

Heterokaryon Analysis of Muscle Differentiation: Regulation of the Postmitotic State

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Abstract. MM14 mouse myoblasts withdraw irreversibly from the cell cycle and become postmitotic within a few hours of being deprived of fibroblast growth factor (Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka, 1987, *J. Cell Biol.*, 105:949–956). To examine the mechanisms that may regulate this developmental state of skeletal muscle, we tested the mitogen responsiveness of various cell types after their polyethylene glycol-mediated fusion with postmitotic myocytes. Heterokaryons containing myocytes and quiescent nonmyogenic cells such as 3T3, L cell, and a differentiation-defective myoblast line (DD-1) responded to mitogen-rich medium by initiating DNA synthesis. Myonuclei replicated DNA and reexpressed thymidine kinase. In contrast, (myocyte x G₁ myoblast) heterokaryons failed to replicate DNA in mitogen-rich medium and became postmitotic. This included cells

with a nuclear ratio of three myoblasts to one myocyte. Proliferation dominance in (myocyte x 3T3 cell) and (myocyte x DD-1) heterokaryons was conditionally regulated by the timing of mitogen treatment; such cells became postmitotic when mitogen exposure was delayed for as little as 6 h after cell fusion. In addition, (myocyte x DD-1) heterokaryons expressed a muscle-specific trait and lost epidermal growth factor receptors when they became postmitotic. These results demonstrate that DNA synthesis is not irreversibly blocked in skeletal muscle; myonuclei readily express proliferation-related functions when provided with a mitogenic signal. Rather, myocyte-specific repression of DNA synthesis in heterokaryons argues that the postmitotic state of skeletal muscle is regulated by diffusible factors that inhibit processes of cellular mitogenesis.

THE differentiation of many tissues is accompanied by a decrease in proliferative activity. While some cell types maintain the potential for replication after their terminal differentiation, a regulatory mechanism operating in tissues like skeletal muscle causes a permanent loss in proliferative capacity. For MM14 mouse myoblasts the postmitotic state is induced by depleting the culture environment of fibroblast growth factor (FGF)¹; within 2–3 h of such treatment G₁ myoblasts become postmitotic (3, 16, 17). Reexposing such cells to FGF and other growth factors fails to stimulate their replication and no longer represses muscle gene expression or myogenic fusion. Although MM14 is a permanent cell line, its behavior with respect to FGF-mediated aspects of replication and differentiation appears similar, if not identical, to primary mouse myoblasts (16).

The mechanism by which myocytes acquire a postmitotic phenotype is unknown. It is not due to an irreversible block in DNA synthesis, per se, because cellular, viral, and/or repair DNA synthesis appears to occur in myonuclei after ex-

posure to oncogenic viruses (6, 9), and after hybridization of muscle with replicating fibroblasts (21). The postmitotic state could, however, be due to the inactivation or loss of one or more components involved in mitogenic signaling. Consistent with this hypothesis are observations that correlate the loss in growth factor receptors with the acquisition of the postmitotic state. MM14 myoblasts permanently lose FGF and epidermal growth factor (EGF) receptors shortly after FGF removal (Olwin, B. B., and S. Hauschka, unpublished results; 14), whereas variant mouse myoblasts, which become quiescent after mitogen deprivation, neither lose growth factor receptors nor become postmitotic (15).

Construction of heterokaryons allows manipulation of the intracellular signals that regulate the postmitotic state of muscle. Because this technique mixes heterologous cytoplasm and surface receptors, yet maintains nuclear integrity, cells of dissimilar physiological states can be combined to determine whether specific traits expressed by either parental cell are maintained, extinguished, or induced in the nonexpressing partner (4). Thus, a heterokaryon analysis tests for the presence (or absence) of diffusible components that control various physiological and developmental processes. Heterokaryon analyses of DNA synthesis have shown that cells in late G₁ and S phase possess factors that induce replication in a variety of nonproliferating cell types (20). In

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1. *Abbreviations used in this paper:* EGF, epidermal growth factor; F10C, Ham's F10 supplemented with 0.8 mM CaCl₂; FGF, fibroblast growth factor; [³H]Tdr, [³H]thymidine; tk, thymidine kinase.

		G ₁ Myoblast Parental Phenotypes		
		True	False	
Myocyte Parental Phenotypes	True = 0.96 ^a	0.82 ^f	0.04 ^g	0.11 ^h
	False = 0.04 ^b	0.03 ⁱ	<0.01 ^j	<0.01 ^k
		0.85 ^c	0.04 ^d	0.11 ^e

Predicted Dikaryon Frequencies

Figure 1. Method for calculating heterokaryon frequencies. a, True myocytes, myocytes that failed to replicate DNA; b, false myocytes; myocytes that replicated DNA (i.e., myoblasts); c, true G₁ myoblasts that replicated DNA; d, false G₁ myoblasts that failed to replicate DNA (i.e., behaved like a myocyte); e, false G₁ myoblasts that had entered S phase at the time of PEG treatment (0.02), and G₁ myoblasts that had responded to mitogens before fusion and had become committed to replicate DNA (0.09); f, frequency of true (myocyte x G₁ myoblast) heterodikaryons (a x c); g, frequency of false heterokaryons in which the G₁ parental cell was actually a myocyte (a x d); h, frequency of false heterokaryons in which the G₁ parental cell had either entered S phase, or had become committed to enter S phase (a x e); i, frequency of false heterokaryons in which the myocyte parental cell was actually a myoblast (b x c); j, frequency of "double-false" heterokaryons (too rare to include in data correction, b x d); k, frequency of false heterodikaryons in which neither cell is a myocyte (insignificant, b x e).

studies of muscle differentiation, heterokaryons have indicated the existence of factors that activate the expression of muscle genes in myoblasts and various nonmuscle cells (1, 2, 22–24), as well as regulators that extinguish muscle-specific gene expression (12, 13).

If FGF deprivation generates an activity in myoblasts which inhibits mitogenesis, then newly differentiated myocytes should prevent the induction of DNA synthesis in heterokaryons containing mitogen-responsive cells. The following experiments test this hypothesis.

Materials and Methods

Cell Lines

The derivation, behavior, and culture conditions for MMI4D mouse myoblasts have been described (3, 8, 17). The experiments in this report used a near tetraploid subclone, MMI4DZ. DD-1 cells are differentiation-defective myoblasts isolated from MMI4DZ myoblasts (15). BALB/c 3T3 mouse cells were a gift from R. Ross (University of Washington), and LMtk⁻ cells were a gift from R. Palmiter (University of Washington). Growth medium (or mitogen-rich medium) contained 81% Ham's F10 supplemented with 0.8 mM CaCl₂ (F10C), 15% horse serum, 3% chick embryo extract, 1% penicillin G (10,000 U/ml), and streptomycin sulfate (0.5 mg/ml). Recent experiments have demonstrated that picomolar amounts of pure bovine fibroblast growth factor substitute totally for embryo extract (3). Differentiation was induced in myoblast cultures by feeding medium lacking FGF or embryo extract (F10C + 5% horse serum), or conditioned medium (17).

Cell Synchronization

G₁ Cells. Cultures were grown exponentially to a density of $\sim 2 \times 10^5$ cells per 100-mm plate. Mitotic cells were detached from the plate surface by gently rocking the dishes, and the culture medium was collected on ice. After centrifugation, cell pellets were resuspended in growth medium, plated for 1 h to allow division, then the synchronized G₁ cells were dis-

sociated and combined with myocytes for heterokaryon construction. The original mitotic cell population was always >95% pure based on the percentage of cells undergoing cytokinesis and the absence of [³H]thymidine ([³H]Tdr) incorporation into DNA.

Myocytes. Proliferating myoblast cultures were rinsed twice with F10C and fed differentiation medium 24 h before harvesting. These cultures were routinely passaged to lower densities (12 h postfeeding) to prevent extensive myotube formation. As a result the myocyte population was enriched for cells containing 1–2 nuclei. After 24 h in mitogen-depleted medium, $\sim 95\%$ of the mononucleated cells expressed myosin and were postmitotic.

Quiescent Cells. Proliferating cultures of DD-1 and 3T3 cells were rinsed twice with F10C and fed F10C plus 1% horse serum for a minimum of 48 h. After this treatment <1% of the culture was replicating DNA, yet >95% resumed proliferation when fed growth medium.

Heterokaryon Formation

Myocytes and G₁ cells were combined in mitogen-rich medium and plated at a concentration of $1-2 \times 10^5$ cells into a 1-cm cloning cylinder placed in the center of a culture dish. Ratios of myocytes to G₁ cells ranged from 1:1 to 3:1. After 1 h the cylinder was removed, the plate surface was rinsed twice with F10C, then covered with 1 ml polyethylene glycol 1,000 (PEG) (Sigma Chemical Co., St. Louis, MO) in F10C (52% wt/vol). 1 min later the plate was rinsed five times with 5 ml F10C and twice with whole medium. The cells were passaged 30–45 min after PEG treatment and incubated in mitogen-rich medium containing 1 μ Ci/ml [³H]Tdr (New England Nuclear, Boston, MA) for a minimum of 30 h. The cultures were then rinsed twice with isotonic saline and fixed with 70% ethanol/glacial acetic acid (7:1).

Cytoplasmic Markers

Green and red fluorescent microspheres (0.25- μ m diameter; Polysciences, Inc., Warrington, PA) were sterilized in 70% ethanol, then diluted into culture medium to give a final bead dilution of 1/500–1/1000. Cultures were given the appropriate color microspheres 24 h before cell fusion. Throughout the experiment >99% of all cells contained microspheres. The number of beads per parental cell always exceeded 10; thus the occurrence of an occasional odd colored bead in a background of the opposite color was not considered sufficient evidence for designating a cell as a heterokaryon. Spurious uptake of microspheres of the opposite color was, in fact, not a serious problem since <1% of the myocyte homokaryons (identified by nuclear markers) contained beads of the opposite color.

Nuclear Markers

Cultures destined to become the G₁ or G₀ parental population were exposed to 0.05 μ Ci/ml [³H]Tdr 24 h before mitotic shake-off, or at the time of serum deprivation. This treatment results in lightly labeled nuclei following the standard autoradiography exposure period. Cultures used for myocyte populations received no [³H]Tdr and therefore remained unlabeled. Autoradiography was performed as follows. Fixed cultures were extracted several times with 70% ethanol, coated with NTB2 emulsion (Kodak, Rochester, NY), and stored at 4°C for ~ 1 wk. Because three different nuclear labeling patterns (unlabeled, lightly labeled, and heavily labeled) are required for this analysis, control plates were used to ensure proper exposure and development times.

EGF Receptor Assay

EGF-binding capacity was measured using the procedure previously described (14). Briefly, cultures were rinsed twice with F10C plus 0.5% horse serum, then incubated for 1 h at 37°C with 2 ml of binding medium containing F10C plus 0.1% BSA and $\sim 1.0 \times 10^6$ cpm/ml of [¹²⁵I]-EGF (Collaborative Research, Lexington, MA). Plates were rinsed thoroughly and fixed with 2% glutaraldehyde. After autoradiography heterokaryons and control homokaryons were identified by fluorescence, photographed under bright field optics, then scored for silver grains. Grains specific for EGF receptor were quantitated by subtracting the average number of grains counted above three comparable areas of plate surface from the total grains scored per cell. Background grains accounted for <20% of the total grains counted.

Heterokaryon Data Analysis

After autoradiography cultures were lightly stained with hematoxylin. Cells were first observed using bright-field optics to score the pattern of nuclear

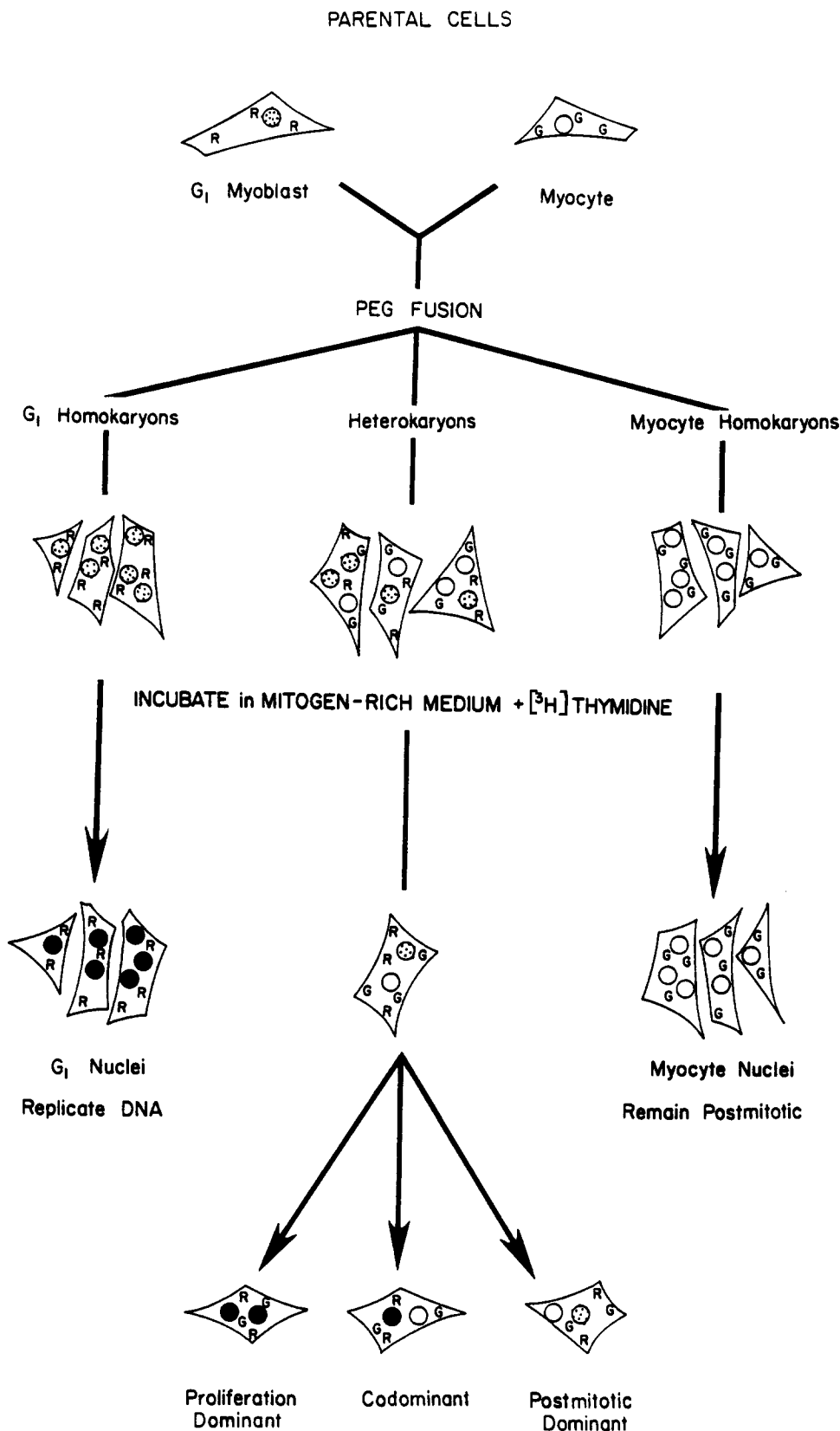
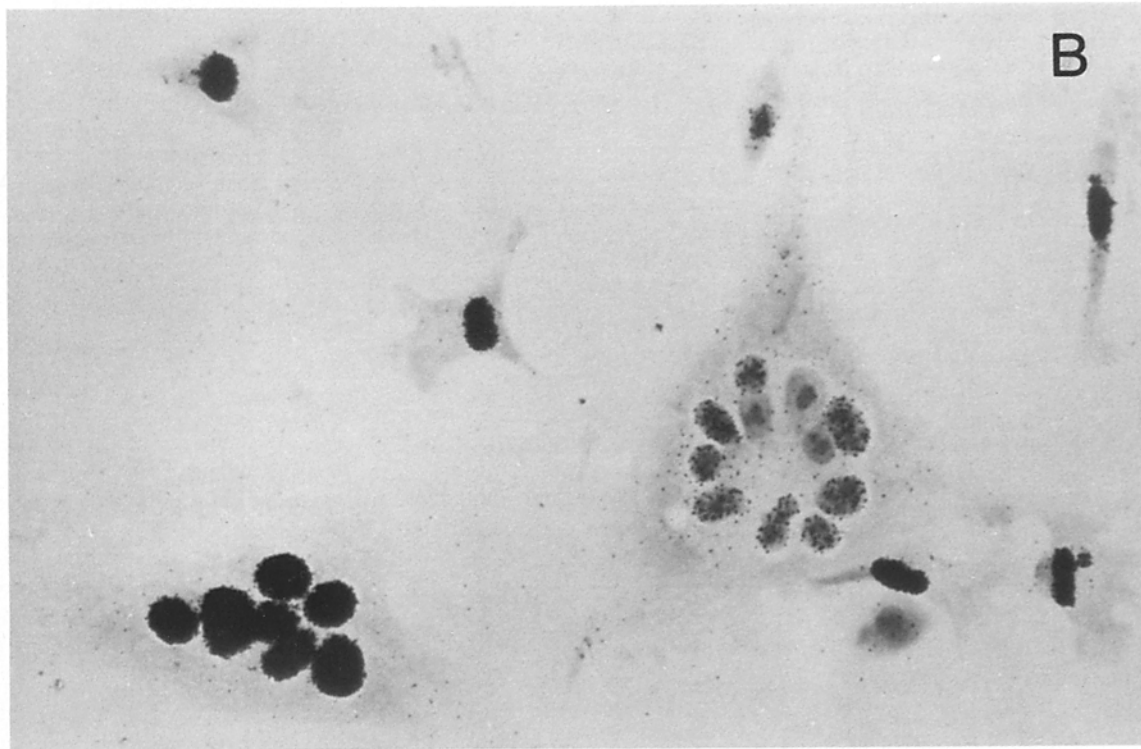
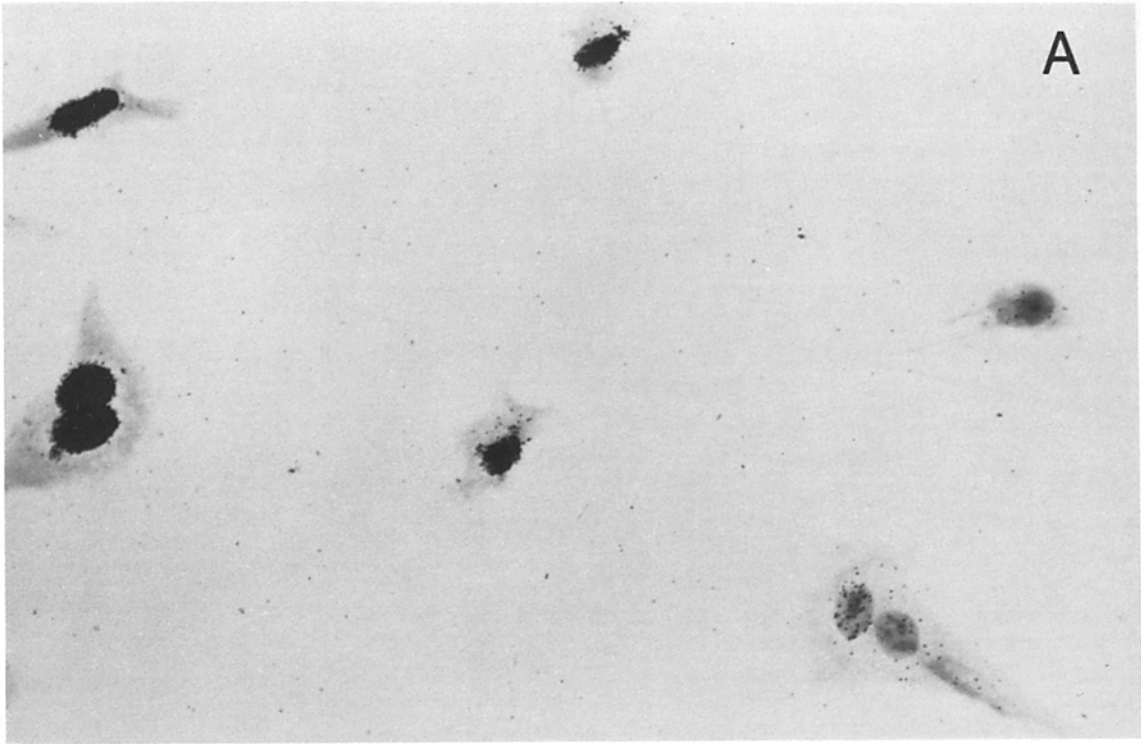


Figure 2. Experimental design. To test for dominance between cell proliferation and the postmitotic state of muscle, G₁ myoblasts and myocytes were given nuclear ($[^3\text{H}]\text{Tdr}$) and cytoplasmic (red or green fluorescent beads) markers, treated with PEG, then incubated for 30 h in mitogen-rich medium containing $[^3\text{H}]\text{Tdr}$. After such treatment the nuclei of all G₁ homokaryons should replicate DNA and become heavily labeled after autoradiography. Myocyte homokaryons, however, should remain postmitotic and unlabeled. The behavior of these and the unfused parental cells acts as an internal control and aids the interpretation of the three possible phenotypic patterns that can be expressed in heterokaryons (see Materials and Methods). The behavior of larger multinucleated heterokaryons in which either parental nucleus may be under or overrepresented is examined for quantitating cell dosage effects.

labeling, then viewed using the appropriate filters for red and green fluorescence. For calculating the frequency of DNA synthesis in parental cells, it was assumed that a proliferative cell would complete one doubling during the 30-h incubation period and thus give rise to two labeled daughter cells.

Thus the percentage of cells replicating DNA equals $(0.5 \times \text{number labeled cells}) / (0.5 \times \text{number labeled cells} + \text{number unlabeled cells})$. This calculation is based on the observation that mitotically synchronized MM14 myoblasts, as well as serum-starved fibroblasts, demonstrated a lag of 10 h be-



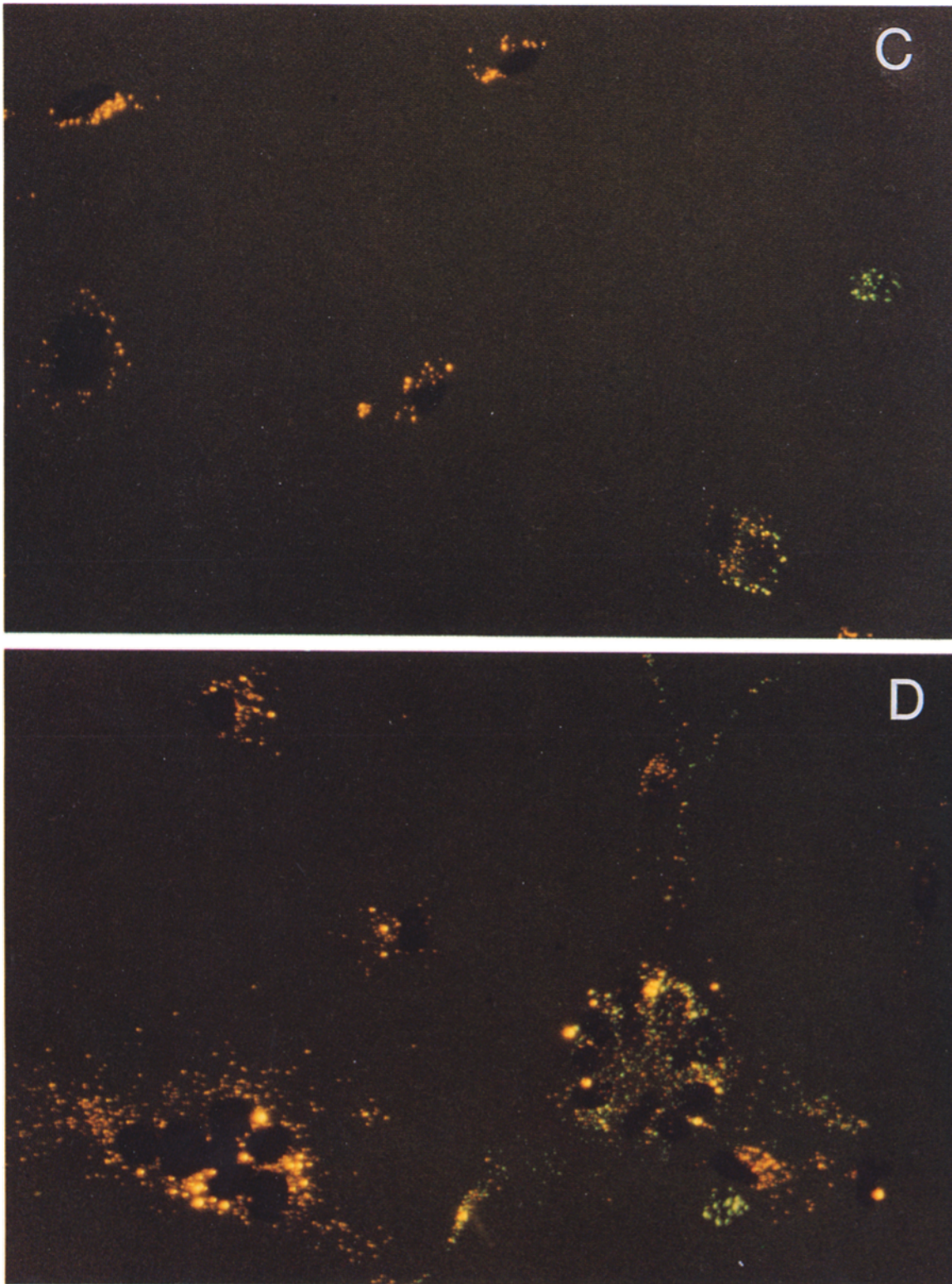


Figure 3. The postmitotic phenotype is expressed in (myocyte x G₁ myoblast) heterokaryons. After the procedure outlined in Fig. 2, cells were assayed for DNA synthesis. (*A* and *B*) Cells containing a mixture of unlabeled and lightly labeled nuclei are identified as heterokaryons that failed to replicate DNA. Nuclei in cells that synthesized DNA became heavily labeled, in this case heterokaryons could be identified by their microsphere labeling pattern. (*C* and *D*) Fields shown in panels *A* and *B*, respectively, photographed under fluorescent light (double exposure). Heterokaryons contain both red and green microspheres, while myoblast (red) and myocyte (green) homokaryons possess only a single bead color. The yellowish color of some of the red beads results from an overexposure after photography with both red- and green-sensitive filters. The results indicate that G₁ myoblast homokaryons containing one, two, or even eight nuclei initiate DNA synthesis when incubated in mitogen-rich medium. Heterodikaryons, and multinucleated heterokaryons containing excess myoblast nuclei, however, express a postmitotic phenotype.

fore entering S phase. The average cell could thus complete one cell cycle after [³H]Tdr addition, but its daughter cells would still be short of completing a second cycle by the 30-h fixation period.

Determination of Dominance Frequencies in Heterokaryons

Theoretically, each parental population should be homogeneous; but in practice this was never achieved. Thus to quantitate dominance effects, apparent heterokaryon behavior was adjusted for contributions due to the heterogeneity within the parental cells. For example, if the parental myocytes contained a low percentage of myoblasts, then when fused with G₁ myoblasts a predictable percentage of the apparent heterokaryons would actually be myoblast "homokaryons". Assuming parental cells fuse randomly to form dikaryons, the false heterokaryon classes were calculated (Fig. 1) and the observed heterokaryon data corrected as described. For the sample calculation, the observed parental cell and heterokaryon frequencies were from the (myocyte x G₁ myoblast) experiment reported in Figs. 3 and 4. (a) The frequencies of all phenotypes in each parental class were determined. For the myocyte parental population this consisted of 0.96 "true" myocytes and 0.04 "false" myocytes that replicated DNA. For the G₁ myoblast parental populations the phenotypic frequencies were 85% true G₁ cells and 15% false G₁ cells. The false category consisted of two types: 4% of the cells that failed to enter S; and (as determined from parallel plates) 11% that had responded to mitogens before PEG treatment by either replicating DNA (2%) or by committing to replicate DNA (9%). None of these false G₁ cells would provide legitimate tests of the effect of this particular heterokaryon combination on DNA synthesis. (b) Fusion frequencies were calculated for each possible phenotype contributing to apparent heterodikaryons (Fig. 1). (c) The predicted frequency of each false heterodikaryon class expected to contribute to a given phenotype was then subtracted from the observed fraction of heterokaryons exhibiting the expected behavior for these cells. For example, in the (myocyte x G₁ myoblast) experiment the observed frequency of heterodikaryons in which neither nucleus replicated DNA was 80%. However, 4% of the total apparent heterodikaryons were actually myocyte-like homokaryons (Fig. 1); thus the corrected frequency of myocyte dominance becomes 76%. (d) The percent dominance of either parental cell phenotype was then calculated as follows: the corrected dominance frequency (for this example 76%) divided by the predicted frequency of true heterokaryons (for this example 82%). Thus the postmitotic state was the dominant phenotype in 93% of (myocyte x G₁ myoblast) heterodikaryons.

Results

The methods used for analyzing the proliferative response of heterokaryons containing postmitotic myocytes are described in Figs. 1 and 2. Myocytes and G₁ myoblasts, whose cytoplasm and nuclei were differentially labeled with fluorescent beads and [³H]Tdr, were combined, then treated with PEG. The resulting hetero- and homokaryons, and the remaining unfused parental cells, were passaged and incubated in mitogen-rich medium containing [³H]Tdr for 30 h. After such treatment the unfused parental cells and multinucleated homokaryons should behave according to their parental class, i.e., G₁ homokaryons (identified by red cytoplasmic beads) should initiate DNA synthesis and become heavily labeled after autoradiography. Myocyte homokaryons (identified by green cytoplasmic beads) should not replicate DNA and should remain unlabeled. The behavior of G₁ myoblast and myocyte homokaryons thus serve as internal controls in this experiment. The percentage of parental cells that fail to behave in the predicted manner is used to calculate the fraction of heterokaryons that contain erroneously classified cell types (see Materials and Methods and Fig. 1).

Heterodikaryons (identified by red and green beads) could exhibit three alternative phenotypes: (a) proliferation domi-

nance (both nuclei heavily labeled) suggests the presence of diffusible factors in the G₁ cell that stimulate DNA synthesis in both myoblast and myocyte nuclei; (b) codominance (one nucleus heavily labeled, the other nucleus unlabeled) suggests that the proliferative and postmitotic states are autonomously regulated; or (c) postmitotic dominance (one nucleus lightly labeled and the other nucleus unlabeled), suggests the presence of diffusible factors in the postmitotic cell that prevent mitogenesis and DNA synthesis. In these experiments the behavior of polynucleated heterokaryons was also examined in order to assess the relative "strength" of any dominance effects observed.

The Postmitotic Phenotype Is Dominant in (Myocyte x G₁ Myoblast) Heterokaryons

The typical labeling pattern of (myocyte x G₁ myoblast) heterokaryons after incubation in mitogen-rich medium is illustrated in Figs. 3 and 4. The parental G₁ myoblasts, as well as most of the multinucleated myoblast homokaryons, initiated DNA synthesis and became heavily labeled after autoradiography (Fig. 4 A). When corrected for the 4% contamination of myocytes in the G₁ population, which would (if the postmitotic phenotype were dominant) render a predictable fraction of such homokaryons postmitotic (see Fig. 1), the values for DNA replication by all nuclei in G₁ homokaryons containing two, three, and four G₁ nuclei should be 92, 88, and 85%. The high frequency of DNA synthesis observed in G₁ homokaryons approximates these theoretical numbers. Thus, multinucleated G₁ myoblasts are minimally affected by the experimental manipulations and are fully responsive to mitogens. In contrast to myoblast behavior, only 4% of the parental myocyte population replicated DNA (Fig. 4 A), demonstrating that almost all of these cells had become postmitotic after the 24-h incubation in mitogen-depleted medium. After the experimental protocol these cells continued to synthesize proteins, and >95% of this cell class stained positively for myosin heavy chain protein.

When heterodikaryons were examined, 80% remained unlabeled, 20% had both nuclei labeled, and <1% exhibited one labeled and one unlabeled nucleus (Fig. 4 B). After making appropriate corrections for false heterokaryons (Fig. 1), 93% of all heterodikaryons exhibited a postmitotic phenotype, whereas only 7% replicated DNA (Fig. 4 C). In four similar experiments the corrected expression of the postmitotic phenotype in heterodikaryons was 86, 97, 90, and 100%. For five experiments of this type (involving >500 heterokaryons), the mean percent expression of the postmitotic state was 93 ± 5%. Clearly, the postmitotic state is the dominant phenotype in (myocyte x G₁ myoblast) heterokaryons.

The behavior of heterokaryons containing more than two nuclei was also examined. DNA replication did not occur in 90–95% of the cells with three and four nuclei, and in no case did three- and four-nucleated cells exhibit mixtures of labeled and unlabeled nuclei. This data supports the myocyte dominance effect observed in heterodikaryons. In fact, it was clear from all of the heterokaryons observed that a single myocyte could block the proliferation signals of three G₁ myoblasts. This is compelling evidence that myocytes contain "trans-acting" factors that inhibit mitogenesis in G₁ myoblasts.

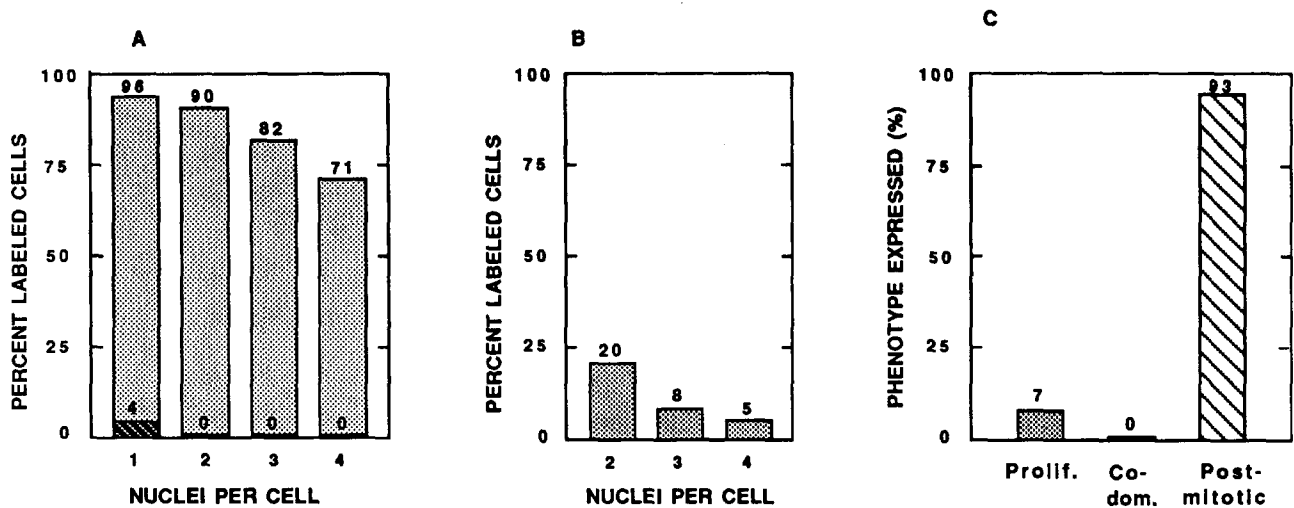


Figure 4. The postmitotic phenotype is dominant in (myocyte x G_1 myoblast) heterokaryons. (A) Controls: parental cell and homokaryon behavior (uncorrected data). The percentage of homokaryons of various sizes in which all nuclei became heavily labeled: myocyte (hatched bars); myoblast (shaded bars). Greater than 300 mononucleated cells and >100 binucleated cells were scored. 75 and 50 cells were scored for the three- and four-nuclei myocytes; and 50 and 25 cells were scored for the corresponding sizes of myoblast homokaryons. (B) Heterokaryon behavior (uncorrected data). The percentage of heterokaryons containing two, three, and four nuclei in which all nuclei became heavily labeled. The number of heterokaryons scored in each category was 100, 30, and 25, respectively. (C) Dominance frequencies of each parental phenotype in heterodikaryons calculated after correction of the data for pseudo-heterokaryons (see Fig. 1; Materials and Methods).

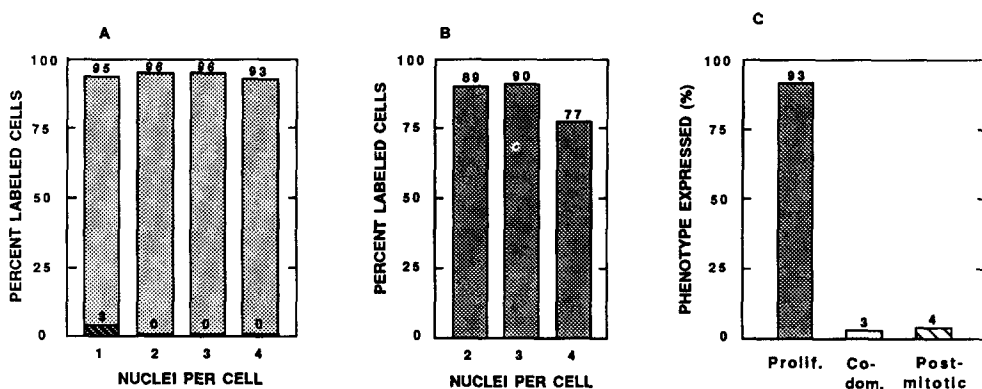


Figure 5. DNA replication is dominant in (myocyte x quiescent 3T3) heterokaryons. (A) Controls: parental cell and homokaryon behavior (uncorrected data). The percentage of homokaryons of various sizes in which all nuclei became heavily labeled with [3 H]Tdr: myocyte (hatched bars); 3T3 (shaded bars). More than 100 cells were scored for each class of one- and two-nuclei cells, and 50 and 25 cells were scored respectively for three-

and four-nucleated homokaryons. (B) Heterokaryon behavior (uncorrected data). The percentage of heterokaryons containing two, three, and four nuclei in which all nuclei have replicated DNA. The number of heterokaryons scored in each category was 90, 60, and 30, respectively. (C) Dominance frequencies of each parental phenotype in heterodikaryons calculated after correcting the data for pseudo-heterokaryons (see Fig. 1; Materials and Methods).

Proliferation Is the Dominant Phenotype in (Myocyte x Quiescent 3T3) Heterokaryons

To determine whether the mitogenic inhibitory activity in myocytes affects only myoblasts or whether it can prevent mitogenesis in cells outside the myogenic lineage, we fused myocytes with mouse BALB/c 3T3 cells, a "fibroblastic cell" type. In preliminary experiments over 25% of mitotically synchronized G_1 3T3 cells were shown to replicate DNA when plated directly into low mitogen medium. For this reason it was necessary to use quiescent (G_0) 3T3 cells. Thus the 3T3 partner would not be predisposed to replicate, and any DNA synthesis that occurred in heterokaryons could only have originated from a mitogenic signal initiated after

heterokaryon formation. In striking contrast to (myocyte x G_1 myoblast) heterokaryons, nearly all (myocyte x G_0 3T3) heterodikaryon nuclei replicated DNA (Fig. 5, B and C). The relative strength of proliferation dominance (93%) in this fusion is demonstrated by heterokaryons containing three and four nuclei; the small fraction that failed to replicate DNA was not biased with respect to the relative ratio of myocyte and 3T3 nuclei. This would suggest that polynucleated heterokaryons replicated DNA even when only one of the nuclei was of 3T3 origin. Thus the proliferative signals generated by a single quiescent 3T3 cell (once mitogens have been restored) can induce DNA synthesis in as many as three myocyte nuclei. 3T3 mitogenesis is clearly dominant in these

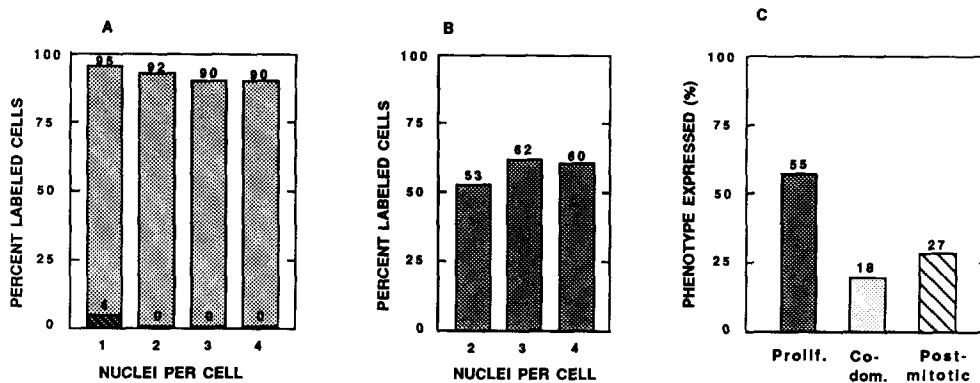


Figure 6. DNA synthesis is dominant in (myocyte x quiescent DD-1 myoblast) heterokaryons. (A) Controls: parental cell and homokaryon behavior (uncorrected data). The percentage of homokaryons in which all nuclei replicated DNA: myocyte (hatched bars); DD-1 (shaded bars). Greater than 100 cells of each parental class of one- and two-nuclei cells, and >50 cells were scored for homokaryons containing three and four nuclei. (B) Heterokaryon behavior (uncorrected data). The percentage of heterokaryons containing two, three, and four nuclei in which all nuclei have replicated DNA. The number of heterokaryons scored in each category was 88, 32, and 20, respectively. (C) Dominance frequencies of each parental phenotype in heterodikaryons calculated after correcting the data for pseudoheterokaryons (see Fig. 1; Materials and Methods).

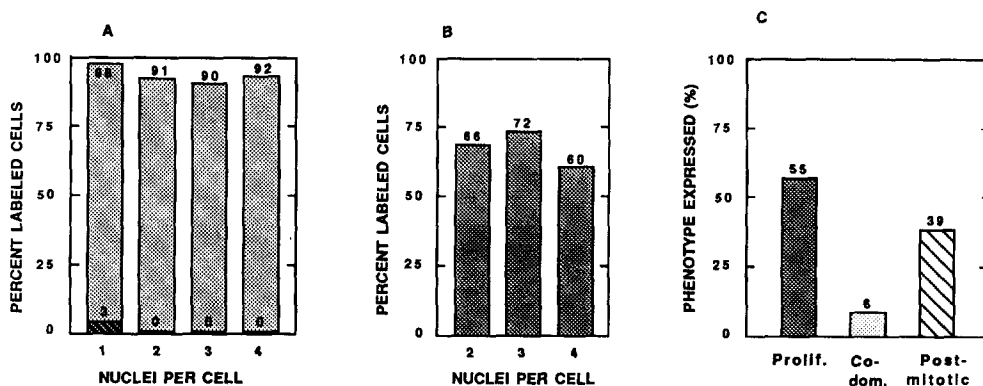


Figure 7. DNA synthesis is dominant in (myocyte x G₁ DD-1 myoblast) heterokaryons. (A) Controls: parental cell and homokaryon behavior (uncorrected data). The percentage of homokaryons in which all nuclei replicated DNA: myocyte (hatched bars) and DD-1 (shaded bars). Greater than 100 cells of each parental class of one- and two-nuclei cells, and >50 cells were scored for homokaryons containing three and four nuclei. (B) Heterokaryon behavior (uncorrected data). The percentage of heterokaryons containing two, three, and four nuclei in which all nuclei replicated DNA. The number of heterokaryons scored in each category was 145, 54, and 20, respectively. (C) Dominance frequencies of each parental phenotype in heterodikaryons calculated after correcting the data for pseudo-heterokaryons (see Fig. 1; Materials and Methods).

heterokaryons. This effect is in direct contrast to the recessive behavior of G₁ myoblasts.

Myonuclear DNA Replication Involves the Reexpression of Down-regulated Myoblast Gene Products

Do nonmyogenic cells provide all of the components required for myocyte DNA replication, or are proliferation-related functions reactivated in myocytes? To approach this question, thymidine kinase (*tk*), whose enzymatic activity is undetectable in myocytes (18), was assayed in heterokaryons containing the *thymidine kinase*-deficient strain of mouse L cells, L^{Mtk} (11). The protocol for this experiment was essentially unchanged. Quiescent L cells were fused with myocytes that were lightly prelabeled with [³H]Tdr, and the culture was then passaged into growth medium for 30 h with [³H]Tdr. If, like 3T3 cells, L cell nuclei are capable of replicating DNA in the presence of myocyte postmitotic factors, only heterokaryons in which myocyte *tk* had been reactivated should incorporate [³H]Tdr into DNA.

67 out of 96 marker-identified heterokaryons (70%) contained two heavily labeled nuclei. Because an independent

marker for DNA synthesis (other than [³H]Tdr incorporation) was not used for L cell homokaryons, the frequency with which quiescent L cells responded to mitogens was unknown. Thus the frequency of true heterokaryons could not be calculated. However, the fact that [³H]Tdr incorporation was only detected in heterokaryons (97% of the parental myocytes remained postmitotic, and no parental L cells became labeled) demonstrates that a mitogenic signal was, indeed, generated in these heterokaryons and that myocyte *tk* was reactivated. It thus appears that the regulatory components responsible for initiating DNA synthesis in these heterokaryons cause the reexpression of myoblast cell cycle gene products that are normally lost during establishment of the postmitotic state.

Proliferation Is the Dominant Phenotype in Heterokaryons Containing Myocytes and Differentiation-defective (DD-1) Myoblasts

MM14 cultures commonly generate variants that are defective in differentiation (approximate frequency = 10⁻³). Only 0.05% of the cells in one such variant clone (DD-1) express myosin and become postmitotic after FGF deprivation.

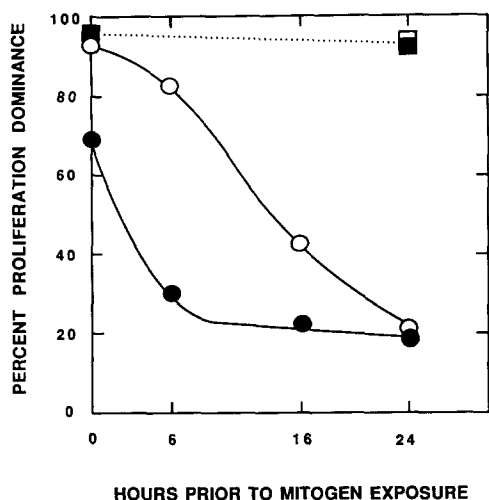


Figure 8. Conditional dependence of postmitotic dominance. (Myocyte x 3T3) and (myocyte x DD-1) heterokaryons acquire a postmitotic phenotype after preincubation in mitogen-depleted medium. Myocytes were fused with either DD-1 or 3T3 cells, then plated in low serum (1% horse serum) containing medium plus [³H]Tdr. At the indicated times cultures were fed growth medium (plus [³H]Tdr), incubated for an additional 30 h, then fixed and processed for autoradiography. (●) Myocyte x DD-1 heterodikaryons; (○) myocyte x 3T3 heterodikaryons. 50 heterodikaryons of each type were scored per data point, and 50 DD-1 (■) and 3T3 homodikaryons (□) were scored for the 24-h time point.

DD-1 cells are also capable of growing in serum-containing medium in the absence of FGF; moreover, unlike normal myoblasts, which possess EGF receptors but lack an EGF growth response, DD-1 cells replicate in medium containing EGF (15). Thus, these variants have not only lost the ability to differentiate but they have acquired new patterns of growth control.

To further characterize the proliferation and differentiation potential of these variant myoblasts, both G₀ and G₁ DD-1 cells were fused to postmitotic myocytes (Fig. 6 and 7). DD-1 nuclei replicated DNA in about two-thirds of these heterokaryons (Figs. 6 C and 7 C). However, unlike 3T3 heterodikaryons (93% proliferation dominance), a substantial myocyte effect was observed. For instance, 33% of all G₁ DD-1 heterodikaryons and 18% of the G₀ DD-1 heterodikaryons failed to replicate DNA. The influence of the postmitotic parent was also demonstrated by the behavior of heterokaryons containing three and four nuclei; almost all cells that exhibited postmitotic dominance (33 and 30%, respectively, of the total heterokaryons monitored) contained 2:1 and 3:1 ratios of myocyte to DD-1 cell nuclei. The results of this experiment suggest that the DD-1 phenotype involves an alteration in mitogenic signaling (relative to normal myoblasts) that circumvents the inhibitory effects of myocyte cytoplasm. A partial response to postmitotic signals is still retained by DD-1 cells, however, particularly when the myocyte regulatory factors are in relative abundance.

The Postmitotic State Is Dominant when (Myocyte x 3T3) and (Myocyte x DD-1) Heterokaryons Are Preincubated in Mitogen-depleted Medium

While myocyte components block the mitogenic response of

G₁ myoblasts, this capacity is greatly diminished with respect to 3T3, LMtk⁻, and DD-1 cells. Assuming that immediate exposure to mitogens after heterokaryon formation might be responsible for overriding the myocyte influence (perhaps by limiting the accumulation of sufficient levels of inhibitor, or limiting the time during which inhibitors can act), we tested whether a more delayed exposure to mitogens would permit the postmitotic state to be implemented.

After PEG treatment, cultures were incubated in low mitogen medium (1% horse serum) for 6, 16, or 24 h before being fed mitogen-rich medium plus [³H]Tdr. Cultures were then analyzed for [³H]Tdr incorporation 30 h later (Fig. 8). The behavior of the homokaryon classes was similar to previous experiments; only a few percent of the myocytes replicated DNA, and almost all mononucleated and binucleated DD-1 and 3T3 cells replicated DNA, even after 24-h incubation in mitogen-depleted medium. In contrast, the DNA synthetic capacity of heterodikaryons was increasingly affected by mitogen-depleted medium. A loss of proliferative ability was detected after only 6 h pretreatment in mitogen-depleted media, and after 24 h of mitogen deprivation only 20% of the heterodikaryons replicated DNA during the subsequent 30-h mitogen exposure. 70 and 95% of the two heterokaryon types replicated DNA when incubated immediately in mitogen-rich media. These results demonstrate that the probability of establishing a postmitotic phenotype in such heterokaryons is related to the time of mitogen deprivation after heterokaryon formation. Although prolonged mitogen-deprivation causes both the 3T3- and DD-1-heterokaryon types to exhibit the same degree of postmitotic dominance, (myocyte x DD-1) heterokaryons acquire this phenotype more rapidly, perhaps due to the fact that DD-1 cells are of myogenic origin.

Absence of DNA Synthesis in (Myocyte x DD-1) Heterokaryons Is Associated with the Loss of DD-1 EGF Receptors

Acquisition of the postmitotic state during MM14 muscle differentiation is accompanied by the loss of EGF receptors (14). DD-1 variant myoblasts, which fail to differentiate, maintain growth factor receptors in the nonproliferative state (15). How do EGF receptors behave in heterokaryons that become postmitotic? To answer this question, (myocyte x DD-1) heterokaryons, which were constructed and maintained in the absence of [³H]Tdr, were incubated in low mitogen medium to induce the postmitotic state. Cultures were then incubated in mitogen-rich medium for an additional 30 h, and assayed for their ability to bind ¹²⁵I-EGF. After autoradiography the EGF-specific grains per cell were quantitated in homo- and heterodikaryons (Fig. 9).

Heterodikaryons exhibited what appears to be a bimodal distribution of ¹²⁵I-EGF grains. Approximately 25% possessed the same apparent number of EGF receptors as mononucleated DD-1 cells (mean = 57 grains per cell); 75% showed a significantly reduced number of receptors (mean = 7 grains per cell), which was more than two standard deviations below the average receptor number of parental DD-1 cells. To determine whether the loss of EGF receptors correlated with the percentage of postmitotic heterokaryons induced in this experiment, DNA replicative behavior was monitored in parallel cultures; 30% replicated DNA and 70% were postmitotic. This experiment suggests that hetero-

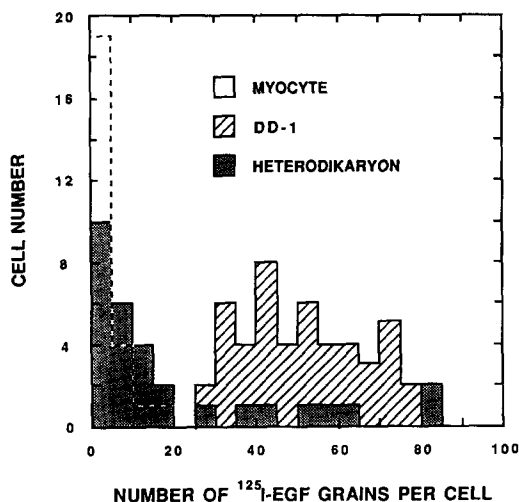


Figure 9. EGF receptor loss correlates with a postmitotic phenotype in heterokaryons. (DD-1 x myocyte) heterokaryons, which were prelabeled with fluorescent beads but not [³H]Tdr, were incubated in 1% serum for 24 h to induce the postmitotic state. After an additional 30 h in mitogen-rich medium cultures were assayed autoradiographically for ¹²⁵I-EGF-binding activity. The percent postmitotic heterokaryons determined in parallel cultures was 70%.

karyons that become postmitotic also become deficient in growth factor receptors. Acquisition of this phenotype of normal differentiated muscle cells (14) suggests that one effect of the diffusible myocyte factors may be to regulate growth factor receptor expression.

Discussion

The DNA replicative behavior of myocyte heterokaryons indicates that the postmitotic state of skeletal muscle is caused by an inhibitor of mitogenesis. Nearly 95% of heterodikaryons containing myocyte and G₁ myoblast nuclei failed to initiate DNA synthesis after incubation in mitogen-rich medium (Figs. 3 and 4). This was not a transient effect since heterokaryons incubated in mitogen-rich medium for as long as 54 h remained postmitotic. Nor was this response due to a general metabolic incompatibility, because heterokaryons maintained protein synthesis throughout the incubation period. The inability of myoblast heterokaryon nuclei to respond to mitogens was also not a result of the various experimental manipulations since all size classes of myoblast homokaryons initiated DNA synthesis. Nor was the lack of DNA synthesis caused by the inability of either cell type to replicate DNA in a foreign environment; myoblasts synchronized at the G₁/S border replicated DNA in myocyte heterokaryons (data not shown), and myocyte nuclei replicated DNA in 3T3 heterokaryons, even when there was a threefold excess of myocyte to 3T3 nuclei (Fig. 5). Rather, when the outcome of these controls is considered in conjunction with the observation that a single myocyte prevents DNA synthesis in heterokaryons containing up to three myoblasts (Figs. 3 and 4), it seems clear that myocytes possess a diffusible inhibitor of myoblast mitogenesis.

Evidence for a mitogenic inhibitor in myocytes is also demonstrated by the conditional dominance of myocytes in

heterokaryons containing nonmyogenic cells. While the majority of (myocyte x quiescent 3T3) and (myocyte x quiescent DD-1) heterodikaryons replicated DNA when exposed directly to mitogens (Figs. 5 and 6), 80% became postmitotic after a 24-h preincubation in low mitogen medium (Fig. 8). Binucleated 3T3 or DD-1 homokaryons within the same culture dish were unaffected by such temporary mitogen deprivation, and replicated their DNA when mitogens were restored. Dominance of the postmitotic phenotype in heterokaryons containing 3T3 and DD-1 cells suggests a degree of universality in the mechanism of the myocyte inhibitor. The fact that the postmitotic phenotype is not expressed unless such heterokaryons are subjected to mitogen deprivation and that its penetrance increases with the time of deprivation suggests that the myocyte inhibitors "compete" less effectively with the mitogenic signaling pathways of non-muscle cells than with those of myoblasts. This could be due to either quantitative or qualitative differences between mitogenic signals of the two cell types.

A further demonstration that myocytes contain factors affecting cell proliferation is the behavior of EGF receptors in heterokaryons. In contrast to quiescent DD-1 and 3T3 cells, which maintain EGF receptors when deprived of growth factors, myocytes lose all EGF-binding activity (14). This differentiation phenotype also appears to be expressed in heterokaryons that become postmitotic. In an experiment in which 70% of (myocyte x DD-1) heterokaryons were postmitotic, nearly the same percentage (75%) exhibited a significantly reduced level of ¹²⁵I-EGF-binding capacity which approximated the low level of binding seen in myocyte controls (Fig. 9). Assuming that all EGF-binding activity in heterokaryons originated from the DD-1 complement, it appears that myocyte factors not only inhibit DD-1 mitogenesis but mediate changes within the receptor biosynthetic, turnover, or internalization pathways, which are specific to differentiated muscle.

The conclusion that myocytes contain inhibitors of DNA synthesis compliments a number of experiments in which the muscle-specific genes of myoblasts and various nonmyogenic cell types have been activated after PEG-mediated fusion with myocytes and myotubes (1, 2, 22-24). Together with our results these data argue that one or more regulators of the skeletal muscle phenotype are present in myocyte cytoplasm and that they are capable of redirecting the developmental program of nonmuscle cells.

The observation that myocyte nuclei can, under certain conditions, be stimulated to replicate DNA (Figs. 5-7) indicates that the normal absence of DNA synthesis observed in myonuclei is not due to an irreversible block at the level of the DNA. To begin characterizing the events associated with the induction of myonuclear DNA synthesis, we tested heterokaryons for proliferation-related functions (in this case, thymidine kinase) that are normally lost during terminal differentiation. DNA synthesis was detected by [³H]Tdr in 70% of (myocyte x quiescent LMtk⁻) heterodikaryons. Since LMtk⁻ cells carry a *tk* gene deletion and are unable to phosphorylate thymidine to make thymidylate, incorporation of [³H]Tdr into DNA must result from the myocyte *tk* gene. Thus, proliferative functions that are inactivated during muscle differentiation remain inducible in myonuclei. The mechanism responsible for *tk* activity in these hetero-

karyons may involve mRNA stabilization, since *tk* gene transcription is maintained in various postmitotic tissues that have no detectable *tk* mRNA (7).

Previous experiments have shown that FGF represses terminal differentiation of MM14 myoblasts during G₁ phase, and that within 2–3 h of mitogen deprivation G₁ cells undergo an irreversible commitment event that initiates processes leading to the postmitotic phenotype, muscle gene expression, and cell fusion (3). Based on the results described above we suggest that the absence of an FGF-mediated activity promotes the appearance of a diffusible factor(s) that inactivates the mitogenic signaling pathway of the cell. This renders the myoblast postmitotic. The mechanism of inhibition is unknown. Conceivably the myocyte regulator blocks FGF-receptor activity directly. However, to explain the inhibition of 3T3 mitogenesis (Fig. 8), this factor would need to inactivate a common feature of the numerous growth factor receptors known to be functional in 3T3 cells (5, 10), perhaps by blocking a step after the convergence of these mitogenic signals. Recent developments in the study of hematopoietic cell differentiation suggest that growth arrest is regulated by the appearance of interferon-related proteins (19).

Alternatively, because postmitotic heterokaryons lose mitogen receptors, the postmitotic state may be implemented by inhibitors of growth factor receptor synthesis. The observation that the rate of EGF receptor loss during terminal differentiation is similar to the receptor half-life (14) is consistent with this hypothesis. An argument against receptor loss being the initial cause of the postmitotic phenotype is, however, the postmitotic behavior of polynucleated heterokaryons that contain a relative excess of myoblasts. In these cells the abundance of mitogen receptors, at least immediately after fusion, should generate a replication signal. Yet no nuclear replication is observed. Similarly, during myotube formation in vivo, myonuclei fail to replicate DNA even though the nascent myotube surface may contain many mitogen receptors derived from recently fused "myoblasts". A unifying explanation for all of these observations would be that the postmitotic phenotype of skeletal muscle is initially caused by changes that block intracellular mitogenic signals. The subsequent loss of mitogen receptors would then provide a simple mechanism for maintaining cells in the postmitotic state. Similar mechanisms may exist in tissues that acquire a postmitotic phenotype in vivo.

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