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# Separation of Simian Virus 40 Large-T-Antigen-Transforming and Origin-Binding Functions from the Ability To Block Differentiation

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**Wild-type simian virus 40 large T antigen is very effective at blocking adipocyte differentiation in 3T3-F442A cells as assayed by triglyceride accumulation, induction of glycerophosphate dehydrogenase activity, and expression of mRNAs for glycerophosphate dehydrogenase, the adipocyte serine protease adipsin, and the putative lipid-binding protein adipocyte P2. Point mutants defective for either origin-specific DNA binding or transformation blocked differentiation as completely as wild type.**

A number of oncogenes and proto-oncogenes (for example, v-*myc*, c-*myc*, v-*erbA*, and those coding for simian virus 40 [SV40] and polyoma large T antigens) have been shown to interfere with differentiation (5, 6, 9, 13, 19, 22, 24, 36), as well as to establish primary cells in culture and enhance transformation of primary cells when coexpressed with other genes (12, 15, 16, 26, 29, 31, 32, 34, 37–39, 41, 42). Little is known, however, about the mechanism(s) underlying these activities and whether these activities are mechanistically related.

We are using preadipocytes derived from 3T3 cells (17, 18) to investigate the relationship between oncogene effects on differentiation and growth regulation. These cells are well suited for such studies because the early events of differentiation occur rapidly after cultures reach confluency (43), a variety of physiological markers may be used to monitor adipocyte differentiation (17, 44), several mRNAs induced specifically during adipocyte differentiation have been cloned (1, 4, 35, 43) and certain aspects of the regulation of these genes have been explored (1a, 4, 8, 10, 11, 11a, 21, 30), and adipocyte differentiation is coupled with growth regulation (40), thus permitting analysis of oncogene effects on growth regulation and regulated differentiation in the same cells. In this work we demonstrate that the SV40 T-antigen-mediated block of adipocyte differentiation is not dependent upon the T-antigen functions of origin-specific DNA binding and induction of transformation (focus formation, loss of anchorage, and density-dependent growth regulation).

**Retrovirus vectors for wild-type or point mutant SV40 T antigen.** Wild-type or point mutant SV40 T antigen was expressed in 3T3-F442A (F442A) preadipocytes (18) by using the PZIPNeoSVX1 (SVX) retroviral vector (3). The wild-type early region of SV40 (*Hin*dIII-*Bam*HI; nucleotides 5171 to 2533) was inserted into the *Bam*HI cloning site of SVX (Fig. 1), after subcloning through a polylinker, to generate a vector plasmid for expression of wild-type SV40 T antigen. Point mutations from SV40 plasmids K1, d10, and U24 (23, 33) were introduced into the SVX plasmid containing the wild-type SV40 early region by exchanging the wild-type *Bst*XI-*Bam*HI fragment (Fig. 1) for the fragment containing the point mutation (see Table 1 for description of mutants and their phenotypes). Stocks of replication-defective, ecotropic retrovirus coding for wild-type or mutant T antigen were generated using *psi* 2 cells (28).

**Expression of T antigen in F442A preadipocytes.** Wild-type or mutant T antigen was expressed efficiently in F442A cells upon infection with recombinant retroviruses. T-antigen-positive populations were established by pooling a minimum of 300 G418-resistant colonies (6) to normalize for clone-to-clone variation in differentiation not due to T-antigen expression.

Essentially every cell in the population was expressing T antigen, as indicated by indirect immunofluorescence assays (Fig. 2) using rabbit anti-SV40-t serum (2). The wild type and K1 and U24 mutants are largely localized in the nucleus, whereas the d10 mutant is largely in the cytoplasm, as expected.

Expression of full-length wild-type or point mutant SV40 T antigen in F442A cells was confirmed by polyacrylamide gel electrophoresis (25) of immunoprecipitates (using pAB419; 20; Fig. 3). All three mutants coprecipitate cellular p53. Slightly less of the K1 and U24 mutant proteins is expressed relative to the wild-type and d10 proteins. Immunoprecipitates were prepared from cell extracts (in 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris [pH 8], 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing equal acid-precipitable counts. Figure 3 shows immunoprecipitates from cells labeled metabolically with [$^{35}$S]methionine (100 μCi/ml, 1,110 Ci/mmol; New England Nuclear Corp.) for 4 h. Relative intensities of large T bands were the same for samples labeled for 24 h (not shown), indicating that the 4-h labeling represents a true indication of the amount of large T in the population.

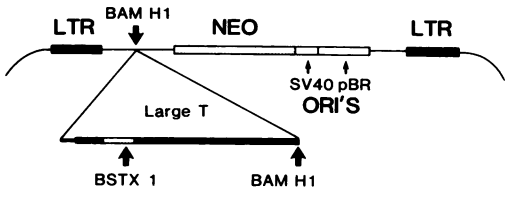


FIG. 1. Construction of retrovirus vectors for wild-type and point mutant SV40 large T antigen.

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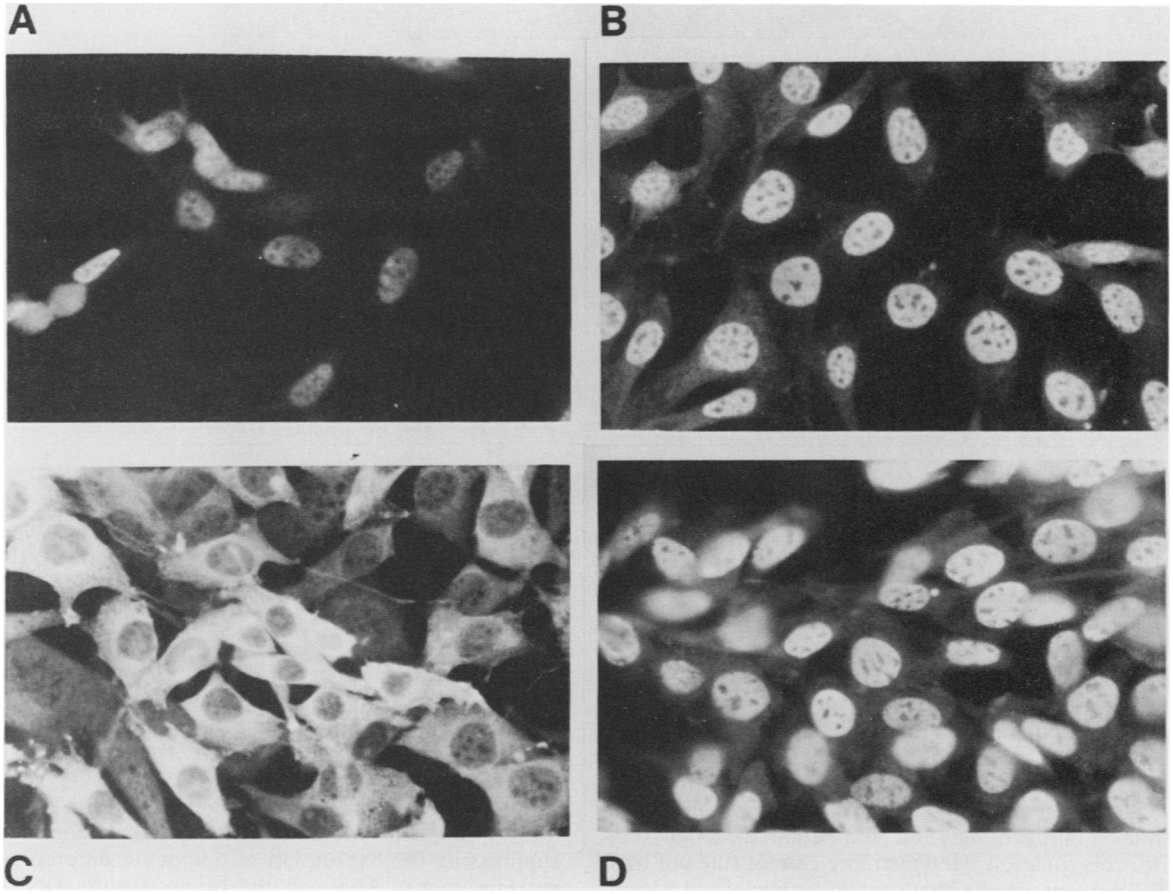


FIG. 2. Expression of wild-type and mutant SV40 large T antigens in F442A cells after retrovirus vector infections. Indirect immunofluorescence of cells expressing T antigen of (A) the wild type or of mutants (B) K1, (C) d10, or (D) U24.

**SV40 T-antigen block of adipocyte differentiation.** Differentiation of F442A cells was blocked in cells expressing either wild-type or mutant T antigen. Cells expressing either wild-type or mutant T antigen did not accumulate triglyceride, as assayed by oil red O staining (17), to the extent observed in uninfected F442A populations or populations infected with a control retrovirus vector not coding for T antigen (Fig. 4). Cells expressing the d10 cytoplasmic mutant appeared to differentiate to a greater extent than cells expressing wild type or the K1 or U24 T-antigen mutants, although differentiation was substantially less than that observed in control populations. The glycerophosphate dehydrogenase (GPD) levels (Table 1) reflect closely the degree of oil red O staining observed in Fig. 4.

These results clearly show that the transformation-defective (K1) and DNA origin binding-defective (U24) mutants block differentiation as completely as does the wild type.

TABLE 1. Differentiation-dependent enzyme induction by F442A cells expressing wild-type and mutant SV40 T antigen

| Large T antigen expressed | Mutation | Phenotype in fibroblasts[a] | Enzyme induction (U of GPD/mg) |
|---|---|---|---|
| None | | | 2,246, 1,851[b] |
| Wild type | None | Wild type | 120 |
| K1 | Glu-107 = Lys | No foci | 21 |
| d10 | Lys-128 = Thr | Cytoplasmic | 210 |
| U24 | Ala-149 = Val | No DNA binding | 54 |

[a] Mutants originally characterized in rat fibroblasts (23, 33).

[b] GPD activity of 2,246 U/mg was obtained from uninfected F442A cells; the activity of 1,851 was obtained from F442A cells infected with retrovirus vector expressing only the drug resistance marker. GPD assays were as previously described (46).

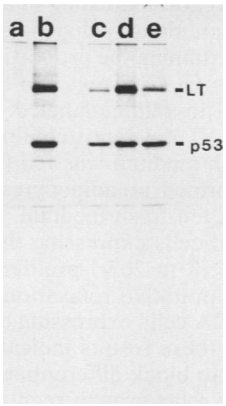


FIG. 3. Fluorography of [$^{35}$S]methionine-labeled immunoprecipitates from F442A cells resolved on 10% polyacrylamide gels. Lane a, F442A cells not infected with retrovirus vectors; lanes b through e, cells infected with retrovirus vectors for SV40 large T antigen of (b) the wild type, (c) K1 mutant, (d) d10 mutant, and (e) U24 mutant.

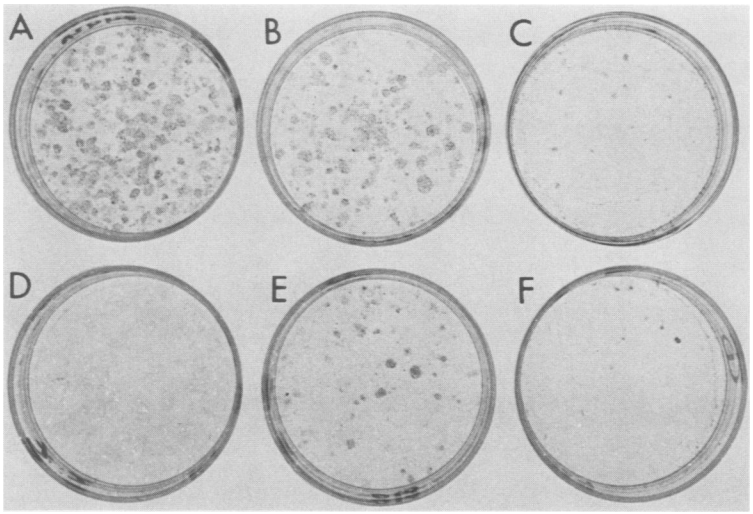


FIG. 4. Block of adipocyte differentiation by wild-type and mutant SV40 large T antigens. Seven-days-postconfluence monolayers of cells grown in Dulbecco modified Eagle medium plus 10% fetal calf serum and 5 μg of insulin per ml and stained with oil red O. (A) Uninfected F442A cells. (B through F) F442A cells infected with (B) retrovirus not coding for T antigen, (C) wild type, (D) K1 mutant, (E) d10 mutant, or (F) U24 mutant.

However, the intermediate result obtained with the d10 cytoplasmic mutant leaves the role of T-antigen cellular localization in blocking differentiation unresolved at this time. The fact that the d10 mutant is expressed at higher levels than, for example, the K1 mutant (see Fig. 3), but may be less effective at blocking differentiation, suggests that cytoplasmic T antigen may be competent although less efficient for this function. However, we cannot rule out that some d10 T antigen may be localized in the nucleus at a level sufficient to block differentiation. Therefore, we cannot rule out the possibility that nuclear localization may be absolutely required.

Although the F442A cells expressing the K1 mutant are blocked for differentiation, they are not transformed to anchorage or density independence. This is consistent with the original characterization of this mutant as defective for focus formation in rat fibroblasts (23). F442A cells expressing either wild-type, d10, or U24 T antigen form colonies in 0.3% agar after 2 weeks (Table 2), whereas preadipocytes expressing no T antigen, or the transformation-defective K1 T-antigen mutant, do not, indicating that they are anchorage dependent. In addition, density-dependent growth regulation was assayed by determining the proportion of the population that progresses from $G_0/G_1$ arrest into S phase, using autoradiography of [$^3$H]thymidine-labeled nuclei. All of the F442A populations, 5 days postconfluency, were largely arrested before fresh medium was fed (Table 2). However, 100% of the transformed preadipocytes (d10 and U24) entered S phase when fed fresh medium, whereas cultures of the control cells and cells expressing the K1 mutant exhibited a more limited (8 to 20%) proliferative response. Although a subtle, quantitative relaxation of the proliferative response in the F442A cells expressing the K1 T antigen has not been ruled out, these results indicate that the ability of the SV40 T antigen to block differentiation is not a function of its ability to fully relax growth regulation.

Several T-antigen functions are not addressed by the mutants included in this study. Such functions include the establishment or immortalization function, the ability to bind and stabilize cellular p53, and T-antigen-associated ATPase activity. The mutants included in this work all immortalize primary mouse fibroblasts (E. Paucha, manuscript in preparation), and all bind p53 (see Fig. 3). The ATPase activity of these mutants has not as yet been assayed, but the domain generally associated with this activity is not altered, so we expect that they retain this activity.

**Suppression of differentiation-dependent gene expression by SV40 T antigen.** SV40 T antigen may block differentiation by suppressing the expression of adipocyte differentiation-dependent genes. Although the origin-specific DNA-binding activity of T antigen, lacking in the U24 mutant, is not required for blocking adipocyte differentiation, this result does not rule out the possibility that T antigen may modulate differentiation-specific gene expression in *trans* by binding cellular DNA or by less direct mechanisms. Three differentiation-dependent adipocyte genes are suppressed in F442A cells producing SV40 T antigen (Fig. 5). RNA was prepared (7) from F442A cells expressing no T antigen or either wild-type T antigen or the transformation-defective K1 mutant. Northern (RNA) blots of RNA resolved on formaldehyde-agarose gels (27) were hybridized to probes (43) for GPD, adipsin, aP2, and actin mRNA. Probes were labeled using the random primer method (14). Differentiation-dependent mRNAs present in adipocytes were essentially absent in parallel cultures of F442A cells expressing wild-type T antigen or the transformation-defective K1 mutant.

TABLE 2. Growth regulation by F442A cells expressing SV40 T-antigen mutants

| Large T antigen expressed[a] | Soft-agar growth[b,c] | % [$^3$H]thymidine-labeled nuclei after 24 h in[c]: | |
|---|---|---|---|
| | | Spent medium | Fresh medium |
| None | − | 4 | 8 |
| Wild type | + | ND | ND |
| K1 | − | 1 | 20 |
| d10 | + | 6 | 100 |
| U24 | + | 21 | 100 |

[a] Mutants described in Table 1.
[b] Two weeks after plating.
[c] All cultures were grown in Dulbecco modified Eagle medium plus 10% fetal calf serum and 5 μg of insulin per ml. ND, Not done.

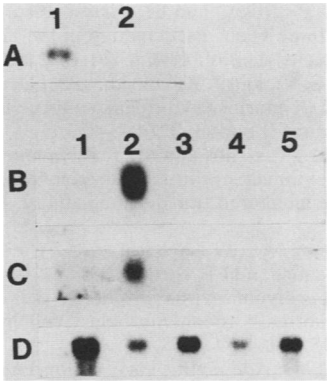


FIG. 5. Suppression of differentiation-dependent adipocyte gene expression by SV40 T antigen. (A) RNA from uninfected (lane 1) or wild-type T retrovirus-infected (lane 2) F442A (grown to 6 days postconfluency in Dulbecco modified Eagle medium plus fetal calf serum and insulin) was blotted onto nitrocellulose (10 μg per lane) and probed with a GPD-specific probe (43). (B through D) RNA (20 μg per lane) from (lane 1) preadipocytes (F442A grown to near confluency in medium containing 10% calf serum); (lane 2) adipocytes (F442A cultured 6 days postconfluency in medium containing 10% fetal calf serum plus insulin); (lane 3) F442A infected with wild-type T retrovirus cultured as described for adipocytes (lane 2); (lane 4) F442A infected with K1 mutant T retrovirus cultured as described for adipocytes (lane 2); and (lane 5) same as lane 4 except cultured in medium containing 10% calf serum. Panels B through D represent the same blot sequentially stripped and hybridized to probes for (B) aP2, (C) adipsin, and (D) actin (43).

Actin mRNA (Fig. 5D) was expressed in differentiated and undifferentiated cells as expected (43). It is interesting that cells expressing the K1 mutant (Fig. 5D, lanes 4 and 5), like control cells (43; Fig. 5D, lanes 1 and 2), down-regulated actin expression under differentiation culture conditions, even though other differentiation-related events were suppressed. This suggests that T antigen may block differentiation by suppressing certain, but not all, differentiation-related events. Differentiation-dependent events, regulated by distinct regulatory pathways (44, 45), may not be equally sensitive to T-antigen suppression.

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