The Amplified Expression of Factors Regulating Myogenesis in L6 Myoblasts

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ABSTRACT A strategy for increasing the expression of the factors regulating myogenesis was developed based upon the observation that increased amounts of regulatory factors could overcome the inhibition of differentiation produced by 5-bromodeoxyuridine (BUdR). L6 rat myoblasts were subjected to multiple cycles of cloning in progressively increasing concentrations of BUdR. The first clones to differentiate were picked and replated for the next cycle of selection. After 28 cycles in BUdR, cells were isolated that could differentiate in the presence of 8 μM BUdR. Cell hybrids between myoblasts subjected to 21 cycles of selection (BU21 cells) and differentiation-defective myoblasts exhibited a high probability of differentiation, consistent with the hypothesis that BU21 cells were overproducing factor(s) involved in the decision to differentiate. The selection of cells able to differentiate in the presence of BUdR may provide a general approach for increasing the expression of the regulatory molecules controlling terminal differentiation.

The process of terminal cell differentiation almost certainly involves multiple events before differentiation is finally initiated. A great deal of progress has been made using molecular approaches toward understanding the factors (enhancers, promoters, methylation sites, etc.) that influence gene regulation at the final stage of cell differentiation: the expression of structural genes. However, the lack of suitable experimental models has made it difficult to apply molecular techniques to earlier stages when the actual decision to differentiate is made. We here report the development of a model system in which the factors influencing the decision to commit to and execute terminal myogenesis should be amenable to study.

The development of this experimental system derived from two series of observations into the behavior of myogenic hybrids and heterokaryons. In the first, cell hybrids were formed between differentiation-defective and differentiation-competent myoblasts. These hybrids exhibited a frequency of differentiation (1%) that was 60-fold less than that seen in competent × competent hybrids (64%) and 20-fold greater than that seen in defective × defective hybrids (0.05%). We proposed a molecular model for the regulation of the probability of differentiation to explain these results (1). This model required that the induced level of a critical regulatory factor must be reduced in differentiation-defective myoblasts. Since differentiation-defective variants arise with a high frequency (2), this in turn suggested that the induced level of this molecule(s) is controlled by a mechanism that also varies with a high frequency. Assuming that there is no intrinsic directionality in this variation, the ability to isolate myoblasts producing very low levels (differentiation-defective myoblasts) implied that it should also be possible to isolate myoblasts producing very high levels (amplified myoblasts).

A second series of cell fusion experiments provided a strategy for isolating such myoblasts with an increased expression of the factors regulating terminal differentiation. 5-bromodeoxyuridine (BUdR)[1] inhibits cell differentiation in a variety of systems, including myogenesis. Although rat skeletal myosin light chain synthesis is normally induced in heterokaryons formed by fusing mononucleated differentiated chick myocytes to undifferentiated rat myoblasts (3, 4), this induction is blocked if the rat myoblasts are grown in BUdR prior to cell fusion (5). However, the ability to induce rat myosin light chain synthesis is regained if binucleated differentiated chick myocytes are fused to BUdR-blocked rat myoblasts (5). This suggested that increased amounts of regulatory factors could overcome the inhibition of differentiation produced by BUdR.

The results of the experiments involving BUdR-blocked myoblasts implied that if L6 myoblasts were cloned in the presence of BUdR, those cells with the highest amounts of the factors regulating cell differentiation should be able to overcome the inhibition of myogenesis produced by a low concentration of BUdR. The variable regulation of the level of these molecules suggested by the experiments involving differentiation-defective myoblasts implied that repeated cycles of selection in increasing concentrations of BUdR

[1] *Abbreviation used in this paper:* BUdR, 5-bromodeoxyuridine.

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might result in a progressively amplified expression (with or without actual gene-amplification [6]) of these molecules. We here report the successful isolation and characterization of myoblasts able to differentiate in the presence of BUdR and which exhibit a dominant behavior when fused to differentiation-defective myoblasts, consistent with the interpretation that they have an increased expression of the factors regulating terminal myogenic differentiation.

MATERIALS AND METHODS

L6 rat myoblasts (7) were grown in medium containing four parts Dulbecco's modified Eagle's medium to one part medium 199, 10% fetal calf serum, 100 μg/ml streptomycin, and 100 μg/ml penicillin. BUdR-resistant cells were isolated during repeated cycles of cloning. At each cycle, approximately 1,000–10,000 colonies in one 150-cm^2 petri dish were visually screened on the day(s) on which previous cycles indicated differentiation would first start to occur. Typically, 10–20 colonies containing myotubes were identified and circled with a fine tipped pen. Because the selection protocol did not require true clones, even colonies with an immediately adjacent colony were marked. Adjacent nonmyotube containing colonies were then scraped off the dish using the fire-polished tip of a drawn out sterile Pasteur pipet under direct observation using a 4 × objective. The marked colonies were trypsinized within glass cloning cylinders sealed to the bottom of the dish with sterile vaseline. All of the trypsinized colonies were then mixed together and replated in 2-3 150-cm^2 petri dishes for the next cycle of selection. A variety of factors motivated the decision to pick and pool multiple colonies at each cycle: (*a*) The identification of a small multinucleated cell as a myotube was often ambiguous, particularly during the initial cycles of selection in BUdR; combining many colonies diluted the effect of any mistaken judgment. (*b*) Since local cell density is one of the most important factors influencing cell differentiation, the probability of differentiation of a well isolated colony might be different from that of two adjacent colonies. The cells were plated at the highest practical density, thus colonies with adjacent colonies were not infrequent. Picking and mixing multiple colonies diluted the effect of choosing a colony that differentiated precociously primarily because it was next to another colony rather than because it was expressing an increased propensity to differentiate. (*c*) The probability of obtaining a variant is proportional to the number of cells studied. Picking 10–20 colonies thus increased the probability of a shift in gene expression 10–20-fold as compared with picking a single colony, without requiring the time delay that would have been incurred by expanding a single colony to an equivalent cell number.

Part of the toxicity of BUdR has been ascribed to its inhibition of thymidylate synthetase and the consequent reduction in intracellular deoxycytidine (8). Since the cells began exhibiting toxic effects (slowed growth rate and a more flattened morphology with apparent stress fibers) under clonal growth conditions in 0.25 μM BUdR, 200 μM deoxycytidine was added to the medium during subsequent cycles. We have shown that this concentration of deoxycytidine only produces a slight decrease in the degree of BUdR for thymidine substitution in the DNA of L6 myoblasts and does not reverse the inhibition of differentiation produced by BUdR (9). The degree of BUdR for thymidine substitution was determined on cesium chloride gradients (9). Manipulations involving BUdR were performed in a room fitted with gold fluorescent lighting (Sylvania gold rapid-start lamps) and using microscopes fitted with filters that blocked the transmission of light of <550 nm.

Cell hybrids were constructed, karyotyped, and stained with antimyosin antibodies as described elsewhere (1). Briefly, highly purified populations of heterokaryons were isolated by exploiting the complementation between the lethal effects of two different irreversible biochemical inhibitors (10, 11). The heterokaryons were then cloned in 96-well plates and expanded into several 2-cm^2 wells, one of which was karyotyped in situ. Clones containing a chromosome number appropriate for hybrids between the parental cells were then replated at clonal density and allowed to grow for 2 wk to form large colonies, then stimulated to differentiate by feeding with medium containing 1% fetal bovine serum and 5 μg/ml insulin (12). 1 wk later the cells were fixed and stained with the monoclonal antimyosin antibody CCM-52(13) (generously provided by R. Zak, University of Chicago, IL) using an avidin-biotin-peroxidase detection system (Vectastain, Vector Laboritories, Burlingame, CA). This permitted both the fraction of myosin-positive subclones and the fraction of myosin-positive cells to be quantitated.

RESULTS

After an initial round of selection of L6 rat myoblasts in the absence of BUdR to obtain a pool of well-differentiating clones, two cycles of selection in 0.5 μM BUdR were performed. This concentration inhibited myotube formation by 50% under mass culture conditions (9). Although some clones containing possible myotubes were picked after 15–16-d growth at each cycle, the extent of myotube formation was so limited under these clonal conditions as to make the identification of myotube-containing clones ambiguous. Several additional cycles of selection in the absence of BUdR were then performed to isolate myoblasts with an increased propensity to differentiate before reinstituting the selection in BUdR. After seven cycles of selection, ED7 cells (early differentiating, seven cycles of selection) were obtained which showed myotube formation as early as 6–7 d after plating in colonies occasionally containing as few as 100 cells. The ED7 cells were then used as the starting cell population for the cycles of BUdR selection. The presence of 0.25 μM BUdR increased the time required for ED7 cells to differentiate from 6–7 d to 12 d. The concentration of BUdR was gradually increased to 8 μM BUdR by alternating cycles in which clones were selected for their ability to differentiate at shorter times with cycles in which they were selected for their ability to differentiate in higher concentrations of BUdR (Fig. 1).

The density of the DNA was determined on cesium chloride gradients at cycle BU25 to verify that the cells were incorporating BUdR normally. DNA from BU25 cells grown in 2 μM BUdR plus 200 μM deoxycytidine (see Materials and

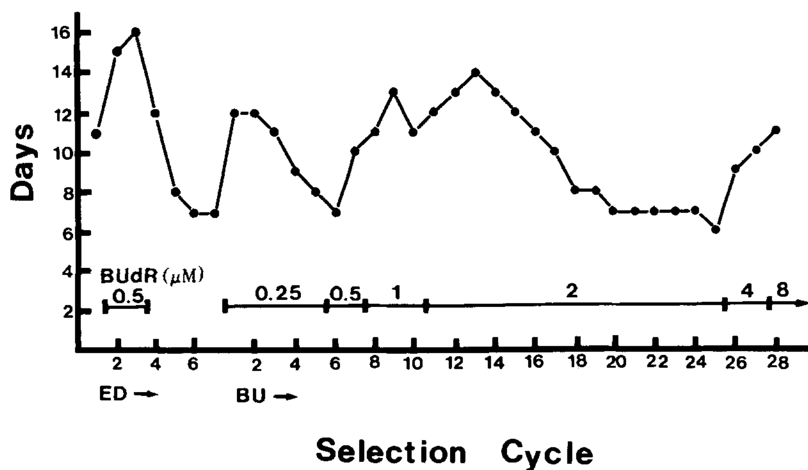

FIGURE 1 Selection history of cells able to differentiate in the presence of BUdR. At each cycle of selection the first 10–20 clones exhibiting myotube formation were picked, pooled, and replated at clonal densities for the next cycle. The myotubes formed during cycles two and three were so small and ambiguous that the use of BUdR was temporarily discontinued and clones were isolated simply based on their capacity to differentiate at low local cell densities *ED*, early differentiating. ED cycle seven cells then formed the starting material for a new series of selection cycles in the presence of BUdR.

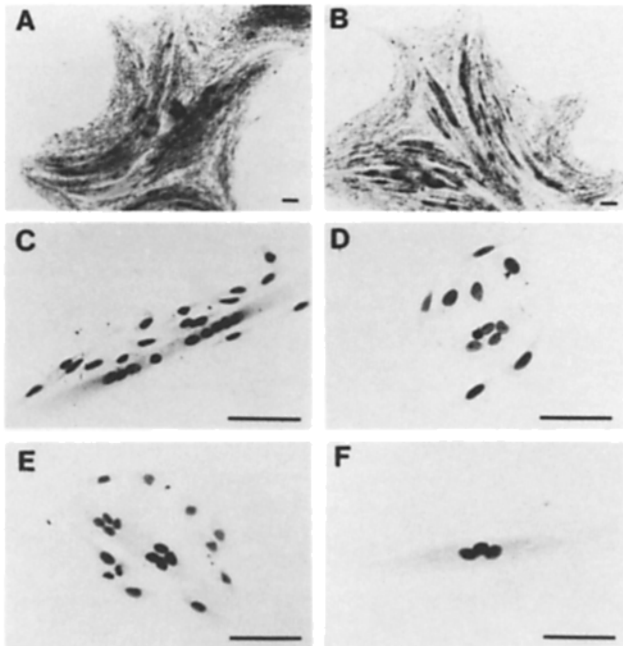

FIGURE 2 BUdR "dependence" of BU21 cells. L6 myoblasts or cells isolated after 21 cycles of selection in BUdR (BU21 cells) were plated at clonal densities in the absence of BUdR, then fixed and giemsa-stained on the day that myotube colonies were first observed. (*A* and *B*) L6 myoblasts fixed 10 d after plating. (*C–F*) BU21 myoblasts fixed 4 d after plating. Although most of the myotube-containing BU21 colonies had mixtures of differentiated and undifferentiated cells (*C–E*), occasional colonies were totally differentiated (*F*). Bar, 100 μm.

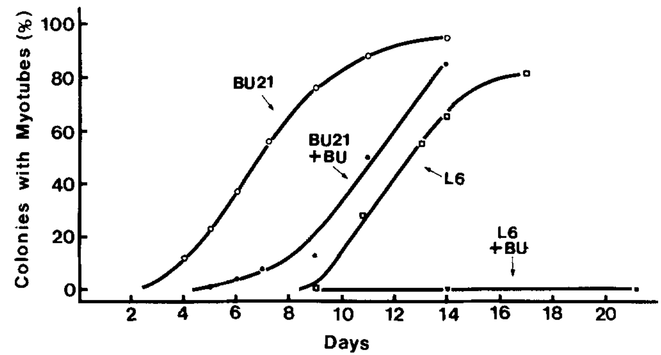

FIGURE 3 Time dependence of clonal differentiation of L6 and BU21 myoblasts. L6 and BU21 myoblasts were plated in multiple dishes at 500 cells per 60-mm petri dish in medium with or without 2 μM BUdR and 20 μM deoxycytidine. Approximately 160 colonies (eight colonies per cm^2) formed per plate. Dishes were fixed, giemsa stained, and counted to determine the percent of colonies containing myotubes. A myotube was defined as a cell containing more than three nuclei. BU21 cells differentiate at earlier times in the presence of 2 μM BUdR than the parental L6 myoblasts do in its absence.

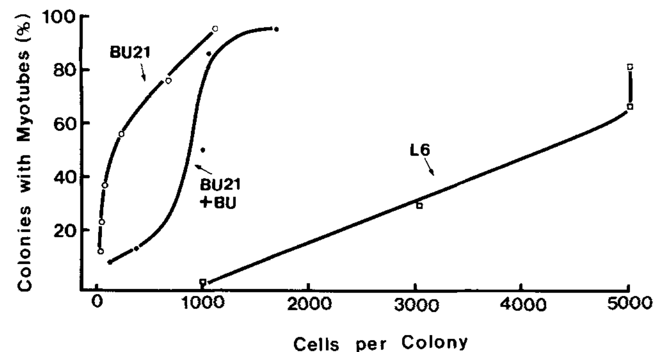

FIGURE 4 Colony size dependence of clonal differentiation of L6 and BU21 myoblasts. Colony sizes from the experiment of Fig. 4 were detemined in two ways. For time points of less than 6 d, all of the nuclei in 10–20 colonies from the fixed and stained dishes were counted, and the average colony size calculated. For later time points, sister dishes were trypsinized and total cell number determined using a Coulter Counter (Model ZBI, Coulter Electronics, Inc., Lehigh Valley, PA). Average colony size was then calculated by dividing total cells by the number of colonies per plate.

Methods) showed a density shift of 0.0239 g/ml, corresponding to a 27% BUdR for thymidine substitution. This was slightly less than the 36% substitution obtained with control L6 myoblasts grown under the same conditions. Although a small part of the resistance to BUdR might have been a result of this lowered incorporation, the cells were nonetheless incorporating 75% of the expected amount of BUdR into their DNA.

Following 21 cycles of selection, cells were obtained that began forming myotubes 6–7 d after being plated at clonal densities in 2 μM BUdR. These BU21 myoblasts were essentially BUdR-dependent for cell growth rather than differentiation. When cloned in the absence of BUdR, small colonies containing fewer than 20 nuclei, half of which were already in a myotube, were formed after 3–4 d growth. Colonies consisting of no mononucleated cells and a single myotube containing 3–4 nuclei were occasionally observed 3 d after cloning. Since no multinucleated myotubes were observed during the first 2 d, these myotubes were not preformed in the initial cell population but represented cells that may have been producing so much of a regulatory factor(s) that they differentiated once the BUdR was removed. Fig. 2 shows some representative colonies of the initial L6 myoblasts and BU21 cells grown in the absence of BUdR. Although L6 parental cells were completely inhibited during clonal growth by 2 μM BUdR, BU21 cells differentiated at shorter times of clonal growth in the presence of 2 μM BUdR than L6 cells did in the absence of BUdR (Fig. 3). This difference is even more impressive when the data of Fig. 4 are replotted as a function of colony size (Fig. 4).

A shift in chromosome number occurred during the isolation of BU21 variants. Whereas our parental L6 myoblasts have a modal number of 41 chromosomes (14), the BU21 cells had 65 (range 56 to 69). The shift to increased ploidy occurred before the cells became resistant to BUdR, since ED7 cells had 75 chromosome (range 66 to 87). The ability to differentiate in the presence of 2 μM BUdR is thus not a simple consequence of a gross increase in chromosome number. However, in the absence of detailed banding studies, we cannot know whether potential gene dosage effects due to increased numbers of specific chromosomes were present. No double minute chromosomes were observed in either ED7 or BU21 cells.

The relative dominance of the BUdR-resistant phenotype was examined in cell hybrids. Because an increase in chromosome number had occurred in BU21 cells, three sets of hybrids were constructed: BU21 × differentiation-defective; L6 × differentiation-defective; and ED7 × differentiation-defective myoblasts. Since ED7 cells had a higher chromo-

TABLE I
*Differentiation Capacity of Hybrid Clones**

Cell combination	Karyotype†	Typical colony size	Colonies counted	Myosin(+) colonies	Total myosin(+) cells	Myosin(+) cells
				%		%
BU21 × DD4a						
Clone 2	86	584/4§	108	100	–§	99
Clone 3	94	2,108/1,232	157	86	–	54
Clone 4	85	2,108/1,860	78	87	–	46
Clone 5	90	2,976/444	104	100	–	87
Clone 6	92	1,460/464	175	98	–	75
Clone 8	82	2,808/236	125	100	–	92
Clone 10	90	2,319/429	275	98	–	82
Clone 11	88	4,732/456	70	96	–	87
Clone 12	86	776/356	58	98	–	68
Clone 14	86	648/133	78	99	–	82
Clone 15	96	380/187	47	96	–	64
Clone 16	89	736/1,416	109	97	–	33
Clone 17	86	380/187	47	96	–	64
Clone 3′	111	2,056/376	76	93	–	79
Clone 9′	85	1,776/340	122	100	–	84
Clone 11′	88	684/272	73	100	–	72
Clone 18′	94	972/560	105	95	–	62
Clone 22′	95	425/93	76	99	–	81
ED7 × DD4a						
Clone 2	88	680	17	88	287	2.5
Clone 4	86	1,001	65	54	8,272	12.7
Clone 6	100	1,021	29	72	2,178	7.4
Clone 11	94	793	20	95	1,923	12.1
Clone 13	84	2,352	10	100	8,815	37.5
Clone 15	95	704	23	87	492	3.0
Clone 25	91	974	17	82	2,850	17.2
Clone 32	98	650	20	90	3,690	28.4
L6 × DD4a						
Clone 2	62	912	21	95	1,471	7.7
Clone 3	67	2,880	55	90	7,356	4.6
Clone 12	64	492	15	67	615	8.3
Clone 13	70	815	45	80	1,555	4.2
Clone 14	57	907	38	74	1,302	3.8
Clone 15	66	2,264	33	91	20,102	27.0
Clone 16	66	1,116	36	72	6,488	16.0
Clone 19	64	8,576	10	90	5,057	5.9
Clone 21	63	1,860	29	90	6,711	12.4
Clone 23	68	2,006	20	100	4,041	10.1

* BU cycle 21 (BU21), early differentiating cycle 7 (ED7), and parental L6 myoblasts were each fused to differentiation-defective clone 4a (DD4a) cells. Each hybrid clone was subcultivated at clonal density, allowed to grow for 2 wk, fed differentiation-stimulating medium for 1 wk, then immunoperoxidase-stained with antimyosin antibodies. The total number of nuclei in myosin(+) cells was counted for all the hybrids except BU21 × DD4a (see §). The percent myosin(+) cells was calculated by dividing the total of myosin(+) cells by the product of the number of colonies counted and the number of cells per typical colony.

† Cells were karyotyped in situ and counted under direct ×400 observation. The number of chromosomes 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. BU21 myoblasts have 65 chromosomes, ED7 cells have 75 chromosomes, and L6 and DD4a myoblasts have ~40 chromosomes. The expected chromosome numbers are thus 105, 115, and 80 for BU21 × DD4a, ED7 × DD4a, and L6 × DD4a hybrids, respectively. As shown elsewhere (1), in situ karyotyping tends to underestimate the actual number of chromosomes, particularly in hybrid cells with more overlapping chromosomes.

§ Typical colony sizes for BU21×DD4a hybrids are expressed as a ratio of myosin(+) to myosin(–) cells. The number of myosin(+) cells in this combination was far too great to permit the total number to be actually counted. Instead, a typical colony was counted, and the total percent myosin(+) cells was estimated by multiplying the percent myosin(+) cells in a typical colony for the percent colonies that were myosin(+).

some number than BU21 myoblasts but lacked the BUdR-resistant phenotype, the potential contribution of "non-specific" gene dosage effects of increased chromosome numbers could be evaluated. The ability of all the sets of hybrid clones to differentiate was analyzed in the absence of BUdR. Table I presents the data from each hybrid clone.

BU21 × differentiation-defective cell hybrids showed a much higher frequency of differentiation (geometric mean = 71%) than L6 × differentiation-defective hybrids (geometric mean = 8%). (The geometric mean is given since the data vary in an exponential fashion [1]). The experimental protocol involved growing each hybrid clone as subclones for 2 wk, then stimulating them to differentiate in low serum plus insulin for 1 wk before analyzing for myosin-positive cells

and clones. The differentiation capacity of the BU21 × differentiation-defective hybrids was so great that most of the subclones had become massively fused after 2-wk growth without ever being exposed to differentiation-stimulating conditions. Since these hybrids had already become maximally differentiated before the end of the experiment, their relative capacity to differentiate was not fully assessed. Nonetheless, they clearly showed an increased probability of differentiation as compared with the L6 × differentiation-defective hybrids. The hybrids between ED7 and differentiation-defective myoblasts exhibited a probability of differentiation that was approximately the same as the L6 × differentiation-defective hybrids (geometric mean = 11%). These hybrids show that an increased gene-dosage effect from the myogenic-competent parent is not sufficient by itself to produce the dramatic rise in the probability of differentiation observed in the BU21 × differentiation-defective hybrids.

DISCUSSION

Cells resistant to the effect of BUdR generally either fail to transport BUdR across the plasma membrane or lack thymidine kinase and fail to incorporate BUdR into DNA. The DNA density shift observed in cesium chloride gradients for myoblasts that can differentiate in the presence of BUdR establishes that BUdR is entering the DNA and, thus, that neither of the above explanations can account for the resistant phenotype. The ability of the cells to differentiate at lower cell densities when cloned in the absence of BUdR shows that BUdR is still having an inhibitory effect on cell differentiation and suggests that the cells are resistant due to a mechanism that overcomes rather than circumvents the action of BUdR. The very high levels of myogenesis observed in cell hybrids formed by fusing BU21 cells to differentiation-defective myoblasts demonstrates that the BUdR-resistant phenotype behaves in a dominant fashion. A variety of possible mechanisms might explain these results; for example, an increased level of phosphorylation of a regulatory molecule or a decreased concentration of an inhibitory factor. However, we believe that the most likely and consistent explanation for the present and previous results (1, 5) is that the mechanism by which they have overcome the inhibitory effects of BUdR is by increasing the expression of the factor(s) regulating the decision to differentiate. This could result from an amplification of the gene(s) coding for this factor(s) or an overproduction of the factor(s) from a single copy gene(s). The variants isolated in this study were obtained without overt mutagenesis. Even though care was taken to avoid exposure of the cells to light of wavelengths <550 nm, we cannot exclude the possibility that the variants obtained are actually mutants caused by the incorporation of BUdR.

Approximately 2,000 colonies were examined and 15 clones were picked during each cycle of selection. Assuming that at least one additional variant was in fact isolated during each cycle, this represents a frequency of roughly 10^{-3}. Since the frequency of gene reduplication is $\sim 10^{-3}$, and can be as high as 40% in the presence of DNA synthesis inhibitors (6), gene amplification could easily be responsible for the effect obtained during this stepwise selection. Stepwise selection has been widely used to amplify genes coding for known enzymes that confer resistance to toxic drugs (reviewed in reference 6). The present experiments provide a novel application of stepwise selection to potentially amplify the expression of unknown regulatory factors. We are attempting to use RNA from BUdR-resistant myoblasts to molecularly clone the factors regulating terminal differentiation. If successful, the probes obtained should permit one to detemine whether the expression of these factors is increased and, if so, whether they are increased as a result of gene amplification or increased transcription/translation/stability.

An alternative to gene amplification as an explanation of the phenotype of BU21 cells derives from the studies of Peterson (15–17) and Pagen (18). They have shown that quantitative variants in gene expression are constantly being generated at essentially all loci, even those for so-called "household functions." Peterson's model (17) states that the mean level of expression of a given product by a population of cells represents the steady state achieved by balancing the generation of variants having decreased levels of expression with those having increased levels of expression. Our previous studies had suggested that differentiation-defective variants expressed decreased levels of the factors regulating the decision to differentiate. Peterson's model suggested that if variants expressing a decreased level of these factors were constantly being generated then variants expressing an amplified level should similarly be present. In addition, should such amplified variants be isolated, they in turn should generate cells with both decreased and further increased levels of expression, so that successive cycles of selection would progressively increase the amplification obtained. The molecular basis for Peterson's mathematical model for quantitative variation is unknown and could well be gene amplification itself. Nonetheless, it provided a major theoretical rationale for the strategy for using successive cycles of selection to isolate amplified myoblasts.

Many steps probably intervene between the decision to differentiate and the expression of differentiated structural proteins. It is likely that BUdR effects multiple steps in this sequence and not just the initial decision to differentiate. Since BU21 cells were selected only for their ability to form morphologically identifiable myotubes, the factors involved in other steps of terminal differentiation may not have been altered and thus may still be inhibited by BUdR. The examination of changes in the coordinate regulation of different myogenic structural proteins during cell differentiation of BU21 cells grown in the presence versus the absence of BUdR may provide some insight into the number of different regulatory stages/sites involved.

There are relatively few experimental models amenable to isolating genes involved in regulatory decisions. The selection for cells able to overcome the inhibition of differentiation produced by BUdR appears to provide such a system. Since BUdR inhibits differentiation in a wide variety of cell lineages (19–25), its use may be generalizable for the isolation of variants with an amplified expression of factors regulating the decision to differentiate along several different pathways. It is hoped that such studies may ultimately shed light on the elusive molecular mechanisms involved in determination and the commitment to terminal differentiation.

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