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Cell Biology

Loss of growth control and differentiation in the fu-1 variant of the L_8 line of rat myoblasts

(muscle development/transformation/tumorigenesis/adhesiveness/soft agar)

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ABSTRACT **A nonfusing variant, fu-1, of the L_8 line of rat myoblasts was isolated and characterized with respect to its growth *in vitro* and developmental properties. Comparative analyses of density-dependent inhibition of growth, serum requirements, cell adhesiveness, colony formation in soft agar, and hexose transport in L_8 and fu-1 cells support the conclusion that the fu-1 cells are transformed. In addition, fu-1, but not L_8, cells promote the development of tumors in athymic *nude* mice. fu-1 cells also do not make increased levels of creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) or myosin and they express an endogenous type-C virus. Both L_8 and fu-1 cells express myokinase (ATP:AMP phosphotransferase, EC 2.7.4.3) activities in single cells. In contrast to fu-1 cells, the parent L_8 line has increased creatine kinase and myosin after fusion and spontaneously contracts; expression of an endogenous virus could not be detected in these cells. These results suggest that loss of the ability to differentiate normally is associated with the loss of the normal control of cell division of myoblasts grown *in vitro* and *in vivo*.**

Myoblasts, the precursors of highly differentiated skeletal muscle, proliferate in cell culture as single cells. Upon reaching a critical stage in their development, the myoblasts withdraw from the cell cycle and fuse into multinucleate cells (myotubes) (1–4). Concomitant with, but not necessarily dependent on, fusion is the synthesis of several proteins characteristic of the differentiated state [e.g., myosin, actin, creatine kinase (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2), etc.] (5–8); acetylcholine receptors and acetylcholinesterase activity likewise appear shortly after fusion (9–13). Several days following the onset of fusion, discrete myofibrillar banding characteristic of muscle fibers develops and the myotubes contract.

Fusion into myotubes usually takes place after withdrawal of the myoblasts from the cell cycle (7, 14, 15), perhaps as the result of the expression or activation of new information during the immediately preceding round of replication (2, 14). Cells that fuse to form myotubes have depleted levels of DNA polymerase and cease replication (16, 17). This termination of DNA synthesis prior to fusion seems to be under stringent control. Analysis of myotube DNA by incorporation of [^{3}H]-thymidine, autoradiography, and Feulgen microspectrophotometry indicate that DNA is not synthesized within myotube nuclei (2, 18). The myotube is thus a "dead-end" with respect to its replicative capacity; indeed, the capacity for continued proliferation appears to be incompatible with the differentiation of myoblasts into myotubes. If this is true, then clearly, transformed myoblasts that have lost their normal growth control mechanisms cannot differentiate into multinucleate fibers. It is not clear whether the converse is true, that cells that have lost the ability to differentiate normally under the appropriate conditions have also lost the mechanisms that normally control growth. To examine these hypotheses we have isolated a line of rat myoblasts (fu-1) that is defective in fusion from the myogenic L_8 line. Analysis of several parameters that are usually attributed to transformed cells *in vitro* suggests that the nonfusing variant fu-1 cells are transformed when compared to the parent L_8 cells. In addition, only fu-1 cells will form tumors in athymic mice. We have also found that the fu-1 cells express an endogenous C-type virus; the L_8 cells do not express this virus.

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MATERIALS AND METHODS

Cell Culture Conditions. The L_8 cells, kindly provided by D. Yaffe, were cloned and maintained by serial passage. Cloned cells were retrieved from frozen stocks as needed. fu-1 cells were selected from the cloned L_8 line as described in the *text*. All cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% horse serum (ISI), 0.5% chick embryo extract, penicillin at 100 units/ml, and streptomycin at 100 μg/ml, at 37° and in a 10% CO_2 atmosphere. Stock cultures of 2×10^5 cells were seeded in 10 ml of medium and grown on 100-mm Falcon tissue culture dishes coated with 0.1% gelatin. To maintain the myogenic capacity of L_8 cells, it is essential to subculture the cells before they become confluent. Cells were routinely harvested by detachment with 0.05% trypsin in Ca^{2+},Mg^{2+}-free Earle's solution. In experimental cultures, 3 ml of medium containing 1 to 2×10^5 cells were seeded in 50-mm dishes; medium was changed every day or as indicated. The cells were found free of mycoplasma.

Uptake of 2-Deoxyglucose. Cells (2×10^5) were plated in 3 ml of medium on 50-mm dishes coated with gelatin. To determine uptake of 2-deoxyglucose (dGlc), the cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), pH 7.4 at 20°. One milliliter of prewarmed PBS containing [^{3}H]dGlc at 1 μCi/ml (New England Nuclear, 7.5 Ci/mmol) was added per plate. The cells were incubated at 37° with the dGlc for various times on day 3 or for 20 min on days 1–5. After incubation the cells were washed three times with 3 ml of cold PBS, drained well, and solubilized for 20 min in 1 ml of 1 M NaOH. Samples (100 μl) from each of the duplicate plates were neutralized in 10 ml of Aquasol with 1 M HCl and were assayed for radioactivity. Protein was determined as described (19).

Colony Foundation in Soft Agar. We adopted the method of MacPherson and Montagnier (20) for the growth of transformed cells in soft agar. The basal layer, consisting of 3 ml of Dulbecco's medium supplemented with 0.5% Bacto agar, 10% horse serum, and antibiotics, in 50-mm dishes, was overlayed with 1.5 ml of cells in 0.33% agar and complete medium. The solutions were prepared on the day of the experiments as described (20) and were kept at 44° till used. Only freshly thawed, cloned cells were used in these experiments. Colonies of cells were readily visible 5–7 days after plating and were counted on day 7.

Abbreviations: CK, creatine kinase; MK, myokinase; PBS, phosphate-buffered saline; dGlc, 2-deoxy-D-glucose.

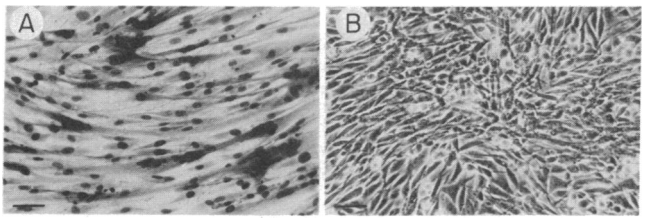

FIG. 1. L_8 and fu-1 cells. Bars equal 50 μm. (*A*) Fused L_8 myoblasts fixed in 70% methanol and stained with Giemsa. (*B*) Phase contrast microscopy of fu-1 cells.

Growth in *Nude* Mice. Specific pathogen-free, athymic, female *nude* (*nu/nu*) mice, 6–7 weeks old (Harlan Industries), were injected subcutaneously with 3×10^6 cloned cells in 0.1 ml of Dulbecco's medium. These mice were maintained in a germfree isolator.

Creatine Kinase and Myokinase Assays. L_8 and fu-1 cells were grown on 50-mm plates in Dulbecco's medium containing 10% horse serum, washed in PBS, and stored at −76°. The cells were scraped from the plates in 10 mM glycylglycine, pH 6.8 at 20°, sonicated at 4°, and assayed for creatine kinase (CK) as modified from Shainberg *et al.* (6). To assay CK activity, conversion of NADP to NADPH was monitored at 340 nm, at 30° in a reaction mixture containing: 20 mM glucose, 10 mM magnesium acetate, 1 mM ADP, 20 mM AMP, 1 mM NADP, 10 mM cysteine, 15 mM creatine phosphate, hexokinase at 1 unit/ml, glucose-6-phosphate dehydrogenase at 0.5 units/ml, and 100 mM glycylglycine, pH 6.8. The same reaction mixture, but without AMP and creatine phosphate, was used to assay myokinase (MK) (ATP:AMP phosphotransferase, EC 2.7.4.3) activity. One unit of CK or MK will convert 1 μmol of NADP to NADPH per min at 30° at pH 6.8. Cell protein was determined as described (19).

Polyacrylamide Gel Electrophoresis. Total cell lysates were electrophoresed in 8–20% polyacrylamide gradient slab gels (0.7 mm thick), for 3 hr at 20 mA, at 4°, in Tris/glycine/sodium dodecyl sulfate buffer (21). The gel was fixed and stained for 2 hr in 0.2% Coomassie blue in 50% (vol/vol) methanol/7% (vol/vol) acetic acid and destained in 25% methanol/7% acetic acid. Electrophoresis-grade reagents were purchased from Bio-Rad. The gels were scanned in a Helena densitometer and the relative amounts of myosin and actin were determined.

RESULTS

The L_8 line of rat myoblasts maintains the capacity to fuse and differentiate in cell culture. This line was isolated by D. Yaffe by procedures analogous to those described for other myogenic lines (22); exogenous mutagens were not used in the selection or establishment of this clone. We have subcloned these cells and have maintained them in culture or as frozen stocks for more than 4 years. Upon reaching a density of approximately 1.0 to 1.5×10^5 cells per cm^2, the L_8 cells fuse and form myotubes (Figs. 1 and 2).

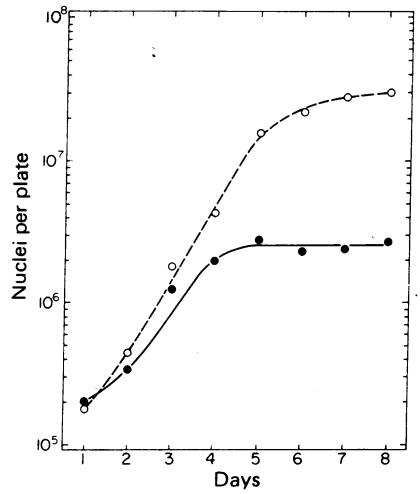

FIG. 2. Growth of L_8 (●—●) and fu-1 (○---○). Inocula of 10^5 cells were plated and grown in 10% serum in 50-mm dishes. Medium was changed daily and nuclei were enumerated as indicated in Table 1.

The myotubes will subsequently assume many of the characteristics of functional muscle, including increased synthesis of myosin and CK. This elevation in CK activity closely parallels fusion and the enzyme is the isozyme characteristic of muscle cells (unpublished observation). Within 2–3 days after fusion has begun these L_8 myotubes will spontaneously contract. This pattern of development rather faithfully mimics myotube formation *in vivo* as well as that which has been reported in primary explants of avian and rat myoblasts (5, 6).

The nonfusing variant (fu-1) of the L_8 line was isolated by passage of the L_8 line 6–10 days after maximum fusion had occurred. This selection for "non-fusers" was repeated four times and the cells were cloned.

Density-Dependent Inhibition of Growth. The growth rates of L_8 and fu-1 cells were compared by enumerating nuclei. The doubling times during exponential growth (determined by linear regression analysis) for L_8 and fu-1 cells were 19.1 hr and 14.8 hr, respectively (Table 1). Upon reaching confluency, L_8 cells align in a parallel juxtaposed array and fuse into myotubes; fu-1 cells also align, but continue to divide. Although the growth of both cell types is inhibited at high densities (Fig. 2), fu-1 cells attain a saturation density up to 10-fold greater than L_8 (Table 1). This is partly the result of overgrowth of the fu-1 monolayer (Fig. 1), and partly due to the difference in size of fu-1 and L_8 cells. In confluent cultures, L_8 cells occupy approximately 3.5 times more area than do fu-1 cells.

Table 1. Comparison of L_8 and fu-1 cells

Cells	Doubling time,* hr		Saturation density,* cells/cm^2		Growth in agar,† % colonies formed	Formation of tumors in *nude* mice‡	Myosin, % total protein§
	10% serum	1% serum	10% serum	1% serum			
L_8	19.1 ± 2.7	25.1 ± 3.5	1.31×10^5	3.05×10^4	0.001	0/13	8.2
fu-1	14.8 ± 1.2	16.6 ± 2.0	1.37×10^6	8.33×10^5	49	11/13	0.6

* 10^5 cells were plated in 50-mm dishes in 10% serum. Eighteen hours later the medium was changed to contain either 10% or 1% serum. Medium was changed daily. Cells were collected by trypsinization and lysed in 0.75% acetic acid. Nuclei were counted in a hemocytometer and doubling times (±SD) in three experiments were determined by linear regression analysis; $r \geq 0.98$.

† Colony formation in 0.3% agar. Cloned L_8 and fu-1 cells were respectively plated at concentrations of 10^4 to 10^7 and 10^4 cells per dish.

‡ Samples containing 3×10^6 cloned L_8 or fu-1 cells in 0.1 ml of medium were injected subcutaneously into *nu/nu* mice. Results are expressed as number of mice developing tumors/number of mice injected. Two injected with fu-1 cells died of infections before developing tumors.

§ Myosin heavy chain in fused L_8 and in fu-1 cells as determined in Fig. 6. Unfused L_8 cells contained approximately 1.1% myosin.

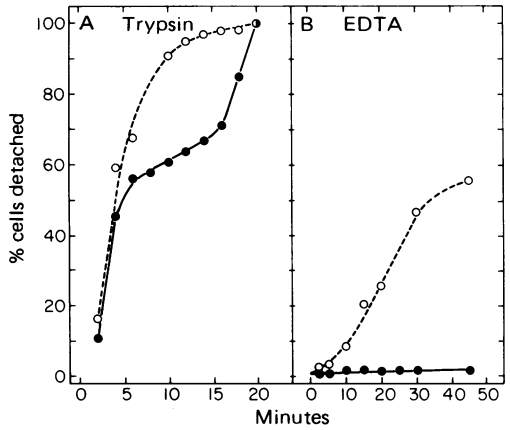

FIG. 3. Detachment of L_8 and fu-1 cells with trypsin and EDTA. Suspensions of 2×10^5 cells in 3 ml were plated on 50-mm gelatin-coated dishes and incubated 2 days. (*A*) The subconfluent monolayers were washed once with Ca^{2+},Mg^{2+}-free Earle's solution and incubated with a prewarmed solution of 0.05% trypsin. At the indicated times, the detached cells were removed from duplicate cultures and counted in a model ZBI Coulter counter. (*B*) Cells were washed with Ca^{2+},Mg^{2+}-free Dulbecco's PBS, pH 7.4, and incubated in the same buffer containing 4 mM EDTA. The numbers of L_8 (●—●) and fu-1 (o---o) cells that detached were compared with the total numbers recovered after trypsinization for 20 min and vigorous agitation.

Serum Requirements. Holley and others have shown that transformants usually require less serum for growth *in vitro* than do untransformed cells (23–25). This may be due to the synthesis by transformed cells of growth-promoting factors that are usually derived from serum. L_8 and fu-1 cells were plated and grown for 18 hr in 10% horse serum; thereafter, they were grown in medium containing either 10% or 1% serum. The growth rate of fu-1 cells in 1% serum was diminished. The doubling times of fu-1 cells in 10% and 1% serum were 14.8 and 16.6 hr, respectively; however, they attained comparable final densities (Table 1). L_8 cells also grew relatively slowly in 1% serum; however, the maximum density attained in 10% serum was 4.3-fold greater than that in 1% serum (Table 1). In 1% serum, the maximum density of fu-1 cells was more than 25-fold greater than that of L_8 cells.

Detachment. Transformed cells growing *in vitro* adhere less tenaciously to their substratum than do their untransformed counterparts (26–29), and this property has been correlated with the induction of tumors *in vivo* (30, 31). Similarly, L_8 and fu-1 cells can be distinguished by their different rates of detachment from plastic tissue culture dishes. The kinetics of detachment of fu-1 and L_8 cells treated with trypsin were significantly different (Fig. 3*A*). Almost all fu-1 cells detached within 10 min, and they appear to be a rather homogeneous population with respect to this criterion. In contrast, at least 40–50% of L_8 cells are not as readily dislodged with trypsin, and two populations of L_8 can be discerned by their relative ease of detachment. The more tightly bound population may represent cells at a different stage of development. This disparity in adhesiveness was even more pronounced when the cells were detached by the removal of divalent cations. More than 50% of fu-1 cells detached within 45 min when treated with 4 mM EDTA, whereas almost all of the L_8 cells remained bound to the dish (Fig. 3*B*). The slower detachment of L_8 cells compared to fu-1 indicates that L_8 cells may be bound to the substratum through more points of adhesion.

Growth in Soft Agar. The growth of cells in soft agar or in methylcellulose correlates well with the growth of transformed cells *in vitro*. Using the procedure of MacPherson and Montagnier (20), we have found that approximately 49% of fu-1 cells will develop into colonies in 0.3% agar; of approximately 2×10^7 L_8 cells plated, only 0.001% formed colonies (Table 1). The growth of fu-1 cells in soft agar is thus another parameter of transformed cells that distinguishes the fu-1 and L_8 lines.

Uptake of 2-Deoxyglucose. An increased rate of hexose transport is one of the earliest events associated with the transformation of cells (32–34). Although at elevated concentrations dGlc inhibits glycolysis, at low levels the uptake of [^{3}H]dGlc is an accurate measure of glucose transport. We have compared the uptake of [^{3}H]dGlc by L_8 and fu-1 cells and found that the transport in both lines progressively diminishes after plating (Fig. 4*A*). The rate of hexose transport in the fu-1 line, however, is 6.75-fold greater than in the L_8 cells (Fig. 4*B*). Elevated glucose transport is thus another characteristic of fu-1 cells.

Growth *In Vivo*. Whereas transformed growth *in vitro* is one measure of the loss of normal growth control, of added significance is the growth of L_8 and fu-1 cells *in vivo*. These cells were injected into congenitally athymic *nu/nu* mice. Recipients of 3×10^6 fu-1 cells developed visible tumors within 10 days at the sites of the subcutaneous injections (Table 1). No tumors were formed in any of the mice receiving either 3×10^6 L_8 cells or medium up to 5 weeks after inoculation. Although these animals have not been maintained for a prolonged period, these results do substantiate and extend our *in vitro* analyses.

Creatine Kinase and Myokinase Activities. In primary cultures of rat myoblasts, CK activity closely parallels fusion; increases in MK occur approximately 4 hr after the onset of fusion (6). In L_8 cells, CK activity also parallels fusion, although under conditions where fusion is inhibited with phospholipase C, the enzyme may be expressed in single cells (35). Elevated levels of CK activity were not detected in fu-1 cells. Unlike primary cultures, MK activity is evident in single L_8 cells and reaches maximum levels by the onset of fusion (Fig. 5). MK activity in fu-1 cells closely parallels that in the L_8 line.

Myosin Synthesis. Myosin levels are also increased in myotubes compared to the amount of myosin in unfused L_8 and fu-1 cells. This was true even when the concentration of fu-1 cells was far greater than that at which L_8 cells would fuse. Total cell lysates of fused and unfused L_8 cells and fu-1 cells were analyzed by sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis. The proportions of the total cell protein that stained with Coomassie blue and that comigrated with purified myosin heavy chain were 1.1% in unfused L_8, 8.2% in fused L_8, and 0.6% in fu-1 cells (Fig. 6). Thus, the increased level of myosin characteristic of the differentiated myotubes was not attained in the nonfusing variant. In contrast, the amounts of actin in myotubes and fu-1 cells are not appreciably different. However, we have not determined whether these are the same actin isotypes.

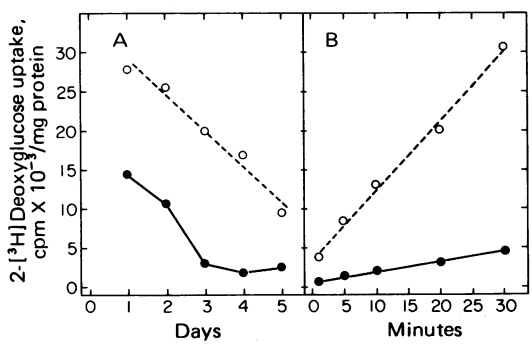

FIG. 4. Hexose uptake in L_8 and fu-1 cells. Uptake of [^{3}H]dGlc was determined in duplicate cultures of L_8 (●—●) or fu-1 (o---o) cells incubated with [^{3}H]dGlc (*A*) daily for 20 min, or (*B*) on day 3 for various time intervals.

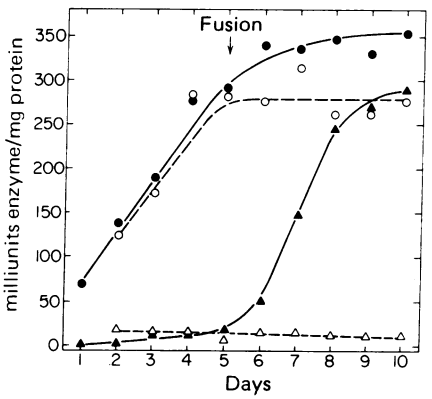

FIG. 5. CK and MK in L_8 and fu-1 cells. Inocula of 2×10^5 cells were plated in 50-mm dishes and the medium was changed daily. L_8 cells began to fuse on day 5. MK activity in L_8 (●—●) and fu-1 (○---○) was present in single cells; CK activity in L_8 (▲—▲) closely paralleled fusion but did not increase in fu-1 (△---△).

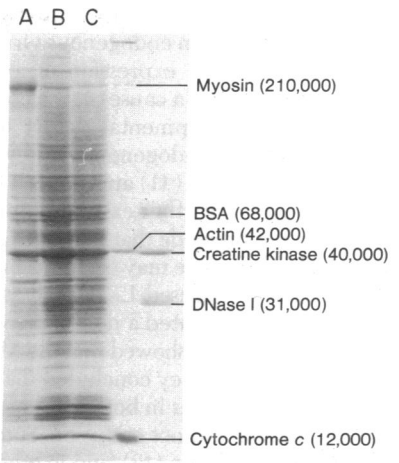

FIG. 6. Electrophoresis of L_8 and fu-1 cell lysates. L_8 myotube (*A*), L_8 myoblast (*B*), and fu-1 cell lysates (*C*) were electrophoresed in an 8–20% gradient slab of acrylamide in Tris/glycine/sodium dodecyl sulfate buffer system (21). The gel was fixed, stained with Coomassie blue, destained, and scanned in a densitometer. The percent of total protein that comigrated with the heavy myosin chain was (*A*) 8.2, (*B*) 1.1, and (*C*) 0.6. The molecular weights of the myosin, bovine serum albumin (BSA), actin, CK, DNase I, and cytochrome *c* markers are indicated.

DISCUSSION

During the development of skeletal muscle there is a stage at which differentiation and continued proliferation are incompatible; myoblasts must cease replication prior to fusing. The mechanism that initiates this phase of development may have a dual role, i.e., turning off replication and promoting further differentiation. This is consistent with experiments demonstrating that myoblasts fuse when the duration of their G_1 period is prolonged (14, 15). In addition, DNA polymerase activity is also diminished in pre-fusion myoblasts. We suggest that a failure to cease myoblast replication represents a commitment away from differentiation into myotubes. A defect at this control point in myoblast development may result in the unrestrained proliferation characteristic of neoplastic cells.

In support of this, we have compared the myogenic line of L_8 cells with a nonfusing variant, fu-1, and have presented evidence which collectively demonstrates that the fu-1 cells have properties characteristic of transformed cells *in vitro*. No single *in vitro* characteristic has yet been shown to be a thoroughly reliable index of either virus-induced or "spontaneous" transformation. Nevertheless, the altered density-dependent inhibition of proliferation of fu-1 cells, the ability of fu-1 cells to grow in soft agar and the reduction in cell adhesiveness, the increased rate of hexose transport, and the reduced serum requirements for growth of fu-1 cells compared to L_8 cells all support the view that fu-1 cells have characteristic properties of transformed cells *in vitro*. Additional proof that the fu-1 cells have lost normal growth control comes from the demonstration that these cells can develop *in vivo* into tumors. L_8 cells did not form tumors. Thus, in selecting for a cell defective in its development, we have selected for a cell that has also lost its normal growth control.

It is important to distinguish whether the fu-1 cell was selected because of its having acquired any of the properties of the transformed phenotype that we have described, or whether it was selected because it could not terminate its proliferative capacity and subsequently differentiate. For example, it could be argued that our isolation procedure had, in fact, selected for cells resistant to density-dependent inhibition of growth. However, fu-1 cells do in fact, become density inhibited and their higher saturation density is in great part due to their smaller size. Because fu-1 cells were selected from cultures that already were density inhibited and fused, it seems unlikely that they were selected simply by overgrowth. Moreover, our analysis of 12 additional nonfusing lines supports this contention (S. J. Kaufman *et al.*, unpublished data). Although these nonfusing lines have some, or all, properties of the *in vitro* transformed phenotype, there is no single *in vitro* characteristic, other than the failure to develop into myotubes, that is indicative of whether these cells will produce a tumor *in vivo*. Thus, although overgrowth of the monolayer is a characteristic of fu-1 cells, neither the two other nonfusing lines selected by serial passage nor the 10 additional lines selected by other procedures share this characteristic. We, therefore, believe that neither the ability to overgrow fused L_8 cells nor any of the other transformed properties of fu-1 cells were directly selected by the procedures used to isolate fu-1. Both L_8 and fu-1 cells attain their respective saturation densities and cease replication; however, only the fu-1 cells maintain the capacity to divide, and this property is sufficient to preclude development and to promote tumors.

In agreement with our results are the recent studies in which viral transformation of myoblasts was shown to render these cells incapable of further differentiation. Fizman and Fuchs (36) and Holtzer *et al.* (37) demonstrated that, when chick myoblasts are transformed with a temperature-sensitive mutant of Rous sarcoma virus, the myoblasts fail to differentiate when the viral *src* gene is expressed but do differentiate into myotubes at the nonpermissive temperature. Their findings indicate that transformed myoblasts cannot differentiate and suggest that the viral *src* gene product may either directly or indirectly preempt the capacity of the myoblasts to differentiate. Hynes *et al.* (38) have shown that when the L_8 rat myoblasts are transformed with avian sarcoma viruses they do not form myotubes, they fail to synthesize levels of myosin characteristic of myotubes, and they show a decrease in a high-molecular-weight membrane glycoprotein. We have shown that the fu-1 cells also do not increase their synthesis of myosin nor do they have increased CK activity.

We have recently found that fu-1 cells liberate a C-type virus that has reverse transcriptase (RNA-dependent DNA polymerase) activity, a density of approximately 1.16 g/cm^3, and a 70S RNA genome [ref. 39; S. J. Kaufman *et al.*, unpublished data). Although fu-1 cells cannot fuse into myotubes, expression of the endogenous virus in fu-1 cells allows them to be fused into syncytia by exogenous murine leukemia virus (39, 40). We have not been able to detect the expression of a virus in the parent

L_8 cells by any of these criteria; thus, fu-1 and L_8 cells are also distinct in their expression of an endogenous virus. At present, we do not know whether the expression of an endogenous type-C virus in the fu-1 cells is a cause of, is a consequence of, or is unrelated to the developmental defect in these cells. However, a possible role for endogenous viruses in muscle development has been suggested (41) and the cell lines we have described may be useful in further exploring this concept.

Our proposal does not preclude that partial expression of the differentiated muscle phenotype may occur in single myoblasts. MK activity is present in unfused L_8 as well as in fu-1 cells. Tarikas and Schubert (42) isolated a nonfusing variant (M3A) from the L_6 myogenic line and showed that the M3A cells make both CK and MK. However, they concluded that the development of these enzyme activities in both M3A and L_6 cells was dependent on cell density and not on fusion. Somite myoblasts synthesize myosin prior to fusion (43), and in other experiments one or more characteristics of fused muscle was detected in single myoblasts in which fusion was reversibly inhibited by Ca^{2+} deprivation (7, 9) or treatment with phospholipase C (35). Thus, fusion per se may not be prerequisite for the partial expression of the muscle phenotype, yet it is obligatory to the formation of functional skeletal muscle; to attain that competence, myoblasts must cease replication. In addition to not fusing, fu-1 cells do not produce the levels of CK and myosin that are found in myotubes. These characteristics may be coordinately regulated at some stage of differentiation.

It is of interest to know the frequency of appearance of nonfusing variants; however, thus far we have not been able to accurately determine this. Although the frequency of colonies of cloned L_8 cells that grow in soft agar is approximately 10^{-5}, this is not an accurate estimate, because not all nonfusing lines grow equally well in agar (S. J. Kaufman, unpublished data).

In our comparison of L_8 and fu-1 cells, it is appropriate to question whether the results obtained reflect absolute differences or differences in degrees of transformation. Because L_8 cells are an established line they may themselves be considered to be transformed cells. However, unlike other established cell lines, L_8 cells will *not* retain the capacity for continual growth *in vitro unless* they are maintained at low cell densities. If this criterion is not met, L_8 cells will behave as do myoblasts in primary cultures; i.e., they will fuse and thereby effectively terminate their existence *in vitro*. In both primary cultures and in established cell lines, myogenic cells share a common "death wish," to fuse into myotubes. In this respect, L_8 cells may be viewed not as an established line, but as terminally differentiating cells that retain most mechanisms by which they control their growth and development. This is supported by the finding that L_8 cells will not induce tumors *in vivo*. In contrast, fu-1 cells cannot fulfill this "death wish" and manifest properties of transformed cells both *in vitro* and *in vivo*.

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