Induction of Muscle Genes in Neural Cells

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ABSTRACT The regulation of skeletal muscle genes was examined in heterokaryons formed by fusing differentiated chick skeletal myocytes to four different rat neural cell lines. Highly enriched populations of heterokaryons isolated using irreversible biochemical inhibitors were labeled with [^{35}S]methionine and analyzed on two-dimensional gels. Rat skeletal myosin light chains were induced in three of the four cell combinations. The one exception, the S-20 cholinergic cell line, not only failed to synthesize rat muscle proteins but also suppressed chick myogenic functions. Experiments with heterokaryons between chick myocytes and cells from whole embryonic rat brain cultures demonstrated that rat skeletal myosin light chains are inducible in normal diploid neural cells as well as in established neural cell lines. In contrast, dividing cell hybrids between rat myoblasts and rat glial cells were nonmyogenic. These results demonstrate that although neural cells may contain factors that prevent the decision to differentiate along myogenic lines in cell hybrids, most neural cell lines do not dominantly suppress the expression of muscle structural genes in heterokaryons. Furthermore, the skeletal myosin light chain genes in most neural cell lines are regulated by a mechanism that permits them to respond to putative chick skeletal myocyte-inducing factors. The "open" state of these myogenic genes may explain many of the reports of apparent "transdifferentiation" to muscle in neural cultures and neural tumors.

Heterokaryons are the initial fusion product between two cells, where both nuclei exist within a common cytoplasm. The interactions in heterokaryons of the regulatory molecules from both parents provide a test system for examining the mechanisms of specific gene activation, the nature of cell determination, and the potential reversibility of cell differentiation. We have shown that differentiated functions can be induced in determined but undifferentiated precursor cells following their fusion to terminally differentiated cells within the same developmental lineage. Fusing undifferentiated rat or quail myoblasts to differentiated chick skeletal myocytes resulted in the induction of rat or quail myosin light chain synthesis (1–3). In contrast, when cells from a different histiotype were used, differentiated functions were suppressed. Neither rat nor chick myosin light chain synthesis occurred in heterokaryons formed by fusing rat fibroblasts to differentiated chick skeletal myocytes (4). These results are consistent with the hypothesis that cell determination and differentiation involve the dominant suppression of alternate developmental pathways. To examine the generality of this phenomenon, we have now examined the regulation of muscle functions in heterokaryons formed by fusing chick myocytes to cells of additional histiotypes.

Various reports indicate that the regulation of alternate pathways might not be rigidly controlled in certain neural derivatives. These results include the apparent transdifferentiation of neural retinal cells to pigment or lens cells (5) and the appearance of striated muscle in various neural cells and tissues: the pineal gland (6), the leptomeninges (7), neural tumors (8), cultures of optic nerve (9) or pituitary tissue (10), and some neural cell lines (11, 12). The observation of aberrant muscle formation by neural cells led us to examine the regulation of the skeletal myosin light chain one (LC1)[1] structural genes in heterokaryons formed by fusing neural cells to differentiated chick skeletal myocytes. The results of these experiments demonstrate that some neural cells activate specific neural differentiated functions without the concomitant dominant suppression of the structural genes of at least some alternate histiotypes. Furthermore, the skeletal myosin light chain genes in many neural cells may be in a chromatin configuration compatible with their induction in the absence of significant DNA synthesis. Both of these observations increase the likelihood that neural to skeletal muscle transdif-

[1] *Abbreviations used in this paper:* BUdR, 5-bromodeoxyuridine; LC1, skeletal myosin light chain one.

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ferentiation events are possible. However, experiments using cell hybrids indicate that neural cells may be producing factors that suppress myogenesis at developmental stages prior to the activation of the muscle structural genes.

MATERIALS AND METHODS

Cells and Culture Conditions: Differentiated chick myocytes were obtained from primary cultures of 12-d-old embryonic chick thigh muscles as described previously (1). 1 d after the establishment of the primary culture, 1.7 mM EGTA was added to inhibit myotube formation (13) and thereby avoid the massive gene-dosage effects that would occur if a multinucleated myotube were fused to a mononucleated neural cell. 1 d later, after most of the muscle cells had become postmitotic, 10^{-5} M cytosine arabinoside was added to kill dividing fibroblasts. The medium was changed on the third day of culture to normocalcemic medium containing 2 μg/ml cytochalasin B as the agent preventing myotube formation, since we have found that differentiated muscle cells survive longer in cytochalasin than in EGTA. Differentiated, mononucleated muscle cells will be referred to as myocytes to distinguish them from both undifferentiated myoblasts and differentiated, multinucleated myotubes. Myocytes were used 7–10 d after the initiation of the culture.

B9 is a rat glial cell line established from a nitrosoethylurea-induced brain tumor. They express S-100 protein and neuron-specific enolase (14). The coexpression of both of these proteins may indicate that B9 cells are a precursor to both neuronal and glial elements (15). B9 cells contain approximately 50 chromosomes. The B9 cells, generously provided by D. Shubert (Salk Institute), were obtained directly from their laboratory of origin and immediately frozen in multiple ampules. The cells were used within 20 passages of being reconstituted. C6 is a near diploid rat astrocytoma cell line that expresses S-100 protein (16, 17). Both the NIE-115 and S-20 lines are clonal derivatives of the mouse C-1300 neuroblastoma line (18). NIE-115 is an adrenergic line with a modal number of 192 chromosomes and S-20 is a cholinergic line with a modal number of 59 chromosomes (18). C6, NIE-115, and S-20 cells were obtained from M. R. Morrison (this institution), who has recently verified that these NIE-115 and S-20 cells are still expressing the appropriate adrenergic or cholinergic functions (19). L6 is a rat myoblast line that remains undifferentiated under growth conditions but that spontaneously forms multinucleated myotubes when confluent (20). Our subclone of L6 contains a modal number of 41 chromosomes.

Primary rat brain cultures were established from 16-d embryos. The cerebral hemispheres were removed, minced with fine scissors, and digested for 1 h at 37°C in 2.5 mg/ml trypsin 1:250 (Gibco Laboratories, Grand Island, NY) in calcium-free saline. Tissue fragments were dispersed by trituration with a 10-ml pipet, and cells were washed with complete medium and then plated in dishes that had been precoated with a sterile solution of 0.01% pig skin gelatin (Sigma Chemical Co., St. Louis, MO). These cells were cultivated in Dulbecco's minimal essential medium containing D-valine (Gibco Laboratories) in order to inhibit "fibroblasts" (21). Cultures were used within two to six passages (4–16 population doublings).

Our laboratory uses a medium composed of four parts Dulbecco's minimal essential medium to one part Medium 199 for chick muscle cultures (1). To simplify cell culture conditions and to have the same medium used before and after heterokaryon formation, we cultivated all of the rodent cell lines in this mixture as well. Rodent cells were grown in 10% fetal bovine serum, and chick myocytes were cultivated in 5% horse serum plus 2% chick embryo extract. Although cell lines were cultured in the absence of antibiotics, primary cultures were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

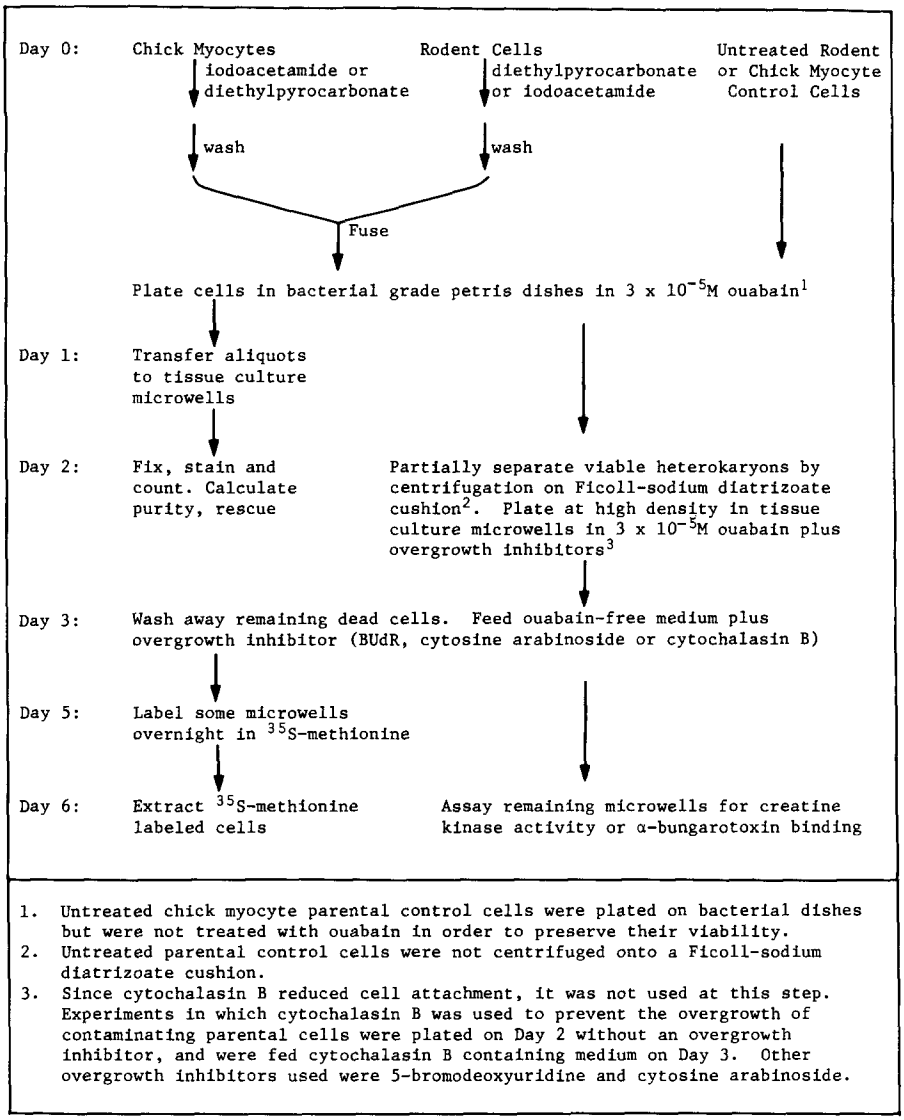
Isolation of Heterokaryons: Populations in which 90–99% of the surviving nuclei were in heterokaryons were obtained by selection using irreversible biochemical inhibitors as described in detail elsewhere (22–24). Cells pretreated with a lethal concentration of iodoacetamide were fused with polyethylene glycol to cells that had been pretreated with a lethal dose of diethylpyrocarbonate. Since different molecules had been inactivated in the two cell populations by the different treatments, each cell contributed active molecules to replace those damaged in its fusion partner and many of the heterokaryons survived whereas unfused parental cells and homokaryons died.

Iodoacetamide and diethylpyrocarbonate inactivate a wide variety of molecules within the cell. The following control experiments suggest that these agents did not have major long-term effects on the regulation of differentiated gene expression. We have shown that treating myoblasts with a dose of either drug that kills 99.9% of the cells does not alter the myogenic potential of the 0.1% surviving cells (22). Homotypic heterokaryons and hybrids formed by fusing iodoacetamide- and diethylpyrocarbonate-treated cells of the same type together retained the ability to differentiate: myocyte × myocyte heterokaryons continued to synthesize myosin light chains (1); myoblast × myoblast hybrids were able to fuse to form myosin containing multinucleated myotubes (22, 25, 26); and adrenal cell × adrenal cell heterokaryons continued to secrete steroids in response to ACTH treatment (27). Inverting the biochemical treatments (i.e., iodoacetamide × diethylpyrocarbonate versus diethylpyrocarbonate × iodoacetamide) produced no difference in the induction of rat myosin light chains in chick myocyte × rat myoblast heterokaryons (1). Finally, in no case has myosin light chain synthesis been induced after treating nonexpressing cells with the biochemical inhibitors (1–3, 27, 28). These combined results suggest that although iodoacetamide and diethylpyrocarbonate have profound short-term effects on viability, their effects on differentiated gene expression is minimal.

This approach using irreversible inhibitors was combined with a partial selection system using the reversible Na/K ATPase inhibitor ouabain (29). Chick cells are much more sensitive to ouabain than rodent cells. Although ouabain did not kill chick cells rapidly enough to be used as the sole selective agent against the chick cells, it did reduce the lethal concentration of the irreversible inhibitors for the chick cells by about threefold (without effecting the lethal dose for treated rodent cells). Plating the heterokaryons in 3×10^{-5} M ouabain for the first 3 d after cell fusion thus permitted the chick cells to be pretreated with a much lower dose of iodoacetamide, which resulted in a much greater efficiency of heterokaryon rescue (24). Approximately 0.5–2% of the initial cells were recovered as viable heterokaryons. Under our fusion conditions (35% vol/vol polyethylene glycol, molecular weight 1,000, 10% vol/vol dimethyl sulfoxide in serum free medium, pH 7.5), ~10% of the cells fuse, of which half should be heterokaryons and the remainder homokaryons. Consequently, about one in five of the heterokaryons actually formed survived the selection protocol. The concentrations of iodoacetamide and diethylpyrocarbonate that gave the highest purity and greatest rescue varied slightly from day to day and between cell types. The specific concentrations used are given in the figure legends.

A flow chart of the experimental protocol is presented in Fig. 1. On day 0, cells were treated with irreversible inhibitors, washed, mixed, fused, and then plated on bacterial-grade plastic to prevent cell attachment during a 2-d recovery period (23). After aliquots (plated on tissue culture grade plastic on day 1) had been analyzed for purity and the efficiency of rescue, the remaining cells in suspension were sedimented onto a Ficoll sodium-diatrizoate cushion in order to remove most of the dead cells (23). The viable cells floating at the interface were harvested, washed, and plated at 60,000 heterokaryon nuclei per cm^2 in gelatinized microtiter wells (0.3 cm^2 surface area). Since ~70% of the heterokaryons were binucleated (4), this represented about 25,000 heterokaryons/cm^2. The following morning (day 3 after fusion) the remaining unattached dead cells were washed away and the heterokaryons were fed fresh ouabain-free medium containing 15% fetal bovine serum. In most experiments this medium also contained 100 μM 5-bromodeoxyuridine (BUdR). BUdR incorporation inhibits cell differentiation (30, 31), and we have shown that the incorporation of BUdR into undifferentiated myoblasts prevents myosin light chain induction after their fusion to differentiated myocytes (28). In high concentrations, BUdR also inhibits cell division. Exposing the cells to 100 μM BUdR thus accomplished the dual objectives of inhibiting the overgrowth of the small proportion of surviving parental cells and inhibiting the differentiation of any cells that did divide. In some experiments these effects were separated by replacing the BUdR with either 10^{-5} M cytosine arabinoside to block DNA synthesis without directly inhibiting differentiation, or with 2 μg/ml cytochalasin B to prevent parental cell overgrowth (by causing dividing cells to become multinucleated [32] which inhibits subsequent division) without inhibiting DNA synthesis. The results of all of these permutations were identical and did not affect the results described below.

Two-Dimensional Gels: The heterokaryons were labeled overnight in 300 μCi/ml of [^{35}S]methionine in methionine-free medium 3–4 d after being plated in microtiter wells (thus on day 5 or 6 of the experiment). The following morning the cells were scraped into low-ionic strength buffer (15 mM KCl, 10 mM Tris, pH 7.5, 0.5% Nonidet P-40, 14 μM 2-mercaptoethanol) in the presence of 15 μg of cold-carrier adult rat cardiac actomyosin. Cell extracts were left in low-ionic strength buffer for several days at 4°C to insure the complete precipitation of even trace amounts of actomyosin. 1–4 d later, the insoluble material was removed by centrifugation and dissolved in O'Farrell's lysis buffer (33). Actomyosin is insoluble at low ionic strength, thus the above procedure removed many of the soluble proteins that migrate in the region of the myosin light chains. Cardiac actomyosin was used as the cold carrier to avoid diluting the radioactive skeletal light chain spots. Cell extracts were analyzed on O'Farrell-type two-dimensional polyacrylamide gels (33) as modified by us (34), employing a mixture of 0.4% pH 3–10 and 1.6% pH 4–6 ampholytes in the isoelectric focusing dimension and a 12½% polyacrylamide Laemmli gel (35) in the second dimension. Dried gels were exposed to Kodak XR-5 medical x-ray film for 12 million cpm × days. The LC1 was chosen as a marker for myogenic expression since the migration of chick fast muscle LC1, rodent fast muscle LC1, and embryonic rodent LC1 are different on two-dimensional gels. Skeletal myosin light chains were identified by their molecular



1. Untreated chick myocyte parental control cells were plated on bacterial dishes but were not treated with ouabain in order to preserve their viability.
2. Untreated parental control cells were not centrifuged onto a Ficoll-sodium diatrizoate cushion.
3. Since cytochalasin B reduced cell attachment, it was not used at this step. Experiments in which cytochalasin B was used to prevent the overgrowth of contaminating parental cells were plated on Day 2 without an overgrowth inhibitor, and were fed cytochalasin B containing medium on Day 3. Other overgrowth inhibitors used were 5-bromodeoxyuridine and cytosine arabinoside.

FIGURE 1 Flow chart of heterokaryon isolation procedure.

weight and isoelectric points, their comigration with the light chains from purified chick or rat thigh actomyosin, their co-precipitation using an antibody against the myosin heavy chain, their co-purification with actomyosin, their absence in undifferentiated myoblasts, and their appearance in differentiated muscle cultures (1).

Acetylcholine Receptor Assays and Creatine Kinase: Total muscle function in the heterokaryons was estimated by two assays that did not distinguish between chick and rat proteins. Acetylcholine receptor was measured by the specific binding of ^{125}I-α-bungarotoxin (2.5×10^{-9} M) in the presence or absence of the competitive inhibitor decamethonium (10^{-5} M) (36). Heterokaryons that were to be analyzed for toxin-binding activity were initially plated overnight in 10^{-8} M unlabeled α-bungarotoxin. This saturates existing receptors essentially irreversibly, so that the appearance of new binding sites during the next 3 d represents the synthesis of new acetylcholine receptors (37).

We measured creatine kinase activity specrophotometrically using a commercial assay (Kit 45-UV, Sigma Chemical Co.) supplemented with 300 μM diadenosine pentaphosphate in order to inhibit adenylate kinase activity (38). To increase the sensitivity, we adjusted the dilution of the kit so that up to 50 μl of a heterokaryon cell extract could be analyzed in a final volume of 175 μl in a microcuvette.

The protein concentration in heterokaryon extracts was determined using fluorescamine (Roche Diagnostics, Nutley, NJ) (39) relative to a bovine serum albumin standard. Since amines such as Tris buffer interfere with this assay, extracts for creatine kinase or acetylcholine receptor activity were prepared in phosphate-buffered solutions. All assays for creatine kinase and acetylcholine activity were performed in triplicate.

Cell Hybrids: Heterokaryons are the initial fusion product between two cells, where both nuclei exist within a common cytoplasm. The dividing progeny of heterokaryons are cell hybrids. Cell hybrids between B9 rat glial cells and L6 rat myoblasts were obtained by first preparing a 95% pure population of heterokaryons using irreversible biochemical inhibitors. The heterokaryons were then plated at a density of two cells per 0.3-cm^2 microtiter well. 3 wk later, ~30% of 200 wells contained colonies. 20 wells containing single colonies were expanded and karyotyped using an in situ technique (40). Four to six metaphase spreads from each colony were counted under direct × 400 observation. Although this approach is not accurate enough to give a precise determination of chromosome numbers, it is easily sufficient to determine whether the colony was roughly diploid or tetraploid. About 50% of the colonies were contaminating parental diploid cells. The remaining tetraploid colonies were then analyzed for their myogenic potential in comparison to homotypic hybrids formed by fusing iodoacetamide-treated L6 myoblasts to diethylpyrocarbonate-treated L6 myoblasts.

Immunoperoxidase Antimyosin Staining: An extremely sensitive clonal immunoperoxidase assay was used to detect myogenic cells. Each hybrid clone was plated at approximately 500 cells per 10-cm dish and allowed to form subclones over a 2-wk growth period. The cells were then fed a differentiation-stimulating medium containing 1% fetal bovine serum and 5 μg/ml insulin (41). The combination of high local cell density and special medium produces a very powerful stimulus to differentiate. Massively fused L6 myotubes frequently detach from the dish within a few days after their formation. The dishes were screened every few days, and dishes in which any areas of myotube detachment were observed were fixed immediately. 1 wk after being fed differentiation-stimulating medium, the remaining dishes were fixed in 50% acetone/ethanol for 3 min at 0°C (42) and processed for immunoperoxidase staining using mouse monoclonal antibody CCM-52 (generously provided by Dr. Rhadovan Zak, University of Chicago). This antibody was raised against purified chick cardiac myosin (43), and cross-reacts with the myosin made in all cultured myogenic cells that we have examined (chick skeletal,

chick cardiac, rat skeletal, and rat cardiac cultures). The following technique was used to stain large surface areas with limited amounts of antibody. A 50 × 20 mm rectangle was scribed onto the surface of each dish using a scalpel blade. This procedure throws up a ridge of plastic that protects the cells from being sheared off the dish after the repeated application and removal of a coverslip. The cells were stained in 50 μl of CCM-52 diluted 1:200 with 1 M lysine pH 7.5 (44) for 90 min at room temperature. The antibody was applied by placing 50 μl of antibody on a 50 × 22 mm glass coverslip, then lowering the inverted dish onto the coverslip until capillarity sucked the coverslip into place. This method reduced the trapping of bubbles under the coverslip. The primary antibody was visualized with a peroxidase-conjugated goat anti-mouse antibody (Cappel Laboratories, Inc., Cochranville, PA) and diaminobenzidine using standard techniques.

The combination of a clonal assay with immunologic staining permits the identification of myogenic cells that differentiate under only the most stringent conditions, where so few adjacent cells differentiate that morphologically identifiable myotubes are not formed. The assay is sufficiently sensitive to identify clones of L6 myoblasts that differentiate with a 10,000-fold reduction in their ability to form myosin-positive cells (companion paper).[2]

RESULTS

Induction of Rat Myosin LC1 Synthesis in Chick Myocyte × B9 Glial Heterokaryons

Although B9 rat glial cells had never differentiated along myogenic lines in their laboratory of origin, myotube formation and muscle protein expression have been observed under the culture conditions of a different laboratory (12). The apparent ability of B9 cells to transdifferentiate led to their selection for our initial studies. We have been unable to obtain spontaneous myotube formation in these cells in our laboratory, even under conditions that normally stimulate myogenic differentiation such as reducing the serum concentration in the presence of 5 μg/ml insulin (41). To determine whether the structural myosin LC1 genes in B9 cells could respond to putative chick inducing factors, we constructed heterokaryons between B9 rat glial cells and differentiated chick myocytes. Purified populations of heterokaryons isolated using irreversible biochemical inhibitors were incubated overnight in [^{35}S]methionine and extracted into a buffer of low ionic strength. The insoluble material which was enriched for actomyosin was then analyzed on two-dimensional polyacrylamide gels. Fig. 2 shows that rat skeletal myosin light chains were induced in these heterokaryons. Because we have previously shown that only a few percent of chick-rodent heterokaryons incorporate [^{3}H]thymidine during the first week after their isolation using irreversible inhibitors (1, 3), and since the cells in the present experiments were cultivated in either 100 μM BUdR or 10^{-5} M cytosine arabinoside following heterokaryon formation, we believe that the induction of rat myosin LCI synthesis in the B9 genome occurred in the absence of appreciable DNA synthesis. These results suggest that structural alterations in chromatin dependent on significant DNA replication may not be prerequisites to the expression of the myosin LC1 genes in B9 glial cells.

Suppression of Myogenesis in Glial × Myoblast Cell Hybrids

The ability of putative chick factors to induce the skeletal myosin LC1 genes in B9 rat glial cells implied that the B9 cells were not producing factors that dominantly suppressed these structural genes. The behavior of the heterokaryons, however, did not indicate whether or not the B9 glial cells were producing dominant suppressive factors that regulated earlier stages in differentiation, such as the decision by undifferentiated but determined myoblasts to initiate the program of terminal differentiation. Preliminary control experiments

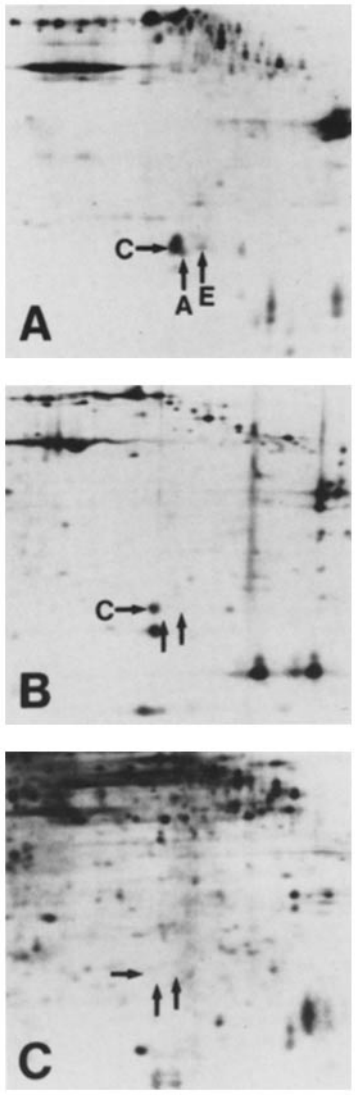


FIGURE 2 Myosin light chain synthesis in heterokaryons formed between B9 rat glial cells and chick skeletal myocytes. Heterokaryons and control cells were cultivated in 10^{-5} M cytosine arabinoside following heterokaryon formation and then labeled with [^{35}S]-methionine and extracted into low ionic strength buffer containing cold-carrier actomyosin. The actomyosin-enriched insoluble material was then analyzed on two-dimensional gels. The region of the gels between pI ~5.6 (*left*) and 4.0 (*right*) is shown. The skeletal myosin light chains are identified as follows: *C*, chick fast muscle LC1; *A*, rat adult fast muscle LC1; and *E*, rat embryonic LC1. Unlabeled arrows indicate the location of light chains that are not present in that particular extract. (*A*) Heterokaryons formed by fusing B9 rat glial cells treated with 0.0045% diethylpyrocarbonate to chick skeletal myocytes treated with 3.8 mM iodoacetamide. (*B*) Chick myocyte control cells. (*C*) B9 rat glial control cells. Rat skeletal myosin light chain synthesis is present only in the heterokaryons. The high concentration of iodoacetamide used to treat the chick cells (compared with the experiments in Fig. 4) is a result of not using ouabain as part of the selective system in this particular experiment.

[2] Wright, W. E. 1984. Control of differentiation in heterokaryons and hybrids involving differentiation-defective myoblast variants. *J. Cell Biol.* 98:436–443.

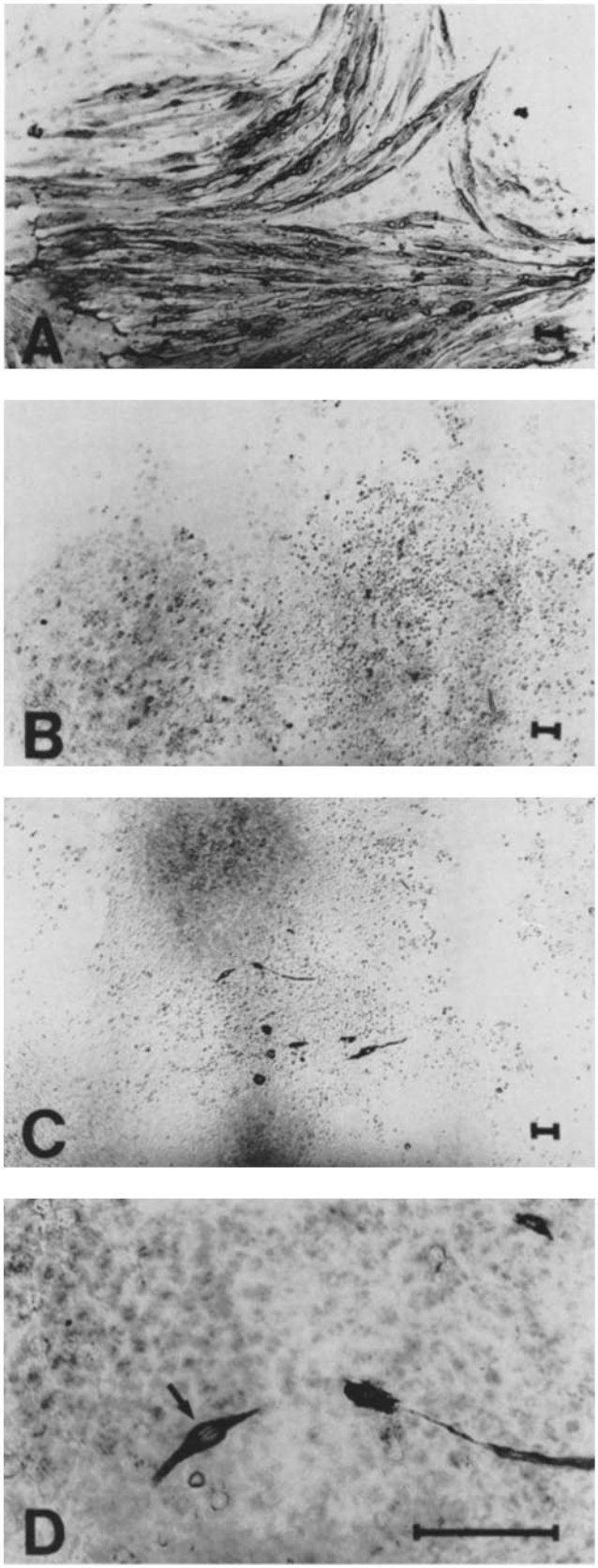


FIGURE 3 Antimyosin staining of hybrid cells. Homotypic and heterotypic hybrid clones were picked and replated at low density. 2 wk later, after large, locally confluent subclones had formed, the cells were fed differentiation-stimulating medium for 1 wk. The cells were then fixed and processed for immunoperoxidase staining using the monoclonal antimyosin heavy chain antibody CCM-52. (*A*) L6 × L6 homotypic hybrid clone 6. (*B*) L6 myoblast × B9 glial cell heterotypic hybrid clone 10. No myosin-positive cells are present. (*C*) L6 myoblast × B9 glial cell heterotypic hybrid clone 3. Nine myosin-positive cells can be seen. (*D*) High power view of the same field shown in (*C*). The arrow indicates a myosin-positive cell in which individual myofibrils can be seen coursing over the cell nucleus. A quantitative analysis of these clones is shown in Table I. Bar, 100 μm. (*A–C*) × 40; (*D*) × 200.

demonstrated that the decision to differentiate required conditions that were not easily obtained using heterokaryons. For example, our differentiation-stimulating conditions employ a decreased serum concentration in the presence of 5 μg/ml insulin (41), which stimulates confluent L6 myoblasts to differentiate to a greater extent than confluence alone. However, the heterokaryon cell density of approximately 25,000 cells per cm^2 that we were able to achieve corresponded to cells that are only about one-quarter confluent. We found that L6 myoblasts plated at 25,000 cells/cm^2 and fed medium containing 1% fetal bovine serum and 5 μg/ml insulin did not differentiate until after they had divided several times and reached confluence. Inhibiting cell division of these freshly plated L6 myoblasts by adding cytosine arabinoside (10^{-6} or 10^{-5} M) to the differentiation-stimulating medium prevented the cells from reaching confluence and also prevented significant numbers of cells from differentiating and forming myotubes. It was thus clear that the experimental conditions we obtained using heterokaryons would not provide an adequate test of the ability of even authentic myoblasts to make the decision to differentiate in the absence of cell division. Heterokaryons that divide to reach confluence are by definition no longer heterokaryons but cell hybrids, thus it was necessary to examine the capacity of cell fusion products to make the decision to differentiate in cell hybrids rather than heterokaryons. We have previously shown that <10% of the heterokaryons formed by fusing chick myocytes to rat fibroblasts were able to incorporate tritiated thymidine (4), even though the myogenic program had been suppressed in these cells. Others have shown that cell hybrids formed between chick and rodent cells rapidly segregate virtually the entire chick genome except the specific gene required for growth in the selection procedure (e.g., chick hypoxanthine-guanine phosphoribosyl transferase) (45, 46). It was thus probable that heterokaryons formed by fusing B9 glial cells to chick myoblasts either would not divide at all or would yield uninformative hybrids that had lost the chick genome. We thus decided to examine the regulation of the decision to differentiate in cell hybrids constructed between B9 glial cells and L6 myoblasts. Since these are both rat cell lines, the frequency of hybrid formation would be high and the preferential loss of one set of parental chromosomes would be avoided. Since the B9 glial cells do not respond to myogenesis-stimulating medium under our laboratory conditions, the decision to differentiate could be examined in the hybrids between responsive L6 myoblasts and nonresponsive B9 glial cells.

Cell hybrids were constructed by first isolating heterokaryons between undifferentiated L6 rat myoblasts and B9 glial cells. The heterokaryons were then cloned and karyotyped to verify that they were hybrids and not contaminating parental cells. The ability of the hybrid clones to initiate terminal differentiation was examined by exposing large, locally confluent subclones to myogenesis-stimulating conditions and then staining them with an antimyosin antibody (Fig. 3).

TABLE I

Differentiation Capacity of Cell Hybrids

Combination	Clone	Number of subclones*		% Myosin (+) subclones	Typical colony size‡	Total number of myosin (+) cells	% Myosin (+) cells§
		Myosin (+)	Myosin (−)				
L6 Myoblast × B9 glial cell	3	9	183	5	8,513	33	0.002
	5	0	87	0	7,821	0	0
	10	0	114	0	5,624	0	0
	13	0	49	0	1,423	0	0
L6 myoblast × L6 myoblast	1	280	1	99	634/230	—‖	73‖
	4	107	6	95	168/333	—	32
	6	62	18	77	892/326	—	56
	8	116	3	97	677/44	—	91

* Dividing cell hybrids were picked, cloned, and karyotyped to verify their hybrid status. Each clone was then subcultivated at low density to form multiple subclones. After large, locally confluent subclones had formed, the cells were fed differentiation-stimulating medium for 1 wk and then fixed and stained with an antimyosin antibody. The number of myosin (+) cells was then determined. Four hybrid clones of each combination were randomly selected for analysis.

‡ Colonies appearing to be of average size were selected under macroscopic observation. The colony was circled, and the number of cells counted under × 400 observation.

§ Total % myosin (+) cells were calculated by dividing the total number of myosin (+) cells by the product of the total number of subclones counted and the typical number of cells per subclone, × 100.

‖ Typical colony sizes are here expressed as a ratio of myosin (+) to myosin (−) cells. Since the number of myosin (+) cells in this combination was too great to permit the total number to be actually counted, the % myosin (+) cells was calculated by first determining the % myosin (+) cells in a typical subclone, and then multiplying this number by the % of myosin (+) subclones.

Table I shows that although L6 × L6 homohybrid controls isolated using the same protocol continued to exhibit high levels of myogenesis, muscle differentiation was suppressed in the B9 × L6 hybrids. Thus, although the B9 genome could respond to internal inducing factors presumably provided by the already differentiated chick myocytes in heterokaryons, dividing cell hybrids of B9 cells fused to undifferentiated myoblasts were unable to respond to the external environmental signals that normally stimulate myogenic differentiation. This suggests that B9 cells were producing factors that dominantly suppressed the early stages of terminal myogenesis but not the later stages involving the activation of the structural myosin LC1 genes. This comparison provides a good illustration of the important functional differences between heterokaryons and cell hybrids and the type of regulatory information that can be obtained from each (24).

Myosin LC1 Synthesis in Heterokaryons between Chick Myocytes and Other Neural Lines

Several other neural cell lines were examined to determine whether the ability to induce the synthesis of skeletal myosin light chains in B9 glial cells was a general characteristic of neural cells or a unique property of this unusual cell line. To do this, heterokaryons between chick myocytes and other neural cells were isolated, then labeled with [^{35}S]methionine, extracted, and analyzed on two-dimensional polyacrylamide gels. Fig. 4*A* shows that both embryonic and adult rat LC1 were induced in heterokaryons between rat C6 astrocytoma cells and differentiated chick myocytes. The induction of the light chains was not a simple consequence of the biochemical treatments, since homokaryons formed by fusing together diethylpyrocarbonate- and iodoacetamide-treated C6 cells did not exhibit any light chain synthesis (Fig. 4*B*). Rodent skeletal myosin light chain was also induced in heterokaryons involving the NIE-115 mouse adrenergic neuronal cell line (Fig. 4*C*). Not all neuronal cell lines were inducible, however. Not only were heterokaryons between S-20 mouse cholinergic neurons and chick myocytes unable to synthesize mouse light chains, but chick light chain synthesis was suppressed in this combination as well (Fig. 4*D*).

The above cell lines were useful for these studies because they represented cloned, relatively homogeneous populations. However, since all of them were derived from tumors and most of them had grossly abnormal chromosome constitutions, it is possible that the expression of differentiated functions in these cells did not reflect normal regulatory controls. To verify that the induction of skeletal myosin light chains in these cell lines also occurred in normal diploid cells, heterokaryons were constructed between differentiated chick myocytes and rat whole brain primary cultures. In spite of the extreme heterogeneity of cell types within these primary brain cultures, both embryonic and adult rat skeletal myosin light chains were induced (Fig. 4*E*). Control extracts from [^{35}S]-methionine-labeled brain cultures demonstrated the absence of low-ionic strength insoluble proteins migrating in the region of the skeletal myosin light chains (Fig. 4*F*). Similar controls for the other cell lines also indicated that the induced light chains were not simply misidentified spots corresponding to endogenous brain proteins (data not shown).

The heterokaryons illustrated in Figs. 2 and 4 were treated with either 100 μM BUdR or 10^{-5} M cytosine arabinoside between days 2 and 6 of the experiment in order to prevent overgrowth of the ~5% contaminating parental cells. Muscle differentiation normally is stimulated by conditions that reduce cell division (e.g., low serum, confluence). To verify that the induction of muscle genes in neural cells was not a consequence of treatment with DNA-synthesis inhibitors, we used cytochalasin B to prevent overgrowth. Cytochalasin B blocks cytokinesis (32), so that cells that synthesize DNA can proceed through karyokinesis and become binucleated. These binucleated cells have a much reduced growth rate, so that treatment with cytochalasin B effectively permits contaminating parental cells to divide at most once during the course of the experiment even though DNA synthesis is not directly affected. The induction of rodent myosin LC1 in B9, C6, and NIE-115 cells also occurred in heterokaryons treated with cytochalasin B between days 3 and 6 of the experiment (data

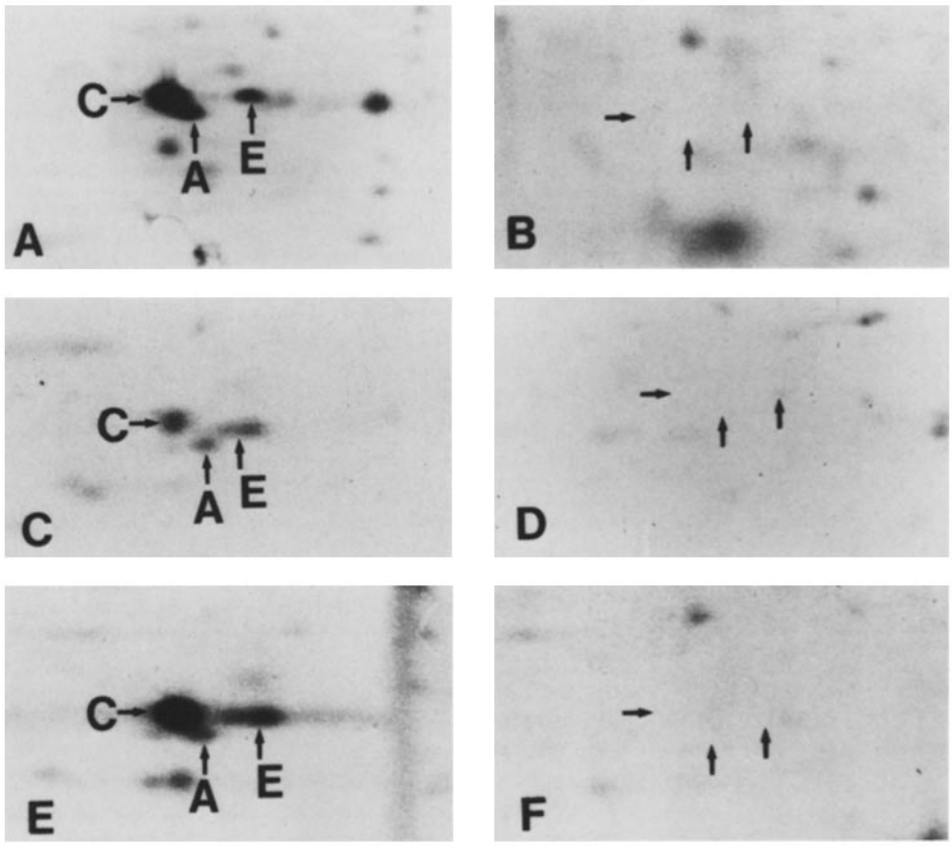


FIGURE 4 Myosin light chain synthesis in heterokaryons involving additional neural cells. Heterokaryons were analyzed as in Fig. 1. Only the skeletal myosin light chain region of the gel is shown. Light chains are labeled as in Fig. 1. (*A*) Heterokaryons plated in 100 μM BUdR formed by fusing C6 rat astrocytoma cells treated with 0.0035% diethylpyrocarbonate to chick skeletal myocytes treated with 1.8 mM iodoacetamide. (*B*) Homotypic heterokaryons plated in 100 μM BUdR formed by fusing 5.5 mM iodoacetamide-treated C6 rat astrocytoma cells to 0.0035% diethylpyrocarbonate-treated C6 astrocytoma cells. (*C*) Heterokaryons plated in 10^{-5} M cytosine arabinoside formed by fusing the mouse NIE-115 adrenergic neuronal cell line treated with 0.0023% diethylpyrocarbonate to chick skeletal myocytes treated with 2 mM iodoacetamide. (*D*) Heterokaryons plated in 100 μM BUdR formed by fusing the mouse S-20 cholinergic neuronal cell line treated with 0.0019% diethylpyrocarbonate to chick skeletal myocytes treated with 1.1 mM iodoacetamide. (*E*) Heterokaryons plated in 100 μM BUdR formed by fusing normal diploid 16-d embryonic rat brain cells treated with 0.0035% diethylpyrocarbonate to chick skeletal myocytes treated with 2 mM iodoacetamide. (*F*) Control unfused 16-d embryonic rat brain cells plated in 100 μM BUdR. Rodent skeletal myosin light chain synthesis is induced in all of the combinations except the homotypic control heterokaryons between C6 astrocytoma cells and themselves, heterokaryons involving the S-20 cholinergic cells, or control unfused cells.

not shown). The induction of myosin LC1 synthesis in neural cells following their fusion to differentiated chick myocytes thus occurs in the presence of DNA synthesis inhibitors but is not a consequence of their presence. In all cases, control parental neural cells were treated with the appropriate agent (BUdR, cytosine arabinoside, or cytochalasin B), and in no instance was myosin LC1 synthesis observed in these controls.

Total Muscle Function in Heterokaryons

The continued synthesis of chick myosin light chains in all of the heterokaryons except those involving S-20 implied that most neural cells were not producing molecules that dominantly suppressed muscle protein synthesis. To determine whether this applied to other muscle proteins, we examined the levels of creatine kinase and acetylcholine receptor activity as a measure of total myogenic expression in the heterokaryons. Table II shows that the total myogenic expression in all of the neural × myocyte heterokaryons except those involving S-20 was equivalent to that found in heterokaryons between undifferentiated L6 rat myoblasts and chick myocytes. These assays did not distinguish between rat and chick proteins, or between muscle and brain forms of creatine kinase. The creatine kinase activity in the heterokaryons between C6 cells and chick myocytes was uninformative, since the parental neural cells had a high background level of creatine kinase activity. Nonetheless, within these limitations, the results indicate that three of the four neural cell lines examined were not actively suppressing myogenic functions.

DISCUSSION

There are at least three possible patterns of muscle gene expression in heterokaryons between differentiated chick myocytes and rodent cells of other developmental lineages. All muscle protein synthesis could be suppressed, chick muscle functions could continue in the absence of rodent muscle gene expression, or chick muscle functions could continue and rodent muscle genes could be induced. The first possibility would suggest the dominant suppression of the structural genes of alternate pathways, the second a gene regulatory mechanism incapable of responding to the putative inducing factors provided by a differentiated muscle cell, and the third an "open" gene. Although part of the program of cell differentiation in some developmental lineages (S-20 cholinergic neurons and fibroblasts [4]) apparently involves the dominant suppression of the structural genes of alternate pathways, other lineages (B9 glial, C6 astrocytoma, and NIE-115 adrenergic neuronal cells) permit some nonexpressed differentiated genes to remain in an inducible, nonsuppressed form.

The present results show that the myosin light chain one structural gene can be induced in neural cells in the presence of inhibitors that reduce DNA synthesis by more than 95%. This suggests that DNA-synthesis–dependent gene modifica-

TABLE II

Creatine Kinase and ^{125}I-α-Bungarotoxin Binding in Heterokaryons

	% of myocyte × myoblast control*	
	Creatine kinase	^{125}I-α-bungarotoxin binding
Parental cells		
Chick myocytes	230	135
L6 Rat myoblasts	3	<1
B9 Rat glial cells	2	<1
C6 Rat astrocytoma cells	180	<1
N1E-115 Mouse adrenergic neurons	3	<1
S-20 Mouse cholinergic neurons	20	<1
Heterokaryons		
Myocyte × L6	100	100
Myocyte × B9	95	70
Myocyte × C6	270	190
Myocyte × N1E-115	145	50
Myocyte × S-20	20	<1

* Each experiment contained an internal positive control fusion between chick myocytes and rat myoblasts. The actual values obtained varied widely between experiments, presumably due to variations in the degree of differentiation in the chick myocytes, which were prepared as primary cultures on a weekly basis. To permit a direct comparison between experiments, the results have been normalized with respect to this internal positive control. Actual values can be estimated from the average results of the myocyte × myoblast control of 110 ± 70 mU/mg of creatine kinase and 40 ± 15 fmol/mg of ^{125}I-α-bungarotoxin binding (±1 SD, six independent experiments).

tion is probably not required before the activation of transcription. If DNA synthesis is required to alter cytidine methylation patterns (47), this result provides evidence that cytidine methylation may not be directly involved in regulating the normal lack of expression of the skeletal myosin light chain genes in neural cells.

Previous somatic cell hybridization studies have indicated that differentiated functions are generally suppressed in dividing cell hybrids formed by fusing cells from different histiotypes (48). The suppression of myogenesis in B9 glial cell × L6 myoblast hybrids was consistent with this observation. In contrast, rat skeletal myosin light chains were induced in the initial fusion product (heterokaryons) formed by combining rat B9 glial cells with differentiated chick myocytes. Although different myogenic cells were used in the two sets of experiments (L6 rat myoblasts versus chick myocytes), it is not necessary to postulate species differences to explain the divergent results. The suppression of myogenesis in the B9 glial × L6 rat myoblast hybrids probably results from either a relatively specific suppression of the commitment to terminal myogenic differentiation or an inability to recognize or respond to differentiation signals from the external environment. Neither of these mechanisms in cell hybrids would allow one to predict the behavior of the heterokaryons, in which the B9 genome is exposed via the internal cytoplasmic environment to putative inducing signals provided by the already differentiated myocyte. This result illustrates the different but complementary information that can be obtained from cell hybrids versus heterokaryons.

The expression of differentiated functions other than those of the tissue of origin in many tumors has contributed to a long-standing controversy over whether or not differentiation is truly irreversible. Our demonstration that at least some genes from alternate developmental pathways are inducible in certain neural cells increases the probability that transdifferentiation events are possible. The lack of a rigid suppression of myogenic genes in these cells would greatly increase the rate at which transdifferentiation events could occur. It thus becomes unnecessary to postulate the presence of primitive stem cells to explain the origin of aberrant tissue. For example, it is known that the muscles of the head and neck are derived from the neural crest (49). The presence of primitive neural crest stem cells has been invoked to explain the appearance of skeletal muscle fibers in some neural tumors (8) and in cultures from optic nerve (9), pituitary (10), and thymus (48) (the thymic stroma is derived from the neural crest [49]). Our results demonstrating the induction of myosin light chain structural genes in clonal cell lines expressing differentiated neural functions indicate that the muscle fibers in the above situations need not have come from primitive stem cells but could have arisen from transdifferentiation events.

These results may also provide insights into the etiology of myasthenia gravis. The presentation of muscle antigens by aberrant muscle fibers forming within the immunologically privileged thymus has been hypothesized to play an important role in the generation of antiacetylcholine receptor antibodies in myasthenia gravis (51). These autoantibodies are thought to be responsible for the muscle weakness in the disease. Our results suggest the possibility that myasthenia gravis may result from the transdifferentiation of thymic stromal cells into skeletal muscle rather than from the abnormal differentiation of primitive stem cells.

It is tempting to postulate a unique regulation of the myosin light chain genes in neural-crest–derived cells. However, recent evidence from this laboratory (27) indicates that myosin light chain genes are inducible in heterokaryons formed by fusing some nonmyoblast mesodermal derivatives to differentiated chick myocytes, whereas other mesodermal derivatives suppress myogenic expression. The regulation of the muscle structural genes in various differentiated ceils thus appears to exhibit a complex pattern that does not reduce to a simple formulation such as neural crest versus mesodermal derivation. This regulation is clearly not uniform, but may reflect many different methods of regulating gene expression. It is hoped that establishing a developmental hierarchy of the control of the skeletal muscle structural genes will help elucidate the different mechanisms by which differentiated cells specifically express their own and not alternate developmental options.

In conclusion, we have shown that chick myosin light chain synthesis is not suppressed and that rodent myosin light chain synthesis is induced in heterokaryons formed by fusing differentiated chick myocytes to a variety of neural cells. These results imply that these neural cells do not produce molecules that dominantly suppress myogenic structural genes in heterokaryons, that the myosin light chain structural gene can respond to inducing factors in the probable absence of DNA synthesis, and that the differentiated program in these cells is considerably more plastic than previously believed.

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