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Effect of oncogenic virus on muscle differentiation

(myogenesis/temperature-sensitive Rous sarcoma virus/transformed myogenic cells/creatine kinase)

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ABSTRACT **Chick muscle cultures infected with wild-type Rous sarcoma virus form myotubes, but these myotubes vacuolate and by day 6 most have degenerated, leaving only large numbers of transformed mononucleated, replicating cells. Muscle cultures infected with a temperature-sensitive mutant (TS) at permissive temperatures behave as cells infected with wild-type Rous sarcoma virus. TS-infected cells reared for 8 days at nonpermissive temperature form contracting myotubes, plus large numbers of fibroblastic cells. If these cultures are lowered to permissive temperature, within 72 hr the myotubes vacuolate and degenerate, whereas the mononucleated cells transform. If replicating TS-transformed cells after 8 days at permissive temperature are shifted to nonpermissive temperature, within 72 hr many cells fuse and form contracting, post-mitotic myotubes. Creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) levels parallel the formation and degeneration of myotubes during these temperature shifts. If the viral transforming gene is expressed in the post-mitotic myotubes it is lethal, whereas it is not lethal if expressed in replicating precursor myogenic cells. The viral gene expression at permissive temperature blocks further myogenesis depending on the position of the cells in the myogenic program. The virus does not cancel the replicating, transformed myogenic cells' commitment to, or position in, the myogenic lineage. When the transforming action of the virus is suppressed, the normal myogenic program resumes.**

The question whether some events which control differentiation can modulate the course of viral-induced transformation is an important one and the answer to this question may throw some light on the mechanism of transformation as well as differentiation. There are several reports that cells in muscle cultures can be infected with either DNA or RNA viruses (1–6). However, muscle cultures, and even "muscle clones" consist of a heterogeneous population of cells in different compartments of the myogenic and fibrogenic lineages, and it is by no means clear whether these different phenotypes respond to the viruses in the same manner (7). For example, when muscle cultures are treated with oncogenic virus, does the virus transform both replicating myogenic and post-mitotic myogenic cells? Will the post-mitotic nuclei in infected myotubes integrate the virus, transcribe the viral genome, and transform? Will normal, post-mitotic myotube nuclei be induced to reenter the cell cycle and undergo mitosis as claimed by some investigators (3, 6)? Clearly, answers to these questions will contribute to understanding myogenesis, and could provide a useful model for following events responsible for transformation.

We have confirmed previous observations on the effect of wild-type Rous sarcoma virus (RSV) on muscle cultures (5). Replicating myogenic cells exposed to RSV fused to form post-mitotic, multinucleated myotubes, but these myotubes invariably vacuolated and degenerated within the next 72–96 hr. We have further demonstrated that the virus had no visible effect on the post-mitotic myotubes at the time of infection. By using a temperature-sensitive mutant (TS) we have also demonstrated that the virus transformed replicating presumptive myoblasts and replicating fibroblasts. Replicating myogenic cells transformed by a TS mutant did not fuse at permissive temperature, but ceased replicating and fused when shifted to nonpermissive temperature. TS-mutant-infected cells reared at nonpermissive temperature fused, but did not vacuolate. However, when such TS-infected cultures were shifted to the permissive temperatures, within 72–96 hr virtually all the myotubes vacuolated and degenerated, whereas all the mononucleated cells transformed. The vacuolization and degeneration in myotubes is probably an expression of the transforming activity of the virus, rather than a response to a deleterious molecule released by transformed fibroblasts, for the vacuolization and degeneration occur in the absence of transformed fibroblasts.

MATERIALS AND METHODS

Breast muscles were dissected from leukosis-free 10-day chick embryos. The tissues were rendered into a suspension of mononucleated cells by incubation in a 0.25% trypsin solution and dissociated with a pasteur pipet. The cells (10^5/ml) were cultured in collagen-coated culture dishes and the uptake of [^{3}H]thymidine ([^{3}H]dThd) was followed radioautographically as described in Bischoff and Holtzer (8). The cultures were infected with the Prague strain of RSV (PR-A), or with a temperature-sensitive mutant (TS mutant) of this strain (9). The titer of RSV in focus-forming units was determined as described by Vogt (10). Cells were infected with approximately 5 focus-forming units per cell. This TS

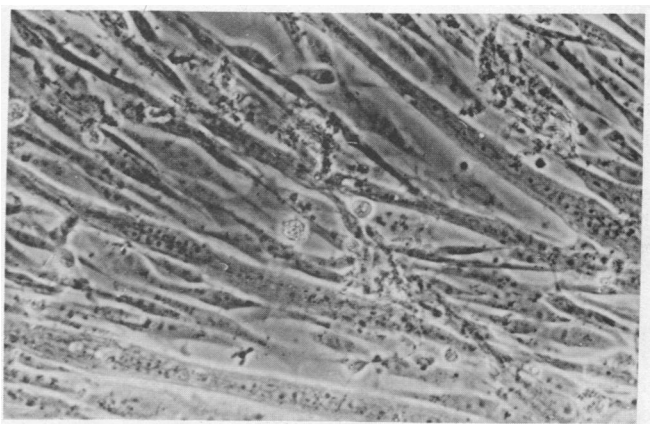

FIG. 1. Observe the huge numbers of mononucleated myogenic and fibrogenic cells interspersed between the immature, multinucleated myotubes. ×300.

Abbreviations: RSV, Rous sarcoma virus; dThd, thymidine; BrdUrd, bromodeoxyuridine; Ara-C, cytosine arabinonucleoside.

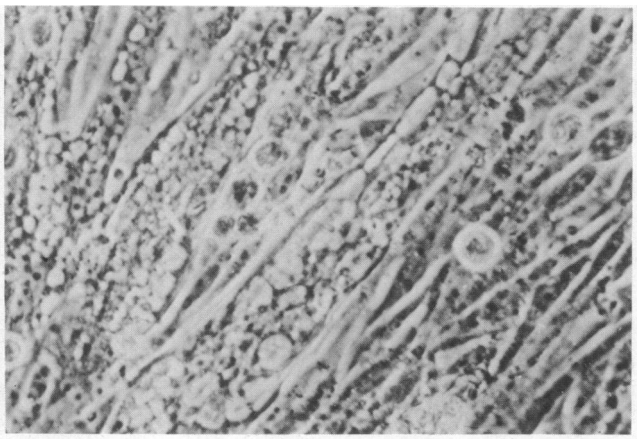

FIG. 2. Virtually all myotubes in the infected cultures display large vacuoles. These vacuoles range up to 10 μm in size. ×300.

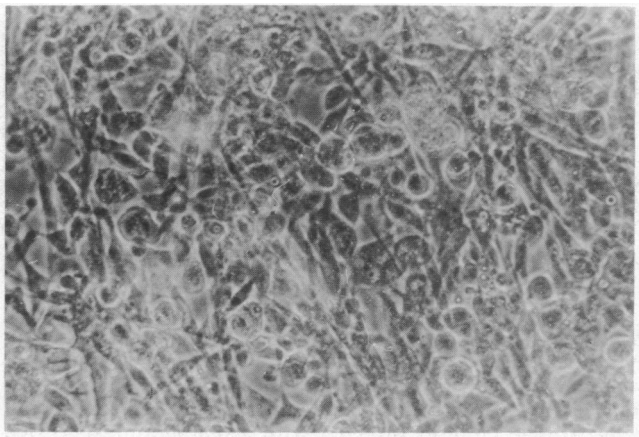

FIG. 3. Eight-day infected cultures consist almost exclusively of mononucleated, replicating, transformed cells. These transformed cells readily detach from the substrate. ×300.

mutant (LA 24) infects and transforms fibroblasts at the permissive temperature of 36°; it will infect and produce virus, but does not transform at the nonpermissive temperature of 41° (9). To determine whether replicating presumptive myoblasts could be transformed, cultures were infected with wild-type or TS mutant RSV immediately after plating. After the cells were exposed to virus for 12 hr the cultures were washed with growth medium and fed daily thereafter. In some experiments 2-day-old cultures were exposed to cytosine arabinonucleoside (Ara-C, 1 μg/ml) for the next 4–5 days, washed, and then grown in normal medium. This treatment kills the great majority of replicating cells, but has no detectable effect on post-mitotic cells (11).

Creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2), which has been shown to be a good marker for fusion (5) was assayed by the method of Oliver (12).

RESULTS

Virus-infected myotubes

Uninfected cultures, and cultures infected with wild-type RSV or the TS mutant reared at permissive temperature were essentially indistinguishable for the first 3 days in culture. The great majority of mononucleated cells replicated at least once in the first 36 hr, and most of them replicated again in the next 12 hr (8). By day 3, many myoblasts withdrew from the cell cycle and began to fuse to form the multinucleated, post-mitotic myotubes (Fig. 1).

The multinucleated myotubes in the infected cultures, though forming on schedule on day 3, invariably formed numerous vacuoles by day 4 or 5 (Fig. 2). These vacuoles, 1 μm to over 4 μm in diameter, filled the myotubes. The fact that the vacuolated myotubes contract spontaneously demonstrated that these cells had synthesized and organized myosin, actin, and tropomyosin into functional myofibrils. As these vacuolated myotubes gradually detached from the substrate and degenerated, by day 5 or 6, few multinucleated myotubes were present in virus-treated cultures (Fig. 3), whereas many thousands of contracting myotubes were present in uninfected cultures. By this time creatine kinase levels in virus-treated cultures have decreased to about 5% of the control level (Fig. 4*a*). Essentially similar observations were made by Easton and Reich (5) using the Schmidt-Ruppin strain of RSV.

Cells transformed by either the Prague strain of RSV or the TS mutant were first detected microscopically toward the end of day 3 and their numbers increased thereafter. Transformed cells were distinguished from untransformed cells by their rounded contours and the fact that they tended to be multilayered, forming grape-like aggregates that were readily dislodged from the substrate. Their cytoplasm was honeycombed with small vacuoles approximately 1 μm in diameter, and upward of 50 vacuoles were present in a cell. By day 5 or 6 the majority of myotubes that had formed degenerated, and all the surviving mononucleated cells in the infected cultures exhibited the cytology of transformed cells. The appearance of cultures transformed by the wild-type RSV was indistinguishable from that of the cultures transformed by the TS mutant at the permissive temperature.

To determine whether RSV would infect and transform post-mitotic myotubes, 2-day-old cultures were exposed to Ara-C for 4 days, washed, and then grown in normal medium. This treatment kills the replicating mononucleated cells, thus yielding cultures consisting almost exclusively of post-mitotic myotubes. RSV was added to the resulting 6 day cultures. The cultures were grown for another 7 days (Fig. 5).

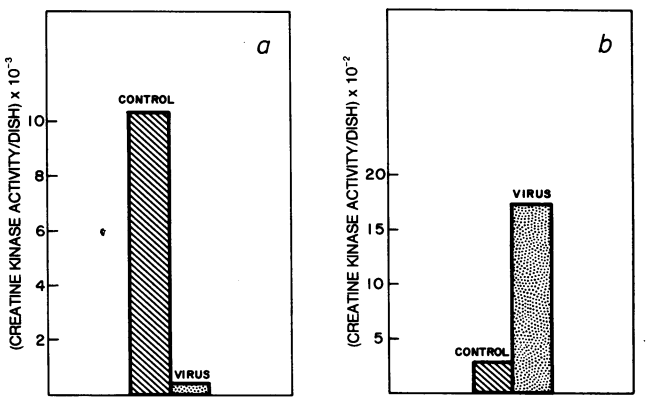

FIG. 4. (*a*) Cultures were initially grown at the permissive temperature (36°) in the presence of the TS mutant (virus) or absence of TS virus (control) for 8 days. Cells were then removed and the creatine kinase was assayed as outlined in *Materials and Methods* section. (*b*) Cultures were initially grown at the permissive temperature in the presence of the TS mutant (virus) or absence of TS virus (control) for 8 days. These cultures were then replated at initial densities, grown at nonpermissive temperature (41°), and fixed after 4 days, and creatine kinase was assayed.

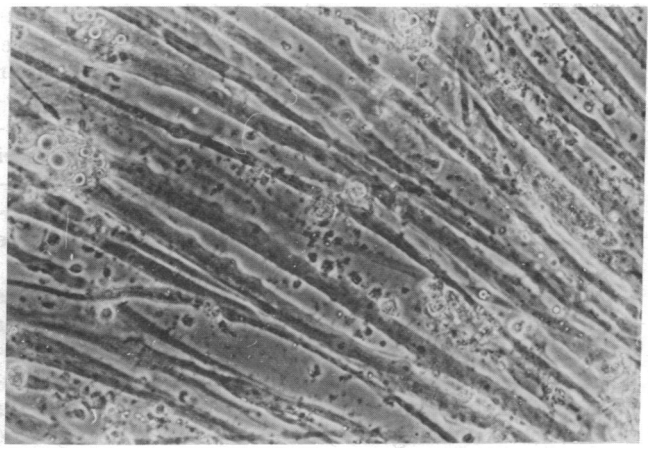

FIG. 5. Eleven-day Ara-C-treated culture that had been exposed to virus 5 days earlier. Note the absence of vacuoles in myotubes, as well as the absence of mononucleated myogenic and fibrogenic cells. ×300.

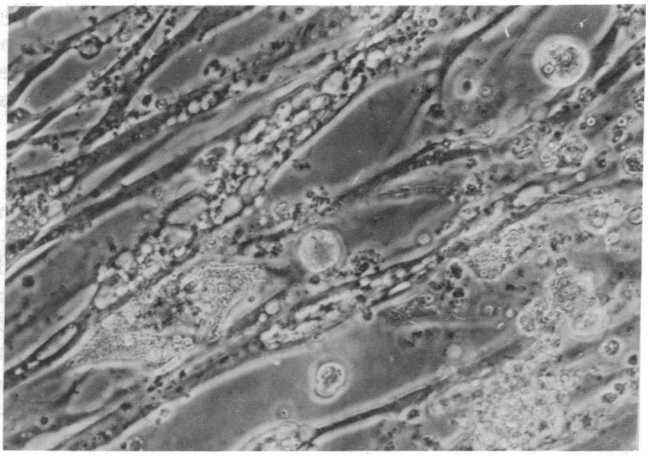

FIG. 6. Cultures were infected with the TS mutant and grown at the nonpermissive temperature with Ara-C. After 8 days at the nonpermissive temperature, the cultures were dropped to the permissive temperature. Note the characteristic vacuoles that appeared after 48 hr. By 96 hr all the myotubes had degenerated. ×300.

During this period the myotubes did not vacuolate and did not degenerate. However, small numbers of mononucleated cells remaining after Ara-C treatment (less than 1% of control cultures) developed into sizable nests of replicating transformed cells which could be observed among the completely normal myotubes. This indicates that the virus successfully infected and transformed the replicating mononucleated cells, though myotubes were not affected.

To determine whether the vacuolization and the degeneration observed in myotubes that had been assembled by infected and/or transformed cells were due to the transforming activity of the virus, the following experiments were performed: Cultures were infected with the TS mutant and grown at 41° (nonpermissive temperature) for 8 days. These cultures contained large numbers of normal-looking mononucleated cells and large numbers of myotubes that contracted spontaneously. Large vacuoles did not form in these myotubes. However, when these cultures were shifted to 36° (permissive temperature) virtually all the myotubes vacuolated and degenerated within 48 hr. All of the mononucleated cells displayed the morphology of transformed cells after 48 hr. Since the transforming gene is temperature sensitive, one can conclude that vacuolization of the myotube is the cell's response to activation of the viral transforming genome. Though TS-infected myotubes at nonpermissive temperature do not form massive vacuoles, nevertheless such myotubes can be distinguished from uninfected myotubes. These differences will be described elsewhere.

To establish that vacuolization and degeneration of the myotubes was due to the transforming activity of the viral genome within the myotube, and not a response of the myotubes to molecules released by adjacent transformed fibroblasts, the following experiment was performed: Cultures were infected with the TS mutant and grown at the nonpermissive temperature. After the cultures were 36 hr old, Ara-C was added and the cultures were grown in its presence for 6 days at the nonpermissive temperature. Myotubes formed in these cultures but few mononucleated cells were present. When these cultures were shifted to permissive temperature, within 48 hr virtually all the myotubes vacuolated and degenerated, although no transformed fibroblasts were present (Fig. 6).

Fusion of TS-transformed cells

Five-day-old infected cultures contain large numbers of replicating transformed cells. To determine whether some of these transformed cells were myogenic, the following experiments were performed: Cultures were infected with the TS mutant and grown at the permissive temperature. By day 5 most of the myotubes that had formed vacuolated and degenerated, so that by day 8 only transformed mononucleated cells were present. These TS-transformed cells incorporated [^{3}H]dThd in a manner indistinguishable from transformed chick fibroblasts. These cultures, which were maintained for another 2 days at permissive temperature, were then subcultured, replated at the initial density, and shifted to nonpermissive temperature. Within the next 48 hr many of the formerly transformed cells fused and formed large numbers of contracting myotubes. By 96 hr such cultures have creatine kinase greater than 6-fold that of subcultures of their uninfected counterparts (Fig. 4*b*). It should be pointed out that in the uninfected muscle cultures fusion peaks around day 4 or 5, and relatively few cells fuse on day 7 or 8. These experiments demonstrate that the active transforming gene keeps the myogenic cells in the cell cycle. Such transformed myogenic cells "remember" their position in the myogenic lineage, and are able to express it, for at the nonpermissive temperature they withdraw from the cell cycle and fuse as would normal myoblasts.

That the myotubes that formed when TS-transformed cells were elevated to nonpermissive temperatures harbored virus was shown by then lowering these cultures to permissive temperatures. Within 48 hr the myotubes that formed by the fusion of transformed cells at the nonpermissive temperature vacuolated and degenerated when shifted down.

The virus used in these experiments has a genetic defect at the gene responsible for transformation so that the TS-infected cells transform only at the permissive temperature. On the other hand, infected fibroblasts produce virus even at the nonpermissive temperatures. Therefore, it was of interest to determine if myotubes that had been infected with the TS virus as mononucleated cells would or would not produce virus at the nonpermissive temperatures. Preliminary experiments with the Ara-C-treated cultures indicate that if

the infected myotubes produce any virus, it is less than $\frac{1}{300}$ of the equivalent transformed fibroblasts. However, even this low value may be due to the virus produced not by the myotubes but by the small numbers of mononucleated cells present in Ara-C cultures.

DNA synthesis

Nuclei in multinucleated myotubes normally never again enter S and so never divide (13, 14). However, there are reports that nuclei in virus-infected myotubes can be induced to synthesize DNA and even enter metaphase (1–3, 6). To determine whether wild-type RSV or the TS mutant could induce normally post-mitotic myoblasts or myotubes to reenter the cell cycle, a series of autoradiographic experiments was performed: Cultures infected with either virus were exposed to [^{3}H]dThd for 1 hr on days 3, 4, 5, or 6 at either permissive or nonpermissive temperatures. In no instance did the nuclei in myotubes incorporate the label.

Normal presumptive myoblasts are obligated to synthesize DNA and pass through a quantal cell cycle to yield post-mitotic myoblasts capable of fusing (19, 20, 23). To determine whether transformed myogenic cells had to replicate prior to fusing, TS-transformed cells were shifted to permissive temperature and [^{3}H]dThd was added to the medium. After 48 hr the cells were prepared for autoradiography. Approximately 25% of the nuclei in the myotubes that formed were unlabeled. However, it is still not clear whether those cells that fused without replicating would have withdrawn from the cell cycle even without fusing. Further experiments are required to clarify this intriguing issue (23).

DISCUSSION

In addition to infecting and transforming fibroblasts, RSV has been reported to infect and transform iris-epithelial cells and pigment cells (15), cells derived from hematopoietic organs (16), and some undefined embryonic retinal cells (17). Our experiments demonstrate that presumptive myoblasts can be infected and transformed. Pending further experiments we cannot state whether failure to observe changes in pre-formed myotubes after exposing them to virus was due to failure of the virus to penetrate the myotubes, or to initiate infection, or to be integrated into the post-mitotic host nuclei.

In replicating myogenic and fibrogenic cells the transforming gene causes the morphological changes typically associated with transformed cells. That vacuolization and degeneration of the myotubes is due to the virus within the myotubes, and is not a secondary response to products of adjacent mononucleated cells, was shown by the behavior of infected cells in Ara-C-treated cultures. The myotubes in these cultures did not vacuolate and degenerate at 41°, but promptly did so when shifted to 36°, although transformed fibroblasts were essentially absent. It is clear that the molecular events responsible for vacuolization and degeneration of the myotubes were caused by the transforming gene product of RSV in the absence of proliferation in the host cells. This point was strengthened by the fact that the time of appearance of the vacuoles in the TS-infected myotubes following temperature shift down roughly corresponded to the time biochemical changes are observed in TS-infected fibroblasts which are subjected to a similar shift to the permissive temperature. In myotubes vacuolization is followed by degeneration. Although vacuolization occurs in mononucleated cells as well, they survive and continue to replicate. This might only mean that the latter could successfully mobilize membranous components to keep up with the transforming gene action, whereas comparable synthetic activity cannot occur in myotubes. Alternatively, activity in the post-mitotic myotubes might lead to forbidden cytoplasmic or nuclear activity. For example, if viral genomes integrated into host nuclei must periodically replicate and if myotube nuclei are incapable of synthesizing DNA, this might constitute a forbidden cytoplasmic-nuclear exchange that would lead to the death of the cell.

It is possible that the viral genome in the myotube exists as a provirus DNA without being integrated into the nuclei, as in the case of ethidium bromide treated fibroblasts (18). Accordingly, it will be of interest to determine by the appropriate hybridization experiments whether the viral genome has or has not been integrated into the post-mitotic myotube chromosomes.

The formation of myotubes during the first 3 or 4 days of the primary virus-treated cultures, but not thereafter, merits discussion, particularly regarding changes in the cell surface of myogenic cells concerned with fusion (19, 20). It is probable that not all of the myogenic cells are infected and transform at precisely the same time. Furthermore it is known that approximately 20% of the original inoculum consists of cells that fuse without replicating (8, 20). From the standpoint of cell differentiation these findings suggest that, depending on the position of the cell in the myogenic program, further development can be superseded by the transforming activity of the virus. If the cell is prepared to fuse, fusion might occur before full expression of the transforming genome so alters the cell surface so as to preclude fusion. Nevertheless, the resulting myotube will vacuolate and degenerate when the transforming genome is finally expressed. In brief, even if suppressed by the action of the transforming gene, the epigenetic program of the transformed myogenic cell is stable enough as a phenotype to persist. These transformed cells do not lose their previously acquired commitment to, and position in, the myogenic lineage.

Phenomenologically, the TS-transformed myogenic cells and bromodeoxyuridine (BrdUrd)-suppressed myogenic cells are markedly similar (8, 21). BrdUrd blocks not only the withdrawal from the cell cycle and fusion into myotubes, but also the initiation of synthesis of such myoblast luxury proteins as skeletal myosin heavy and light chains, tropomyosin, and possibly the actin destined for the thin filaments of myofibrils (14, 23, 26, 29). The block by these agents is readily reversible, and it is unlikely that BrdUrd or a viral transformation product suppresses the individual structural genes for myosin, actin, creatine kinase, and the genes regulating the events required for fusion. These observations coupled with those made on the effects of BrdUrd on erythrogenic cells (21, 22, 27, 28) suggest the existence of BrdUrd-sensitive "master switch" loci (21–23, 26, 28) on chromosomes. Such loci, it has been postulated, would make available for transcription just those unique groupings of structural genes which characterize the metabolic options of that particular cell type. Neither the blocking effect of BrdUrd nor the activity of the transforming gene impair the position in the lineage of the myogenic cell. Thus, when the transforming action of the virus is suppressed by shifting to the nonpermissive temperature, or when BrdUrd in the cell's chromosomes is diluted by new DNA synthesis, in both instances read-out of the normal myogenic program resumes. TS-transformed myogenic cells and Friend erythroleukemic cells (30), to a degree, mimic BrdUrd-suppressed myogenic

and BrdUrd-suppressed erythrogenic cells. It will be of interest to determine whether the sites of viral gene integration into the chromosomes of myogenic cells and erythrogenic spleen cells correspond to those sites sensitive to BrdUrd and whether such sites have the properties of "master switch" loci (23).

Previous reports that nuclei in myotubes infected with RSV or simian virus 40 were induced to enter S and that such nuclei proceeded into mitosis must be reconsidered (1–3, 6). What these investigators interpreted as DNA synthesis and metaphase chromosomes, others (13, 23) have interpreted as the incorporation of [^{3}H]dThd associated with DNA repair and/or nuclear pulverization (24).

The data in this paper contribute two points regarding basic controls in neoplastic cells: (*1*) The epigenetic program of RSV-transformed myogenic cells is blocked, not abrogated. Blocking viral oncological activity by shifting temperature allows the previously transformed, continuously replicating cell to withdraw from the cell cycle; (*2*) If cells in the terminal compartment of the myogenic lineage are obligated to irreversibly turn off DNA synthesis, then the cell of origin of the rhabdomyosarcoma is likely to be a transformed presumptive myoblast, rather than a terminal myoblast (25).

Note Added in Proof. Transformation of myogenic cells by Rous sarcoma virus has recently been carried out in other laboratories also (31, 32).

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