Transformation by *myc* Prevents Fusion But Not Biochemical Differentiation of C2C12 Myoblasts: Mechanisms of Phenotypic Correction in Mixed Culture with Normal Cells

M. Crescenzi, D. H. Crouch,* and F. Tatò

Dipartimento di Biologia Cellulare e dello Sviluppo, Universitá di Roma "La Sapienza", 00185 Roma, Italy; and *Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow G61 1BD, Scotland

Abstract. To study the effects of *myc* oncogene on muscle differentiation, we infected the murine skeletal muscle cell line C2C12 with retroviral vectors encoding various forms of avian c- or v-*myc* oncogene.

myc expression induced cell transformation but, unlike many other oncogenes, prevented neither biochemical differentiation, nor commitment (irreversible withdrawal from the cell cycle). Yet, myotube formation by fusion of differentiated cells was strongly inhibited. Comparison of uninfected C2C12 myotubes with differentiated *myc*-expressing C2C12 did not reveal consistent differences in the expression of several muscle regulatory or structural genes. The present results lead us to conclude that transformation by *myc* is compatible with differentiation in C2C12 cells.

myc expression induced cell death under growth restricting conditions. Differentiated cells escaped cell death despite continuing expression of *myc*, suggesting that the muscle differentiation programme interferes with the mechanism of *myc*-induced cell death.

Cocultivation of v-*myc*-transformed C2C12 cells with normal fibroblasts or myoblasts restored fusion competence and revealed two distinguishable mechanisms that lead to correction of the fusion defect.

SKELETAL muscle cell differentiation involves irreversible withdrawal from cell cycle (also referred to as commitment) and coordinate activation of a number of unlinked, muscle-specific genes (biochemical differentiation) (Pearson and Epstein, 1982). Terminally differentiated myocytes have the option to fuse with one another, thus generating multinucleated myotubes. The mutual exclusion between cellular proliferation and expression of differentiation markers is maintained by myogenic cells in vitro and is particularly evident in myogenic cell lines that proliferate indefinitely in mitogen-rich culture medium and are induced to differentiate in mitogen-poor medium (Clegg et al., 1987). The ordered sequence of events that leads to myogenic terminal differentiation appears to be controlled by a small number of regulatory genes that include the MyoD family of muscle-specific transcription factors and their positive and negative coregulators E2A and Id (Olson, 1990; Weintraub et al., 1991).

During the last decade, a growing number of genes involved in cell transformation and tumorigenesis, the oncogenes, have been identified. When expressed in fibroblasts, a large subset of these genes promote proliferation, thus contributing to transformation (Weinberg, 1989). By promoting proliferation and partially upsetting its regulation, the oncogenes allow cells to proliferate in conditions normally not supporting cell growth (e.g., confluence, or absence of exogenous growth factors). *myc* constitutes an interesting exception to this paradigm. The proto-oncogene c-*myc* is ubiquitously expressed and required for cell proliferation (Lüscher and Eisenman, 1990). Its oncogenic form can transform cells alone (Vennström et al., 1984) or in conjunction with other oncogenes (Land et al., 1983). However, when mammalian fibroblasts are forced to express *myc* in culture conditions not sustaining growth, they undergo apoptosis (Evan et al., 1992).

Constitutive expression of viral oncogenes or cellular proto-oncogenes has been shown to prevent myogenic differentiation in early passage avian myoblasts (Alemà and Tatò, 1987) as well as mammalian cell lines (Schneider and Olson, 1988). In particular, transformation of the mouse C2C12 cell line by activated *ras* (Konieczny et al., 1989), (Lassar et al., 1989), v-*fos* (Lassar et al., 1989), c-*jun* (Bengal et al., 1992), or v-*src* (Gauzzi, C., and S. Alemà, personal communication; Zanetti, A., unpublished work) results in the extinction of MyoD transcription, and this represents one mechanism to explain the inhibition of differentiation. On the other hand, transformation of unestablished quail myoblasts by v-*src* (Falcone et al., 1991) or v-*jun* (Grossi et al., 1991) prevents terminal differentiation without requiring transcriptional silencing of MyoD, as also

Address all correspondence to F. Tatò, Dipartimento di Biologia Cellulare e dello Sviluppo, Universitá di Roma "La Sapienza", Via degli Apuli, 1-00185 Roma, Italy.

observed in murine myoblasts transformed by Polyoma large-T antigen (Maione et al., 1992). In the case of v-*src*–transformed quail myoblasts, it has been shown that the transactivating function of MyoD appears to be impaired, thereby providing a second mechanism through which transformation can inhibit myogenic differentiation (Falcone et al., 1991).

While there is a general consensus that the expression of most known oncogenes prevents myogenesis, the effects of the *myc* oncogene in muscle cells appear to be quite variable, ranging from partial inhibition by exogenous c-*myc* in BC3H1 cells (Schneider et al., 1987), a non-fusing mouse myogenic cell line (Schubert et al., 1974), to very strong inhibition by v-*myc* in quail myoblasts (Falcone et al., 1985). Given the differences between experiments with continuous cell lines and those with unestablished myoblasts and the widespread use of the C2C12 myogenic cell line as a model for myogenesis, we have decided to analyze the effects of c-and v-*myc* oncogene expression in C2C12 cells.

The results presented here indicate that v-*myc* and c-*myc* disrupt growth control in C2C12 cells. Contrary to the more common findings, *myc*-transformed C2C12 cells retain the ability to undergo commitment and biochemical differentiation. However, they are strikingly unable to fuse into multinucleated myotubes, in spite of conspicuous induction and accumulation of muscle-specific structural proteins. The unusual fusion-defective phenotype of *myc*-transformed C2C12 cells is readily suppressed by cocultivation with either fusion-competent heterologous myoblasts or normal fibroblasts via two distinguishable mechanisms. One mechanism appears to involve the phenotypic reversion of *myc*-transformed cells and leads to the formation of multinucleated myotubes whose nuclei derive exclusively from *myc*-transformed cells. The other mechanism is represented by the recruitment of differentiated, mononucleated *myc*-expressing cells into myotubes formed by fusion competent myoblasts, that leads to the formation of "hybrid" myotubes, containing nuclei derived from both normal and transformed cells. The present data suggest that fusion of differentiated myocytes, although usually sequentially linked to commitment and biochemical differentiation, can be specifically uncoupled from the rest of the myogenic program by the action of *myc* oncogene.

Materials and Methods

Cells and Tissue Culture Media

C2C12 cells and Rat-2 fibroblasts were kindly provided by S. Alemà (Inst. of Cell Biology, C. N. R., Rome, Italy) and P. Amati (Università "La Spienza", Rome, Italy). L6 and L8 cells are a generous gift of S. Adamo (Università "La Spienza", Rome, Italy) and D. Yaffe (Weizmann Institute, Rehovot, Israel). Growth medium was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Serum-free (SF)[1] medium was Dulbecco's modified Eagle's medium supplemented with 10 μg/ml insulin, and 5 μg/ml transferrin. *myc*-expressing cells were routinely cultured in collagen-coated dishes, due to their poor adherence on plastic. All experiments were performed in coated dishes.

Soft-agar assays for anchorage-independent growth were carried out as described (Bignami et al., 1988*b*).

1. *Abbreviations used in this paper*: AraC, cytosine β-D-arabinofuranoside; BrdU, 5-bromo-2'-deoxyuridine; IF, immunofluorescence; MHC, myosin heavy chain; SF, serum-free.

Viruses and Infections

MMCV is a replication-defective recombinant retrovirus carrying the avian v-*myc*^{OKI0} oncogene (Vennström et al., 1984). The MLV helper virus is a kind gift of T. Graf (EMBL, Heidelberg, Germany).

A schematic representation of the pBabe viruses is depicted in Fig. 1. pBabe-c-*myc* was generated by cloning an artificial cDNA containing an exonII/exonIII avian c-*myc* (Crouch et al., 1990) into the replication defective retrovirus pBabeneo (Morgenstern and Land, 1990), using BamHI linkers. A c/v-*myc* chimaera was constructed by fusing the 5' end of avian c-*myc* to the 3' end of MC29 v-*myc* at the SstI site. The resulting chimaera, which contains the point mutations associated with MC29 v-*myc* (Walter et al., 1986) but lacks any *gag* sequences, was subsequently cloned into pBabeneo using BamHI linkers, generating pBabe-c/v-*myc*.

ψ-2 cells expressing the pBabe viruses were generated by transfection using standard calcium phosphate precipitation (Crouch et al., 1990), allowing the production of helper-free pBabe virus stocks (Mann et al., 1983). Growth conditions were as described for C2C12 cells (see above), with *neo* selection performed using 1 mg/ml G418.

Infections were performed in 60 mm dishes. Cells (2×10^5) were seeded and treated for 1–3 h with 2.5 μg/ml polybrene in growth medium. 1 ml of viral stock was added and infection was allowed to proceed overnight. On the next day, cells were fed growth medium. For replication-defective viruses, G418 selection began on the second day after infection.

Immunofluorescence and DNA Staining

The following antisera and mAbs were used for immunocytochemistry and immunofluorescence (IF): MyoD, rabbit polyclonal antiserum kindly provided by H. Weintraub (Fred Hutchinson Center, Seattle, WA); myogenin, mAb F5D (Wright et al., 1991), made available by G. Cossu (Università "La Spienza," Rome); 5-bromo-2'-deoxyuridine (BrdU): mAb-DNAse mixture (Amersham, Amersham, UK); myosin heavy chain (MHC): mAb MF-20 (Bader et al., 1982) (a kind gift of D. Fischman, Cornell University, New York); Troponin T, mAb (Amersham); Myc, rabbit polyclonal antiserum raised against avian v-Myc (generously provided by K. Moelling, Max-Planck Institut, Berlin, Germany). mAbs were detected by affinity-purified goat anti-mouse IgG (Organon Teknika, West Chester, PA). Rabbit antisera were detected by affinity-purified goat anti-rabbit IgG (Organon Teknika). Secondary antisera were conjugated with either rhodamine or fluorescein. In experiments involving simultaneous detection of BrdU and MHC by two mAbs, the anti-BrdU mAb was detected first by a peroxidase-conjugated anti-mouse Ig antiserum supplied in the Cell Proliferation kit (Amersham), that was reacted with diaminobenzidine; anti-MHC IF was then carried out as described above. Nuclei were stained after IF treatments by incubating the permeabilized cells for 3 min with a 0.1 μg/ml solution of Hoechst 33258 dye in phosphate-buffered saline.

Cocultivations

Mixed cultures of proliferating v-*myc*–transformed C2C12 cells (C2C12-[v-*myc*]) and normal cells were set up by seeding simultaneously the cells into collagen-coated, 35 mm dishes as follows: 10^3 C2C12(v-*myc*) + 2×10^5 Rat-2 or 3×10^4 C2C12(v-*myc*) + 10^5 quail myoblasts. The next day, the cultures were shifted to serum-free medium. 3 d later, the differentiated cultures were fixed in 50% methanol/acetone (vol/vol) and stained for fluorescent detection of MHC and DNA. Rat and quail nuclei were stained homogeneously by the Hoechst 33258 dye, while mouse nuclei showed

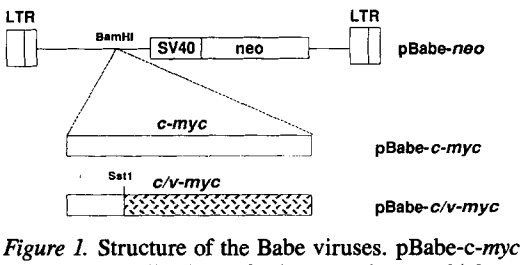

Figure 1. Structure of the Babe viruses. pBabe-c-*myc* and pBabe-c/v-*myc* are replication defective retroviruses which encode an exon II/exon III avian c-*myc* and a c-*myc*/v-*myc* chimaera, respectively. The c-*myc* sequence is represented by the open box, while the MC29 v-*myc* sequence is indicated by the hatched area.

characteristic bright chromocenters. Alternatively, 0.5–1.5 × 10^5 C2C12-(v-*myc*) cells were induced to differentiate in SF medium for 3–4 d, adding 50 μM cytosine β-D-arabinofuranoside (AraC) on day 2 to eliminate undifferentiated, proliferating cells. After washing out AraC, quail myoblasts (2 × 10^5) or Rat-2 fibroblasts (4 × 10^5) were added in growth medium and after a few hours mixed cultures were shifted to SF medium and incubated for further 3 d. Cultures were then analyzed as above.

Results

Transformation by myc Prevents Fusion But Not Differentiation of C2C12 Myoblasts

To study the effects of the constitutive expression of *myc* on muscle differentiation, we infected the mouse myoblast cell line C2C12 with the *v-myc*–expressing retroviral vector MMCV, along with the MLV helper virus. Since the presence of the helper virus allowed replication of MMCV, the vast majority of the cells became rapidly infected. Accordingly, the cells became smaller, more round in shape, loosely attached, and showed prominent nucleoli. Their doubling time was evidently shorter and their saturation density higher, compared to those of MLV- and mock-infected cells. v-*myc* expression was verified by Northern blot and ascertained to be present in the great majority of the cells by IF studies (see below).

Because the MMCV-infected cells (C2C12[MMCV]) showed morphological conversion, we determined whether they were capable of anchorage-independent proliferation. Cells were seeded in soft agar and colony formation was assessed. Table I shows that C2C12(MMCV) readily formed colonies in suspension, while parental cells failed to grow. Based on these data and on the other growth properties of these cells, we regard C2C12(MMCV) as transformed.

Since transformation virtually always antagonizes muscle differentiation, we assessed the ability of C2C12(MMCV) to differentiate. Muscle differentiation was induced by feeding the cells serum-free medium, depriving them of growth factors. Biochemical differentiation was determined by IF staining of MHC, a muscle-specific marker. C2C12(MMCV) cells underwent biochemical differentiation with an apparent efficiency similar to that of MLV-infected C2C12 cells. However, fusion was severely inhibited (Fig. 2 and Table II). Very few of the MHC-positive cells had more than one nucleus, even when grown at high density to promote fusion. Table II shows a representative experiment in which the differentiation index and the fusion index of C2C12(MMCV) and control cells were compared. In spite of similar differentiation rates, fusion was virtually absent in C2C12(MMCV). The fusion index of C2C12(MMCV) never exceeded one percent in several independent experiments.

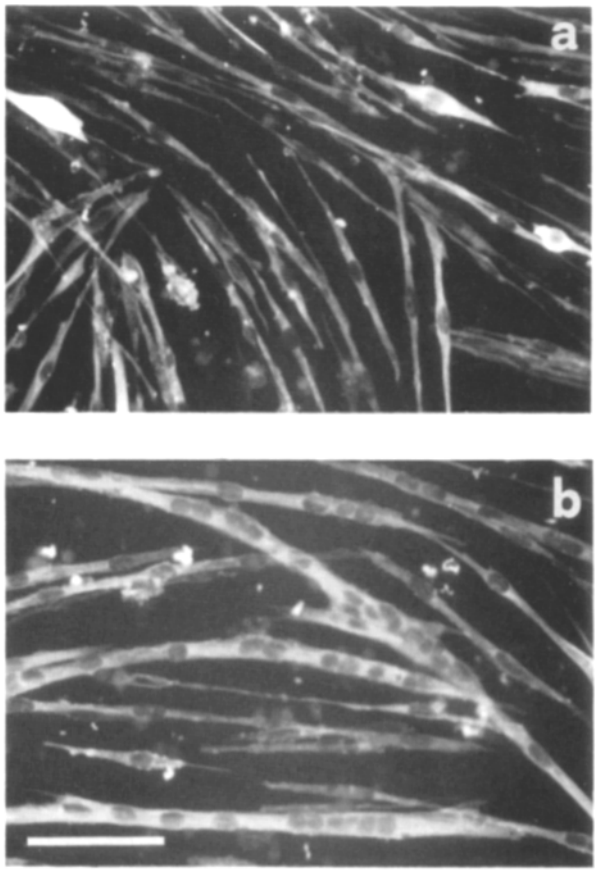

Figure 2. Phenotype of C2C12(MMCV) and parental C2C12 cells. Cells were induced to differentiate in serum-free medium. 3 d later they were subjected to IF staining for MHC. (*a*) C2C12(MMCV); (*b*) C2C12. Bar, 100 μm.

Despite the absence of fusion, a striking result was the ability of transformed cells to differentiate at all. One possibility was that, within C2C12 cells, a subset of cells was not transformed, e.g., due to low v-*myc* expression levels. To disprove this, we randomly isolated 14 independent C2C12-(MMCV) clones from soft agar. They all behaved very similarly to the uncloned transformed cell line and showed differentiation indices up to and higher than 50% (data not shown). Thus, anchorage-independent, clonal cell lines were capable of differentiating efficiently in the appropriate conditions. Furthermore, double IF staining showed high ex-

Table I. Anchorage-independent Growth of myc-infected C2C12

Cells	Colonies (Percent ± SD)	
C2C12(MMCV)	40.5	±4.5
C2C12(−)	<0.1	
NIH 3T3(v-*src*)	34.6	±1.7

Cells were seeded in duplicate 60 mm dishes as specified in Materials and Methods, at 1,000 cells/dish. Colonies were counted 23 d later. C2C12(−), mock-infected cells; NIH 3T3(v-*src*), NIH 3T3 cells transformed by *v-src*, added as positive controls. Results are presented as percentage of colonies/number of cells seeded ±SD.

Table II. Fusion Index of C2C12(MMCV)

Cells	Differentiation index	Fusion index
C2C12(MMCV)	28	<1
C2C12(MLV)	30	27
C2C12(−)	40	37

Cells were seeded at 10^5 cells/35 mm dish, serum was removed on the next day, and 4 d later cells were fixed. MHC was stained by indirect IF; nuclei were stained with Hoechst 33258 dye. Differentiation index, percentage of nuclei belonging to MHC-positive cells; fusion index, percentage of nuclei belonging to cells with three or more nuclei. At least 100 nuclei were counted for each data point.

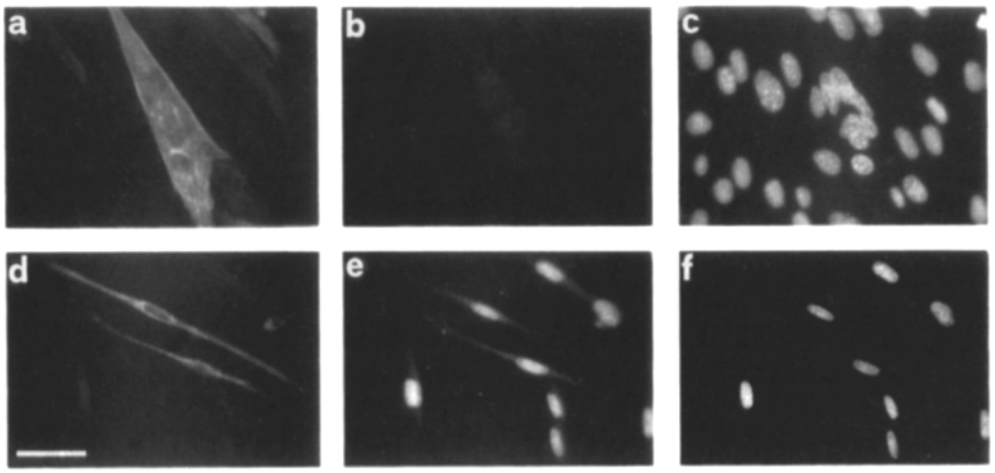

Figure 3. myc expression in differentiated cells. C2C12-(MMCV) and C2C12 cells were induced to differentiate for 3 d, and then stained by double IF to detect troponin T and v-Myc protein and by Hoechst 33258 to highlight the nuclei. Three views of the same microscopic field showing C2C12 cells (*a–c*). Three views of the same microscopic field showing C2C12(MMCV) (*d–f*). Photographs show MHC staining (*a* and *d*), v-Myc staining (*b* and *e*), and DNA staining (*c* and *f*). Bar, 50 μm.

pression of v-*myc* in MHC-positive, differentiated cells (Fig. 3). In conclusion, in C2C12 cells transformation by v-*myc* is compatible with differentiation.

The BC3H1 cell line derives from a mouse brain tumor but behaves in many respects as a skeletal muscle cell line (Schubert et al., 1974). It has been reported that these cells can undergo biochemical differentiation, but are unable to fuse. More importantly, differentiated cells lose muscle-specific expression and reenter the cell cycle when stimulated by growth factors (Lathrop et al., 1985). We asked whether C2C12(MMCV) would behave similarly. C2C12-(MMCV) were induced to differentiate in serum-free medium for 3 d and then were fed medium containing 10 or 20% fetal bovine serum and 20 μM BrdU. Cells were fixed after 12, 24, and 48 h and stained for MHC and BrdU, the latter as a marker of DNA synthesis. No double-positive cells were found at any time. Moreover, differentiated cells did not give rise to colonies upon serum stimulation (not shown). Thus, despite their impaired ability to fuse, C2C12-(MMCV) fully undergo commitment.

Other myc-expressing Vectors

The phenotype displayed by C2C12(MMCV) was unexpected and somewhat at variance with previous results obtained in other systems by us and others, in that differentiation was allowed to occur. Consequently, we made an effort to verify whether our results applied only to this particular system or were applicable to other *myc* vectors and mammalian cells. Two other retroviral vectors were used to infect C2C12 cells. Babe-c-*myc* expresses the chicken proto-oncogene; Babe-c/v-*myc* encodes a chimaeric molecule lacking the *gag* but retaining most of the *myc* moiety of *v-myc* (see Materials and Methods). Both viruses are replication-defective and encode the *neo* selectable marker. G418-selected C2C12 infected with either virus, referred to as C2C12(c-*myc*) or C2C12(v-*myc*), displayed a phenotype virtually indistinguishable from that of C2C12(MMCV), with Babe-c-*myc* allowing slightly more fusion than MMCV (data not shown). In particular, cells infected with either virus differentiated efficiently, fused very poorly, and formed colonies in soft agar, although Babe-c-*myc*-infected cells gave rise to smaller colonies.

We have attempted to extend our findings to other mammalian cell lines such as L6 and L8, using the Babe-*c/v-myc* vector, but we could not reach firm conclusions. The major drawbacks were the different time and cell density requirements displayed by these other cell lines to undergo differentiation after mitogen removal. While C2C12 can differentiate within 2–3 d, even at relatively low density, both L6 and L8 require at least twice as long a time after reaching confluence. As a consequence, v-*myc*–transformed L6 and L8 cells underwent massive cell death (see below) before attaining the conditions necessary for their differentiation. Differentiated, mononucleated myocytes—but virtually no myotubes—were indeed observed among the cells surviving at the end of the experiments; however, the cell density was too low to rule out that the lack of fusion was merely the result of the inability of the cells to contact one another.

Differentiation Protects from myc-induced Cell Death

Recently it has been reported that forced expression of *myc* in fibroblasts induces apoptosis when they are cultivated in conditions in which normal cells would not proliferate (Evan et al., 1992). A similar phenomenon was evident in *myc*-transformed myogenic cells. C2C12(MMCV) proliferated rapidly until they became confluent, then a wave of cell death ensued. The same phenomenon was accurately reproducible upon serum starvation. Many cells detached from the substrate within a few hours, and eventually up to 50% of the cells were lost. Interestingly, however, those cells that differentiated following serum starvation did not die despite continued incubation in serum-free medium for as long as 1 wk. This was true even though the differentiated cells continued to express high levels of *myc* (see Fig. 3 for protein and Fig. 5 for mRNA). In differentiation-inducing conditions, extensive cell death was observed in c- and c/v-*myc*–transformed C2C12. Since in these cells exogenous *myc* mRNA levels do not vary significantly upon differentiation (see below), we conclude that differentiation protects from *myc*-induced cell death without selecting for cells that express low levels of *myc*.

Muscle-specific Gene Expression in myc-transformed Myoblasts

Given the absence of fusion in differentiated cells, we asked whether the expression of a subset of muscle-specific structural genes was suppressed in *myc*-expressing myocytes. C2C12 cells infected with each of the three viruses were analyzed by Northern blot and IF for expression of a number of muscle-specific proteins. Upon differentiation, MHC, myo-

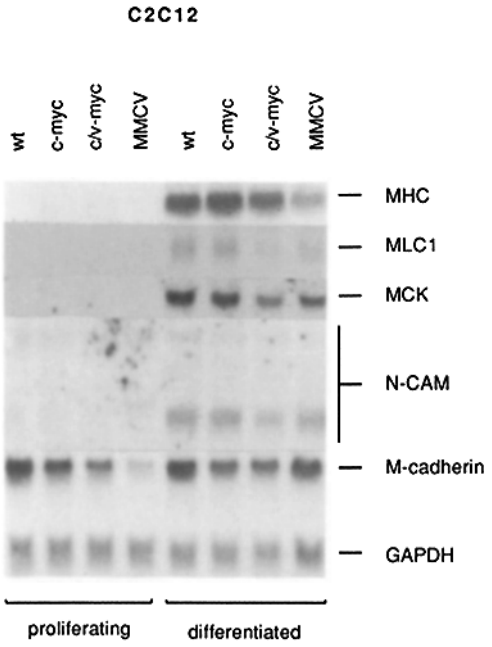

Figure 4. Expression of structural genes. Total RNA was purified from cells exponentially growing (*left*) or induced to differentiate for 3 to 4 d (*right*). 25 μg from each sample were loaded, separated on a 1.2% agarose gel, transferred to reinforced nitrocellulose paper, and hybridized with the indicated probes. All probes were hybridized to the same filter except MCK, which was used on a different blot.

sin light chain 1, and muscle creatine kinase mRNAs were expressed at levels comparable with those of wild-type C2C12 (Fig. 4). MHC and Troponin T were also analyzed by IF and found to be expressed similarly in all cell lines (see Fig. 3 for Troponin T and Fig. 6 for MHC). The expression of two surface molecules possibly involved in fusion was analyzed. M-cadherin, a muscle-specific cadherin (Donalies et al., 1991), and N-CAM, a widely expressed cell-adhesion molecule (reviewed in Edelman and Crossin, 1991). Fig. 4 shows that M-cadherin mRNA was indeed reduced in proliferating cells, but was at wild-type level in differentiated cells. The pattern of N-CAM did not show significant variations (Fig. 4). Thus, in differentiated cells, none of the structural genes we examined showed significant alterations due to *myc* expression.

We also analyzed the expression of muscle transcription factors belonging to the MyoD family. At the RNA level, MyoD seemed somewhat down-regulated by *myc* in proliferating myoblasts, more evidently in C2C12(MMCV). However, similar MyoD levels were found in all differentiated cells, irrespective of their *myc* expression levels (Fig. 5). Myogenin, virtually only expressed in differentiated cells, did not show significant variations (Fig. 5). Myf-5 was only detected in exceedingly small amounts in our conditions (not shown). MRF4 was undetectable, in agreement with the literature (Rhodes and Konieczny, 1989; Braun et al., 1990; Miner and Wold, 1990). Thus, although *myc* significantly altered the phenotype of myocytes, it did not appear to affect transcription of the known muscle regulatory genes, at least after differentiation. IF staining confirmed the presence of MyoD in proliferating cells and MyoD and myogenin in differentiated ones (not shown). Altogether, the data suggest that variations of the exogenous *myc* levels are not required for the differentiation of *myc*-transformed myoblasts. The

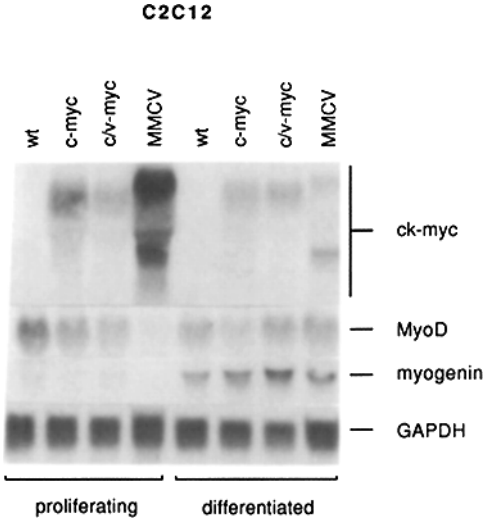

Figure 5. Expression of regulatory factors. Experiments performed as described in the legend to Fig. 4.

steady-state levels of exogenous *myc* transcripts were comparable in proliferating and differentiated cells, with the exception of C2C12(MMCV) (Fig. 5). However, the reduced level of *v-myc* mRNA in differentiated C2C12(MMCV) is still comparable with that of C2C12(c-*myc*) and C2C12-(v-*myc*).

Phenotypic Reversion of C2C12(v-myc) in Mixed Culture with Normal Myoblasts or Fibroblasts

In an attempt to correct the defective phenotype of *myc*-transformed cells, we cocultivated C2C12(v-*myc*) along with primary quail myoblasts. Cells were seeded together and serum starved. After differentiation had taken place, the cells were fixed and stained to visualize MHC expression and nuclear morphology. As shown in Fig. 6 *a*, many myotubes contained both quail and mouse nuclei, demonstrating that the *myc*-expressing cells were not impaired in fusing with normal myoblasts. Hybrid myotubes were also observed when C2C12(v-*myc*) were cocultivated with rat L8 myoblasts (not shown). Interestingly, we also found that many myotubes contained only mouse nuclei. These results suggested that the presence of normal myoblasts in the culture was also able to drive the phenotypic reversion of the transformed cells, thus allowing them to fuse with one another. To prove this contention, C2C12(v-*myc*) were seeded together with Rat-2 fibrobasts. As shown in Fig. 6 *b*, Rat-2 cells induced phenotypic reversion of C2C12(v-*myc*) that formed large multinucleated myotubes. Correction of the fusion-defective phenotype could not be achieved by feeding C2C12(v-*myc*) medium conditioned by normal fibroblasts, even when normal and transformed cells were in close proximity, but separated by a porous membrane. Thus, it appears that in the present system, analogously to previous reports (Bignami et al., 1988; La Rocca et al., 1989; Mehta et al., 1986), contact between normal and transformed cells is required to override Myc effects.

Normal Fibroblasts Fail to Promote Fusion of Differentiated C2C12(v-myc) Myocytes

We next asked whether phenotypic correction of C2C12-

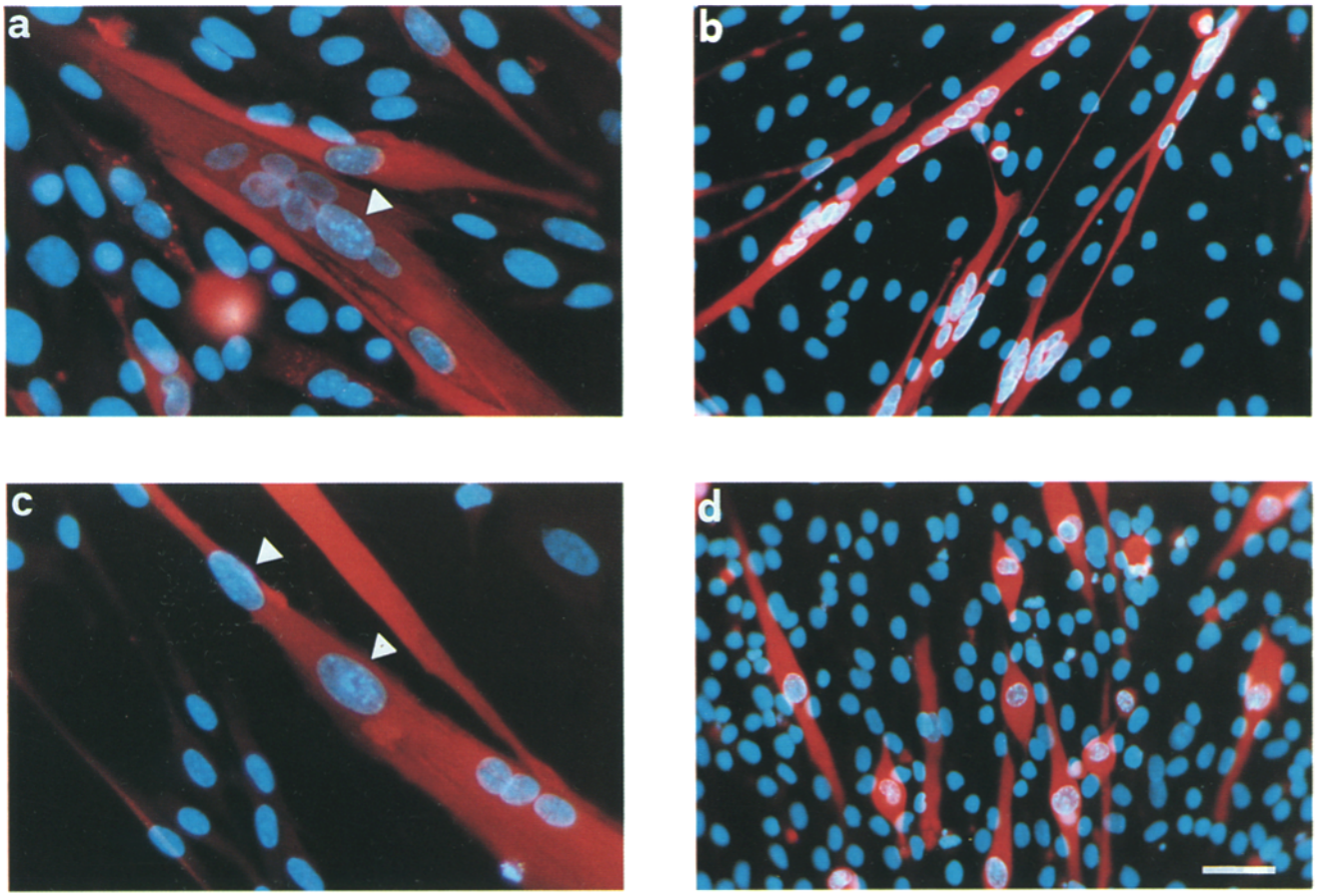

Figure 6. Cocultivation of C2C12(v-*myc*) and normal cells. Mixed culture of proliferating C2C12(v-*myc*) seeded together with quail myoblasts (*a*) or Rat-2 fibroblasts (*b*), as described in Materials and Methods, and induced to differentiate for three days. (*c*) Mixed culture of pre-differentiated C2C12(v-*myc*) myocytes and quail myoblasts. (*d*) Mixed culture of pre-differentiated C2C12(v-*myc*) myocytes and Rat-2 fibroblasts. Cells were then stained by IF for MHC detection and with Hoechst 33258 to distinguish mouse (speckled) from rat or quail (homogeneous) nuclei. Note the presence of myotubes containing mouse and quail nuclei in *a* and *c*, the presence of multinucleated myotubes in *b* but not in *d*. Triangles point at mouse nuclei in hybrid myotubes. Bars: (*a* and *c*) 25 μm; (*b* and *d*) 50 μm.

(v-*myc*) in mixed culture takes place before or after differentiation. To distinguish between these two alternatives, we analyzed mixed cultures where the normal counterparts, quail myoblasts or Rat-2 fibroblasts, were added after C2C12-(v-*myc*) had terminally differentiated. As shown in Fig. 6 *c*, quail myoblasts were capable of recruiting C2C12(v-*myc*) myocytes, and quail–mouse hybrid myotubes were formed. On the contrary, Rat-2 fibroblasts did not induce the fusion of pre-differentiated C2C12(v-*myc*) myocytes (Fig. 6 *d*), thus suggesting that myotube formation observed when C2C12(v-*myc*) and Rat-2 cells were coseeded (see Fig. 6 *b*) represented the correction of *myc*-induced effects and that such correction is no longer possible after differentiation has occurred in C2C12(v-*myc*).

Discussion

In the present report, we describe the effects of forced expression of the *myc* oncogene in mammalian myoblasts. Several aspects of the resulting phenotype deserve discussion. First, despite the cells behaving as fully transformed in vitro, they could still differentiate efficiently. Second, although *myc* did not significantly affect biochemical differentiation or commitment, it prevented cell fusion. Third, we found that muscle differentiation could prevent *myc*-induced cell death in spite of the continued expression of the oncogene. Finally, cocultivation experiments with either fibroblasts or fusion-competent myoblasts showed that correction of the *myc*-induced phenotype in mixed culture can be ascribed to two distinguishable mechanisms.

Several types of myoblasts, transformed by a variety of oncogenes, have been shown to lose their differentiation competence (reviewed in Alemà and Tatò, 1994). This appears to reflect a common finding in in vivo tumors, where malignancy is usually associated with reduction or loss of differentiation. C2C12 cells infected with c- or v-*myc*–transducing viruses showed a transformed phenotype, as judged from faster growth rate, higher saturation density, and anchorage-independent proliferation. However, *myc*-transformed C2C12 cells retained the ability to differentiate efficiently in the appropriate conditions. We could show that 14 individual MMCV-transformed clones, selected for growth in soft agar, all retained the ability to differentiate as efficiently as the transformed polyclonal cell population. We detected continued expression of v-Myc protein in differentiated cells. Moreover, differentiated cells maintained high expression

levels of exogenous *myc*, with the exception of the MMCV-infected ones. This strongly suggests that *myc* expression does not interfere with biochemical differentiation and that differentiation does not occur selectively in low *myc* expressors. Altogether, these findings demonstrate the coexistence of transformation and differentiative potential within the same cell. The fact that, upon serum withdrawal, differentiation and commitment take place indicates that the differentiative mechanism prevails over the transforming activity of *myc*.

Our findings also represent an instance of uncoupling of myotube formation from biochemical differentiation by means of an oncogene. A fusion-defective phenotype is physiologically displayed by early differentiating myoblasts in the somites (Vivarelli and Cossu, 1986). Fusion can also be prevented in biochemically differentiated myocytes by manipulating environmental conditions, for instance, lowering calcium levels in the medium (Paterson and Strohman, 1972). These manipulations presumably impair the activity of effector molecules necessary for syncitium formation. In this case, however, we show that *myc* specifically interferes with that part of the differentiation programme. Myc proteins are currently viewed as transcriptional regulators that, upon heterodimerization with the appropriate partners (Amati et al., 1993), can either induce (Eilers et al., 1991) or inhibit (Yang et al., 1991) the transcription of specific genes. Thus, it is conceivable that the fusion defect of differentiated C2C12- (v-*myc*) myocytes might depend on the absence of a critical gene product specifically required for the fusion process. Alternatively, a functional reduction of gene product(s) required for fusion might prevent homotypic fusion, while the residual function might suffice when fusion into myotubes is driven by differentiating normal myoblasts. It should be stressed that such a phenotype is displayed in the presence of presumably functional MyoD and myogenin proteins—as inferred from the activation of muscle gene transcription—thus suggesting that *myc* may exert its effects downstream of these molecules and/or that fusion is under the direct control of different regulators.

Our results are in agreement with the reported inability of deregulated c-*myc* to prevent biochemical differentiation in BC3H1 cells (Schneider et al., 1987), but appear at variance with other reports in the literature. In our laboratory, primary quail myoblasts infected with *myc*-transducing viruses became transformed but completely lost differentiative potential (Falcone et al., 1985). MyoD-converted NIH 3T3 cells were not transformed by *myc* but were inhibited from differentiating (Miner and Wold, 1991). The phenotype described here is still different, since C2C12 cells were transformed, but retained the ability to differentiate, though without fusing into myotubes. It should be noticed that these observations have been made in quite different systems, and might reflect the phenotypical heterogeneity found in skeletal muscle cells at different developmental stages (Cusella-De Angelis et al., 1992). Quail myoblasts, in fact, represent fetal myoblasts (Smith et al., 1993), while the C2C12 cell line was derived from adult satellite cells (Yaffe and Saxel, 1977). We are currently investigating whether the phenotypes of *myc*-transformed quail myoblasts and C2C12 cells are prototypical for *myc* effects in fetal myoblasts and satellite cells, respectively, and whether differential modulation of the transcription of myogenic regulatory genes can account for the different phenotypes. In the case of MyoD-converted NIH 3T3 (Miner and Wold, 1991), the use of an inducible vector for *myc* expression might have allowed acute induction of high levels of *myc* that would prevent differentiation but might be incompatible with long term cell viability. Our data do not exclude that the same phenomenon might also occur in C2C12 cells when using similar inducible vectors.

The fact that in C2C12 cells expression of v-*myc* does not inhibit biochemical differentiation allowed us to make an interesting observation. In agreement with recent results obtained using mammalian fibroblasts (Evan et al., 1992), *myc* expression caused extensive cell death after shift to serum-free medium, i.e., in conditions not sustaining proliferation. However, in the same conditions, differentiated myocytes did not die. This finding leads us to conclude that the differentiation programme interferes with the mechanism of *myc*-induced cell death. Mutual exclusion between programmed cell death and terminal differentiation was also reported in hemopoietic cells (Yonish-Rouach et al., 1991).

In a series of experiments we explored the possibility of correcting the fusion defective phenotype of the *myc*-expressing cells by cocultivating them with non-transformed cells. We set up mixed cultures of either replicating C2C12(v-*myc*) or differentiated C2C12(v-*myc*) myocytes and either rat fibroblasts or quail and rat myoblasts. When replicating C2C12(v-*myc*) were coseeded with rat fibroblasts, extensive fusion was observed after mitogen removal; cocultivation with fusion-competent quail or rat myoblasts gave rise to quail/mouse or rat/mouse hybrid myotubes, respectively. However when rat fibroblasts were added to differentiated C2C12(v-*myc*) myocytes, myotube formation could not be rescued; on the contrary, addition of fusion-competent quail myoblasts led to quail/mouse hybrid myotube formation. These findings suggest that correction of the fusion defective phenotype can be achieved via two mechanisms: one, involving the passive recruitment of C2C12(v-*myc*) myocytes into myotubes formed by differentiating normal myoblasts; the other, involving phenotypic reversion of *myc*-induced transformed phenotype, actively induced by surrounding normal cells, as previously reported for *myc*-transformed quail myoblasts (La Rocca et al., 1989). In the case of mixed cultures of replicating C2C12(v-*myc*) and normal myoblasts the two mechanisms might concur to produce the observed phenotypic correction.

The data obtained from the analysis of mixed cultures have other interesting implications. The rescue of myotube formation in mixed culture of rat fibroblasts and replicating C2C12(v-*myc*) confirms and extends our previous results with *myc*-transformed quail myoblasts to a system that is more amenable to further manipulation and experimentation. At the same time, these data strongly argue that myotube formation in mixed cultures of *myc*-transformed myoblasts and normal fibroblasts cannot be accounted for by mere induction of growth inhibition by surrounding normal cells, as C2C12(v-*myc*) are perfectly competent to withdraw from cell cycle by themselves. This conclusion, in turn, reinforces our previous proposal (La Rocca et al., 1989) that fusion of *myc*-transformed myoblasts in mixed culture with normal cells entails the triggering of phenotypic reversion from the transformed state. Finally, the inability of normal fibroblasts to correct the fusion defective phenotype of

differentiated C2C12(v-*myc*) myocytes indirectly lends some support to the postulated mechanism underlying phenotypic reversion of *myc*-transformed cells in mixed culture with normal cells. We had previously suggested that junctional intercellular communication between *myc*-transformed cells and surrounding normal cells might play a role in mediating the susceptibility of transformed myoblasts to undergo phenotypic reversion. While replicating myoblasts can establish junctional communication, differentiated skeletal muscle cells display a loss of junctional competence (reviewed in Loewenstein, 1979). Such an important physiological difference might be responsible for the striking difference between replicating C2C12(v-*myc*) and differentiated C2C12(v-*myc*) myocytes in the ability to fuse in mixed culture with normal fibroblasts.

Further work is still required to clarify the mechanism of and the molecules actually involved in this intriguing issue. Using the system and the approach here described, it will be soon possible to assess whether intercellular communication and phenotypic reversion are more generally and mechanistically involved in the growth inhibition of transformed cells in mixed culture with normal cells (Bignami et al., 1988; Mehta et al., 1986; Stoker et al., 1966).

We thank the following persons for providing valuable reagents used in this study: S. Alemà, G. Cossu, D. Fischman, C. Goridis, T. Graf, K. Moelling, A. Starzinski-Powitz, B. Vennström, W. Wright, and D. Yaffe. We also thank A. Calconi and F. Falzini for technical support.

This work has been supported by grants from Associazione Italiana per la Ricerca sul Cancro (progetto Oncosoppressori); Consiglio Nazionale delle Ricerche (progetto finalizzato Applicazioni Cliniche della Ricerca Oncologica); Fondazione Cenci-Bolognetti.

Received for publication 19 January 1994.

References

Alemà, S., and F. Tatò. 1987. Interaction of retroviral oncogenes with the differentiation programme of myogenic cells. *Adv. Cancer Res.* 49:1–28.

Alemà, S., and F. Tatò. 1994. Oncogenes and muscle differentiation: multiple mechanisms of interference. *Semin. Cell Cancer Biol.* In press.

Amati, B., M. W. Brooks, N. Levy, T. D. Littlewood, G. I. Evan, and H. Land. 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell.* 72:233–245.

Bader, D., T. Masaki, and D. A. Fischman. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* 95:763–770.

Bengal, E., L. Ransone, R. Scharfmann, V. J. Dwarki, S. J. Tapscott, H. Weintraub, and I. M. Verma 1992. Functional antagonism between c-Jun and MyoD proteins: a direct physical association. *Cell.* 68:507–519.

Bignami, M., S. Rosa, G. Falcone, F. Tatò, F. Katoh, and H. Yamasaki. 1988*a*. Specific viral oncogenes cause differential effects on cell-to-cell communication, relevant to the suppression of the transformed phenotype by normal cells. *Molecular Carcinogenesis.* 1:67–75.

Bignami, M., S. Rosa, S. A. La Rocca, G. Falcone, and F. Tatò. 1988*b*. Differential influence of adjacent normal cells on the proliferation of mammalian cells transformed by the viral oncogenes *myc*, *ras* and *src*. *Oncogene.* 2:509–514.

Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold. 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:821–831.

Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka. 1987. Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G_1 phase and is repressed by fibroblast growth factor. *J. Cell Biol.* 105:949–956.

Crouch, D. H., C. Lang, and D. A. F. Gillespie. 1990. The leucine zipper domain of avian cMyc is required for transformation and autoregulation. *Oncogene.* 5:683–689.

Cusella-De Angelis, M. G., G. Lyons, C. Sonnino, L. De Angelis, E. Vivarelli, K. Farmer, W. E. Wright, M. Molinaro, M. Bouche', M. Buckingham, and G. Cossu. 1992. MyoD, myogenin independent differentiation of primordial myoblasts in mouse somites. *J. Cell Biol.* 116:1243–1255.

Donalies, M., M. Cramer, M. Ringwald, and A. Starzinski-Powitz. 1991. Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. *Proc. Natl. Acad. Sci. USA.* 88:8024–8028.

Edelman, G. E., and K. L. Crossin. 1991. Cell adhesion molecules: implications for a molecular histology. *Annu. Rev. Biochem.* 60:155–190.

Eilers, M., S. Schirm, and J. M. Bishop. 1991. The Myc protein activates transcription of the α-prothymosin gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:133–141.

Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell.* 69:119–128.

Falcone, G., F. Tatò, and S. Alemà. 1985. Distinctive effects of the viral oncogenes myc, erb, fps, and src on the differentiation program of quail myogenic cells. *Proc. Natl. Acad. Sci. USA.* 82:426–430.

Falcone, G., S. Alemà, and F. Tatò. 1991. Transcription of muscle-specific genes is repressed by reactivation of $pp60^{v\text{-}src}$ in postmitotic quail myotubes. *Mol. Cell. Biol.* 11:3331–3338.

Grossi, M., A. Calconi, and F. Tatò. 1991. v-*jun* oncogene prevents terminal differentiation and suppresses muscle-specific gene expression in ASV-17-infected muscle cells. *Oncogene.* 6:1767–1773.

Konieczny, S. F., B. L. Drobes, S. L. Menke, and E. J. Taparowsky. 1989. Inhibition of myogenic differentiation by the H-*ras* oncogene is associated with the down regulation of the MyoD1 gene. *Oncogene.* 4:473–481.

La Rocca, S. A., M. Grossi, G. Falcone, S. Alemà, and F. Tatò. 1989. Interaction with normal cells suppresses the transformed phenotype of v-*myc*-transformed quail muscle cells. *Cell.* 58:123–131.

Land, H., L. F. Parada, and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (Lond.).* 304:596–602.

Lassar, A. B., M. J. Thayer, R. W. Overell, and H. Weintraub. 1989. Transformation by activated *ras* or *fos* prevents myogenesis by inhibiting expression of MyoD1. *Cell.* 58:659–667.

Lathrop, B. K., E. N. Olson, and L. Glaser. 1985. Control by fibroblast growth factor of differentiation in the BC_3H1 muscle cell line. *J. Cell Biol.* 100:1540–1547.

Loewenstein, W. R. 1979. Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta.* 560:1–65.

Lüscher, B., and R. N. Eisenman. 1990. New light on Myc and Myb. Part I. Myc. *Genes Dev.* 4:2025–2035.

Maione, R., G. M. Fimia, and P. Amati. 1992. Inhibition of *in vitro* myogenic differentiation by a polyomavirus early function. *Oncogene.* 7:85–93.

Mann, R., R. C. Mulligan, and D. B. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell.* 33:153–159.

Mehta, P. P., J. S. Bertram, and W. R. Loewenstein. 1986. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell.* 44:187–196.

Miner, J. H., and B. Wold. 1990. Herculin, a fourth member of the *MyoD* family of myogenic regulatory genes. *Proc. Natl. Acad. Sci. USA.* 87:1089–1093.

Miner, J. H., and B. J. Wold. 1991. c-*myc* inhibition of MyoD and myogenin-initiated myogenic differentiation. *Mol. Cell. Biol.* 11:2842–2851.

Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587–3596.

Olson, E. N. 1990. MyoD family: a paradigm for development? *Genes Dev.* 4:1454–1461.

Paterson, B., and R. Strohman. 1972. Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. *Dev. Biol.* 29:113–138.

Pearson, M. L., and H. F. Epstein. 1982. Muscle development: molecular and cellular control. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 581 pp.

Rhodes, S. J., and S. F. Konieczny. 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev.* 3:2050–2061.

Schneider, M. D., and E. N. Olson. 1988. Control of myogenic differentiation by cellular oncogenes. *Mol. Neurobiol.* 2:1–39.

Schneider, M. D., M. B. Perryman, P. A. Payne, G. Spizz, R. Roberts, and E. N. Olson. 1987. Autonomous expression of c-*myc* in BC_3H1 cells partially inhibits but does not prevent myogenic differentiation. *Mol. Cell. Biol.* 7:1973–1977.

Schubert, D., J. Harris, C. E. Devine, and S. Heineman. 1974. Characterization of a unique muscle cell line. *J. Cell Biol.* 61:398–413.

Smith, T. H., N. E. Block, S. J. Rhodes, S. F. Konieczny, and J. B. Miller. 1993. A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newborn mouse myogenic cells. *Development.* 117:1125–1133.

Stoker, M. G. P., M. Shearer, and C. O'Neill. 1966. Growth inhibition of polyoma-transformed cells by contact with static normal fibroblasts. *J. Cell Sci.* 1:297–310.

Vennström, B., P. Kahn, B. Adkins, P. Enrietto, M. J. Hayman, T. Graf, and P. Luciw. 1984. Transformation of mammalian fibroblasts and macrophages

in vitro by a murine retrovirus encoding an avian v-*myc* oncogene. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3223–3229.

Vivarelli, E., and G. Cossu. 1986. Neural control of early myogenic differentiation in cultures of mouse somites. *Dev. Biol.* 107:2191–2198.

Walter, N., C. Jansen, C. Trachmann, and K. Bister. 1986. Nucleotide sequence of the CMII *v-myc* allele. *Virology.* 154:219–223.

Weinberg, R. A. 1989. Oncogenes and the molecular origins of cancer. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 307–326.

Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, K. T. Blackwell, D. Turner, R. Rupp, S. Hollenberg, Y. Zhuang, and A. Lassar. 1991. The *myoD* gene family: nodal point during specification of the muscle cell lineage. *Science (Wash. DC).* 251:761–766.

Wright, W. E., M. Binder, and W. Funk. 1991. Cyclic amplification and target selection (casting) for the myogenin binding site. *Mol. Cell. Biol.* 11: 4104–4111.

Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (Lond.).* 270:725–727.

Yang, B.-S., T. J. Geddes, R. J. Pogulis, B. de Crombrugghe, and S. O. Freytag. 1991. Transcriptional suppression of cellular gene expression by c-Myc. *Mol. Cell. Biol.* 11:2291–2295.

Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature (Lond.).* 352:345–347.