This author did not indicate the ratio of chicken to rat nuclei in these polykaryons, but, given the severalfold difference in cell size of chicken and rat myoblasts, the selective poisoning technique used, and the heterokaryons illustrated in his report (see reference 31; Fig. 1), it is likely that they contain an excess derived from the chicken myocyte parent. Similarly, Blau et al. (50) reported that the average heterokaryon myotube in their experiments contained a two- to threefold excess of nuclei derived from the mouse parental muscle cell. The range of nuclear ratios was not specified, but one would expect from a Poisson distribution that a substantial number of heterokaryon myotubes with a larger than threefold excess of mouse nuclei was present. Since only mass populations were

analyzed, rather than individual cells, activation may have occurred in a selected population of heterokaryons.

We therefore suggest that activation and extinction of genes that are regulated during differentiation is dosage dependent in heterokaryons and, further, that both these phenomena reflect regulatory interactions active in differentiation-competent cells. When nuclear and cytoplasmic input ratios are approximately equivalent, the phenotype of the determined, undifferentiated cell predominates and extinction of differentiated functions results. When the differentiated component is present in sufficient excess, activation can occur even when the undifferentiated parental cell is from a different developmental pathway (50). This hypothesis is testable and has significant implications with respect to regulatory processes involved in the developmental transition from the determined to the differentiated state.

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