Control of Differentiation in Heterokaryons and Hybrids Involving Differentiation-defective Myoblast Variants

WOODRING ERIK WRIGHT
Department of Cell Biology and Internal Medicine, University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, Texas 75235

ABSTRACT Clones of differentiation-defective myoblasts were isolated by selecting clones of L6 rat myoblasts that did not form myotubes under differentiation-stimulating conditions. Rat skeletal myosin light chain synthesis was induced in heterokaryons formed by fusing these defective myoblasts to differentiated chick skeletal myocytes. This indicates that the structural gene for this muscle protein was still responsive to chick inducing factors and that the defective myoblasts were not producing large quantities of molecules that dominantly suppressed the expression of differentiated functions. The regulation of the decision to differentiate was then examined in hybrids between differentiation-defective myoblasts and differentiation-competent myoblasts. Staining with antimyosin antibodies showed that the defective myoblasts and homotypic hybrids formed by fusing defective myoblasts to themselves could in fact differentiate, but did so more than a thousand times less frequently than the 64% differentiation achieved by competent L6 myoblasts or homotypic competent × competent L6 hybrids. Heterotypic hybrids between differentiation-defective myoblasts and competent L6 cells exhibited an intermediate behavior of ~1% differentiation. A theoretical model for the regulation of the commitment to terminal differentiation is proposed that could explain these results by invoking the need to achieve threshold levels of secondary inducing molecules in response to differentiation-stimulating conditions. This model helps explain many of the stochastic aspects of cell differentiation.

During the past twenty years cultured cells have become one of the most important tools for the study of cell differentiation. Unfortunately, the expression of differentiated functions is frequently unstable under conditions of continuous cell culture (1-7). Although overgrowth of the differentiated cell type by "fibroblastoid cells" is often invoked as the cause in primary cultures, there is good evidence in some systems that differentiated diploid cells can "dedifferentiate" and reduce their synthesis of differentiated products (2, 3, 7). This loss of differentiated functions is also observed in cloned established cell lines where, if overgrowth is the cause, it is overgrowth by a dedifferentiated variant arising within the cloned population.

The mechanisms by which cells lose the capacity to express differentiated functions and the nature of the differentiated program in nonexpressing variants are important issues for understanding the regulation and maintenance of cell differentiation. In the present study we have approached this problem by examining the regulation of muscle functions in heterokaryons and cell hybrids involving differentiation-defective myoblasts. We have previously shown that rat skeletal myosin light chain synthesis is induced when undifferentiated rat myoblasts are fused with polyethylene glycol to mononucleated differentiated chick skeletal myocytes (8, 9). This suggests that differentiated chick myocytes contain factors that induce muscle functions in the undifferentiated rat myoblast. In contrast, muscle functions are suppressed in heterokaryons when rat fibroblasts are fused to chick skeletal myocytes (10), implying that fibroblasts produce a muscle suppressing factor. These observations enabled us to test the hypothesis that differentiation-defective myoblasts had become a fibroblastoid cell type by examining the regulation of myosin light chain synthesis in heterokaryons between the defective myoblasts and differentiated chick myocytes.

The control of myosin light chain synthesis in these nondividing heterokaryons provides information concerning only the regulation of the structural gene itself, not about earlier stages in terminal differentiation such as the transition from a dividing myoblast to a postmitotic differentiated cell. The mechanisms controlling this process were investigated by

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 FEBRUARY 1984 436-443
© The Rockefeller University Press · 0021-9525/84/02/0436/08 $1.00

forming dividing cell hybrids between differentiation-defective and undifferentiated but differentiation-competent myoblasts. The regulation of the decision to differentiate was examined by testing the capacity of the cell hybrids to respond to differentiation-stimulating conditions. The ability of the hybrids to differentiate was evaluated using a technique that permitted a 10^5-fold reduction in the frequency of cell differentiation to be identified and quantitated. These heterokaryon and cell hybrid approaches indicate that differentiation-defective myoblasts have not transdifferentiated into an alternate cell type, but, rather, have a 10^3–10^4-fold reduction in their capacity to differentiate in response to the external culture environment. Their myosin light chain structural genes are nonetheless responsive to putative differentiated chick inducing factors. Our results suggest that most of the "nondifferentiating" myoblast variants described in the literature would prove to be "poorly differentiating" variants if their behavior was analyzed using techniques that could detect levels of differentiation of 0.001% rather than only using the relatively insensitive criteria of morphologic myotube formation or the biochemical differentiation of mass populations. On the basis of these observations, we present a model that provides a molecular mechanism for the regulation of myogenesis in differentiation-defective myoblast variants.

MATERIALS AND METHODS

Cells and Culture Conditions: L6 rat myoblasts (11) were originally obtained from D. Shubert at the Salk Institute. Immediately after receipt in our laboratory, a well differentiating subclone was isolated, expanded, and frozen in multiple ampules for later use. Cells were maintained in medium composed of four parts Dulbecco's modified Eagle's medium to 1 part medium 199, supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 20% newborn bovine serum. L6 cells were subcultivated before confluence three times per week in order to maintain the cells in exponential growth and prevent cell differentiation. Cells were usually split 1:4 or 1:8 on Mondays and Wednesdays and 1:16 on Fridays, with minor adjustments made as needed to insure that the cells did not become confluent before the next subcultivation. In spite of these precautions, after 2–3 mo of continuous cultivation (60–100 population doublings), the capacity of the cells to differentiate gradually decreased. Fresh early passage ampules of L6 cells were thus routinely reconstituted every 6–8 wk.

Primary cultures of myoblasts from 12-d embryonic chick thighs were prepared as described elsewhere (8). Mononucleated, differentiated chick myocytes were obtained by first blocking the spontaneous fusion to form multinucleated myotubes with EGTA (12) and then maintaining the differentiated myocytes in medium containing normal calcium and 2 μg/ml cytochalasin B (13).

Isolation and Analysis of Heterokaryons: The selective system used to isolate highly enriched populations of heterokaryons has been described in detail elsewhere (14–16). Briefly, each of two cell populations to be fused is treated for 30 min in an ice bath with a lethal concentration of one of two irreversible inhibitors, iodoacetamide or diethylpyrocarbonate. The unreacted inhibitor is then washed away and the cells are mixed and fused with polyethylene glycol. Parental cells and homokaryons die from the lethal treatments. Because different molecules have been inactivated by each agent, each cell participating in a heterokaryon contributes active molecules to replace those damaged in its fusion partner, complementation occurs, and the heterokaryons survive. Using this technique, populations in which 95–99% of the nuclei are in heterokaryons can routinely be prepared. The heterokaryons were plated at 60,000 rescued nuclei per square centimeter in 0.3-cm^2 microtiter wells. The cells were labeled overnight with 300 μCi/ml of [^{35}S]methionine on the fifth day after heterokaryon formation. The next morning the heterokaryons were lysed in a low ionic strength buffer containing 15 mM KCl, 10 mM Tris-HCl pH7, 0.1% 2-mercaptoethanol, and 0.5% Nonidet P-40 in the presence of 12 μg of cold carrier cardiac actomyosin. 2 d later the lysate was centrifuged for 10 min at 4°C in a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, CA) and the supernatant was discarded. The residue, enriched for actomyosin which is insoluble under these conditions, was then extracted into O'Farrell's lysis buffer (17) and analyzed on two-dimensional polyacrylamide gels as modified by us (18).

The synthesis of rat skeletal myosin light chain one was chosen as a marker for rat myogenic differentiation since chick and rat forms can be distinguished on two-dimensional gels. The localization of chick fast, rat adult fast, and rat embryonic skeletal myosin light chains on these gels has been described elsewhere (8).

Isolation of Hybrids: Hybrids were produced by first isolating 95–99% pure populations of heterokaryons as described above. The heterokaryons were then plated at five rescued nuclei per 0.3 cm^2-microtiter well (thus at approximately two cells per well). 1–2 wk later the wells were examined under phase-contrast microscopy. Approximately 20 wells in which only one clone was growing were then trypsinized from each group of putative hybrids. Contaminating diploid parental clones were identified by karyology and discarded. We (16, 19) and others (20) have shown that homotypic hybrids isolated using irreversible biochemical inhibitors retain the capacity to differentiate normally.

In Situ Karyotyping: In these experiments, the purpose of karyotyping the different clones was to distinguish diploid parental cells from tetraploid hybrid cells. A relatively inaccurate but rapid method was thus chosen to facilitate the screening of large numbers of clones. Hybrid cells were karyotyped in situ according to the method of Cox et al. (21). Three to five metaphase chromosome spreads were counted under × 400 observation.

Cell Differentiation Assay: We determined the differentiation capacity of cells by plating cells at 100, 300, or 1,000 cells per 100-mm dish and allowing large colonies to develop for 2 wk. The medium was then changed to a myogenesis-stimulating medium containing 2% newborn bovine serum and 5 μg/ml insulin (22). Competent L6 cells exhibit massive myotube formation within 3 d in this medium. The cells were fixed after 6 d in order to provide adequate time for even differentiation-resistant cells to express their phenotype. The combination of 2 wk of clonal growth followed by 6 d in differentiation-stimulating conditions resulted in a long-term assay of the ability to differentiate that was relatively insensitive to differences in growth rate or the number of colonies per dish. The cells were either fixed for immunoperoxidase staining using antimyosin antibodies (see below) or fixed in 95% ethanol, Giemsa's stained, and analyzed for myotube formation. Myotubes were defined as cells containing more than three nuclei. In some cases, clones were isolated by trypsinization within glass cloning cylinders before the remaining clones were fixed.

Myosin-containing cells were identified using a murine monoclonal antibody CCM-52 (generously provided by Dr. Radovan Zak, University of Chicago) produced against embryonic chick cardiac myosin that is specific for an epitope in the light meromyosin fragment of V3 cardiac myosin (23, 24). We have found that this antibody cross-reacts with the myosin in cultured chick cardiocytes, chick skeletal myotubes, rat cardiocytes, and rat skeletal myotubes. The following technique was devised to permit the staining of large surface areas using small amounts of antibody. 10-cm dishes containing several hundred colonies were fixed for 3 min on an ice bath with 50% ethanol/acetone, washed briefly, and air dried. A rectangle was then scribed onto the surface of the dish using a scalpel blade. This produces a ridge of plastic that serves as a spacer so that the cells are protected from the shearing forces produced by the repeated application and removal of a coverslip. 50 μl of antibody was sufficient to stain the 10-cm^2 surface area covered by a 50 × 22 mm coverslip. The most efficient method for applying the antibody, which minimizes the trapping of bubbles, was to place 50 μl on the coverslip, invert the dish, and lower it onto the coverslip until capillary force sucked the coverslip into place. Pressing the bottom of the dish to induce a curved surface caused the coverslip to raise slightly off the dish, and several repetitions of this procedure produced sufficient turbulence to adequately mix the antibody with residual saline on the dish and cause a uniform exposure of the cells to antibody. After 90 min at room temperature, the coverslip was floated off the dish by adding 10 ml of saline. Binding of the primary antibody was visualized using a peroxidase-conjugated goat anti-mouse antibody (Cappel Laboratories, Cochranville, PA) and diaminobenzidine according to standard protocols.

RESULTS

Isolation of Differentiation-defective Myogenic Variants

Clones were prepared from both a freshly thawed ampule of L6 rat myoblasts and a culture that had been under continuous subcultivation for 2 mo in which <10% of the cells formed myotubes after becoming confluent. This culture was allowed to remain confluent for 1 wk before cloning to further reduce the number of differentiation-competent cells.

Table I shows that most of the clones from the poorly differentiating culture nonetheless retained the capacity to differentiate under clonal conditions. However, whereas the clones from the freshly thawed cells became massively fused after being placed in a differentiation stimulating medium, most of the myogenic clones from the poorly differentiating culture formed only a few myotubes.

Six clones that did not contain morphologically identifiable myotubes under phase-contrast observation were isolated. Table I also shows the myogenic capacity of these clones. Clones 3 and 4 were recloned again to yield clones 3c and 4a, which together with clones 5 and 6 were used in subsequent experiments.

Heterokaryons Involving Differentiation-defective Myoblasts

To determine whether differentiation-defective myoblasts lacked the capacity to differentiate because they lacked inducing factors or produced suppressing factors, we constructed heterokaryons by fusing the various defective myoblast subclones to differentiated chick skeletal myocytes. Heterokaryons were isolated using irreversible biochemical inhibitors, labeled with [^{35}S]methionine, and analyzed on two-dimensional gels. Fig. 1 presents the results from three such experiments. In each experiment, heterokaryons between chick myocytes and undifferentiated but differentiation-competent L6 myoblasts were also formed (Fig. 1, *a*, *e*, and *g*) to provide an internal control for variations due to the quality of the chick primary culture or other parameters. Fig. 1, *b*, *c*, *f*, and *h* show that both embryonic and adult rat skeletal myosin light chain one were induced in heterokaryons formed by fusing chick myoblasts to the different clones of defective myoblasts. Chick myosin light chain synthesis continued in all of the heterokaryons. Fig. 1 *d* demonstrates that the selec-

TABLE I

*Characterization of Differentiation-defective Myoblast Variants**

Cell type	Number of subclones		Estimated myotube formation
	Fused	Unfused	
			% of total cells
Competent L6 culture	250	11	70
Defective L6 culture	116	77	17
Defective L6 clones			
1	14	114	0.04
2	2	114	0.007
3	11	102	0.04
3c	0	282	0
4	3	159	0.008
4a	0	253	0
5	0	114	0
6	10	50	0.07

* The differentiation capacity of a freshly thawed ampule of competent L6 myoblasts was compared under clonal conditions with a culture that had been in continuous cultivation for 2 mo and had "gone off", and to clones and subclones derived from that culture. The assay consisted of plating cells at clonal densities, allowing large colonies to form, and then feeding them with differentiation-stimulating medium. 1 wk later the cells were fixed and stained with Giemsa's. The extent of differentiation was estimated as the % of total cells that had fused to form myotubes containing three or more nuclei. "Typical" fused clones were counted to determine the actual % myotube formation and then the myotube formation as a % of total cells was determined by multiplying this value by the proportion of total subclones that had fused. The average number of cells per colony was 1,000–5,000 for the competent L6 cells and 5,000–15,000 for the defective cells.

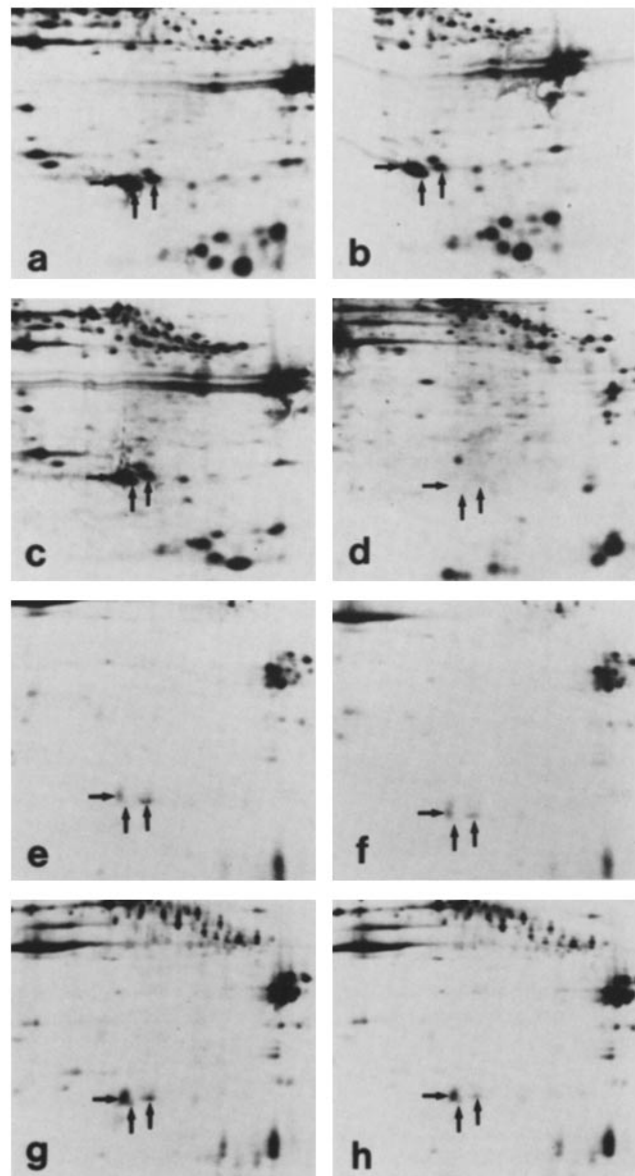

FIGURE 1 Myosin light chain one synthesis in heterokaryons. Highly enriched populations of heterokaryons formed by fusing mononucleated differentiated chick myocytes to different clones of differentiation-defective myoblasts were labeled with [^{35}S]methionine 5 d after heterokaryon formation and then analyzed on two-dimensional polyacrylamide gels. Only the region of each gel between the approximate pI of 5.5 (*left*) and 4.7 (*right*) is shown. Each of three independent experiments contained an internal positive control fusion between differentiated chick myocytes and undifferentiated (but differentiation competent) L6 rat myoblasts (*a*, *e*, and *g*). The gels shown are of heterokaryons between the following pairs of cells. (*a*) Myocyte × competent L6 myoblast control for gels *b*–*d*; (*b*) myocyte × defective myoblast clone 3c; (*c*) myocyte × defective myoblast clone 4a; (*d*) homokaryons between defective myoblast clone 4a cells and themselves; (*e*) myocyte × competent myoblast control for gel *f*; (*f*) myocyte × defective myoblast clone 5; (*g*) myocyte × competent myoblast control for gel *h*; (*h*) myocyte × defective myoblast clone 6. The myosin light chain one proteins are identified as follows: horizontal arrow, chick fast skeletal myosin light chain one; vertical arrows, rat embryonic (right arrow) and rat adult fast (left arrow) skeletal myosin light chain one. The rat skeletal myosin light chains are inducible in the differentiation-defective myoblast clones.

tion procedure itself did not induce rat light chain synthesis, since heterokaryons isolated by fusing subclone 4a to itself did not synthesize muscle products. The defective myoblasts are thus not producing dominant factors that actively suppress chick differentiated functions, and contain inducible myosin light chain one structural genes.

Hybrids Involving Differentiation-defective Myoblasts

The above experiments indicated that defective myoblasts were capable of responding to putative inducing factors provided by already differentiated chick myocytes. This response could result from regulation at various levels, from the initial decision to differentiate involving the entire myogenic program to a direct induction of the light chain structural gene itself. The behavior of these heterokaryons demonstrated that the myosin light chain gene was not defective and that dominant suppression of myogenic functions did not occur, but did not indicate the location of the lesion in defective myoblasts. To examine earlier stages in myogenesis, we studied the decision to differentiate. Cell hybrids between undifferentiated myoblasts and defective myoblasts provide access to the regulation of the decision to differentiate, since any response of undifferentiated hybrids to differentiation-stimulating conditions is dependent on the successful completion of at least the decision to differentiate. Hybrids were constructed by first producing 95–99% pure populations of heterokaryons, cloning the heterokaryons, and then picking colonies of dividing cell hybrids. Three sets of hybrids were compared: differentiation-competent parental L6 cells fused to themselves ($L6^2$), differentiation-defective myoblast subclone 4a cells fused to themselves ($DD4a^2$), and competent L6 cells fused to defective myoblast subclone 4a cells (L6 × DD4a). All of the newly picked colonies were initially plated in two different 16-mm wells. After 1 wk, one set of wells was processed for in situ karyotyping in order to identify tetraploid hybrids and diploid parental cells. Although counting the chromosomes directly under × 400 observation in only three or four spreads from this type of preparation is much less accurate than counting from photographs of spreads prepared from suspended cells, it was sufficient to decide whether a colony was roughly diploid or roughly tetraploid. Table II presents the results from all of the colonies examined. Many of the colonies were diploid, as would be expected since the initial populations were not 100% pure. Since fewer heterokaryons than parental cells successfully divide and give rise to growing colonies, the proportion of contaminating parental colonies is increased with respect to the initial parental contamination (14, 16). Nonetheless, 50–80% of the colonies were tetraploid. 10 tetraploid colonies from each combination were then trypsinized and replated at clonal density. After 2 wk of growth, most the these secondary colonies were 2–3 mm diam and were tightly packed with a high localized cell density. The cells were fed differentiation-stimulating medium for one additional week and then fixed and processed for immunoperoxidase staining using a monoclonal antibody against myosin (Fig. 2). Massively fused clones were fixed after 4 or 5 d because many myotubes in these cultures detached after longer periods. 20–200 subclones were examined from each clone. The total number of myosin-positive cells was counted, and the total number of cells was estimated by counting the cells in an average-sized colony and multiplying by the number of colonies scored. (In the case of $L6^2$, the number of myosin-positive cells was so large that only one or two average subclones were counted for myosin-positive cells.) This analysis thus yielded information as to both the percentage of cells capable of giving rise to differentiated progeny (percent myosin-positive clones) and the percentage of cells that actually differentiated under the conditions of the experiment (percent myosin-positive cells). Several conclusions can be drawn from the data presented in Table III. First, even tetraploid differentiation-defective myoblasts are capable of differentiating. The number of differentiated cells is so low that individual myosin-positive cells were almost invariably surrounded by large numbers of undifferentiated cells. The inability of these cells to form morphologically identifiable myotubes is thus probably a consequence of the rare differentiated cell's not finding other differentiated cells with which to fuse, rather than an inability to fuse to form myotubes per se. The defective myoblasts are thus very poorly differentiating rather than nondifferentiating variants. Second, the capacity to differentiate was roughly homogeneous among each set of subclones. For example, the presence of 0.9% myosin-postive cells in L6 × DD4a clone 16 subclones was not due to 0.9%

TABLE II

*Karyotype of Hybrid Clones**

$DD4a^2$		L6 × DD4a		$L6^2$	
Clone	Chromosomes	Clone	Chromosomes	Clone	Chromosomes
	No.		No.		No.
1*	66	1	34	1*	75
2	37	2	63	2	32
3	—	3*	66	3	39
4	38	4	35	4*	70
5	—	5*	58	5	39
6*	91	6	61	6*	71
7*	77	7*	64	7*	62
8	—	8	57	8*	70
9	34	9	—	9*	57
10	99	10	55	10	40
11	—	11*	65	11*	71
12*	87	12	66	12	36
13	39	13	36	13*	64
14*	78	14*	61	14	34
15	30	15*	66	15	58
16*	64	16*	64	16	34
17	40	17*	67	17	—
18	36	18	—	18	36
19*	84	19	47	19	—
20*	71	20*	62	20	—
21	38	21	61	21	—
22*	69	22	64	22	42
23*	68			23*	63
24	102			24	34

* Putative hybrids between differentiation-defective myoblast subclone 4a cells and themselves ($DD4a^2$), competent L6 myoblasts and differentiation-defective myoblast subclone 4a (L6 × DD4a), and competent L6 myoblasts and themselves ($L6^2$) were karyotyped in situ and counted under direct × 400 observation. The number of chromosomes listed is the average of 3–6 metaphase spreads. Occasional spreads that were grossly different from the average for that particular colony were excluded from the analysis. Parental L6 myoblasts have a modal number of 41 chromosomes. In situ karyotyping tends to underestimate the actual number of chromosomes, particularly in hybrid cells with more overlapping chromosomes. Since some contaminating diploid cells had in situ determinations of as few as 30 ($DD4a^2$ clone 15) or 32 ($L6^2$ clone 2) chromosomes, clones containing more than approximately 60 chromosomes were considered to be hybrids. Hybrid clones selected to be used in the analysis of Table III and Fig. 3 are indicated by an asterisk.

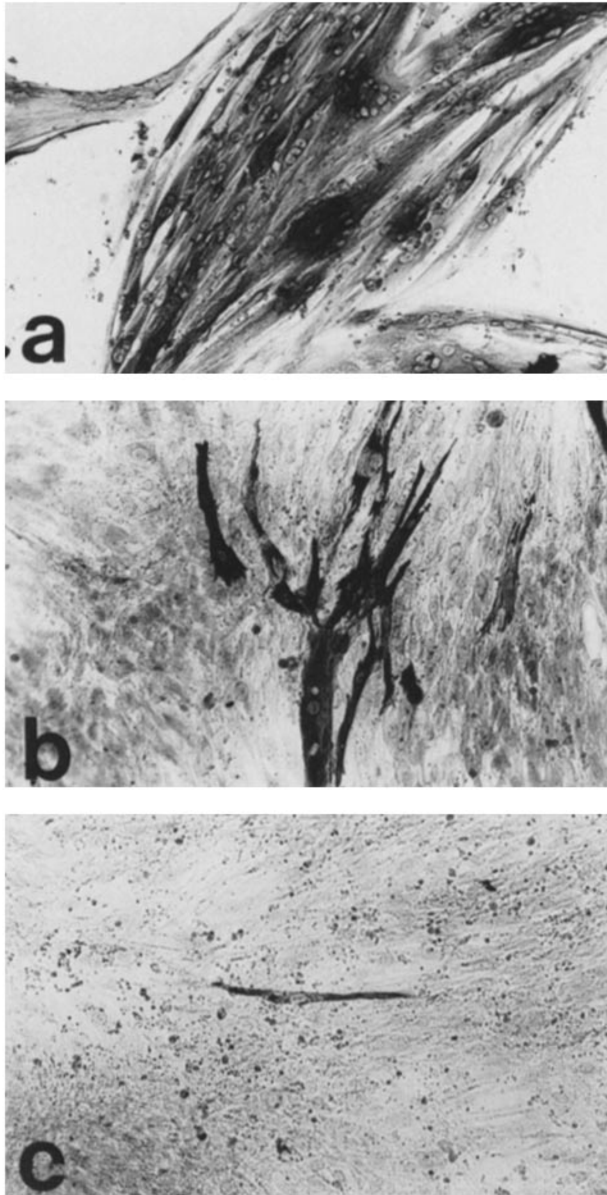

FIGURE 2 Antimyosin immunoperoxidase staining of cell hybrids. Cell hybrids were subcloned, allowed to grow to a high local cell density, fed differentiation-stimulating medium, and then fixed and processed for immunoperoxidase staining using the murine monoclonal antimyosin antibody CCM-52. Cells were lightly counterstained with Giemsa's in order to visualize cell nuclei. (a) L6 × L6 homotypic hybrid clone 1; (b) L6 × defective myoblast hybrid clone 16; (c) defective myoblast × defective myoblast homotypic hybrid clone 1. See Table III for a quantitative analysis of these specific clones. × 80.

of the subclones being massively fused, but rather was the result of a few myosin-positive cells being present in most of the subclones (although an occasional subclone contained 10% myosin positive cells and many contained only 0.1% positive cells). Finally, the ability to differentiate appears to have been largely suppressed in the L6 × DD4a hybrids in comparison to the homotypic hybrids formed by fusing differentiation-competent L6 cells to themselves.

This last conclusion is based on the strictly arithmetic analysis presented in Table III. However, if the data are reanalyzed on a logarithmic scale as in Fig. 3, it is possible to reach a very different interpretation. Under these conditions, the heterotypic hybrids appear to exhibit a behavior intermediate to that of either homotypic hybrid, suggesting a coexpression of the parental phenotypes rather than a suppression by the defective parent of the capacity to differentiate. The essentially nonoverlapping behavior of the three cell populations confirms that the tetraploid cells isolated in the L6 × DD4a cross were true hybrids and not the progeny of parental cell homokaryons. The intermediate frequency of differentiation exhibited by the L6 × DD4a hybrids can be contrasted to the complete suppression we observed when the same technique was applied to hybrids between competent L6 myoblasts and B9 rat glial cells (25). In these hybrids, <0.002% myosin-positive cells were seen when subclones were stimulated to differentiate and stained with antimyosin antibodies. The present result we are describing as an intermediate value is almost 1,000-fold greater than that seen in the suppressed L6 × B9 hybrids.

DISCUSSION

These results indicate that the clones used in the heterokaryon experiments were poorly differentiating myoblasts. Nonetheless, fusion to differentiated chick myocytes was able to induce rat myosin light chain synthesis in these cells despite the 10^3–10^4-fold reduction in their capacity to differentiate spontaneously. Since this result is based on the biochemical analysis of the entire population of heterokaryons, a large fraction of the heterokaryons must have been induced. Furthermore, although not quantitated, the amount of induction observed was roughly the same as that in control heterokaryons using parental competent rat myoblasts. The genes for the structural myosin light chains in these poorly differentiating cells thus retain a relatively normal capacity to respond to putative chick inducing factors.

The mechanism regulating the reduced capacity to differentiate is one that results in an apparently intermediate behavior of 1.2% differentiation in the heterotypic hybrids between poorly differentiating and fusion competent myoblast as compared with the 0.048 and 64% differentiation seen in the two sets of parental homotypic hybrids. It is not yet known what molecular mechanisms could regulate a probability function covering three to four orders of magnitude, such that a combination of a 64% response and a 0.048% response could yield a 1.2% response. One speculative possibility is presented in Fig. 4. If myogenic cells produce inducing molecules in response to differentiation-stimulating medium and if the number of molecules produced must exceed a threshold value before the commitment to differentiate is executed, then the average of the outer curves in Fig. 4 would yield an intermediate response illustrated by the middle curve. In this model the co-expression average of a 64 and a 0.048% response would be a 7% response. This analysis has not considered any of the multiple complexities known to effect myogenic differentiation. For example, the number of colonies per dish might influence the rate of media conditioning and thus the rate of cell differentiation; cell growth rate affects colony size which might also affect the commitment to differentiate; different clones exhibit different degrees of cell migration and thus different local cell density in the center of the clones which in turn might modify the propensity to differentiate; the data represent the results from a single point (3 wk after plating the cells including 6 d in differentiation-stimulating medium) and thus might be somewhat influ-

Table III
*Differentiation Capacity of Hybrid Clones**

Cell combination	Typical colony size	Number of colonies: Myosin (+)	Number of colonies: Myosin (−)	% Myosin (+) colonies	Total number of myosin (+) cells	% Myosin (+) cells
Defective clone 4a × defective clone 4a						
Clone 1	3,542	13	72	15	52	0.017
Clone 6	795	0	51	0	0	0
Clone 7	785	12	87	27	12	0.034
Clone 12	750	34	33	51	2,177	4.3
Clone 14	2,210	1	67	1.4	3	0.002
Clone 16	3,540	31	56	36	96	0.031
Clone 19	2,196	41	78	34	630	0.23
Clone 20	826	17	72	19	132	0.13
Clone 22	2,228	27	32	46	113	0.035
Clone 23	3,550	21	36	37	238	0.12
Defective clone 4a × competent L6						
Clone 3	612	54	18	75	1,398	3.2
Clone 5	1,326	25	64	27	229	0.13
Clone 7	326	39	39	50	653	1.0
Clone 11	5,720	29	1	97	5,455	3.1
Clone 14	298	19	33	37	318	2.1
Clone 15	333	35	59	37	697	2.2
Clone 16	3,432	93	25	79	3,654	0.9
Clone 17	627	34	29	54	419	1.1
Clone 20	540	76	101	43	666	0.7
Competent L6 × competent L6						
Clone 1	634/230‡	280	1	99	—‡	73‡
Clone 4	168/333	107	6	95	—	32
Clone 5	892/326	62	18	77	—	56
Clone 7	622/334	67	0	100	—	62
Clone 8	677/44	116	3	97	—	91
Clone 9	1,818/1,262	19	1	95	—	56
Clone 11	804/25	83	6	93	—	90
Clone 13	260/79	159	4	98	—	75
Clone 23	270/103	71	3	96	—	69

* Subclones from each hybrid colony were analyzed as described in Table I, except for the critical difference that cells were scored as differentiated on the basis of their staining with antimyosin antibody rather than their presence in myotubes. This proved a far more sensitive assay. The total number of nuclei in myosin (+) cells was counted for all the hybrids except the competent L6 × competent L6 hybrids.

‡ Typical colony sizes are here expressed as a ratio of myosin (+) to myosin (−) cells. The number of myosin (+) cells in these hybrids was far too great to permit the total number to be actually counted. Instead, a typical colony was counted, and the total % myosin (+) cells was estimated by multiplying the % myosin (+) cells in a typical colony by the % of colonies that were myosin (+).

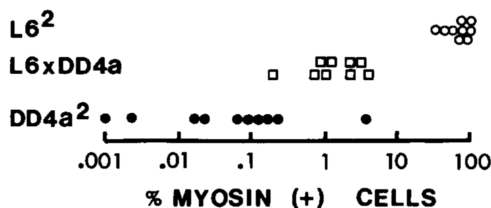

Figure 3 Differentiation capacity of hybrid clones. The data from the last column of Table III are presented on a logarithmic scale. Each symbol represents one of the clones in Table III. *L6²*, competent L6 × competent L6 hybrids; *L6 × DD4a*, competent L6 × differentiation-defective myoblast clone 4a hybrids; *DD4a²*, differentiation-defective myoblast clone 4a × differentiation-defective myoblast clone 4a hybrids. The heterotypic hybrids appear to exhibit an intermediate level of differentiation.

enced by factors affecting the rate rather than the capacity to differentiate, etc. The cumulative effects to these factors probably account for the wide scatter of the data over several orders of magnitude within each hybrid cell type (see Fig. 3).

Given the limitations of the data and the fact that the model treats only one of the many contributing factors, as a first approximation the observed level of 1.2% differentiation in the heterotypic hybrids is in general agreement with the 7% differentiation predicted by the model.

Threshold effects suggest that multiple sites need to be affected before a response occurs. The presence of multiple nuclear binding sites for steroid hormone-receptor complexes is well established (26, 27). Our model for the production of multiple copies of internal inducing molecules in response to differentiation-stimulating conditions with a threshold effect is essentially based upon the same concept. It predicts that the commitment to differentiate will depend upon a threshold occupancy of multiple receptor sites, which occurs only after a sufficient concentration of inducing molecules has been produced.

The reduced capacity of the defective myoblasts to differentiate could also be explained by the presence of suppressive factors. In this case, the dilution of the suppressor in the heterokaryons or hybrids could give the observed results.

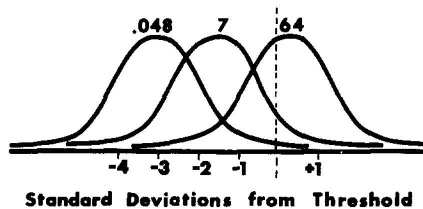

FIGURE 4 Model for the commitment to differentiate. The geometric means for the frequency of myosin (+) cells in the parental and hybrid cells (Table III and Fig. 3) are 64% ($L6^2$), 1.2% (L6 × DD4a), and 0.048% ($DD4a^2$). The following speculative model attempts to provide a rationale for this result. Statistical tables for the area under a standard distribution (37) were used to determine the proportion of a population that would exceed a particular value, expressed in units of standard deviations from the mean. The vertical dashed line represents the threshold number of molecules of some factor that must be exceeded before the commitment to terminal differentiation is made. The number above each curve represents the proportion of cells in that population that will exceed the threshold and thus differentiate. If the average number of molecules produced per cell were 0.36 SD greater than the required threshold, 64% of the cells would exceed that threshold and differentiate (*right curve*). Similarly, if the average number of molecules produced per cell in a second population were 3.3 SD less than the threshold value, then only 0.048% of the cells would produce sufficient quantities of the inducer to exceed the threshold and differentiate (*left curve*). If cells from these two populations were hybridized and each genome continued to express its original response to differentiation-stimulating medium, then the average concentration of molecules produced would be $[(0.36\ SD) + (-3.3\ SD)] \div 2 = -1.47$ SD, or 1.47 SD less than the threshold. Assuming that the standard deviation remained constant, 7% of this hybrid population should be able to differentiate (*middle curve*). Thus, the co-expression average of a 64 and a 0.048% response would be a 7% response.

However, the dominant behavior of fibroblasts in suppressing myogenic functions in heterokaryons (10) suggests that fibroblast suppressive factors are quantitatively and probably qualitatively different from putative suppressive factors in defective myoblasts.

There are numerous reports of the isolation of clones of nondifferentiating myoblasts (6, 28–33). Although the high frequency of their phenotype has led most authors to be cautious about describing them as mutants, the mechanism responsible for their appearance has remained obscure. Our results suggest that most of these "nondifferentiating" variants would probably prove to be "poorly differentiating" variants if their behavior was analyzed using in situ techniques that could detect individual differentiated cells, rather than only using the relatively insensitive criteria of morphologic myotube formation or the biochemical differentiation of mass populations.

Cell density is one of several important factors influencing the commitment of myogenic cells to differentiate. Mass cultures of poorly differentiating cells probably never achieve the extremely high local cell density that is obtained at the center of a newly formed subclone. We believe factors such as this are responsible for our observation that almost all of the cells from a culture of L6 cells that had largely lost the capacity to differentiate as a mass population nonetheless gave rise to colonies that contained myotubes under clonal conditions. However, the extent of myotube formation per clone was substantially reduced with respect to the degree of differentiation observed in colonies derived from competent L6 myoblasts. It was only by picking the colonies so resistant to differentiation that no myotubes were formed even after 3 wk of culture including 1 wk in differentiation-stimulating medium that our differentiation-defective myoblast clones were isolated. However, antibody staining later demonstrated that even these clones could produce rare myosin positive cells. Although the decision to differentiate exhibits many aspects of an all-or-none phenomenon, our results indicate that the process regulating this decision can apparently vary in a relatively continuous fashion to produce rates of differentiation differing by at least three to four orders of magnitude. The ability of different clones to inherit these extremely different probabilities of differentiation is consistent with our proposed model.

Our results differ from two recent reports involving myogenic cell hybrids (33, 34). Lawrence and Coleman found that hybrids between competent and differentiation-defective L6 myoblasts exhibited high rather than intermediate levels of differentiation (33). We believe that this is due to their use of moderately rather than severely differentiation-defective myoblasts. Their defective myoblasts retained sufficient capacity to differentiate so that rare morphologic myotube formation was still present even under mass culture conditions. The intermediate result we would predict under these circumstances (the model presented in Fig. 4 predicts that hybrids between cells exhibiting a 74 and a 1% frequency of differentiation would show a 30% response) would be very difficult to distnguish from a dominance of the competent phenotype. It was only by using an extremely differentiation-resistant clone that we were able to identify an intermediate result above the scatter of our data. Konieczny and Coleman (34) have also reported that myogenesis was dominant in crosses between myoblasts and muscle-derived fibroblasts. We believe this is due to an inadequate definition of a "fibroblast." They functionally defined fibroblasts as those cells remaining after several postconfluent passages of a primary muscle culture. Such cultures are likely to include large numbers of differentiation-resistant cells that are nonetheless myoblasts, in that they would retain the capacity to exhibit some myogenic potential under appropriate conditions. Most of the cell hybrids they isolated were thus probably crosses between myoblasts and differentiation-resistant myogenic cells rather than true myoblast × fibroblast hybrids.

Quantitative differences in the expression of certain enzymes between different cell lines (35) or mouse strains (36) have been found to vary according to a geometric progression. The models proposed to explain these observations involve the doubling of certain "regulatory elements" in one or both relevant chromosomes of a diploid cell, with the total cell expression being the arithmetic sum of the contribution of both chromosomes. These models were designed to explain the variation in constitutively expressed enzymatic activities, and did not specifically address the regulation of the apparently all-or-none decision to express new functions. If combined with the threshold effects described in Fig. 4, these models could also explain the intermediate expression of myogenesis that we obtained in the cell hybrids, where the level of expression appears to be a type of geometric mean of the parental cells rather than an arithmetic mean.

In conclusion, we have shown that most clones of myoblasts that have lost the capacity to form myotubes retain the capacity to differentiate but do so only with a very low frequency. Although the mechanism regulating this pheno-

type remains unknown, we have proposed a model that can explain how cells stably regulate their probability of differentiation. This model is consistent with the intermediate behavior of hybrids between poorly differentiating and competent cells, and provides a rationale for how the co-expression of a 0.048 and a 64% response could give a 1.2% response. The hypothesis that a threshold occupancy of multiple receptor sites is required to trigger terminal differentiation should provide a useful conceptual model to explain many of the stochastic aspects of cell differentiation.

This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association.

Received for publication 4 April 1983, and in revised form 31 October 1983.

REFERENCES

1. Eagle, H. 1965. Metabolic controls in cultured mammalian cells. *Science (Wash. DC).* 148:42–50.
2. Cahn, R. D., and M. B. Cahn. 1966. Heritability of cellular differentiation: clonal growth and expression of differentiation in retinal pigment cells *in vitro. Proc. Natl. Acad. Sci. USA.* 55:106–114.
3. Coon, H. G. 1966. Clonal stability and phenotypic expression of chick cartilage cells *in vitro. Proc. Natl. Acad. Sci. USA* 55:66–73.
4. Cotton, R. G. H., D. S. Secher, and C. Milstein. 1973. Somatic mutation and the origin of antibody diversity. Clonal variability of the immunoglobulin produced by MOPC 21 cells in culture. *Eur. J. Immunol.* 3:135–140.
5. Harrison, P. R., T. Rutherford, T. D. Conkie, N. Affara, J. Sommerville, P. Missey, and J. Paul. 1978. Analysis of erythroid differentiation in friend cells using non-inducible variants. *Cell.* 14:61–70.
6. Pearson, M. C. 1980. Muscle differentiation in cell culture: a problem in somatic cell and molecular genetics. *In* The Molecular Genetics of Development. T. Leighton and W. F. Loomis, editors. Academic Press, Inc. New York. 361–419.
7. Bissell, M. J. 1981. The differentiated state of normal and malignant cells or how to define a "normal" cell in culture. *Int. Rev. Cytol.* 70:27–100.
8. Wright, W. E. 1981. The synthesis of rat myosin light chains in heterokaryons formed between undifferentiated rat myoblasts and chick skeletal myocytes. *J. Cell Biol.* 91:11–16.
9. Wright, W. E. 1982. The regulation of myosin light chains synthesis in heterokaryons between differentiated and undifferentiated myogenic cells. *In* Muscle and Cell Motility. R. M. Dowbin and J. W. Shay, editors. Plenum Press, New York. 177–184.
10. Wright, W. E., and J. Arnoff. 1983. The suppression of myogenic functions in heterokaryons formed by fusing chick myocytes to diploid rat fibroblasts. *Cell Differ.* 12:299–306.
11. Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA.* 61:477–483.
12. Vertel, B. M., and D. A. Fischman. 1976. Myosin accumulation in mononucleated cells of chick muscle cultures. *Dev. Biol.* 48:438–446.
13. Sanger, J. W. 1974. The use of cytochalasin B to distinguish myoblasts from fibroblasts in cultures of developing chick striated muscle. *Proc. Natl. Acad. Sci. USA.* 71:3621–3625.
14. Wright, W. E. 1982. The selection of heterokaryons and cell hybrids using the biochemical inhibitors iodoacetamide and diethylpyrocarbonate. *In* Techniques in Somatic Cell Genetics. J. Shay, editor. Plenum Press, New York. 48–65.
15. Wright, W. E. 1981. Recovery of heterokaryons at high cell densities following isolation using irreversible biochemical inhibitors. *Somatic Cell Genet.* 7:769–775.
16. Wright, W. E. 1978. The isolation of heterokaryons and hybrids by a selective system using irreversible biochemical inhibitors. *Exp. Cell Res.* 112:395–407.
17. O'Farrell P. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
18. Wright, W. E. 1982. Equilibration in aqueous methanol increases the resolution of two-dimensinal polyacrylamide gels. *Separation Science and Technology.* 17:1689–1696.
19. Wright, W. E., and Gros, F. 1981. Coexpression of myogenic functions in L6 rat × T984 mouse myoblast hybrids. *Dev. Biol.* 86:236–240.
20. Lawrence, J. B., S. F. Konieczny, M. Shaffer, A. W. Coleman, and J. R. Coleman. 1982. Analysis of myogenesis by somatic cell hybridization. I. Myogenic competence of homotypic hybrids derived from rat L6 myoblasts. *Exp. Cell Res.* 142:261–272.
21. Cox, D. M., V. Niewczas-Late, M. I. Riffel, and J. L. Hamerton. 1974. Chromosomal mosaicism in diagnostic amniotic fluid cell cultures. *Pediatr. Res.* 8:679–683.
22. Mandell, J. L., and M. L. Pearson. 1974. Insulin stimulates myogenesis in a rat myoblast line. *Nature (Lond.).* 251:618–620.
23. Clark, W. A., Jr., R. A. Chizzonite, A. W. Everett, M. Rabinowitz, and R. Zak. 1982. Species correlations between cardiac isomyosins. A comparison of electrophoretic and immunological properties. *J. Biol. Chem.* 257:5449–5454.
24. Clark, W. A., K. S. Frogner, and R. Zak. 1982. Analysis of proteolytic fragments of cardiac myosin with monoclonal antibodies. *J. Cell Biol.* 95(2, Pt. 2):369*a*. (Abstr.)
25. Wright, W. E. 1984. The induction of muscle genes in neural cells. *J. Cell Biol.* 98:427–435.
26. Shapiro, D. 1982. Steroid hormone regulation of vitellogenin gene expression. *CRC Crit. Rev. Biochem.* 12:187–203.
27. Hayward, M. A., Mitchell, R. A., and D. J. Shapiro. 1980. Induction of estrogen receptor and reversal of the nuclear-cytoplasmic receptor ratio during vitellogenin synthesis and withdrawal in xenopus laevis. *J. Biol. Chem.* 255:11308–11312.
28. Loomis, W. F., J. P. Wahrmann, and D. Luzzati. 1973. Temperature-sensitive variants of an established myoblast line. *Proc. Natl. Acad. Sci. USA.* 70:425–429.
29. Somers, D. G., and M. L. Pearson. 1975. Myoblast mutant cell line possessing α-amanitin-resistant RNA polymerase II. *J. Biol. Chem.* 250:4825–4831.
30. Ng, S. K., J. Rogers, and B. D. Sanwal. 1976. Alterations in differentiation and pyrimidine pathway enzymes in 5-azacytidine resistant variants of a myoblast line. *J. Cell. Physiol.* 90:361–374.
31. Kaufman, S. J., and C. M. Parks. 1977. Loss of growth control and differentiation in the fu-1 variant of the L8 line of rat myoblasts. *Proc. Natl. Acad. Sci. USA.* 74:3888–3892.
32. Tarikas, H., and D. Schubert. 1974. Regulation of adenylate kinase and creatine kinase activities in myogenic cells. *Proc. Natl. Acad. Sci. USA.* 71:2377–2381.
33. Lawrence, J. B., and J. R. Coleman. 1983. Analysis of myogenesis by somatic cell hybridization. II. Retention of myogenic competence and suppression of transformed properties in hybrids between differentiation competent and incompetent L6 myoblasts. *J. Cell. Physiol.* 114:99–110.
34. Konieczny, S. F., and J. R. Coleman. 1983. Analysis of myogenesis by somatic cell hybridization. III. Myogenic competence of hybrids derived from rat L6 myoblasts and mouse primary fibroblasts and myoblasts. *Somatic Cell Genet.* 9:25–42.
35. Peterson, J. A. 1983. The widespread nature of phenotypic variability in hepatomas and cell lines, in the form of a geometric series. *J. Theor. Biol.* 102:41–53.
36. Paigen, K., and J. Felton. 1971. Genetic factors affecting enzyme activity. *In* Drugs and Cell Regulation. E. Mihich, editor. Academic Press, Inc. New York. 185–196.
37. Huntsberger, D. V., and P. E. Leaverton. 1970. Statistical Inference in the Biomedical Sciences. Allyn and Bacon, Boston. 237–239.