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Distinctive effects of the viral oncogenes *myc*, *erb*, *fps*, and *src* on the differentiation program of quail myogenic cells

(transformation mechanism/myogenesis/Rous sarcoma virus/virus MC29)

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ABSTRACT **The relationship between susceptibility to transformation *in vitro* by different oncogenes and terminal differentiation was analyzed in embryonic quail myogenic cells. Infection with Rous sarcoma virus (RSV), Fujinami sarcoma virus (FSV), avian erythroblastosis virus (AEV), and the avian myelocytomatosis virus MC29 led to rapid and massive transformation. Transformed cells had distinctive morphological alterations, increased proliferation rates, and the ability to grow in agar suspension. Furthermore, homogeneously transformed cultures failed to fuse into multinucleated myotubes and to express muscle-specific genes. However, cloned populations of RSV-, FSV-, and AEV-transformed myogenic cells could, under appropriate culture conditions, partially differentiate into atypical "revertant" myotubes. In contrast, competence for terminal differentiation was completely and irreversibly suppressed on transformation by MC29. The specificity of action of a given oncogenic sequence on the inhibition of differentiation was further studied by using conditional and nonconditional transformation mutants. Myogenic cells infected with temperature-sensitive (*ts*) mutants of RSV and FSV exhibited a fully reversible block of differentiation after shift to restrictive temperature, while cells infected with *ts*34 AEV were not temperature sensitive for differentiation. Cultures infected with the partially transformation-defective mutant of MC29 *td*10H were morphologically transformed and acquired anchorage independence for proliferation but maintained a residual competence for terminal differentiation.**

Retrovirus-induced transformation *in vitro* is often accompanied by derangement in the expression of cell type-specific differentiated functions (1–4). The available evidence suggests that the block of differentiation is under continuous control of the transforming gene products: when temperature-sensitive (*ts*) transformation mutants of Rous sarcoma virus (RSV) or avian erythroblastosis virus (AEV) were used, a simple shift to the nonpermissive temperature suppressed the transformed state and allowed the expression or reexpression of differentiated functions in infected cells belonging to different lineages (5–9). Viral infection, however, does not inevitably result in cell transformation and block of differentiation. In a few cases, transforming retroviruses are without detectable effects on host cells: (*i*) macrophages infected with RSV (10) or AEV (11) remain untransformed and functionally differentiated, albeit producing transforming viral progeny and synthesizing the transforming proteins, and (*ii*) *ts* AEV-transformed erythroblasts superinfected by the myelocytomatosis virus MC29 (11) can nonetheless differentiate after a shift to the restrictive temperature.

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In RSV-transformed myogenic cells (5, 6), differentiation is prevented by the continuous action of $pp60^{src}$, the RSV transforming gene product (12). The block of differentiation, however, is reversible and a variable proportion (≤50%) of the transformed cells can, under appropriate culture conditions, fuse into atypical multinucleated myotubes that express muscle-specific markers such as acetylcholine receptors, myosin, desmin and MM-creatine kinase (referred to as "revertant" myotubes, refs. 13 and 14).

We have further investigated the competence for myogenic differentiation in RSV-transformed cells and in cells infected with three independently isolated strains of transforming retroviruses—Fujinami sarcoma virus (FSV), AEV, and MC29—that differ in target cell specificity, oncogenic sequences, intracellular localization of the transforming proteins, and putative mechanism of transformation (1). Accordingly, experiments were made to test whether (*i*) myogenic cells can be transformed by oncogenes other than *src*, (*ii*) the differentiation program can be affected by the various transforming proteins, (*iii*) there are distinctive features in the block of differentiation imposed by different oncogenes, and (*iv*) the integrity of the viral sequences required for leukemogenesis *in vivo* is also required for block of myogenic differentiation *in vitro*.

MATERIALS AND METHODS

Quail embryo myoblasts were prepared from the breast muscles of 10-day-old Japanese quail embryos as described (14). Growth medium for all transformed cells consisted of Dulbecco's modified Eagle's medium supplemented with 10% tryptose phosphate broth/10% fetal calf serum/1% chicken serum. To assay myogenic differentiation, cells were plated on collagen-coated 35-mm dishes at 10^5 cells per dish in growth medium. Twenty-four hours later, cultures were fed with differentiation medium, which consisted of F14 (15) medium supplemented with 2% fetal calf serum and insulin (Sigma) at 5 μg/ml, and then incubated at the appropriate temperature. The permissive temperature for *ts* transformation mutants was 35°C; the restrictive temperature was 41°C.

Stocks of MC29 (RAV-1), MC29 (*td*B77), *td*10H (RAV-1) (td, transformation defective), and *ts*34 AEV (RAV-1) were prepared by rescue with the appropriate helper from quail and chicken nonproducer cells, kindly provided by M. Hayman. FSV (RAV-1) and *ts*225 FSV (RAV-1) were prepared from transformed rat fibroblasts (obtained from P. M. Comoglio and C. DeGiuli-Morghen) by polyethylene glycol-mediated fusion with RAV-1-infected chicken embryo fibro-

Abbreviations: ts, temperature sensitive; RSV, Rous sarcoma virus; AEV, avian erythroblastosis virus; FSV, Fujinami sarcoma virus; *td*, transformation defective; wt, wild type.

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blasts. Original stocks of RAV-1, *td*B77, RSV-PR-A, and *ts*LA29-PR-A were kindly provided by J. A. Wyke. AEV (RAV-1) strain R and *ts*GI201-PR-A were obtained from G. Calothy and R. R. Friis.

Infected and transformed cells were assayed for colony formation in soft agar by suspending 10^2, 10^3, and 10^4 cells in Dulbecco's modified Eagle's medium containing 10% fetal calf serum/10% tryptose phosphate broth/2% chicken serum, twice the normal amount of vitamins, four times the normal amount of folic acid, and 0.35% Bacto-Agar (Difco) and layering the mixture in triplicate on a hard base of 0.7% agar with the same supplements.

Cells were labeled for 2–3 hr with methionine-free medium supplemented with 100–250 μCi (1 Ci = 37 GBq) of [^{35}S]-methionine (New England Nuclear) per ml. After washing, cells were harvested and lysed with 1.5 ml of RIPA buffer (12) supplemented with 0.2 mM phenylmethylsulfonyl fluoride/0.1% Trasylol (for further details, see Fig. 2 legend).

The viral pp60^{src} and pP140$^{gag\text{-}fps}$ products were assayed using the immunocomplex assay of Collett and Erikson (16) as modified (17, 18). The tumor-bearing rabbit sera, which precipitate RSV pp60^{src}, were the gift of P. Enrietto.

Immunofluorescence experiments were performed as described (14). Myosin was visualized by incubation with a 1:100 dilution of fluorescein-conjugated rabbit antiserum against muscle-specific light meromyosin, obtained as a gift from H. Holtzer. P110$^{gag\text{-}myc}$ was stained in nuclei by indirect immunofluorescence with rabbit anti-*gag* antiserum (1:30) and fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (Miles).

RESULTS

Oncogene-Induced Alterations in Quail Myogenic Cells Infected with RSV, FSV, AEV, and MC29. When primary or secondary cultures of quail myogenic cells are cultivated in differentiation medium (see *Materials and Methods*) on collagen-coated dishes, the majority of cells readily withdraw from the cell cycle, differentiate to form multinucleated myotubes, and express muscle-specific products such as myosin, desmin, and acetylcholine receptors. Moreover, normal myotubes further maturate, assembling sarcomeres and showing spontaneous contractions. Myogenic differentiation can be delayed by plating cells at low density on uncoated dishes and cultivating in growth medium (see *Materials and Methods*). Under these conditions, many myogenic cells can proliferate for several generations, thus allowing rapid spread of infecting viruses.

Myogenic cells were infected by the following viruses: the Prague strain of RSV (oncogene *src*), AEV (oncogene *erb*), FSV (oncogene *fps*), MC29 (oncogene *myc*), and the nontransforming Rous-associated virus 1 (RAV-1). RAV-1-infected myogenic cells had no detectable alterations in morphology, proliferation, and differentiation. Transforming virus-infected cells exhibited characteristic morphological conversion and increased proliferation rates (data not shown). Morphologically transformed cells had also acquired anchorage independence for growth. The relative efficiencies of plating in soft agar of virus-infected cells are summarized in Table 1. RAV-1-infected cells, which could not be cloned in suspension, formed colonies on plastic dishes with a plating efficiency of 10–15%. Quantitative differences in the number and size of colonies were observed among the various transformed cells; MC29 infection, which caused the highest increase in proliferation rate on plastic, induced the highest efficiency of plating in soft agar as well as the highest ratio of large to small colonies.

The most striking feature of transformed quail myogenic cells was the severe reduction in the percentage of cells undergoing differentiation. However, as reported for RSV (13, 14), homogeneously transformed cultures of FSV- and AEV-infected cells showed a residual competence for differentiation, as evidenced by the persistent presence of atypical, revertant myotubes. MC29-transformed cells, on the contrary, appeared to be irreversibly incapable of differentiating (see below).

Table 1. Agar colony formation and expression of differentiated functions by retrovirus-infected quail myogenic cells

Virus	*t*, °C	Colonies,* no. per 10^3 cells: Total	Large	Small	Fusion†	Sarcomere organization‡
RAV-1	37	0	0	0	NA	NA
RSV	37	130	54	76	+	−
ts-RSV	35	ND			+	−
	41	ND			+++	+
FSV	37	28	5	23	+	−
ts-FSV	35	ND			+	−
	41	ND			+++	+
AEV-	37	115	10	105	+	(+)
ts-AEV	35	ND			+	−
	41	ND			+	(+)
MC29	37	173	116	57	−	−
td-10H	37	131	37	94	+	−

NA, not applicable; ND, not done.
*Uncloned fifth-passage quail myogenic cells were seeded in triplicate and scored after 2 weeks. Small, <0.25 mm; large, >0.25 mm.
†Clonal strains were cultured in differentiation medium and scored at day 3. +, 3–30% nuclei in revertant myotubes; +++, >90% fusion; −, no fusion.
‡Clonal strains were cultivated in differentiation medium and scored at day 5 by immunofluorescence. +, Sarcomeres in the majority of myotubes; (+), abortive sarcomere in revertant myotubes; −, no sarcomeres.

Infection of myogenic cells with *ts* transformation mutants of RSV, FSV, and AEV at permissive temperature (35°C) produced the same pattern of alterations described for parental viruses. On shift to restrictive temperature (41°C), >90% of *ts* RSV- and *ts* FSV-transformed cells differentiated into elongated, myosin-positive, multinucleated myotubes that further maturated, organizing sarcomeres and showing spontaneous contractile activity (Fig. 1 *E* and *F*), as described for uninfected cells. *ts*34 AEV-transformed cells, on the contrary, did not resume a fully normal phenotype after shift to 41°C and, resembling wild-type (*wt*) AEV-transformed cells, could differentiate only into revertant myotubes (Fig. 1*G*). Moreover, increased deoxyglucose uptake was not temperature sensitive (data not shown), suggesting that the lesion in *ts*34 AEV might be less penetrant in myogenic cells than in fibroblasts or erythroblasts (19).

Reversible Block of Myogenic Differentiation in Clonal Strains of Transformed Cells. To demonstrate that revertant myotubes arise from previously transformed cells, we have developed clonal strains of transformed myogenic cells from soft-agar colonies. Myogenic clones of RSV-, FSV-, and AEV-transformed cells synthesized the putative transforming proteins, pp60^{src} for RSV, pP140$^{gag\text{-}fps}$ for FSV, and P75$^{gag\text{-}erbA}$ for AEV (Fig. 2 *A* and *B*). p68^{erbB}, the other putative transforming product of the *erb* oncogene, was not assayed in this study, but it is known to be produced by the AEV strain used (20). As described for uncloned cells, clonal strains of RSV-, FSV-, and AEV-transformed cells retained a residual competence for expression of the myogenic phenotype. The incidence of differentiated cells was increased 10- to 20-fold on cultivation in differentiation medium and varied considerably among the clones, occasionally reaching >50% of the population. Revertant myotubes formed in

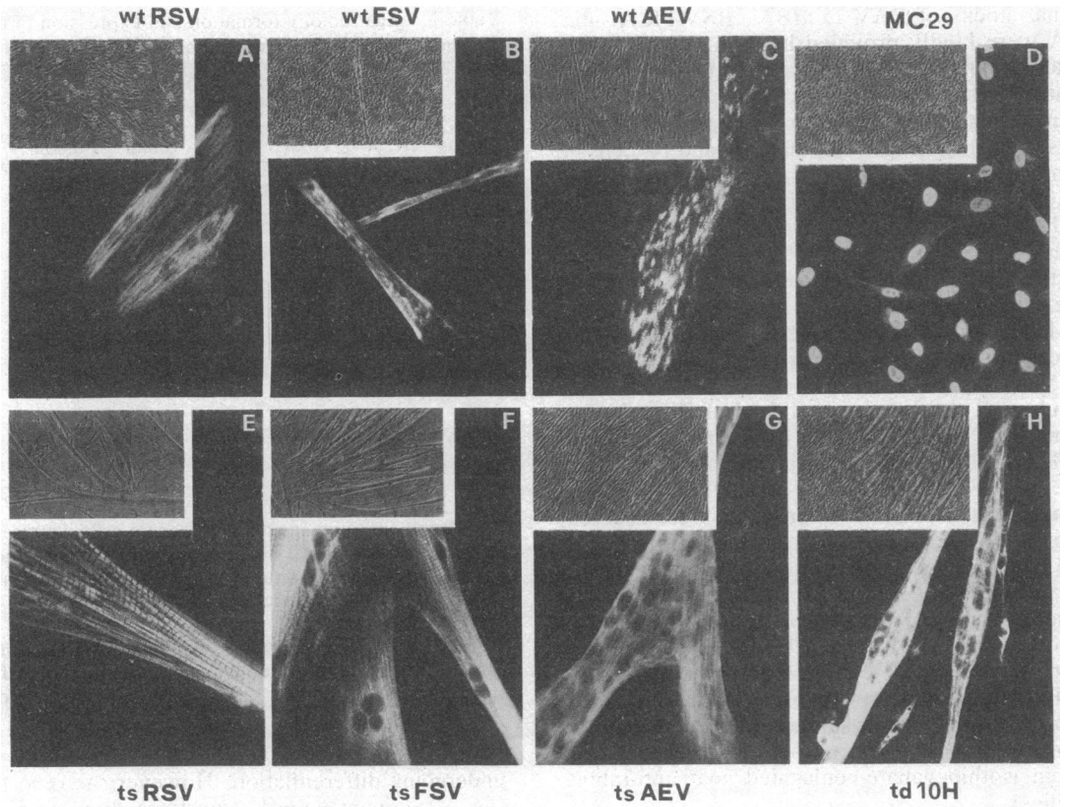

FIG. 1. Fusion and myosin expression in myogenic cells transformed by various oncogenes. Phase-contrast (*Insets*) and immunofluorescence micrographs of cultures grown for 4 days at 37°C (*A–D*, *H*; clonal strains) and 41°C (*E–G*; uncloned cells) in differentiation medium. Phase-contrast micrographs (×21) refer to living cultures showing formation of phenotypically normal myotubes in *E* and *F* and revertant myotubes in *A*, *B*, *G*, and *H*. Note the complete absence of fusion in *D*. Cultures shown in *D* were stained with anti-*gag* serum to visualize the nuclear localization of $P110^{gag\text{-}myc}$. Those in *A–C* and *E–H* were stained for muscle-specific myosin by direct immunofluorescence with fluorescein isothiocyanate-conjugated anti-light meromyosin. Note the extensive cross-striation in myotubes formed in *ts* RSV- and *ts* FSV-transformed cultures at 41°C (*E*, ×260; *F*, ×185), in sharp contrast to the irregular organization of myosin in revertant myotubes from *wt* RSV and *wt* FSV cultures (*A*, ×165; *B*, ×130). Note in *wt* AEV (*C*, ×230) the atypical myotube with myosin assembled into peculiar structures resembling abortive sarcomeres. In *ts* AEV at 41°C (*G*, ×165) myotubes appear more elongated than those shown in *C* but myosin staining is diffuse and only partly organized in myofibrils. Note in *H* (×130) revertant myotubes brightly stained by anti-light meromyosin as compared to *A* and *B*.

differentiation medium, while expressing a number of muscle-specific markers (data not shown), were atypical in shape, with centrally clustered nuclei, and failed to assemble normal striated myofibrils (Fig. 1*A–C*). In revertant myotubes arising from AEV-transformed cells, myosin appeared to be assembled into abortive sarcomere-like structures (Fig. 1*C*). Clonal strains of MC29-transformed cells were also developed but, although selected by the same criteria, were uniformly negative for expression of all differentiation markers tested. The MC29 clonal strains synthesized the product of the *myc* oncogene, $P110^{gag\text{-}myc}$ (Fig. 2*C*), which showed the characteristic nuclear localization (Fig. 1*D*) (21, 22).

Irreversible Block of Myogenic Differentiation in MC29-Transformed Cells. Two independent lines of evidence suggest that the *myc* oncogene can transform myogenic cells and block their differentiative potential. First, three independent *ts* RSV myogenic clones were superinfected with MC29 (*td*B77) or with *td*B77 alone and passaged three times to allow spreading of the superinfecting virus. Cells superinfected with MC29 became smaller, had prominent nucleoli, and proliferated faster than helper-superinfected cells. Fig. 3 shows that MC29 superinfection completely abolished both the temperature shift-induced differentiation and the appearance of revertant myotubes at 35°C in differentiation medium. One of the *ts* RSV clones used was characterized by a limited lifespan. Strikingly, on superinfection with MC29, the senescent cells resumed a high proliferation rate, whereas controls could not be passaged further. Second, we made use of a partially transformation-defective mutant of MC29, *td*10H. This mutant has a 600-base-pair deletion in the *myc* sequence that results in a *gag-myc* fusion protein of reduced size ($P90^{gag\text{-}myc}$) and in loss of pathogenicity *in vivo* (23). It no longer transforms macrophages *in vitro*, but it still transforms fibroblasts (24). Myogenic cells were infected with *td*10H and analyzed as described above for other viruses. *td*10H-infected cells formed colonies in soft agar (Table 1) and clonal strains derived therefrom synthesized the expected transforming product (Fig. 2*C*). Unlike MC29, however, *td*10H-transformed cells could differentiate into revertant myotubes (Fig. 1*H*). A peculiar feature of *td*10H noted in the soft agar cloning experiments is worth mentioning. When freshly infected cells were plated in soft agar, there was a 5- to 7-day lag before they began to grow, whereas no such lag was observed for parental MC29-infected cells. On the contrary, there was no detectable lag when *td*10H homogeneously transformed cells were assayed for efficiency of plating in soft agar, and colonies developed in times comparable with those of MC29, albeit with some reduction in number and size (Table 1).

DISCUSSION

In the present experiments, we have analyzed and compared the effects of functionally different oncogenes—*src*, *fps*, *erb*,

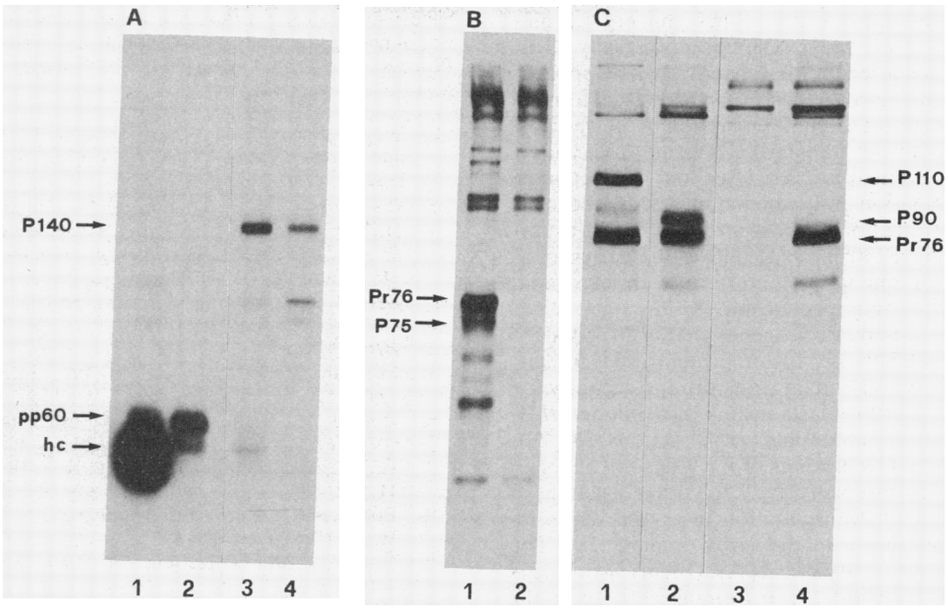

FIG. 2. Expression of protein kinase activity and *gag*-related proteins in transformed myogenic clones. (*A*) Analysis of the pp60^{src}- and pP140$^{gag\text{-}fps}$-specific kinase activities of Prague A RSV- (lanes 1 and 2) and *wt* FSV- (lanes 3 and 4) transformed clonal strains. Tissue culture cells were washed, lysed in 1.0% Nonidet P-40/0.3 M NaCl/50 mM Tris·HCl, pH 7.5, clarified and immunoprecipitated with appropriate antisera, and assayed for kinase activity as described (16–18). Samples were electrophoresed on 7.5% $NaDodSO_4$ gels and processed for autoradiography. Lanes 1 and 2: extracts of Prague A RSV-transformed cells were challenged with tumor-bearing rabbit sera that preferentially favor phosphorylation of IgG heavy chains (lane 1) and pp60^{src} protein (lane 2), respectively. Lanes 3 and 4: extracts of *wt* FSV-transformed cells were immunoprecipitated with two different anti-*gag* sera (obtained from M. Hayman). (*B*) Lanes 1 and 2: extracts of *wt* AEV (RAV-1)-transformed clonal cells were immunoprecipitated with anti-*gag* serum (lane 1) and avian myeloblastosis virus-adsorbed anti-*gag* serum (lane 2). The immunoprecipitates were analyzed on 7.5% polyacrylamide gels, dried, and processed for autoradiography. Molecular weights were calculated by comparison with ^{14}C-labeled markers (Amersham) run on adjacent lanes. (*C*) Tissue culture cells of MC29 (RAV-1)- (lane 1), *td*10H (RAV-1)- (lane 2), and RAV-1- (lanes 3 and 4) infected cells were treated as described in *Materials and Methods*. Equal amounts of 10% trichloroacetic acid-precipitable radioactivity were immunoprecipitated with anti-*gag* serum (lanes 1, 2, and 4) and with normal rabbit serum (lane 3).

and *myc*—on the differentiation program of quail myogenic cells infected by the appropriate transforming retrovirus. These oncogenes were selected as prototypes of different strategies of transformation, as inferred from the properties of their products: (*i*) membrane-associated protein kinases specific for tyrosine residues (*src*, *fps*) (1); (*ii*) nuclear proteins that bind to DNA *in vitro* (*myc*) (21, 22); and (*iii*) proteins structurally homologous to members of the *src* family, yet without detectable enzymatic activity *in vitro* (*erbB*) (20, 25). The data show that the expression of each of these oncogenes resulted in rapid and massive transformation of infected cells. Transformed cells were anchorage independent for proliferation and synthesized the specific putative transforming proteins. The most striking consequence of transformation, irrespective of the oncogene involved, was a dramatic reduction in the percentage of cells attaining terminal differentiation. Although in RSV-, FSV-, and AEV-transformed clonal strains, a variable proportion of the cells could overcome, under appropriate culture conditions, the block of differentiation, MC29 had the distinctive property of completely and irreversibly preventing myogenic differentiation.

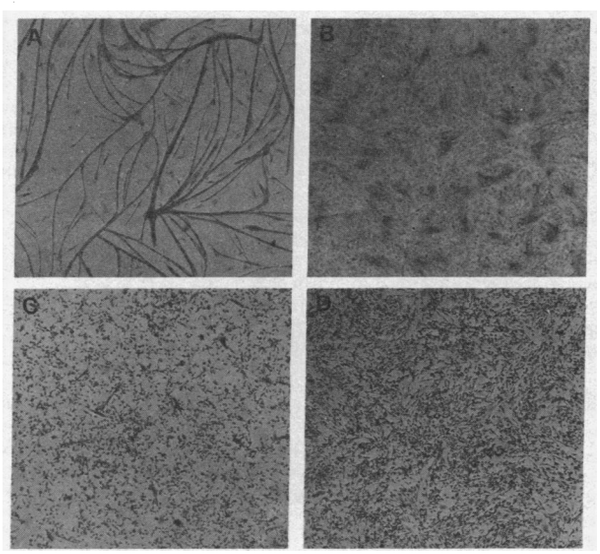

FIG. 3. Superinfection of a *ts*-RSV transformed myogenic clone with MC29 (*td*B77). *ts* GI201-transformed cells superinfected with MC29 (*td*B77) (*C*, *D*) or with *td*B77 alone (*A*, *B*) were incubated at 41°C (*A*, *C*) or 35°C (*B*, *D*) and scored for myogenic differentiation at day three by fixation and staining with Giemsa. (×45.)

In principle, terminal differentiation of transformed myogenic cells might be blocked either by a *direct* effect on the expression of the developmental program or by an *indirect* effect resulting from disruption of the control of proliferation that would prevent the necessary withdrawal from the cell cycle. pp60^{src}, the *src* gene product, appears to affect the regulation of differentiated functions in *ts* RSV-transformed chicken myogenic cells directly: *ts* transformed cells can differentiate at the restrictive temperature without DNA synthesis requirement, but DNA synthesis inhibition at 35°C is not *per se* sufficient to allow expression of the differentiated phenotype (26). Moreover, if *ts* transformed cells are allowed to differentiate at 41°C and then shifted to 35°C, reactivation of pp60^{src} in myotubes selectively reduces the synthesis of muscle gene products (27). Altogether, these findings indicate that pp60^{src} interferes with both initiation and maintenance of the differentiated state in myogenic cells. The question now arises as how this conclusion may be reconciled with the presence of revertant myotubes in *wt*

RSV-transformed cultures (13, 14). Since the normal differentiation program is promptly resumed in virtually all *ts* RSV-transformed cells after thermal inactivation of $pp60^{src}$, activation of the same set of genes in *wt* RSV-transformed cells might ensue from a transient interruption in the cascade of events that maintain the transformed phenotype. Such a postulated transient event appears to affect only a fraction of the transformed population and to be modulated by the local environment. The strong homology in structure and function between $pp60^{src}$ and $pP140^{gag\text{-}fps}$ (14) allows the provisional conclusion that both oncogenes interfere with myogenic differentiation via the same mechanism.

AEV contains two distinct oncogenes that are separately expressed, *erb*A as a $P75^{gag\text{-}erbA}$ fusion protein and *erb*B as a $p68^{erbB}$ protein (20, 28). The main transforming capacity for both fibroblasts and erythroblasts seems to reside in *erb*B, while *erb*A appears to potentiate *erb*B action (28, 29). Although the lesion(s) in *ts* mutants of AEV has not yet been mapped, their temperature sensitivity for transformation in fibroblasts and block of differentiation in erythroblasts indicate a probable mutation in the *erb*B domain (28). The partial temperature sensitivity of *ts*34 AEV in myogenic cells could reflect either a residual transforming activity of the *erb*B product or a specific role played by the *erb*A product in control of differentiation of AEV-transformed myogenic cells. Clarification of this point will require use of the recently developed nonconditional mutants in *erb*A and *erb*B (29).

The data reported here show that *myc* is the most potent oncogene, affecting both control of proliferation and competence for differentiation irreversibly. The presence of revertant myotubes in myogenic clones transformed by *td*10H suggests that the *myc* sequence, which controls leukemogenesis and pathogenicity, is required in its entirety for the irreversible block of myogenic differentiation. The expression of c-*myc*, the cellular homologue of v-*myc*, has recently been shown to be coordinately regulated with cell cycle progression in normal cells (30). In addition, recent studies have shown that introduction of v-*myc* into quail and rat embryo fibroblasts results in an enhanced rate of proliferation (31, 32). Our data indicate that expression of v-*myc* conferred to myogenic cells two altered growth properties: a greatly expanded lifespan (immortalization?) and a very high proliferation rate at clonal density either on plastic or in suspension. Hence, the v-*myc*-induced block of differentiation may be envisaged as an indirect consequence of relaxed growth control, because expression of the myogenic differentiation program requires previous withdrawal from the cell cycle (33). Intriguingly, in chicken chondroblasts (unpublished work) and macrophages (10), where, at variance with myogenic cells, expression of the differentiation program is compatible with cell proliferation, MC29 transformation leads to growth alterations yet leaves synthesis of the specific differentiated products unaffected.

The present results and the above considerations are consistent with the existence of two groups of oncogenes primarily acting either on the expression of differentiation (*src* and *fps*) or on the control of proliferation (*myc*). The first group is further characterized by a distinctive instability in maintenance of the transformed phenotype in myogenic cells that can be modulated by environmental cues.

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