A Temperature-conditional Mutant of Simian Virus 40 Large T Antigen Requires Serum to Inhibit Myogenesis and Does Not Induce DNA Synthesis in Myotubes[1]

Giovanni Salvatori, Laura Lattanzi, Pier Lorenzo Puri, Roberta Melchionna, Carlo Fieri, Massimo Levrero, Mario Molinaro, and Giulio Cossu[2]

Istituto di Istologia ed Embriologia Generale [G. S., L. L., R. M., M. M., G. C.]; I° Clinica Medica, Fondazione A. Cesalpino, Università di Roma "La Sapienza" [P. L. P., M. L.]; and Casa di Cura Columbus [C. F.], Rome, Italy

Abstract

The temperature-conditional mutant tsA58 of SV40 large T antigen (Tag) increases the proliferation rate and the number of cell divisions in primary murine and human myogenic cells when expressed under permissive conditions (*i.e.*, at 33°C in medium containing high levels of serum). Under these conditions, Tag also prevents terminal differentiation. Under nonpermissive conditions (*i.e.*, at 39°C in medium containing low levels of serum) in which Tag is largely inactive, proliferation is arrested, and differentiation occurs. However, even at a permissive temperature, the removal of serum induced myosin expression and the fusion of myogenic cells, which continued to express functional Tag. Although Tag was complexed with pRb, as expected from a functional protein, proliferation was nevertheless arrested, and differentiation was induced. Consistent with these findings, the exposure of Tag-expressing differentiated myotubes to serum at 33°C did not reinduce DNA synthesis in these cells. Thus, in myogenic cells, temperature-conditional mutants of Tag stimulate proliferation in the presence of serum but neither prevent terminal differentiation in the absence of serum nor induce DNA synthesis once complete withdrawal from the cycle has occurred.

Introduction

Proliferation and terminal differentiation are mutually exclusive processes in several cell types such as neurons and muscle cells (1). Consequently, a strict and complex control must ensure that the correct number of precursor cells is attained in the right place and time during embryogenesis before differentiation is triggered (2) because in differentiated cells, DNA synthesis and cell division are no longer possible.

The mechanism that activates terminal differentiation and triggers the irreversible arrest of DNA synthesis has been partially clarified. The retinoblastoma gene product Rb participates in the induction and maintenance of the irreversible arrest of DNA synthesis during myogenic differentiation (3). This protein controls cell cycle progression by inactivating the transcription factor E2F/DP, which is required to induce many genes involved in the G_1 to S-phase progression (4). For pRb, a physical association with the MyoD/E12 heterodimer to form an active transcriptional complex has also been described (3). pRb functions as a growth suppressor when it is dephosphorylated, and this in turn depends upon complex regulatory networks involving the cyclin-dependent kinases and their specific inhibitors (5, 6). Thus, signals such as growth factors, by acting through cyclin/cyclin-dependent kinases, can ultimately dictate the choice between proliferation and differentiation in myogenic cells. Viral and cellular oncogenes, which force myogenic cells through the cell cycle, can inhibit terminal differentiation (7). Large Tags[3] of both polyoma and SV40 as well as E1A of adenovirus are all able to bind the "pocket region" shared by pRb, p107, and p130 (4, 8–11), disrupting their association with E2F(s)/DP(s) transcription factors and thereby preventing withdrawal from the cell cycle in differentiating muscle cells.

Recent studies have used temperature-conditional mutants of Tag (in which the protein is inactive at nonpermissive temperatures) on established myogenic cell lines whose choice between proliferation and differentiation is tightly controlled by extracellular cues such as growth factor concentration. These studies have led to the conclusion that in the presence of active Tag, proliferation continues, and differentiation is inhibited, whereas temperature-dependent inactivation of Tag leads to terminal differentiation (3, 9, 12–14). However, two important points were overlooked in these studies: (*a*) established myogenic cell lines differ from primary myoblasts in that they are already immortal, a crucial difference in this respect, suggesting different intracellular regulatory mechanisms of cell division; and (*b*) even when primary cells were used, Tag function was always assayed in the presence of growth factors, whereas the temperature-dependent inactivation of Tag was carried out in the absence of or with a reduced concentration of growth factors. Thus,

Received 9/6/96; revised 11/14/96; accepted 12/13/96.
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[1] Supported by Telethon Grant A.53, EC Biotech-2 CT-95-0228, and grants from the Fondazione Cenci-Bolognetti (to G. C.), Associazione Italiana per la Ricerca sul Cancro and P. F. Oncologia (to M. M.), and Ministero dell'Università e Ricerca Scientifica e Tecnologica (to G. C. and M. M.).
[2] To whom requests for reprints should be addressed. Phone: 396-49766757; Fax: 396-4462854.

[3] The abbreviations used are: Tag, T antigen; GM, growth medium; DM, differentiation medium; BrdUrd, bromodeoxyuridine; TBS-T, Tris-buffered saline.

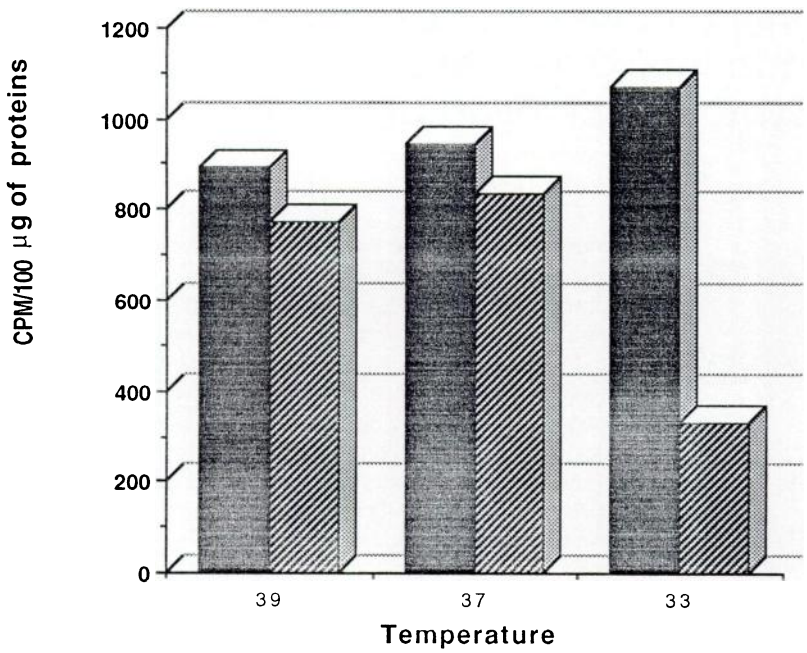


Fig. 1. Thymidine incorporation in murine satellite cells transduced with the tsA58 mutant of SV40 Tag. MSC-tsA58/3 (■) and control cells (▨) grown at three different temperatures in GM for 24 h were incubated for 4 h with 2 µCi/ml [^{3}H]thymidine. The incorporated radioactivity was measured as described in "Materials and Methods." Results represent the average of three independent experiments, each conducted on triplicate samples.

two important variables were modified at the same time to maximize the yes/no response expected from Tag.

Tag has been proposed as an immortalizing agent to increase the yield of primary human myogenic cells to be used in *ex vivo* protocols of gene transfer, and in this context, it is especially important to clarify the relationships between Tag and the growth stimulation to which a primary myogenic cell is exposed to understand the basic interference of this oncoprotein with myogenic proliferation and differentiation.

In this study, we report that, at variance with previous observations, a *ts* mutant of SV40 Tag does not inhibit differentiation in the absence of serum. Moreover, it is not able to reinduce DNA synthesis in myotubes that have completed withdrawal from the cell cycle.

Results

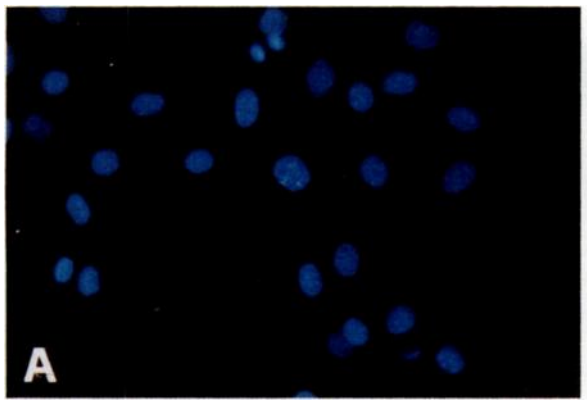
Growth and Differentiation of MSC-TsA58/3 Infected Myogenic Cells. Mouse satellite cells were isolated from the hind limb skeletal muscle of 2-week-old mice, grown in GM, and infected by daily addition of the supernatant of Ψ2-tsA58 packaging cells for 5 consecutive days. The cells were then selected with neomycin, and different isolates were analyzed for their capacity to differentiate into myotubes at 39°C in DM. Five of nine murine clones showed myogenic differentiation under nonpermissive conditions, and one of these, termed MSC-TsA58/3, was chosen for further analysis.

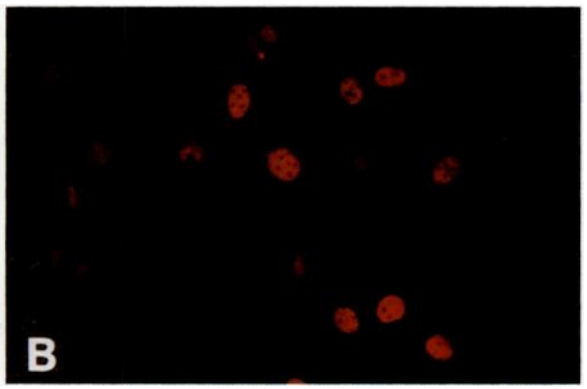
MSC-TsA58/3 cells continue to proliferate for at least 20 passages, corresponding to more than 50 cell divisions, whereas primary satellite cells slow down their growth rate and reach senescence after 5–10 passages, depending upon culture conditions (data not shown). Even at early passages, MSC-TsA58/3 showed a different behavior with respect to uninfected primary satellite cells when grown in GM at 33°C, 37°C, or 39°C (Fig. 1); specifically, thymidine incorporation measured 1 day after passage was similar between MSC-TsA58/3 and control cells at 37°C or 39°C, when the activity of Tag is probably inactivated by the temperature, but it was three times higher in MSC-TsA58/3 at 33°C, thus showing that Tag promotes the growth rate under permissive conditions. It is obvious that low temperature reduces the growth rate in normal cells. When grown at clonal density in GM at different temperatures, MSC-TsA58/3 constantly gave higher numbers of clones and higher numbers of myogenic cells per clone as compared to uninfected controls (Table 1). The fact that even at nonpermissive temperatures, a higher clonogenicity was observed in MSC-TsA58/3 than in control cells suggests that tsA58 may not be fully inactive at 39°C (15) and, under critical clonal conditions, may still confer an advantage to expressing cells. The table also shows that the highest clonogenicity is attained at a permissive temperature. These data are in good agreement with previous reports for both human and murine myogenic cells expressing Tag under a variety of experimental conditions (11–14).

Table 1 Clonal growth of murine satellite cells transduced with tsA58 SV40 Tag

Cell type	Temperature	No. of clones	Average no. of cells/clone
MSC-LT	33°C	65	63 ± 7
	37°C	42	53 ± 4
	39°C	16	33 ± 8
MSC	33°C	4	12 ± 2
	37°C	18	25 ± 4
	39°C	5	10 ± 2

Cells were cloned by limited dilution in GM at the indicated temperature. After 10 days, the number of clones and the number of cells/clone were analyzed in triplicate samples.





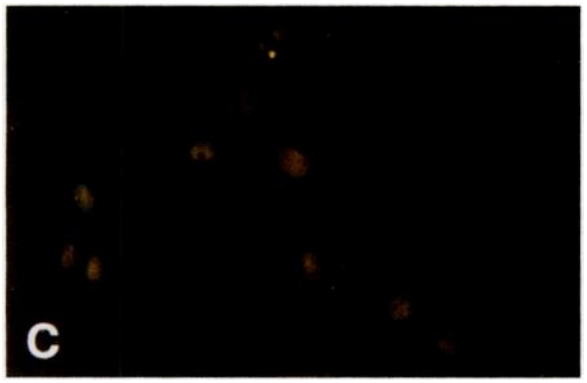
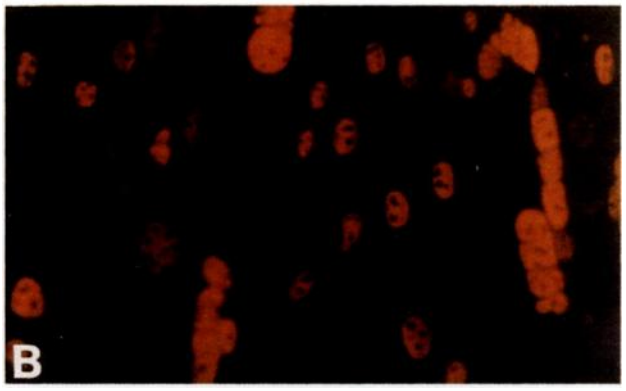


Fig. 2. Double immunofluorescence of a 3-day-old culture of MSC-tsA58/3 grown in GM at 33°C and stained with anti-Tag monoclonal antibody (*B*) and with an anti-MyoD polyclonal antibody (*C*). Virtually all nuclei (revealed by Hoechst staining in *A*) of the culture are stained by both antibodies.



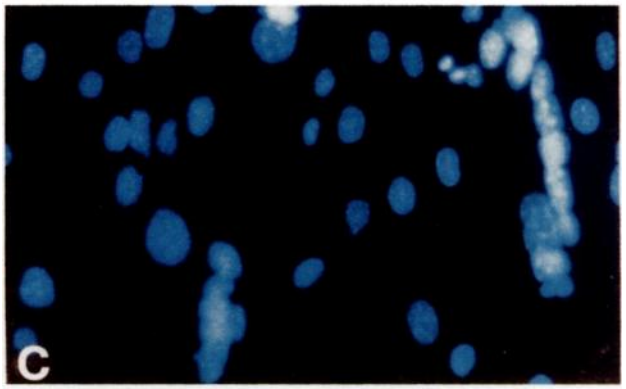


Fig. 3. Double immunofluorescence of MSC-TsA58/3 grown for 10 days at 33°C in DM and stained with a polyclonal antibody against myosin heavy chains (*A*) and with an anti-Tag monoclonal antibody (*B*). Clusters of Tag-positive nuclei are localized within myosin-positive myotubes. Nuclei are stained with Hoechst (*C*).

We next investigated the differentiation potential of MSC-TsA58/3 cells under various conditions. Specifically, we were puzzled by the fact that in all previous studies using conditions that lead to the inactivation of Tag, this was always accomplished by simultaneously reducing the amount of growth factors or serum to which cells were exposed. In this case, differentiation invariably occurs, similar to what occurs in untransduced myogenic cells. We therefore performed experiments aimed at dissociating the effect of Tag from that of serum and analyzed fusion as well as the expression of both Tag and muscle-regulatory and structural proteins. For this purpose, MSC-TsA58/3 cells were exposed to both GM and DM at either 33°C or 39°C for 3 or 7 days and then processed for immunofluorescence. At 33°C in GM, virtually all proliferating cells coexpressed Tag and MyoD in their nuclei (Fig. 2). As expected, multinucleated myotubes appeared after 3 days in DM at 39°C but not in GM at 33°C. Surprisingly, myotubes developed after about 5 days at 33°C in DM, whereas very little fusion was observed at 39°C if the cells were left in GM. Double immunofluorescence analysis with antibodies against Tag and sarcomeric myosin revealed that at 33°C in DM, the presence of an active Tag did not

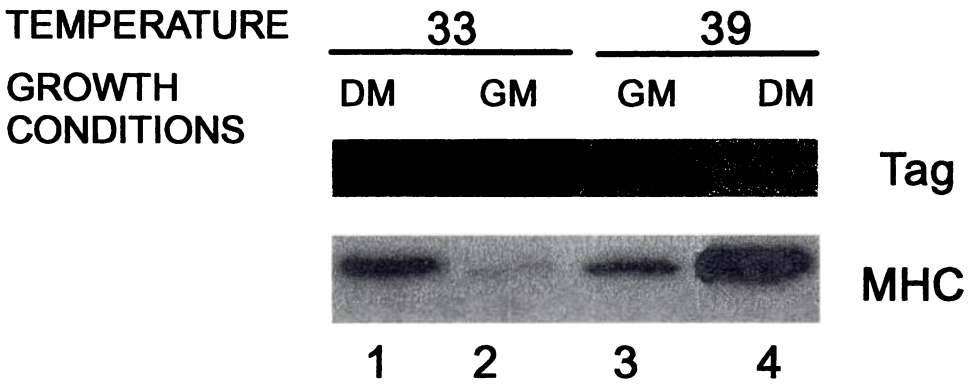


Fig. 4. Expression of Tag and sarcomeric myosin in MSC-TsA58/3 cells. Western blot analysis with Tag and MHC antibodies of protein extracts of MSC-TsA58/3 cells grown at permissive (*Lanes 1* and *2*) and nonpermissive (*Lanes 3* and *4*) temperatures in GM (*Lanes 2* and *3*) and DM (*Lanes 1* and *4*).

prevent fusion into myosin-positive myotubes, most of which readily expressed Tag in all of their nuclei (Fig. 3). Thus, the presence of an active Tag does not block differentiation if the cells are not simultaneously exposed to growth factors.

To quantify the expression of Tag and sarcomeric myosin heavy chains in the experimental conditions described above, extracts of MSC-TsA58/3 were separated by electrophoresis, transferred to nitrocellulose filters, and reacted with antibodies against Tag and MHC. Fig. 4 shows that the level of Tag was similar under all the conditions tested; in contrast, at 33°C very little MHC could be detected in GM, whereas a significant amount was observed in DM. At 39°C, the amount of MHC expressed was higher in cells grown in DM than in cells grown in GM; the presence of MHC in the latter condition may be explained by the high density that cells reach with serum and high temperature, so that differentiation begins, probably because of growth factor depletion from the medium. Thus, it seems that serum dictates the choice between differentiation and proliferation in myogenic cells.

tsA58 Mutant of Tag Binds to pRb. We then investigated the ability of Tag to bind pRb under several conditions of growth and temperature. This experiment was aimed at assessing whether formation of the protein heterodimer pRb-Tag (9–11) would occur during myogenic differentiation of Tag-positive primary myogenic cells. Protein extracts of MSC-TsA58/3 grown at 33°C in GM or DM were immunoprecipitated with a monoclonal antibody for Tag. Immunoprecipitates were separated by SDS-PAGE, electroblotted on nitrocellulose filters, and subsequently revealed with monoclonal antibodies for pRb and Tag. As shown in Fig. 5, the hypophosphorylated form of pRb was prevalently coimmunoprecipitated with Tag in samples from cells that were maintained in DM for 3 days (*Lane 1*). In extracts from cells grown in GM, Tag binds approximately the same amount of both the hypophosphorylated and phosphorylated forms of pRb (*Lane 2*). These results are consistent with the idea that a population of dividing cells expresses both the phosphorylated and hypophosphorylated form of pRb, whereas serum-starved or G_0/G_1 cells prevalently express hypophosphorylated pRb. However, no dramatic differences were observed in the total amount of pRb bound to Tag in cells grown in both GD and DM. Furthermore, the total amount of

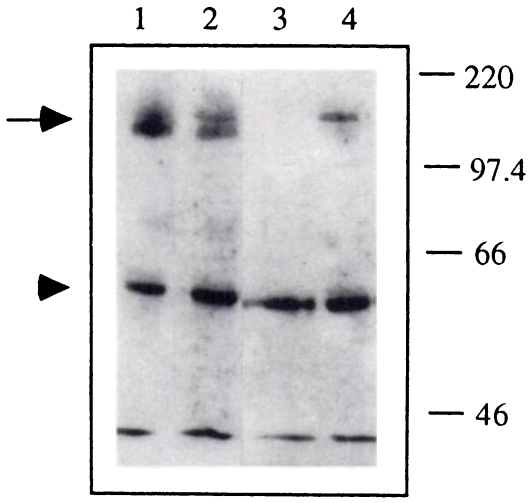
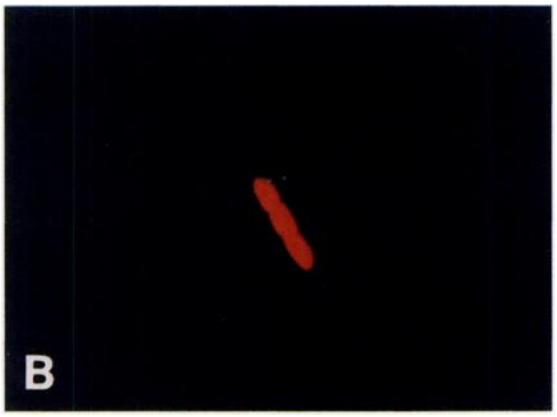


Fig. 5. Binding of Tag to pRb in MSC-TsA58/3 cells. Extracts from MSC-TsA58/3 cells grown at permissive temperature in DM (*Lane 1*) and GM (*Lane 2*), uninfected satellite cells (as negative control; *Lane 3*), and Tag-expressing COS cells (as positive control; *Lane 4*) were immunoprecipitated with anti-Tag antibody. The immunoprecipitated extracts were separated by electrophoresis and subjected to Western blot analysis with anti-pRb antibodies. *Arrow*, a doublet corresponding to the differentially phosphorylated forms of pRb. *Arrowhead*, the migration of immunoglobulin heavy chains.

pRb did not differ significantly between cells grown in DM and in GM (data not shown).

Tag does not prevent differentiation of human myogenic cells. We next investigated whether the results obtained with murine myogenic cells were also relevant to human cells. For this purpose, we electrophoresed human satellite cells with the same ts mutant of Tag into a plasmid configuration under transcriptional control of the SV40 promoter (see "Materials and Methods"), and after 2 days, the cultures were shifted to DM at 33°C. Transfected cells were identified by nuclear expression of Tag, and half of these cells coexpressed myosin heavy chains in their cytoplasm (an example is shown in Fig. 6). Because half of the nontransfected cells (Tag negative) also expressed myosin heavy chains (the remainder being probably nonmyogenic cells), it can be concluded that the majority of human myo-



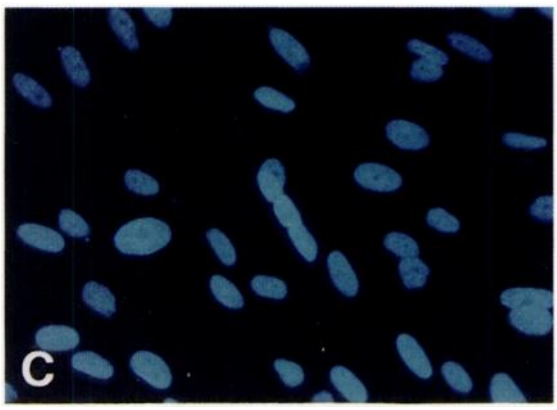
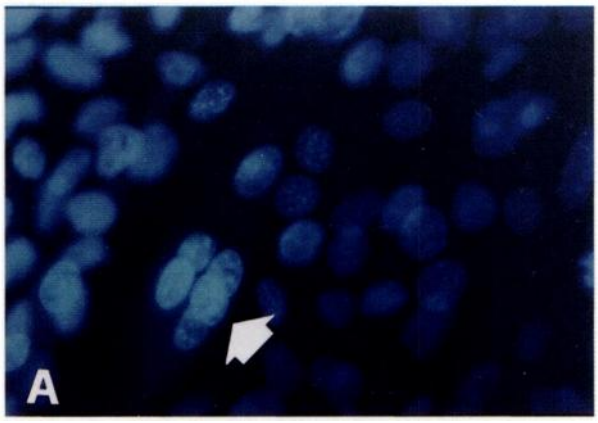


Fig. 6. Effect of Tag on differentiation of human myogenic cells. Human fetal myoblasts were electrophoresed with pAOtsA58 plasmid containing the same ts mutant of Tag of retroviral vector under SV40 promoter. Cells were grown for 3 days in GM at 33°C and subsequently shifted to DM for 4 days at 33°C. The cells were then double-stained with a polyclonal antibody against myosin heavy chains (*A*) and with an anti-Tag monoclonal antibody (*B*). Nuclei are stained with Hoechst (*C*). Note the myosin-positive myotube containing three Tag-positive nuclei.



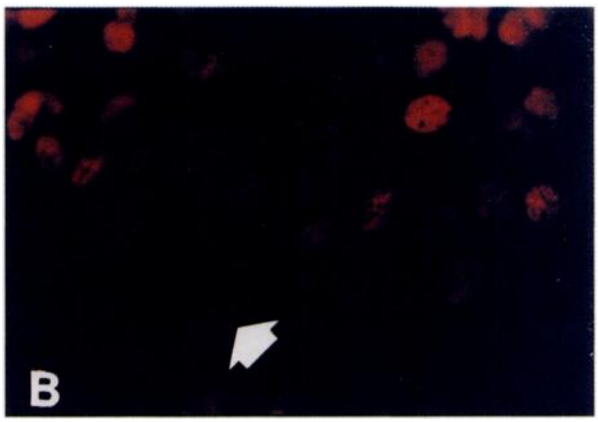


Fig. 7. Effect of serum on DNA synthesis in differentiated myotubes from MSC-TsA58/3 cells. MSC-TsA58/3 cells were differentiated into myotubes at 39°C in DM and then shifted for 24 h at 33°C in GM in the presence of 10 μM BrdUrd. Cells were then fixed and double-stained with an anti-BrdUrd monoclonal antibody (*B*) and with a polyclonal antibody against myosin heavy chains. Nuclei are revealed by Hoechst (*A*). *Arrow*, cluster of BrdUrd-negative localized within a myosin-positive myotube.

genic cells expressing fully active Tag in their nuclei undergo terminal differentiation in the absence of growth factors.

Effect of Tag on DNA Synthesis in Terminally Differentiated Primary Myotubes. If Tag does not prevent terminal differentiation in the absence of serum, then it may be asked whether it is capable of reverting differentiation and forcing the differentiated muscle cell to reenter the cell cycle, as reported previously (3, 12). For this purpose, MSC-TsA58/3 cells were induced to differentiate by shifting the temperature to 39°C in the absence of serum. At various times after myotube formation, cultures were shifted back to 33°C in the presence of serum and pulse-labeled with BrdUrd for 24 or

48 h. In five independent experiments, not a single nucleus of multinucleated myotubes incorporated BrdUrd, whereas most of the mononucleated cells showed a strong fluorescence, indicating the occurrence of DNA synthesis (Fig. 7). Thus, MSC-TsA58/3 cells cannot reenter DNA synthesis once they have differentiated into myotubes. It is still possible that a threshold intracellular concentration of Tag must be reached to titrate and inactivate Rb. Although Western blot analysis had shown levels of Tag roughly comparable to an average level within cells raised at a permissive temperature, we decided to compare different myogenic cells expressing Tag under a variety of regulatory contexts. We therefore used primary myogenic cells from two different immortomouse strains, Vimentin-Tag and H2K-Tag, as well as primary myogenic cells transfected with tsA58 under the control of the metallothionin promoter (13, 14). In all these cases, the exposure of differentiated myotubes to conditions that activate Tag in the presence of serum did not induce nuclei to reenter DNA synthesis (data not shown).

Discussion

The experiments described here were undertaken to evaluate the interference of a temperature-conditional mutant of the SV40 Tag with the control of proliferation and differentiation in primary mammalian myogenic cells. The results obtained show that Tag neither prevents or grossly perturbs myogenic differentiation in the absence of growth stimulation nor maintains proliferation or allows DNA synthesis in differentiated myotubes.

Tag Does Not Prevent Myogenic Differentiation. Tag-expressing primary myogenic cells proliferate in the presence of serum at a higher rate than uninfected controls raised at permissive temperatures. They differentiate as uninfected controls when shifted to nonpermissive temperatures in the absence of serum. This is in agreement with all previous reports (3, 12–14). However, when serum is withdrawn under a permissive temperature when Tag is presumably fully active, differentiation occurs in infected cells at levels comparable to those of uninfected controls raised under the same conditions. Similarly, when infected cells are raised at nonpermissive temperatures but serum is maintained, little differentiation occurs, once again, as in uninfected controls. Thus, in primary mammalian myogenic cells, it is serum concentration and not Tag that dictates the choice between proliferation and differentiation, and this decision cannot be overruled by an active Tag.

In established cell lines such as C2C12, Tag has been shown to prevent fusion into myotubes and expression of certain muscle-specific proteins but not of the muscle-regulatory transcription factors MyoD and myogenin (16). The *in vitro* interaction between MyoD and pRb proteins described by Guy *et al.* (3) strongly suggests that pRb requires MyoD to mediate its inhibitory effect on cell growth. Because Tag is able to bind pRb (11), it may inhibit myogenesis by subtracting pRb and preventing its interaction with MyoD. In agreement with these data, our results show that Tag does not suppress the expression of MyoD, as shown by the coexpression of both antigens in the same nucleus. On the other hand, it was also shown that Tag blocks MyoD and myogenin expression (9, 17). So it seems that Tag-mediated inhibition of muscle differentiation may depend upon at least two alternative mechanisms. However, the fact that in primary murine myogenic cells, Tag and MyoD proteins are coexpressed and Tag binds to pRb suggests the existence of other pRb-independent molecular pathways to myogenic differentiation, probably mediated by other members of pRb family such as p107 and p130 (18).

In human myogenic cells, Tag under the control of the vimentin promoter does not prevent myogenic differentiation (19). Thus, it is conceivable that the effects of Tag on muscle differentiation can differ depending on its regulatory context and on the myogenic cell background. The actual concentration of active Tag within the cell may be important, and we have determined that, at least as protein accumulation detectable by Western blots, there is no appreciable difference between the level of Tag present in several different primary myogenic cells expressing Tag under a variety of regulatory contexts and the level present in isolates of the C2C12 myogenic cell line expressing Tag under similar regulatory contexts.[4] Therefore, the interpretation of previous reports has probably been biased by the use of established cell lines as well by the experimental conditions used. The more likely result of Tag activity is to prolong the duplicative potential of primary cells by allowing them to escape from a first senescence crisis and to divide for a number of additional cycles until a second and irreversible senescence crisis occurs. Thus, Tag does not immortalize myogenic cells but simply expands their proliferative life span, and this effect cannot be seen in established cell lines, which are already immortal.

Tag Does Not Reactivate DNA Synthesis in Differentiated Myotubes. A second point that is raised by the data reported here is the effect of Tag on DNA synthesis in terminally differentiated myotubes. At variance with previous reports (3, 12), we did not observe DNA synthesis in terminally differentiated myotubes; thus, we need to explain the discrepancy with the other data and the currently held opinion. The old data cannot be directly compared to ours because the SV40 virus used in these studies also contained other transforming genes. More recently, reexpression of the ts mutant tsA58 in the presence of serum has been shown to induce cell cycle reentry and S-phase progression in myotubes derived from the fusion of Tag-expressing C2C12 myoblasts (20, 21). As stressed above, C2C12 myoblasts are immortal cells; thus, they have undergone a disruption of the elements controlling life span, a situation clearly different from that of primary myogenic cells, whose life span is tightly controlled and whose cell cycle is, for example, longer than that of C2C12 cells. In the case of myogenic cells derived from Tag-expressing immortomice, reactivation of DNA synthesis has not been investigated or has been found not to occur.[5] However, we cannot exclude the possibility that concentrations of Tag higher than those obtained in our experiments might be required to inactivate proteins important for

[4] G. Salvatori, unpublished observations.
[5] T. Partridge, personal communication.

the maintenance of a differentiated phenotype, such as pRb, and to reinduce DNA synthesis.

In conclusion, our results suggest that reactivation of DNA synthesis, with the known catastrophic consequences for cell cytoarchitecture and life, may only occur in a subset of immortalized, aneuploid, and potentially tumorigenic myogenic cell lines. Thus, although the mechanism by which Tag interferes with myogenesis remains to be fully elucidated, Tag-mediated *in vitro* amplification of myogenic cells from myopathic patients (19, 22) may represent a potentially relevant experimental strategy for *ex vivo* therapy of myopathies. Obviously, additional strategies for irreversible inactivation or excision of Tag from the genome will have to be devised before the cells may be reintroduced into the patient, but it is likely that with the increasing pace of gene manipulation technologies, this will become feasible in the near future.

Materials and Methods

Cell Cultures. Primary myogenic cells from mouse muscle and human biopsies were prepared as described (23). Primary cells and the C2C12 myogenic cell line were grown in GM consisting of DMEM medium (Life Technologies, Inc.) supplemented with 15% FCS (Flow), 0.3 mM β-mercaptoethanol, and 50 μg/ml gentamicin or in DM (*i.e.* DMEM supplemented with 2% horse serum from Flow and 50 μg/ml gentamicin).

Primary murine satellite cells were infected with recombinant retrovirus (24), which expresses the ts-A58 mutant of SV40 Tag under the control of the Moloney leukemia virus long terminal repeat and the Neo-resistance gene essentially as described (23). Briefly, growing cells were cultured with supernatant from Ψ2 packaging cells (24) for 2 days and selected in G418 (0.7 mg/ml) at 33°C in GM. Surviving clones were tested for myogenic differentiation by shifting them to 39°C in DM.

One × 10^7 primary human myogenic cells were electrophoresed with the pAOtsA58 plasmid at 320 V and 960 mF capacitance. Electrophoresed cells were grown for 24 h in GM at 33°C and then shifted to DM for 6 days at 33°C.

When indicated, control and MSC-tsA58/3 cells were plated on 35-mm dishes and grown at three different temperatures in GM for 24 h. Cells were then incubated for 4 h with 2 μCi/ml [^{3}H]thymidine. At the end of the incubation period, cells were washed three times with ice-cold PBS, precipitated with 5% perchloric acid, and solubilized in 2N NaOH. The incorporated radioactivity was measured in a β-counter. Results represent the average of three independent experiments, each conducted on triplicate samples. At the times indicated, cultures were fixed and immunostained with different antibodies or processed for biochemical analysis.

Immunocytochemistry. The following antibodies were used in this study: (*a*) MF20, a monoclonal antibody that recognizes all sarcomeric myosins (25), donated by D. Fischman; (*b*) a rabbit antibody against sarcomeric proteins that was produced in our laboratory (26); (*c*) an anti-MyoD polyclonal antibody donated by S. Alemà (27); (*d*) Pab 419, a monoclonal antibody that reacts with SV40 Tag (11); and (*e*) an anti-BrdUrd monoclonal antibody from Amersham.

Cells were fixed with fresh 4% paraformaldehyde, washed three times in 1% BSA (Sigma) in PBS (BSA/PBS), and incubated overnight at 4°C with different polyclonal and monoclonal antibodies. After the incubation, cells were washed three times in 1% BSA/PBS and incubated with a fluorescein-conjugated goat anti-rabbit immunoglobulin and with a rhodamine-conjugated goat anti-mouse immunoglobulin for 1 h at room temperature (both second antibodies, from Cappel, were used at a 1:30 dilution). After three final washes, culture dishes were mounted in 75% glycerol/PBS (pH 8) and observed under an epifluorescence Zeiss Axiophot microscope.

Immunoprecipitation and Immunoblotting. Cells were lysed with ABC buffer [50 mM Tris-Cl (pH 8), 120 mM NaCl, 0.5% NP40, and 0.1 TIU aprotinin (Sigma)] for 20 min on ice. The lysates were then cleared by centrifugation in a microfuge for 15 min at 12,000 × *g* at 4°C. Aliquots of the supernatants were mixed with 2 volumes of NET buffer [20 mM Tris-Cl (pH 8), 100 mM NaCl, 1 mM EDTA, and 0.5% NP40], and Pab 419 antibody was then added at a 1:50 dilution. The mixture was gently agitated for 2 h at 4°C. Then 50 μl of protein A-Sepharose (Pharmacia) preincubated with rabbit anti-mouse immunoglobulin (Zymed) were added, and incubation was continued for 1 h. Protein A-Sepharose beads were washed five times in NET buffer and finally resuspended in sample buffer, boiled for 5 min, and subjected to SDS-PAGE on 8% gels.

After electrophoretic separation, immunoprecipitates or total protein extracts were transferred to nitrocellulose filters for 2 h at 4°C with 200 mAmp. The nitrocellulose filters were blocked by incubation with TBS-T [Tris-Cl (50 mM; pH 8), NaCL (137 mM), and 0.1% Tween 20 containing 5% dried milk (Carnation)] for 2–14 h and then incubated overnight with various antibodies in TBS-T. The filters were washed five times in TBS-T and incubated for 2 h with horseradish peroxidase-conjugated second antibodies, washed again, and treated with the enhanced chemiluminescence kit from Amersham.

Acknowledgments

We thank Drs. T. Partridge and D. Paulin for the gift of the myogenic cells from the H2K/Tag and Vimentin/Tag immortomice, respectively. We also thank P. Amati, M. Crescenzi, and F. Tatò for helpful discussions and critical reading of the manuscript.

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