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The Inhibition of Cultured Myoblast Differentiation by the Simian Virus 40 Large T Antigen Occurs after Myogenin Expression and Rb Up-Regulation and Is Not Exerted by Transformation-Competent Cytoplasmic Mutants

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We have investigated the mechanism by which the simian virus 40 large T antigen (SVLT) interferes with the differentiation of C2 myoblasts. SVLT mutants, defective either in the Rb binding site, near the N-terminal end, in a region that affects binding to p53, or in the nuclear transport signal, were also employed to determine whether the interference was especially dependent on these functional domains. It was found that wild-type (wt) SVLT strongly inhibited the terminal differentiation of mouse C2 myoblasts, but this arrest occurred only after the synthesis of myogenin, an initial step in biochemical differentiation. Neither the synthesis nor some basic activities of MyoD appeared to be affected by wt SVLT. In these transformants, mitogen depletion elicited an increase in the Rb level comparable to that in normal C2 cells; wt SVLT, however, promoted the phosphorylation of a large part of the induced Rb. Mutations affecting nuclear transport were far more critical for the ability to interfere with myogenic differentiation than were those affecting the transforming potential; cytoplasmic SVLT expression was fully compatible with the terminal differentiation of C2 cells, despite enabling them to grow in semisolid medium, thus showing that the myogenesis-inhibiting property can be dissociated from transforming competence. The remaining SVLT mutants presented different degrees of ability to inhibit differentiation (as shown by the expression of tissue-specific markers in transformants). The inhibiting mutants, including the Rb binding site mutant, were able to promote a higher state of Rb phosphorylation than that observed in either normal cells or cytoplasmic-SVLT transformants.

The simian virus 40 (SV40) oncogene large T antigen (SVLT) is a nuclear phosphoprotein of 708 amino acids and is able by itself to transform a broad range of cell types and species, including precrisis mammalian cells (26, 27, 46). The full transforming competence of SVLT appears to be due to the combined actions of several functions, which depend on the integrity of particular domains of this protein. Defectiveness in any of these functions results in only partial impairment of the SVLT transforming activity (46, 63, 67, 68, 81). The complexing of SVLT to the tumor suppressor Rb is probably the best understood of these functions. The required binding region is between amino acids 102 and 115 of SVLT (17, 18), and binding prevents the normal growth-repressing interactions of Rb, such as Rb-E2F, by a sequestering effect and/or by favoring Rb phosphorylation (10, 12, 60, 70). A similar interaction occurs between SVLT and p107, which shares structural homology with Rb (7, 21, 24, 59) but does not have completely identical functional properties (58, 82). The biochemical and functional aspects of SVLT association with the cell growth suppressor p53 have also been fairly well elucidated. The SVLT binding region for p53, much larger than that for Rb, has been mapped to a bipartite region spanning amino acids 351 through 626 (35, 79). This binding strongly decreases the amount of p53 free to activate the expression of genes, including those of cyclin-dependent kinase regulators, and to promote apoptosis (19, 41, 69). Several studies have shown the critical importance of the region corresponding to the first exon of SVLT (amino acids 1 to 82) for its oncogenic activity (54, 65, 80). The specific tasks performed by this region have not yet been fully clarified, but this region is known to be essential for transactivation by SVLT (80) and is involved in the binding of α-DNA polymerase and probably of cellular protein p300 (20, 46, 54, 76). The transport of SVLT to the nucleus depends on the functionality of the nuclear location signal (NLS), formed by 7 amino acids occupying positions 126 to 132 (34). The SVLT transforming activity is not very sensitive to lesions of the NLS that change this protein's subcellular localization; SVLT cytoplasmic mutants can even fully transform precrisis rat embryo fibroblasts with only somewhat lower efficiencies than that of wild-type (wt) SVLT (38, 67). Numerous studies have dissected the contributions of the functions discussed above to the SVLT transforming potential in fibroblasts; the expression of SVLT mutants in these cells can release them from primary-cell crisis, density inhibition, and/or anchorage dependence (11, 62, 63, 67, 68, 81).

In cells that undergo differentiation, this often occurs with an initial phase in which cells stop dividing, with a subsequent phase in which the synthesis of specific products takes place. In general, with many exceptions, the expression of oncogenes able to interfere with withdrawal from the cell cycle can be expected to hinder the process of differentiation (1, 45, 51).

C2 cells are a line of murine myoblasts which allow the reproduction in culture of the basic events of muscle differentiation (77). In C2 cultures of suitable density, the deprivation of serum growth factors results in the arrest of cell division, the synthesis of tissue-specific proteins, and cell fusion into multinucleated myotubes (1, 16, 52, 73). It is known that this growth arrest involves the function of general down-regulators of proliferation (such as Rb) active in all cell types (13, 17, 56, 61); in myoblasts, however, this negative control has to be

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coordinated with the activation of tissue-specific genes (14, 30, 44, 47, 66). In C2 cells, MyoD is the most upstream acting myogenic factor and is constitutively expressed. It belongs to a family of factors (with homologous basic/helix-loop-helix domains) binding to the E-box-containing promoters of muscle-specific genes; during embryonic development, MyoD shares tasks with Myf-5 and (in a first approximation) their activities can be considered equivalent (16, 52, 57, 72, 73). The expression of myogenin, a more downstream acting, essential myogenic factor, depends on MyoD (or Myf-5) activity and is induced in C2 cells only when they stop dividing (1, 32, 52, 73, 78). Other transcription factors participating in the process of myogenesis include the late acting factor MRF4 and the nonhomologous myogenin transactivator MEF2 (22, 52); it should also be added that the functioning of myogenic factors includes not only cascade-like but also self- and feedback regulation (51, 72, 73).

Oncogenes of different classes have been previously investigated for the ability to interfere with myoblast differentiation. From these results, it appears that myogenesis is almost invariably inhibited. A very frequent case is that of oncogenes whose expression in C2 cells prevents the expression of MyoD, as happens with H-*ras*, v-*mos*, v-*src*, v-*jun*, c-*fos*, and E1A, whereas a few oncogenes affect MyoD function rather than its expression (polyomavirus large T antigen, polyomavirus middle T antigen, and v-*myc*) (reviewed in reference 1). Myoblast transformation by wt SVLT has also been previously investigated, with the inhibition of differentiation observed in mouse cells (31) but not in rat primary muscle cells (33).

The SVLT mutants employed in this work were altered in four domains directly or indirectly relevant to transformation: the site of Rb binding, a region involved in p53 binding, a region close to the N-terminal end, and the NLS. Mutant and wt SVLTs were transfected into C2 cells to determine at which step of myogenesis SVLT interference with C2 differentiation occurred and whether it was particularly dependent on any of the functions listed above. The anchorage dependence of the transformants' growth was examined to see if a correlation between the mutants' transforming potential and their effects on cell differentiation existed. Morphological differentiation and myosin expression were strongly inhibited in clones expressing wt SVLT, but the inhibition occurred downstream of myogenin expression. The increase in the level of Rb, typical of myoblasts induced to differentiate, was not prevented by wt SVLT, but a high proportion of Rb was in a highly phosphorylated form. As for the SVLT mutants, the SVLT function most critical for the inhibition of C2 cell differentiation was found to be transport to the nucleus. Although clones expressing cytoplasmic SVLT could form agar colonies with an efficiency close to that of wt SVLT, their monolayer cultures could also be induced to synthesize tissue-specific proteins and to form large multinucleated myotubes. In contrast, all of the nucleus-localized mutants conserved notable inhibiting capacity on morphological differentiation, including the mutant altered in the Rb binding site.

MATERIALS AND METHODS

Plasmids. The SVLT genes were SV40 genomic DNA delimited by the *Bam*HI and *Kpn*I sites (through the ori region) and cloned in a modified pAT153 vector. Mutants M1, NKT1, and *dl*259-684 have been previously described (67). Mutant *d*10, a construct of Kalderon et al. (34), was the gift of S. H. Cheng. Mutants T1/R and T1/Y were obtained by recombining at a *Pst*I site mutant NKT1 with two mutants (pRLT3 and pYLT3) in which the NLS had been inserted during previous work (28). The remaining mutants were constructed by temporarily subcloning the 5′ and 3′ halves of SVLT in a vector (a recombinant of pUC18 and pACYC184) that rendered two *Ava*II sites of SVLT unique. To construct *d*412A and *d*412H, synthetic double-stranded oligomers replaced the wt SVLT nucleotide sequence between the *Ava*II and the *Nsi*I sites at nucleotides 3538 and 3583 of the SV40 genome, respectively; *dl*412-426 was obtained by directly joining the ends of these two cuts, with the help of an eptamer complementary to the protruding nucleotides. Similarly, synthetic oligomers replaced the *Ava*II-*Stu*I tract (between nucleotides 5118 and 5190) for the construction of *dl*3-15 and *dl*7-9. The nucleotide sequence was verified through the altered segments of these constructs.

Plasmid pSV2hyg, conferring hygromycin B resistance to transfectants (5), was originally obtained from S. Pellegrini. The probes for the mRNAs of myosin heavy chain and α-actin, excised with *Pst*I (8) from plasmids pMHC2.2 (74) and pAM91.200 (48), respectively, and the *Eco*RI fragment of a W. E. Wright construct containing the myogenin cDNA were all kindly donated by A. Felsani (47). The MyoD expression plasmid pEMC11s (16), used to obtain stable C3H10t½ transfectants (47), was originally obtained from H. Weintraub.

Cell cultures, transfections, and transformation assays. The C2 cell line (clone 7) of murine myoblasts (77), originally obtained from M. Buckingham, was cultured in Dulbecco's medium with 20% fetal calf serum, also referred to as growth medium. To induce differentiation, 2×10^5 cells seeded in 10-cm-diameter dishes and allowed to grow for 3 days were then exposed for 2 days to differentiation medium, made of Dulbecco's medium with 2% newborn calf serum. The C3H10t½ line of mouse fibroblasts was cultured in the same way.

Transfections were carried out by the Polybrene method (9), modified by substituting 15% glycerol for dimethyl sulfoxide, with 0.6 μg of pSV2hyg DNA, 6 μg of SVLT plasmid DNA, and 2×10^5 cells on 6-cm-diameter dishes. After 48 h, cells were replated in selective medium containing 200 μg of hygromycin B per ml. Resistant clones were then picked, expanded, and screened by anti-T indirect immunofluorescence. The full names of the clones used in this work were WTSV/5a, M1/2c, *dl*7-9/1, *dl*3-15/1, NKT1/3c, *d*10/1, *d*412A/1, *d*412H/6, *dl*412-426/14, *dl*259-684/4, T1/R-1a, and T1/Y-1d.

Transformation was assayed by measuring the ability of clones to grow in semisolid medium. Cells were resuspended at a concentration of 2×10^4 cells per 6-cm-diameter dish in growth medium containing 0.3% agar, with fetal calf serum lowered to 10%. Colonies of at least 0.1 mm in diameter were counted in the microscope about 4 weeks later.

Immunoreactions and Western blots (immunoblots). The following monoclonal antibodies were employed: anti-T PAb101 (ATTC culture TIB-117) and PAb 419 (Oncogene Science, Uniondale, N.Y.), anti-p53 PAb122 (ATTC culture TIB-116), anti-Rb PMG3-245 (Pharmingen, San Diego, Calif.), anti-myosin heavy chain MF20 (2), and anti-myogenin IF5D (75). The polyclonal anti-MyoD antibody was a rabbit serum, prepared against a MyoD fusion protein, kindly donated by S. Alemà. This serum could be used in radioimmunoprecipitations and indirect immunofluorescence (32) but could not be used in Western blots (because the need to concentrate MyoD by immunoprecipitation could not avoid the presence of immunoglobulin G chains migrating very close to MyoD). Then we obtained a sample of anti-MyoD made in chickens (a kind gift of A. Felsani), which we immobilized on Affi-Gel 15 (Bio-Rad) and employed in the initial immunoconcentration.

For radioimmunoprecipitations, cell cultures on 10-cm-diameter dishes were labeled for the indicated times with 0.15 mCi of [^{35}S]methionine (Tran ^{35}S-label; ICN) in 1.5 ml of methionine-free medium. Cell extracts were prepared by lysis with 1% Triton in 20 mM Tris (pH 8.0)–0.5 M NaCl–0.5 mM EGTA–5 mM ATP–5 mM $MgCl_2$–0.05 mg of leupeptin per ml–1 mM phenylmethylsulfonyl fluoride (PMSF), with a subsequent 5-s sonication. Extracts (1 ml) were cleared by centrifugation at 20,000 × *g* for 20 min and incubated (with agitation in a cold room) for 30 min with nonimmune serum and protein A-Sepharose (Pharmacia). After the pellet was discarded, extracts were incubated for 2 h with the indicated antibodies and then for another hour with protein A-Sepharose. The pellet was washed five times with 0.05% Triton in 150 mM NaCl–10 mM EDTA–50 mM Tris (pH 8)–0.01 mg of leupeptin per ml; samples were eluted by boiling for 90 s in buffer containing 1% sodium dodecyl sulfate (SDS) and 5 M urea and electrophoresed on Laemmli 10% polyacrylamide gels (U.S. Biochemicals). Detection was either by standard autoradiography or by using a PhosphorImager (Molecular Dynamics) and CL5000S Palette system (Polaroid).

Western blots were carried out by immunoprecipitating extracts (prepared as above) with PMG3-245 followed by protein A-Sepharose for Rb detection and with Affi-Gel-immobilized chicken antibody for MyoD detection (see above). Electrophoresis on 6.5 and 10% gels, respectively, and semidry electric transfer of proteins to Hybond-C filters (Amersham) were monitored by using prestained protein markers (Sigma). Each sample represented half of a 10-cm-diameter dish culture. After incubation with the same anti-Rb antibodies or rabbit anti-MyoD serum, filters were then processed for enhanced chemiluminescence detection (Boehringer).

Indirect immunofluorescence with C2 cells was carried out as previously described (28).

Northern (RNA) blots. Northern blots were carried out by electrophoresing 0.02 mg of total cell RNA from each sample, extracted as previously described (8), on 1.2% agarose–10% formaldehyde gels. RNA was vacuum blotted to Hybond N+ filters (Amersham) and probed with specific DNA fragments that had been ^{32}P labeled by random priming. Gels and filters were stained with ethidium bromide to monitor the presence of equal, intact rRNA bands. Hybridization was carried out overnight at 42°C in 50% formamide–5× SSPE (1×

TABLE 1. Structures of the SVLT mutants employed[a]

Transfected oncogene	Mutation(s)	Location	Remark(s) (reference)
WTSV	None	Nucleus	Normal SVLT protein
M1	Asp-102, Glu-105, Arg-107, Gly-109, Ile-111, Leu-114	Nucleus	Rb binding site erased; able to transform only established cells (67)
*dl*7-9	Ser-6 and amino acids 7–9 deleted	Nucleus	New construct
*dl*3-15	Amino acids 3–15 deleted	Nucleus	New construct
NKT1	Thr-128, Ser-130, Ser-131, Arg-132, Ser-133	Cytoplasm	NLS erased; able to transform precrisis rat embryo fibroblasts (67)
*d*10	Thr-128	Cytoplasm	NLS inactive; transforms precrisis rodent cells (34)
*dl*259-684	Amino acids 259–684 deleted	Nucleus	Transforms established cells and immortalizes primary rat embryo fibroblasts (67)
*d*412A	Glu-418, Asn-419, Ser-420	Nucleus	New construct; p53 binding defective
*d*412H	Glu-418, Asn-419, Ser-420, Asp-415, Gln-425	Nucleus	New construct; p53 binding defective
*dl*412-426	Amino acids 412–426 deleted	Nucleus	New construct; p53 binding defective
T1/R	Triple NLS inserted after amino acid 682 of mutant NKT1	Nucleus	New construct; mutant NKT1 relocated to the nucleus
T1/Y	Similar to T1/R, but the added NLSs are defective	Cytoplasm	New construct; isogenic to T1/R but remaining cytoplasmic

[a] All constructs, except *dl*3-15 and *dl*7-9, encode a normal small t antigen.

SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.5]) 5× Denhardt solution, 0.5% SDS, 0.1 mg of herring sperm DNA per ml–10^6 cpm (10^8 cpm/μg) of labeled probe per ml. Washing was done three times in 0.1× SSPE–0.1% SDS for 20 min at 65°C. Radioactivity was detected with a PhosphorImager as described above.

RESULTS

Mutant structure and expression. Table 1 shows the basic properties of the SVLT mutants employed in this work. Some of them have been previously described and characterized (67) (M1, with mutations in the Rb binding site; NKT1, with mutations in the NLS; *dl*259-684, with the residues indicated in its name deleted) as indicated in last column of this table. The list also includes an NLS mutant (*d*10) constructed by Kalderon et al. (34), the most frequently used cytoplasmic mutant of SVLT. After each mutant was transfected into C2 cells, cell clones were isolated and screened by anti-T immunofluorescence and two positive clones of each mutant were chosen for further analysis.

Figure 1 illustrates the expression of SVLT mutants in clones labeled with radioactive methionine, extracted, and immunoprecipitated with anti-T antibody. Each mutant showed a clear SVLT band, with an intensity somewhat higher or lower than that of the wt (but not significant functionally since it did not correlate with the differentiation phenomena) and with the minor migration differences expected of mutants with small deletions or insertions. This figure also shows anti-p53 immunoprecipitates from clones expressing three mutants, not previously described, defective for p53 binding (*dl*412-426, *d*412A, and *d*412H). p53 was not detectably coimmunoprecipitated by the deletion mutant *dl*412-426, whereas the coimmunoprecipitation of p53 by substitution mutants *d*412A and *d*412H was

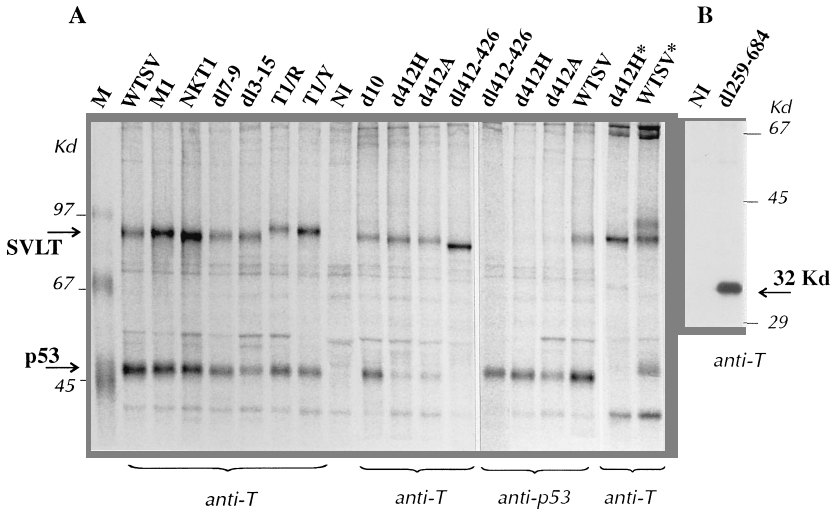

FIG. 1. Polyacrylamide gel electrophoresis analysis of anti-T immunoprecipitates from transformant extracts. C2 cell clones transformed with the indicated SVLT genes were labeled for 3 h with [^{35}S]methionine and extracted as detailed in Materials and Methods. Similar extracts were also prepared from two lines of rat embryo fibroblasts, one transformed by wt SVLT and the other transformed by mutant *d*412H. SVLT was immunoprecipitated with PAb 419, and p53 was immunoprecipitated with PAb 122, as indicated. Electrophoresis was carried out on 10% polyacrylamide–SDS gels, and radioactivity was detected with a PhosphorImager and recorded on film with a Palette apparatus. Lane M, molecular weight markers. The two designations with asterisks are for samples from rat embryo fibroblast transformants; all other samples are from C2 transformants. In panel B the sample migration was suitably shortened relative to that in panel A. Kd, kilodalton.

notably decreased. The affinity of p53 for SVLT appeared to be stronger in C2 cells than in rat fibroblasts, since p53 complexes with *d*412H were not detectable in the latter cells (last two lanes of Fig. 1A).

Distinct inhibition of C2 cell differentiation by SVLT mutants. Figure 2 shows microphotographs of the transformants displaying their homogeneity and T-antigen localization (upper panel) and their ability to differentiate morphologically (lower panel). These morphological data visualized the disorder introduced by this oncogene in the overall myogenic process, which would normally end with all cells fused into large myotubes. The pictures show that in most transformants, myotube formation was strongly inhibited, although a certain number of cells that escaped the block were present in all cases, including cells inhibited by wt SVLT, which showed some rare bi- and trinucleated elements (Fig. 2 [WTSV]). This behavior persisted after recloning. Clones expressing the SVLT mutants exhibited different abilities to differentiate, but the most dramatic difference was between the clones expressing cytoplasmic mutants and those expressing the remaining mutants localized in the nucleus. The former (NKT1 and *d*10), as shown by pictures taken at a lower magnification, formed long, thick myotubes, essentially similar to those (not shown) of untransformed cells. In contrast, none of the nuclear mutants formed more than abortive syncytia, ranging from oligonucleated myosin-expressing elements (*dl*7-9, *d*412A, and *dl*259-684) to longer, thin, abnormally fused structures (*dl*3-15, M1, *d*412H, and *dl*412-426). Syncytia were often irregularly distributed in cultures, increasing in quantity with prolonged time in differentiation medium but never forming normal myotubes. The surprising fact was that none of the defects carried by nuclear mutants were specifically apt to suppress the block of differentiation; to different extents, all of these mutants caused the derangement of morphological differentiation.

The primary importance of the mutants' nuclear localization for the inhibition of differentiation was confirmed by the following experiment. The cytoplasmic mutant NKT1 was altered by inserting three NLSs near its C terminus, and a parallel construct containing nonfunctional NLSs (Lys-128 replaced by Thr) was made. Clones transformed by these two mutants were isolated and tested for the ability to differentiate. The clone expressing the renuclearized mutant (T1/R) was markedly inhibited, whereas the one expressing the cytoplasmic cognate (T1/Y) was not (Fig. 2).

The experiment shown in Figure 3A was carried out to determine whether SVLT synthesis selectively decreased in clones transformed by the cytoplasmic mutants (relative to the nuclear mutants) when differentiation was induced. Duplicate confluent cultures of the cytoplasmic *d*10 and NKT1 transformants and of the nuclear M1 transformant were either kept in growth medium or exposed to differentiation medium for 2 days; then cultures were labeled and extracted. Analysis of anti-T immunoprecipitates showed that the medium change did not cause a particular decrease in SVLT synthesis for the two cytoplasmic-SVLT clones (NKT1 and *d*10). Figure 3B shows that in cells transformed either by a partially inhibiting mutant, such as *dl*412-426, or by a noninhibiting mutant, such as cytoplasmic *d*10, the synthesis of myosin conserved a normal proportion of light chains to heavy chains. (To display the light chains from *dl*412-426 cells, this sample was fivefold more concentrated than the other.) Figure 3C shows that (i) the rare oligonucleated elements detectable in wt SVLT transformants were not due to an inability to express SVLT and that (ii) cytoplasmic SVLT was present in perfectly differentiated myotubes (in this case, double staining was avoided to prevent the ambiguity caused by the spillover of colocalized signals).

The expression of two muscle-specific genes, encoding the myosin heavy chain and α-actin, was examined by Northern hybridization with the nine most representative transformants (Fig. 4). For the myosin heavy chain, the highest induction was shown by cytoplasmic SVLT transformants NKT1 and T1/Y, whereas WTSV and *dl*259-684 were totally negative. Substantial induction was shown by *dl*3-15, and somewhat less induction was shown by *dl*412-426 and *d*412H (Fig. 4A). Such clones also showed some ability to differentiate morphologically, frequently forming oligonucleated structures. In the case of α-actin (Fig. 4B), the transcriptional increase upon induction was more general; it involved WTSV (at a minimal level) and M1 (at a higher level) as well as the clones already seen to be induced for myosin. The fact that the expression of α-actin, unlike that of myosin, partially escaped even wt SVLT inhibition is an indication that genes activated at approximately the same stage of differentiation have differences in their dependence on cell factors with which SVLT can interfere.

Transforming and myogenesis-inhibiting capacities can be dissociated. Each clone whose ability or inability to differentiate was illustrated above was tested for anchorage-independent growth in semisolid medium. The results in Table 2 and Fig. 5 show that the plating efficiencies of clones transformed by SVLT mutants in soft agar varied considerably. The highest values, between 10 and 15%, were shown by clones expressing wt SVLT, mutants with altered NLSs (NKT1, *d*10, T1/R, and T1/Y), and the mutant lacking the entire C-terminal region (*dl*259-684); the lowest value, 0.5%, was shown by clones expressing the two p53 binding-defective mutants (*d*412H and *dl*412-426); intermediate values of 2 to 5% were shown by clones expressing the remaining mutants (Table 2). Figure 5 depicts the colonies formed by some representative clones; the sizes and aspects of colonies from the transformants able to differentiate (NKT1 and *d*10) were similar to those from WTSV. The plating efficiencies of cells expressing nuclear mutants may suggest a direct correlation between the ability of mutants to inhibit differentiation and the ability to release cells from anchorage dependence. However, the highly transformed phenotypes of clones NKT1, *d*10, and T1/Y clearly indicated that the inhibition of differentiation and transforming competence were separable functional properties of this oncogene.

Mutants *dl*412-426 and *d*412H, the first with a minor deletion and the second with five substitutions in the p53 binding domain, were very weak transformers and moderate inhibitors of differentiation. This contrasted with the fact that for the deletion mutant *dl*259-684 (lacking a much larger region that encompasses this domain) both transforming and differentiation-inhibiting abilities were notably higher. Some possible explanations are mentioned in Discussion.

Expression of myogenic factors and Rb in C2 transformants. Clones transformed by SVLT and its mutants were analyzed for the expression of myogenin, a tissue-specific factor which has a unique role in myogenesis and is not expressed in growing C2 cells. Cultures were induced to differentiate, labeled with radioactive methionine, and lysed. Figure 6A shows electrophoretograms of immunoprecipitates from lysates. All of the samples from induced cultures, with the exception of *dl*259-684, displayed the presence of myogenin (the upper band was confirmed to be the hyperphosphorylated form by its relative decrease upon phosphatase treatment [Fig. 6B]). Cells transformed by wt SVLT or mutants either blocking or allowing differentiation showed no striking difference from untransformed C2 cells, except for slightly higher proportions of the hyperphosphorylated band in the WTSV, M1, and *d*412A samples. In the *dl*259-684 sample, a minor amount of myogenin became visible only after the gel was overexposed (result not

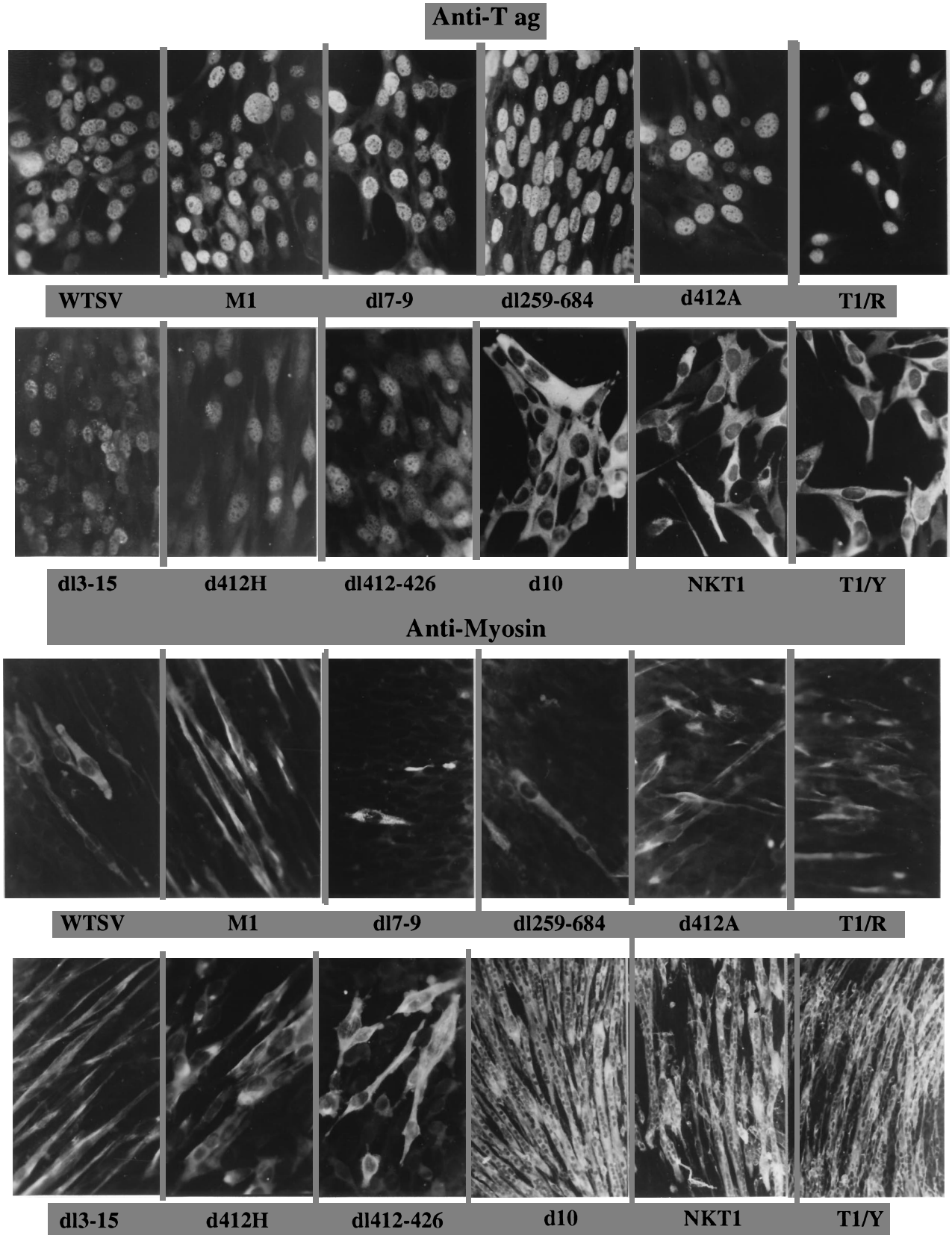

FIG. 2. Immunofluorescent staining of C2 myoblasts transformed by SVLT mutants. (Upper panel) Anti-T antigen (Ag) staining of the indicated clones carried out on growing cultures; (lower panel) anti-myosin heavy-chain staining carried out on the same clones cultured for 2 days in differentiation medium. Magnification, ×400 for all pictures, except the last three of the lower panel for which the magnification was ×160. Note that the fields (magnification, ×400) in the lower panel were chosen to show the presence of multinucleated elements or abortive myotubes in all samples, overrepresenting the frequency of these structures in the most inhibited samples.

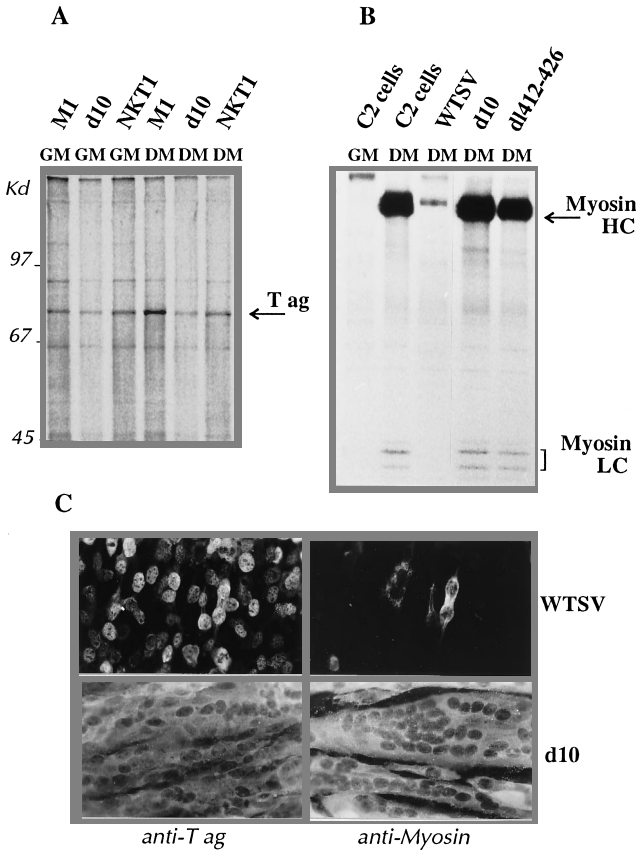

FIG. 3. (A) SVLT synthesis in differentiation medium (DM) compared with that in growth medium (GM). Duplicate cultures of two transformants able to differentiate (*d*10 and NKT1) and of one transformant inhibited to differentiate (M1) were either exposed for 2 days to DM or kept in GM. Then cultures were labeled for 3 h with [^{35}S]methionine, and anti-T immunoprecipitates from lysates were electrophoresed. T ag, T antigen; Kd, kilodaltons. (B) Myosin light-chain (LC) synthesis in a transformant with partially inhibited differentiation. Coimmunoprecipitation of myosin heavy chain (HC) and myosin LC from an extract of partially inhibited transformant *dl*412-426 was compared with those from extracts of normally differentiating C2 cells and transformant *d*10. The *dl*412-426 sample was five times more concentrated than the others to display the myosin LCs. Extracts of C2 cells (in GM) and of WTSV (in DM) served as negative controls of myosin absence. Cell labeling was done as described for panel A. The antibody was anti-myosin HC MF20, and electrophoresis was carried out on a 6.5 to 12% polyacrylamide gel. (C) SVLT presence in both abortive and regular syncytia. Immunostaining of WTSV cells in DM was carried out by double labeling cells with hamster anti-T and anti-myosin HC MF20 and subsequently with fluorescein isothiocyanate- and tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies, respectively. Immunostaining of myotubes by transformant *d*10 with either anti-T ag or anti-myosin HC antibodies was carried out on parallel cultures (to avoid the spillover by colocalized signals). Magnification, ×276.

shown); presumably, myogenin was nearly absent because MyoD was repressed, as shown below. The Northern blots in Fig. 6C also show myogenin mRNA induction in the most representative transformants.

Figure 7A shows the synthesis of MyoD, as detected by electrophoresis of labeled immunoprecipitates. C3H10t½-MyoD cells, expressing a transfected MyoD cDNA, and parental C3H10t½ cells (not expressing MyoD) were analyzed together with the C2 transformants to identify MyoD unambiguously; C3H10t½-MyoD cells displayed a band missing in the parental C3H10t½ cells. The MyoD band was somewhat weaker but still detectable in all of the C2 clones, except *dl*259-684 (which also did not express myogenin). To confirm that MyoD was normally expressed in wt SVLT transformants, untransformed C2 cells and wt and cytoplasmic-SVLT transformants were compared by Western blot, with a chicken anti-MyoD antibody for immunoprecipitation and a rabbit antibody for detection. The results (Fig. 7B) indicated that MyoD was present at similar levels in untransformed C2 cells and wt and cytoplasmic-SVLT transformants. Detection of the expected two bands of MyoD (hyper- and hypophosphorylated) by Western blot but not by radioimmunoprecipitation (Fig. 7A) could be due either to insufficient resolution of the radioactive bands or to different levels of phosphorylation in the newly synthesized molecules (labeled for 110 min). The anti-MyoD immunostaining of wt and cytoplasmic-SVLT transformants were essentially identical (Fig. 7C).

It has previously been shown that the Rb level increases in several types of cells undergoing differentiation (14, 61, 64, 66) and that Rb is involved in the process of myogenesis (8, 30, 44, 47). It was thus interesting to establish whether C2 cells transformed either by the cytoplasmic mutant NKT1 (which are allowed to differentiate) or by wt SVLT (which are not) presented this kind of Rb induction. It was equally important to detect the level of Rb phosphorylation in these and other transformants with different degrees of myogenic inhibition. Figure 8 shows the results of analysis (by Western blotting) of the Rb extracted from these cells. The Rb from both WTSV and NKT1 cells exposed to the differentiation medium displayed a very evident increase over the Rb from parallel cultures kept in growth medium; the increase was similar to that in untransformed C2 cells. In the WTSV sample, however, the highly phosphorylated form (the upper band) represented about half of the augmented Rb. In contrast, normal C2 and NKT1 cells showed only the lower band. As for other transformants in differentiation medium, *dl*259-684 cells showed the presence of both Rb forms at barely detectable levels, whereas M1, *d*412A, and *dl*412-426 showed proportions of the highly phosphorylated form that were midway between those of normal C2 and WTSV cells. From these data, it was concluded that wt SVLT does not block the induction of Rb that occurs in C2 cells upon mitogen depletion; however, it seems to be able to control a system of kinases that phosphorylate a good portion of the induced Rb. The degree of Rb phosphorylation in mutant SVLT transformants roughly paralleled the degree of the differentiation block; it was absent in NKT1 and lower than that of wt SVLT in the others, with an overall low Rb level in *dl*259-684, presumably because of the need for MyoD expression to induce Rb (47).

DISCUSSION

Inhibition of C2 myoblast differentiation by wt SVLT. The indication emerging from our results was that SVLT strongly decreased the morphological differentiation of C2 myoblasts; however, analysis of the expression of muscle-specific genes showed that SVLT was a less drastic inhibitor of myogenic differentiation than many other oncogenes, since it blocked neither the expression of MyoD nor some of its main functions. One remarkable aspect of the inhibition of myogenesis by wt SVLT was that it occurs at a relatively late step, after the synthesis of two main muscle-specific transcription factors, MyoD and myogenin. Cell level examination of transcription factor MEF2 by Western blotting showed an increase for wt SVLT transformants as much as for normal C2 cells, roughly paralleling the increase in myogenin (66a). The following oncogenes have previously been studied for their interference with the myogenic differentiation of rodent myoblasts: H-*ras* (29, 37, 40), adenovirus E1A (6, 8, 23, 71), c-*fos* (40, 42), v-*jun*

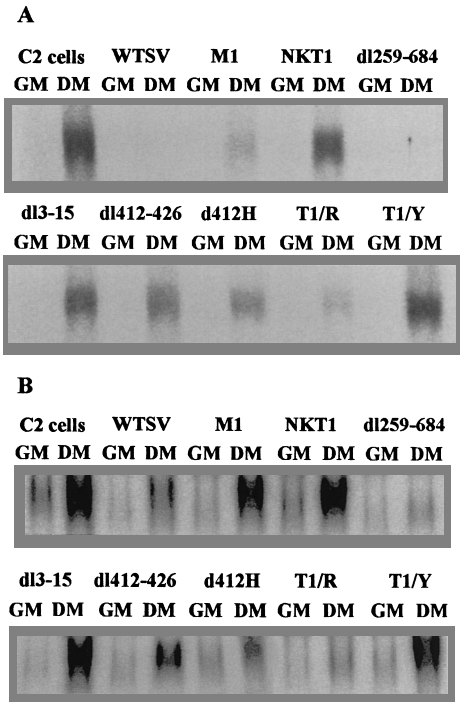

FIG. 4. Northern blot analysis of myosin heavy-chain (A) and α-actin (B) expression. Total RNAs (0.02 mg each) extracted from the indicated cells, either kept in growth medium (GM) or exposed to differentiation medium (DM) were electrophoresed, blotted, and probed with ^{32}P-labeled DNA fragments, as specified in Materials and Methods. Equal loading of RNAs was assessed by ethidium bromide staining of rRNA. Hybridized radioactivity was detected with a PhosphorImager.

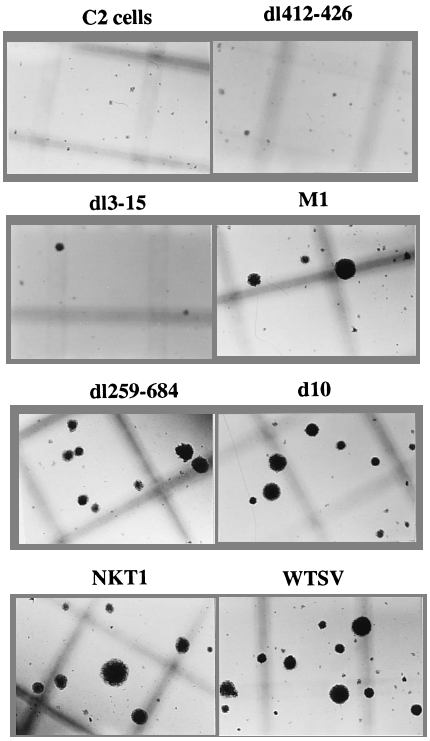

FIG. 5. Colonies on soft agar formed by various transformants. The indicated cells (2×10^4), resuspended in agar-containing medium, were plated on 6-cm-diameter dishes as detailed in Materials and Methods and photographed 20 days later. The squares in the background have sides of 2 mm. Colonies formed by the two clones able to differentiate (*d*10 and NKT1) were essentially similar in size and aspect to those formed by wt SVLT (WTSV).

(3, 42), v-*src* (1, 25), v-*mos* (4), v-*myc* (15), polyomavirus large T antigen (43, 44), and polyomavirus middle T antigen (1). In all of these transformants, except those of polyomavirus large T antigen and middle T antigen, the block of morphological differentiation was accompanied by the inhibition of MyoD expression (only partial in the case of v-*myc* [15]). A very modest decrease in MyoD mRNA, accompanied by a considerable decrease in myogenin mRNA, was found in C2ts11 cells upon the induction of SVLT (31), whereas SVLT allowed morphological differentiation in rat satellite myoblasts (33).

TABLE 2. Plating efficiencies of transformants in soft agar[a]

Transformant	Plating efficiency (%)
WTSV	15.2
*d*10	15.0
*dl*259-684	12.5
T1/R	10.6
T1/Y	10.2
NKT1	9.9
*d*412A	5.1
M1	3.7
*dl*7-9	3.2
*dl*3-15	2.1
*d*412H	0.5
*dl*412-426	0.5
Untransformed C2 cells	<0.01

[a] The percentages of cells forming colonies after 2×10^4 cells were resuspended in growth medium containing 0.3% agar and 10% serum and plated on 6-cm-diameter dishes. About 4 weeks later, colonies of greater than 0.1 mm in diameter were scored from duplicate samples and averaged.

We found that MyoD synthesis was active in wt SVLT transformants and that the exposure of these cells to differentiation medium promoted an increase in the Rb level comparable to that shown by untransformed C2 cells. This second finding was consistent with the recent demonstration that MyoD activates the Rb1 promoter, a function that is required for Rb induction and does not involve E box binding (47). Thus, in SVLT transformants, at least two important MyoD functions, the induction of Rb and of myogenin, are not blocked. (The level and migration of p107 appeared to be essentially unchanged in both wt SVLT transformants and normal cells in differentiation medium [data not shown].) An arrest of C2 cell differentiation without MyoD down-regulation was recently observed with the E1A mutant *pm*563; this mutant inhibits MyoD transactivation of muscle-specific genes but leaves Rb induction unaffected (8, 47). Under differentiating conditions, an augmented Rb level has been previously described for polyomavirus large T antigen transformants (44). Our results allow us to conclude that the initial steps of myogenesis are compatible with the expression of the wt SV40 oncogene (whose normal functionality was shown by the growth of the same myoblasts in agar). Two tissue-specific structural genes, the myosin heavy chain and α-actin, were repressed in wt SVLT transformants (the first was repressed completely, and the second was repressed with minor "leakiness"); this placed the point of effective SVLT interference in C2 cell differentiation between the expression of myogenin and that of myosin.

Inhibition of C2 differentiation by SVLT mutants. SVLT mutants differed in the ability both to transform C2 cells and to inhibit myogenic differentiation. Predictably, the effects on

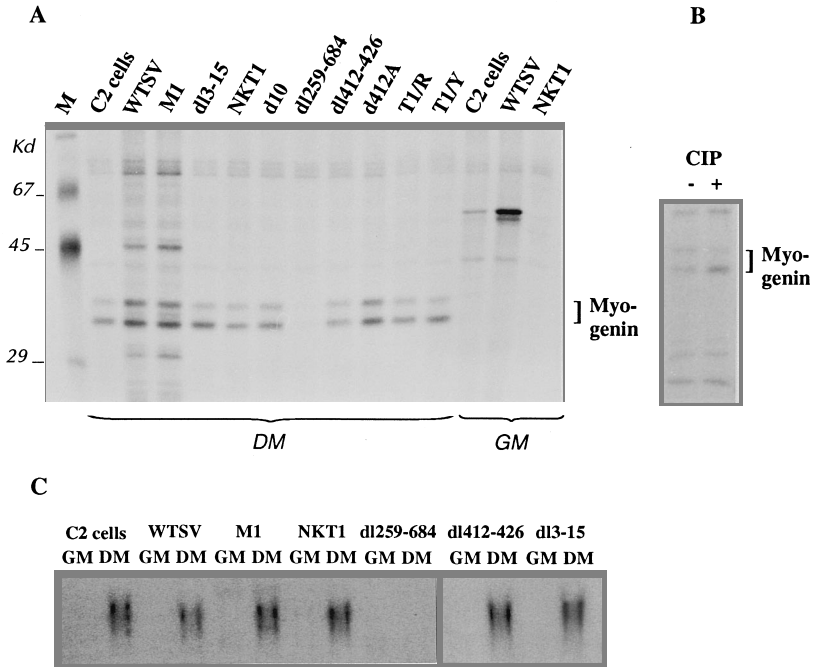

FIG. 6. Myogenin expression in transformed myoblasts. (A) The indicated cultures were induced to differentiate in differentiation medium (DM), except for three cultures which were kept in growth medium (GM) to serve as controls. All cells were labeled for 2.5 h with [^{35}S]methionine, extracted, and analyzed by immunoprecipitation with monoclonal antibody IF5D; immunoprecipitates were then electrophoresed. (B) An aliquot of an immunoprecipitate was incubated for 30 min with (+) and without (−) 280 U of calf intestine phosphatase (CIP; Boehringer) per ml to verify the relative increase in the faster-migrating myogenin band upon dephosphorylation. (C) Northern blots of myogenin mRNAs. Total RNAs (0.02 mg each) extracted from the indicated cells, either kept in growth medium (GM) or exposed to differentiation medium (DM), were electrophoresed, blotted, and probed with ^{32}P-labeled DNA. Equal loading of RNAs was monitored by ethidium bromide staining of rRNA. Hybridized radioactivity was detected with a PhosphorImager. Kd, kilodaltons.

myogenesis were more evident for morphological differentiation than for the expression of single genes (such as the myosin heavy chain and α-actin). The former is a complex process, requiring the coordinate activation of an entire set of muscle-specific genes, whereas the latter is likely to be more variably affected by oncogene interference. The expression of α-actin appeared to be less sensitive than that of myosin to the inhibitory effects of most mutants and the wt.

The most remarkable finding concerning the SVLT mutants was that subcellular localization of the oncoprotein was more important for the inhibition of myogenic differentiation than was the integrity of some of the functions involved in transformation. Interference by cytoplasmic-SVLT mutants in the myogenic differentiation of C2 cells was so scarce that even full morphological differentiation was allowed; transformants could form large multinucleated myotubes that were not observed with the least-inhibited transformants of nuclear mutants. The compatibility between cytoplasmic-SVLT expression and C2 cell differentiation indicates that (i) the oncoprotein must be directly present in the nucleus to interfere with differentiation and that (ii) the transforming potential and the differentiation-inhibiting potential can be neatly dissociated, since the cytoplasmic mutants conserved a transforming competence close to that of wt SVLT. A similar dissociation has been recently reported for a c-Myc mutant (39).

Among the nuclear mutants, a lower-level ability to release cells from anchorage-dependent growth roughly coincided with a lower-level ability to inhibit differentiation. It should be noted, however, that the transforming activity could be almost totally lost in mutants conserving nonnegligible differentiation-inhibiting power (*d*412H and *dl*412-426), in agreement with the conclusion that the two properties are separable.

Mutant *dl*3-15 had low-level transforming and myogenesis-inhibiting activities, as expected for mutations in this region which impair the SVLT transactivating ability (80). Transactivation thus appears to be necessary but not sufficient for the SVLT block of myogenesis, since cytoplasmic mutants conserve transactivating competence (27). The *dl*3-15 mutation also altered the N terminus of small t; however, the small t relevance to the differentiation block cannot be great, since we examined the properties of SVLT small t-minus transformants and found that such cells were as differentiation inhibited as wt SVLT transformants (66a).

The mutation in the Rb binding site (mutant M1) unexpectedly brought about only a relatively minor decrease in the ability of the oncoprotein to inhibit myogenesis and to transform C2 cells. The induction of anchorage-independent growth in C2 cells was not severely impaired, as had been observed for NIH 3T3 cells (67). The proportion of hyperphosphorylated Rb under differentiating conditions was lower for M1 than for wt SVLT transformants; however, it was not lower than those for transformants of other mutants. These data suggest that the conservation by SVLT mutants of the competence to promote Rb phosphorylation, regardless of direct physical association between the two molecules, is of primary importance for interference in C2 myoblast differentiation. The induction of Rb-phosphorylating enzymes can be promoted by wt SV40 (30, 50, 53, 70) as well as by Rb binding site mutants (49, 53). E1A domain 2 mutants cannot bind Rb but conserve the competence to promote cdc2 expression and Rb-phosphorylating activity at wt levels (70).

Mutant *dl*259-684 behaved differently from the other mutants. Despite its large deletion, it showed high-level transforming and myogenesis-inhibiting abilities, unlike those of

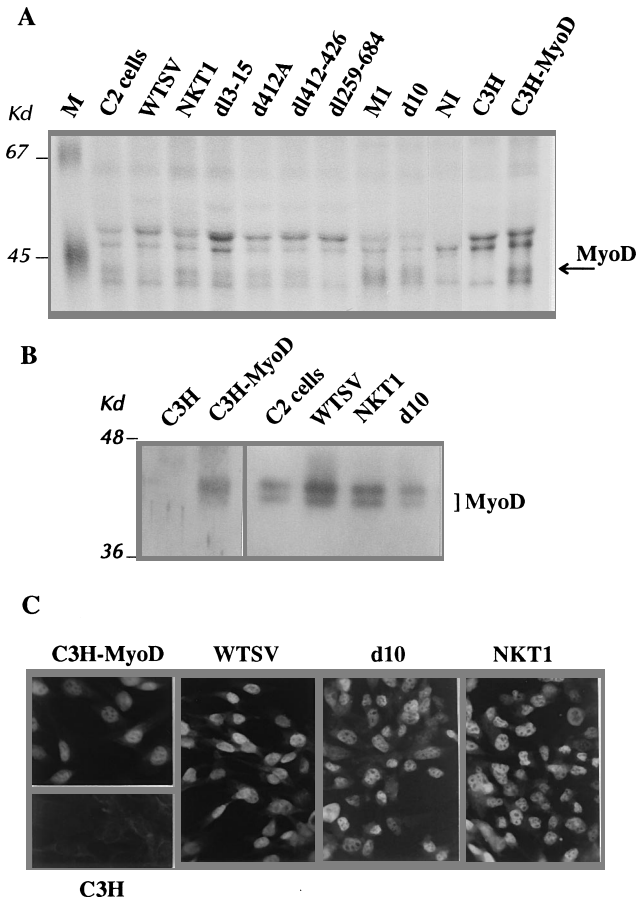

FIG. 7. MyoD synthesis in transformed myoblasts. Subconfluent cultures of the indicated cells in growth medium were labeled for 110 min with [^{35}S]methionine, extracted, and analyzed by immunoprecipitation with anti-MyoD rabbit serum, as detailed in Materials and Methods. Positive and negative controls were represented by extracts from C3H10t$^{1/2-}$ cells stably transfected with MyoD cDNA (C3H10t½-MyoD) and from their parental cells. (A) Electrophoretogram of labeled immunoprecipitates. (B) Western blot analysis of MyoD from wt SVLT (WTSV) and cytoplasmic-SVLT transformants. Unlabeled immunoprecipitates, prepared with anti-MyoD made in chickens (whose immunoglobulin Y heavy chain is about 68 kDa), were electrophoresed, blotted, and incubated with the anti-MyoD rabbit serum used in panel A. Bands were visualized by enhanced chemiluminescence. (C) Indirect immunofluorescence staining with rabbit anti-MyoD serum of the transformants examined in panel B.

less severely altered mutants of the C-terminal half *dl*412-426 and *d*412H. This particular effectiveness was not totally surprising in view of the known transforming potential of truncated SVLTs; for instance, REF52 cells are not transformed by mutants of the p53 binding region (81) but are transformed by an SVLT N-terminal fragment (62). The most important difference concerning *dl*259-684, however, was that it also inhibited myogenin and MyoD expression (unlike wt SVLT and other mutants). A tentative hypothesis to explain the behavior of *dl*259-684 is that the effects of large deletions in the SVLT C-terminal part are not exclusively negative; for instance, some interactions might be carried out more efficiently by the N-terminal part of SVLT alone. Thus, the preferred interactions with factors important for the myogenic block may differ between wt SVLT and *dl*259-684 because the former physically interacts with a larger number of regulatory molecules (mostly related to other tasks, such as viral replication) than does the latter. It is worth noting that in the case of E1A, myoblast differentiation is arrested because MyoD expression is repressed, but mutant *pm*563 reveals a second block at a step following MyoD expression (8).

A balance of antagonistic programs. wt SVLT stopped the differentiation process after the synthesis of myogenin, which is a step in differentiation, with tighter inhibition of myosin expression and looser inhibition of α-actin expression. All of the inhibited transformants contained variable amounts of myosin-expressing cells, either mono- or polynucleated; although this principally depended on the type of mutant, in all cases cells that fused and/or expressed myosin were unevenly distributed as if minor local changes, perhaps due to more ordered intercellular contacts, sufficed to overcome inhibition. This did not reflect clonal heterogeneity, as it also occurred after recloning. Taken together, these data are suggestive of a nearly balanced antagonism between two programs, one elicited by density inhibition and mitogen depletion and leading to differentiation and the other promoted by SVLT and leading to cell proliferation. This is well reflected in the behavior of Rb. Exposing wt SVLT transformants to the differentiation medium had two effects, a strong increase in the Rb level and a high proportion of the hyperphosphorylated form of Rb. The first effect also occurs in normal C2 cells and has to be attributed to the normal process of differentiation, induced by mitogen depletion and cell contact and mediated by MyoD (47). The second effect occurs only in cells expressing SVLT and should be attributed to the known SVLT effect of promoting Rb phosphorylation in many cell types (30, 53, 70). Any mutant (M1 included) conserving myogenesis-inhibiting ability shared at least in part the property of phosphorylating a sizable portion of the augmented Rb. It is conceivable then that by sustaining high levels of Rb phosphorylation, SVLT tends to keep cells cycling and antagonizes the stabilization of basic/helix-loop-helix proteins on E boxes, which appears to be due to hypophosphorylated Rb (30, 58). Furthermore, it was recently reported that during myoblast differentiation, the expression of cyclin D1 is down-regulated, the expression of cyclin D3 is up-regulated, and both events are prevented by the basic fibroblast growth factor (55). We have observed that wt SVLT transformants in differentiation medium maintain normal levels of cdk2 and cdk4, do not prevent the down-regulation of

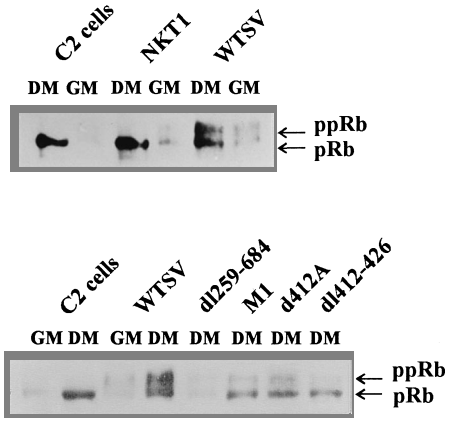

FIG. 8. Rb levels and phosphorylation in transformants able or unable to differentiate. Cultures of transformants and normal C2 cells were exposed to differentiation medium (DM) for 2 days, while some parallel cultures were kept in growth medium (GM), as indicated. Rb was immunoprecipitated from cell lysates with PMG3-245, and samples (each equivalent to half of a 10-cm-diameter dish culture) were electrophoresed and examined by Western blotting, as detailed in Materials and Methods. The two strips are from two separate experiments. pRb and ppRb, the low-level and high-level phosphorylated forms of Rb, respectively.

cyclin D1, but cannot induce cyclin D3 at the normal level (66a). Given the special dual role of cyclin D3 (36, 55), this might be a way through which an advantage for cdk complex phosphorylation of Rb is produced. Lastly, the up-regulation of Rb in wt SVLT transformants in the absence of subsequent differentiation is further indication that the Rb increase does not merely help to maintain myogenic differentiation but is involved in starting such a process (8, 30, 44, 66).

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