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Use of Transgenic Mice Reveals Cell-Specific Transformation by a Simian Virus 40 T-Antigen Amino-Terminal Mutant

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We have used the multifunctional transforming protein, simian virus 40 T antigen, as a probe to study the mechanisms of cell growth regulation in the intact organism. T antigen appears to perturb cell growth, at least in part, by stably interacting with specific cellular proteins that function to maintain normal cell growth properties. Experiments in cultured cells indicate that at least three distinct regions of simian virus 40 T antigen have roles in transformation. Two regions correlate with the binding of known cellular proteins, p53, pRB, and p107. A third activity, located near the amino terminus, has been defined genetically but not biochemically. By targeting expression of wild-type and mutant forms of T antigen to distinct cell types in transgenic mice, we have begun to systematically determine which activities play a role in tumorigenesis of each cell type. In this study, we sought to determine the role of the amino-terminal transformation function with such an analysis of the T-antigen mutant *dl*1135. This protein, which lacks amino acids 17 to 27, retains the p53-, pRB-, and p107-binding activities yet fails to transform cells in culture. To direct expression in transgenic mice, we used the lymphotropic papovavirus transcriptional signals that are specific for B and T lymphocytes and the choroid plexus epithelium of the brain. We show here that although defective in cell culture, *dl*1135 specifically induced the development of thymic lymphomas in the mouse. Expression of the protein was routinely observed in B- and T-lymphoid cells, although B-cell abnormalities were not observed. Choroid plexus tumors were observed only infrequently; however, *dl*1135 was not consistently expressed in this tissue. Within a given transgenic line, the penetrance of T-cell tumorigenesis was 100% but appeared to require secondary events, as judged from the clonal nature of the tumors. These experiments suggest that the amino-terminal region of T antigen has a role in the transformation of certain cell types (such as fibroblasts in culture and B lymphocytes) but is dispensable for the transformation of T lymphocytes.

Simian virus 40 (SV40) large T antigen efficiently disrupts normal cell growth control in a variety of cell types in culture and in test animals (reviewed in references 17 and 29). The diversity of cells susceptible to the effects of T antigen has been demonstrated most dramatically in transgenic mice, in which the growth properties of over 20 cell types have been altered by this protein (reviewed in references 1 and 22). Current hypotheses suggest that T antigen induces neoplastic transformation by direct interaction with cellular proteins that normally function to regulate cell proliferation (18, 20, 27). Genetic analyses of T antigen in cultured cells indicate that the protein possesses at least three transforming functions (10, 35, 42, 52). One of these functions corresponds to the ability of T antigen to bind both pRB (9) and p107 (12, 15) and requires amino acids 105 to 114. A second activity correlates with the p53-binding function of T antigen (amino acids 271 to 517) (38, 39, 46, 52). Since both pRB and p53 can inhibit the proliferation of cells in culture and are often lost or altered in certain human tumors (reviewed in references 28 and 34), T antigen may induce transformation simply by interfering with their normal functions. The function of p107 is not yet known, although it shares several properties with pRB, including binding to the same region of T antigen (12, 15), interaction with some of the same cellular proteins (reviewed in reference 37), and homology with a region referred to as the pocket (16).

A third transforming function of T antigen, which appears to reside near the amino terminus, is distinct from pRB/p107 and p53 binding, but its cellular target(s) is not yet known. Evidence for the existence of this activity includes the following observations: first, elimination of pRB/p107 and p53-binding reduces but does not abolish transformation of certain cell lines (31, 42, 45); second, mutants with deletions in the first exon (amino acids 1 to 81) that retain the ability to bind pRB/p107 and p53 are defective for transformation in culture (6, 15, 35, 36, 39, 52); and third, a stable fusion protein containing the pRB-binding region of polyomavirus large T antigen and the p53-binding domain located within the carboxyl region of SV40 T antigen does not transform cells in culture (32).

The role in transformation of each T-antigen activity, either alone or in concert with one another, is not completely understood. In cultured cell assays, some cell types (such as REF52) seem to require all three activities for transformation, while others (such as C3H10T1/2) do not (35, 41, 42, 47, 52). Thus, the type of cell and/or its state of differentiation may be important parameters in determining the response to oncogene action. Since T antigen encodes multiple transformation-related activities, the subset of T-antigen functions needed and the corresponding target proteins could differ among cell types. Alternatively, since transformation assays have largely been carried out in cultured cells, the observed differences in requirements for transformation may reflect different growth conditions and/or the accumulation of ab-

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normal genetic changes. We have developed transgenic mouse models that explore the role of T-antigen transforming activities in defined cell types. By introducing T-antigen mutants into transgenic mice under the control of a cell-specific promoter, we have begun to test the hypothesis that different subfunctions of T antigen are required for transformation depending on the cell type or developmental stage.

Previously, we analyzed wild-type and mutant SV40 T antigens for their ability to induce tumors in three cell types with distinct normal growth properties. One cell type, the choroid plexus epithelium of the brain, normally differentiates during fetal life and stops dividing within 2 weeks after birth (33). The other cell populations, the B- and T-lymphoid cells, undergo proliferation and differentiation throughout the life of the animal (24). We targeted transgene expression to all three cell types by using the lymphotropic papovavirus (LPV) transcriptional signals (3). Under LPV control, wild-type SV40 T antigen induces choroid plexus tumors in the majority of founder mice within 3 to 4 weeks of life and does not require secondary genetic changes (5). A direct correlation exists between T-antigen expression, morphological transformation, and proliferation of the choroid plexus epithelial cells, resulting in uniform expansion of the entire tissue (5). Lymphoproliferative disorders of both B and T cells are also evident in these mice (3, 4).

Our previous examination of T-antigen mutants defective in pRB/p107 and/or p53 binding in this transgenic model system indicates that the relevant targets of T antigen could differ depending on the cell type (4). While loss of the C-terminal four-fifths of the protein (including the p53-binding region) eliminates the appearance of lymphoma, the remaining N-terminal fragment of 121 amino acids still induces the formation of choroid plexus tumors. A mutant with a single amino acid change that simultaneously disrupts pRB/p107 and p53 binding is unable to induce both lymphoma and choroid plexus tumors (4). Interestingly, this protein induces hyperplasia in the choroid plexus, an observation which supports the notion that a T-antigen activity other than pRB/p107 and p53 binding can alter cell growth control.

In this report, we explore the role of the amino-terminal activity in this transgenic model system by using a deletion mutant of SV40 T antigen that is defective for transformation in cultured cells. This mutant protein, *dl*1135, contains a deletion of amino acids 17 to 27 yet retains the ability to bind pRB, p107, and p53 (15, 35, 39). This approach uncovered the first known susceptible target cell for this mutant protein, in that *dl*1135 specifically induced the development of T-cell lymphomas.

MATERIALS AND METHODS

Construction of pLST1135. Plasmid LST1135 was constructed from two intermediate plasmids, p1135T/t and pLST1135T/t. To create p1135T/t, a plasmid containing the complete SV40 mutant viral genome in pBR322, pdl1135 (39), was digested with *Stu*I and *Bam*HI. The 2.4-kb T-antigen coding fragment was isolated and inserted into the *Sma*I site of pGEM3. An *Asp* 718-to-*Eco*RI fragment, containing the LPV control region (3), was inserted into the same sites of p1135T/t. pLST1135T was generated by substituting the 1,590-bp *Eco*NI fragment of pLST1135T/t for the corresponding fragment of pSVT (5). Since this fragment of pSVT harbors a deletion of bp 4854 to 4586 that removes the splice acceptor site for small t antigen, pLST1135T encodes only large T antigen.

Generation of transgenic mice. The LST1135 fusion gene was isolated after digestion of pLST1135T with *Eco*RI and *Bam*HI and was used to generate transgenic mice as described previously (3). Transgenic mice were screened either by dot blot analysis as previously described (4) or by polymerase chain reaction analysis. For polymerase chain reaction analysis, 1 to 2 μg of genomic tail DNA was used as a template. A 215-bp fragment was amplified with primers that flank the *dl*1135-specific deletion (GAATCTTTGCAGC TAATGGACC and GCATCCCAGAAGCTCCAAAG; nucleotides 5140 to 5119 and 4946 to 4926 of SV40). LST mice harbor the wild-type T-antigen protein under LPV control and have been previously described (5).

Antibodies. Monoclonal antibodies specific for SV40 T antigen, PAb101 (21) and PAb419 (23), have been described previously. The epitope recognized by PAb419 maps to within the first 100 amino acids of T antigen and is altered in the *dl*1135 protein. Since PAb419 interacts poorly with *dl*1135, this antibody readily distinguishes the *dl*1135 protein from the wild type. PAb101 recognizes an epitope within the carboxyl one-fourth of T antigen which is unaltered in *dl*1135. Monoclonal antibodies were also used for detection of p53, PAb421 (23) was used in immunoprecipitation reactions, and a combination of PAb421 and RA32C2 (7) was used in immunoblotting analyses. A monoclonal antibody which recognizes the T-lymphocyte surface antigen, Thy1.2, was used for T-cell depletion. All monoclonal antibodies were prepared as tissue culture supernatants. For immunofluorescence, protein A-purified polyclonal anti-T-antigen hamster antibody (44) was used as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster immunoglobulin G (Cappel) was used as the secondary antibody. The following conjugated antibodies that recognize murine cell surface antigens were used for flow cytometry: FITC–anti-Thy1.2 (Becton Dickinson, Mountain View, Calif.), FITC–anti-CD4 (Pharmigen, San Diego, Calif.), biotin–anti-CD8 (Becton Dickinson), FITC–anti-CD3 (Boehringer Mannheim, Indianapolis, Ind.), biotin–anti-B220 (Pharmigen), phycoerythrin (PE)–anti-T-cell receptor (TcR) γ/δ (Pharmigen), biotin–anti-TcR α/β (Pharmigen), biotin–anti-TcR Vβ2, Vβ3, Vβ5 to -11, and Vβ14 (Pharmigen), and avidin-PE (Caltag, San Francisco, Calif.).

Immunoprecipitation and immunoblotting analysis. Tissues were either used fresh or stored frozen at −80°C prior to analysis. Samples were suspended in lysis buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40. A cocktail of protease inhibitors (Boehringer Mannheim), including phenylmethylsulfonyl fluoride (25 mg/ml), leupeptin (0.5 mg/ml), and pepstatin (0.7 mg/ml), was added to the lysis buffer. Tissues were homogenized and sonicated on ice. The tissue extracts were cleared twice by centrifugation at 3,000 rpm for 10 to 15 min. Protein determinations, immunoprecipitation reactions, and polyacrylamide denaturing gel electrophoresis were performed as described previously (44).

Proteins were transferred to nitrocellulose overnight at 25 V in a buffer consisting of 20 mM Tris, 150 mM glycine, and 20% methanol. Filters were air dried for 1 to 4 h, rinsed in Tris-buffered saline (TBS; 0.2 M NaCl, 50 mM Tris-Cl [pH 7.4]), and blocked with 3% bovine serum albumin (BSA) in TBS for 1 h at 37°C. After each incubation, filters were washed three times with TBS containing 0.1% Tween 20 and once with TBS. The filters were incubated for 1 h with the appropriate primary antibodies. For the experiment in Fig. 3A, filters were rinsed as described above and then incubated at 25°C for 30 to 45 min with 2 μCi of ^{125}I-protein A

(New England Nuclear) in TBS. Radioactive filters were rinsed as described above, air dried, and exposed to Kodak film at −80°C with an intensifying screen. A chemiluminescent detection method was used to obtain the results in Fig. 3B. The filter was incubated for 1 h at room temperature with a 1:3,000 dilution of protein A-horseradish peroxidase (Amersham). Following incubation, the filter was washed with TBS and Tween 20 as described above. Specific reactions were detected as recommended, using the ECL kit supplied by Amersham. The filters were exposed to Kodak film at room temperature for 1 min.

Flow cytometry. Fresh spleens and thymuses from nontransgenic (BDF1) and LST1135-11 transgenic mice were forced through a sterile screen to disperse cells. Erythrocytes were removed from spleen samples by treatment with ACK lysing buffer (NH_4Cl, 8.29 g/liter; KCO_3, 1 g/liter; EDTA, 37.2 mg/liter) for 30 s. Cell suspensions were washed twice with Hanks' buffered saline containing 0.1% sodium azide and 0.1% BSA. For flow cytometry, 10^6 cells were stained with Hanks' buffered saline containing saturating concentrations of antibody at 4°C. Cells were stained with combinations of the FITC- and biotin-labeled antibodies listed above. Biotin-labeled antibodies were detected after incubation with avidin-PE. FITC-, biotin-, and PE-conjugated forms of an anti-human CD3 monoclonal antibody, Leu4, were used as negative controls since human anti-CD3 does not cross-react with mouse cell surface antigens. Fluorescence data were obtained for 1×10^4 to 2.5×10^4 viable cells as determined by forward and 90° light scatter, using logarithmic signal amplification on a FACScan (Becton Dickinson).

T-cell depletion of spleen cells. Spleen cell suspensions were prepared as described above for flow cytometry. The cells were incubated at 10^7 cells per ml with anti-Thy1.2 for 30 min at 4°C, washed once, and resuspended at 10^7/ml in rabbit complement (Cedar Lane) for 40 min at 37°C. The cells were washed extensively, recounted, and pelleted for use in immunoprecipitation reactions as described above. The composition of cells after T-cell depletion was verified by flow cytometry as described above.

Immunofluorescence. Thymocyte suspensions were prepared as described above for flow cytometry. Cell suspensions or 6-μm tissue sections were placed on polylysine-coated slides (0.5×10^6 cells per slide), air dried, and stored at −80°C. Slides were fixed with 2% paraformaldehyde in 50 mM Tris-HCl (pH 7.6)–150 mM sodium chloride (TS) for 90 s and were then washed three times for 5 min each time with TS. The cells were permeabilized with 0.1% Nonidet P-40 in TS at 0°C for 15 min. Slides were washed three times for 5 min each time with TS and were incubated for 1 h at 25°C with 10% normal goat serum (Boehringer Mannheim) and a 1:250 dilution in TS of unlabeled goat anti-mouse secondary antibody (Boehringer Mannheim). After three washes (5 min each time) with TS, slides were incubated for 1 h with protein A-purified anti-T-antigen polyclonal hamster antibody (50 to 75 μg/ml) in TS. Slides were then washed four times for 5 min each time with TS and incubated with FITC-conjugated goat-anti-hamster secondary antibody (1:2,000; Cappel) for 1 h at 25°C. After four rinses (5 min each time) with TS, samples were mounted in a solution of TS containing 80% glycerol and 100 mg of 1,4-diazabicyclo [2.2.2] octane (DABCO; Sigma Chemical) per ml. The percentage of thymocytes in a suspension which expressed T antigen was taken as the number of fluorescing cells divided by the number of cells in a particular bright-field view. Values from 26 fields (approximately 30 cells each) from five different LST1135-11 mice were averaged. Cell suspensions from each mouse contained similar numbers of T-antigen-positive cells.

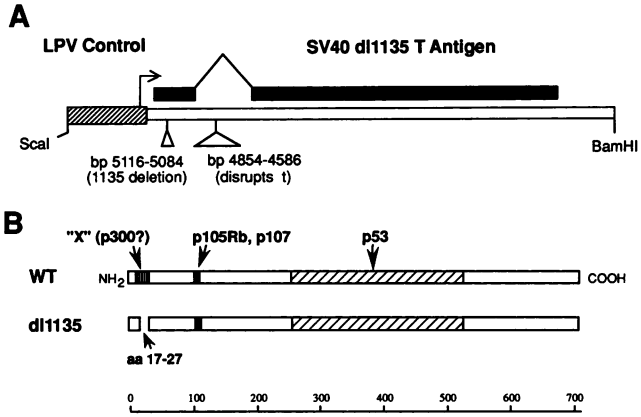

FIG. 1. Diagrams of the LST1135 transgene (A) and the *dl*1135 protein (B). The *Sca*I-to-*Bam*HI fragment containing the LST fusion gene was used for the generation of the LST1135 transgenic mice. The fragment consists of the 580-bp LPV control region (▨) and the mutant SV40 T-antigen coding region (□) containing two deletions, the *dl*1135 amino-terminal deletion and a deletion within the intron which abolishes the production of small t antigen. Shaded boxes represent exons. Linear diagrams of wild-type (WT) and *dl*1135 T antigens are shown in panel B. Regions involved in binding pRB, p107, and p53 are indicated. The putative amino-terminal transforming activity is labeled "X" to reflect the fact that biochemical functions which correlate with this activity have not been fully characterized; p300 binding represents one candidate.

RESULTS

***dl*1135 T-antigen activity in transgenic mice.** To determine the role of the amino-terminal transforming activity of T antigen in tumorigenesis, transgenic mice that harbored the LST1135 transgene were generated. This fusion gene encodes the mutant SV40 large T-antigen protein, *dl*1135, under the control of the LPV enhancer and early promoter (Fig. 1). In transgenic mice, the LPV regulatory region directs expression to the B and T lymphocytes and to the choroid plexus epithelium (3). Previously, we described the analysis of similar hybrid genes encoding the wild-type protein and three other mutant forms of T antigen (3, 4). As with our other studies, the LST1135 transgene harbors a deletion from nucleotides 4854 to 4586 of SV40 that disrupts the expression of small t antigen as a result of removal of the small t-specific splice donor site (8). This mutation does not affect the expression of large T antigen since it lies entirely within the large T intron. The *dl*1135 mutation deletes nucleotides 5116 to 5084, resulting in the in-frame loss of amino acids 17 to 27 (Fig. 1B [39]). The *dl*1135 protein is unable to transform the cultured cells which have so far been tested, including C3H10T1/2 (42a), REF52 (35, 39), primary baby rat kidney (49), and CREF (35) cells. However, it retains the ability to bind pRB (15), p107 (15), and p53 (36). The LST1135 fusion gene was introduced into the mouse germ line by pronuclear microinjection. Twenty-six founder mice were generated. Mice that developed life-threatening abnormalities and all remaining mice at 13 months of age were sacrificed for analysis.

In previous experiments, wild-type T antigen driven by the LPV control region (the LST transgene) predominantly

TABLE 1. Summary of phenotypes in the LST1135 transgenic founder mice and the LST1135-11 line

Mouse no.[a]	Sex[b]	Tumor type	Age (days) at death
10	F	Thymus	290
11	F	Thymus	150
12	M	Choroid plexus	222
13	M	Choroid plexus	250
14	M	Thymus	183
16	M	Thymus	270
18	M	Sarcoma[c]	390
19	F	Thymus	180
20	M	Sarcoma[c]	390
21	M	Thymus	212
22	M	Thymus	180
24	F	Thymus	181
27	M	Thymus[c]	390
28	F	Thymus	210
30	F	ND[d]	205
34	M	Thymus	220
36	F	Thymus	288
37	M	Thymus	318
LST1135-11 F_2[e]		Thymus	132–212

[a] The 18 LST1135 founder mice that developed phenotypes are listed. Another eight founder mice were normal after 1 year of age (see Fig. 2).

[b] F, female; M, male.

[c] The mouse appeared normal and was sacrificed at 390 days of age. However, upon dissection, the indicated abnormalities were observed.

[d] ND, not determined. The mouse was lost to autolysis but displayed the typical restricted breathing pattern observed in the other mice with thymomas.

[e] Mice in the LST1135-11 lineage consistently develop thymic lymphoma. Data for a representative generation (F_2) in which all 16 transgenic mice developed this phenotype are given.

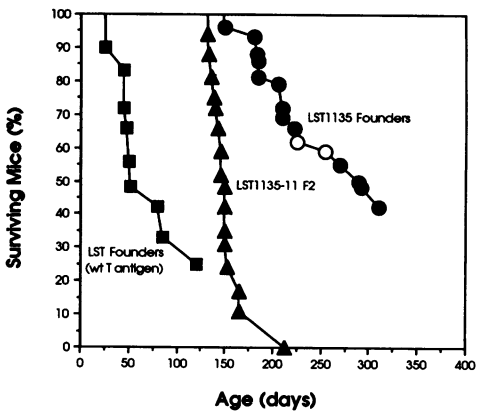

FIG. 2. Survival of LST and LST1135 transgenic mice. Survival of the LST founder mice (expressing wild-type [wt] T antigen), LST1135 founder mice, and mice of the LST1135-11 lineage is shown. Animals 12 months of age or less were sacrificed for analysis when death appeared imminent. The age of sacrifice versus the percentage of animals remaining was plotted to give the survival curve. Each point represents the death of one animal in which an abnormal phenotype was grossly detectable (see Table 1 for the LST1135 phenotypes). Open circles indicate LST1135 founder animals that developed choroid plexus tumors rather than T-cell lymphoma (filled circles). Mice which were sacrificed at the end of the study are considered survivors. Mice of the LST1135-11 F_2 generation were born in several different litters at various times.

induced rapid choroid plexus tumorigenesis (5). Signs of lymphoproliferation also appeared in these mice, although early death precluded assessment of malignant lymphoma (see the introduction) (3, 5). In contrast to wild-type T antigen, the predominant phenotype induced by *dl*1135 was thymic lymphoma. The LST1135 founder mice that developed abnormal phenotypes are shown in Table 1, and a graph of their survival is shown in Fig. 2. Thirteen of the twenty-six founder mice (50%) were sacrificed upon showing signs of distressed breathing due to extensive thymic enlargement. The spleens of some of these mice were also enlarged. Histological examination of abnormal thymuses and spleens showed extensive population by morphologically similar lymphocytes (not shown). Only two of the LST1135 founder mice developed choroid plexus tumors (9% of these founder mice compared with 75% with wild-type T antigen) (5). These tumors also arose much later (222 and 250 days) than did those induced by wild-type T antigen (between 21 and 35 days of age). The remaining founder mice were sacrificed at 13 months of age and examined. Eight of these mice showed no gross abnormalities, one had an enlarged thymus characteristic of thymoma, and two had developed sarcomas (Table 1).

For further analysis of the thymic phenotype, a line of mice was established from the LST1135-11 founder (Table 1). All transgenic mice in this lineage developed thymic lymphomas that became life threatening by about 5 months of age (Table 1 and Fig. 2). While the LST1135 founder mice died within a rather broad period of time (150 to 318 days), the mice of the LST1135-11 lineage developed disease within narrow time frame (132 to 212 days; Fig. 2). The fact that only 50% of the founder mice had developed the thymic phenotype could have indicated that the need for additional genetic changes limited the penetrance of tumorigenesis. However, the transgene demonstrated 100% penetrance of the phenotype in the LST1135-11 lineage (Fig. 2). The lower frequency of tumor development in the LST1135 founder mice most likely reflects the influence of some insertion sites on transgene expression. Indeed, founder mice that did not develop an abnormal phenotype did not detectably express the *dl*1135 protein (see below).

Expression of *dl*1135 in transgenic mice. A variety of tissues from all founder mice and several mice of the LST1135-11 lineage were examined for *dl*1135 expression by immunoblotting analysis. In mice which developed phenotypes, the *dl*1135 protein was commonly expressed in thymuses and spleens, consistent with the specificity of the LPV regulatory region (3, 5). A representative immunoblotting analysis is shown in Fig. 3A. In this experiment, T antigen was immunoprecipitated from tissue extracts with the indicated monoclonal antibodies prior to immunoblotting. The *dl*1135 protein was abundant in spleen and thymus but was undetectable in the brain of a 5-month-old LST1135-11 mouse (lanes 13 to 18). Monoclonal antibody PAb419 was used to confirm that the protein expressed in LST1135 mice was *dl*1135. While the C-terminal-specific antibody PAb101 recognizes both wild-type and *dl*1135 T antigens with equal affinity (lanes 1, 15, and 17), PAb419, an antibody directed to an amino-terminal epitope (23), reacts poorly with *dl*1135. Immunoprecipitation using PAb419 failed to detect the T antigen in LST1135 mice (lane 19) but efficiently precipitated the wild-type protein from the LST tumor cell extract (lane 5). The same result was obtained with a different monoclonal antibody directed against the amino terminus, PAb108 (data not shown).

In previous experiments, the LPV control region directed expression to both B- and T-lymphoid cells (3). To determine whether expression in the spleen originated from B cells as

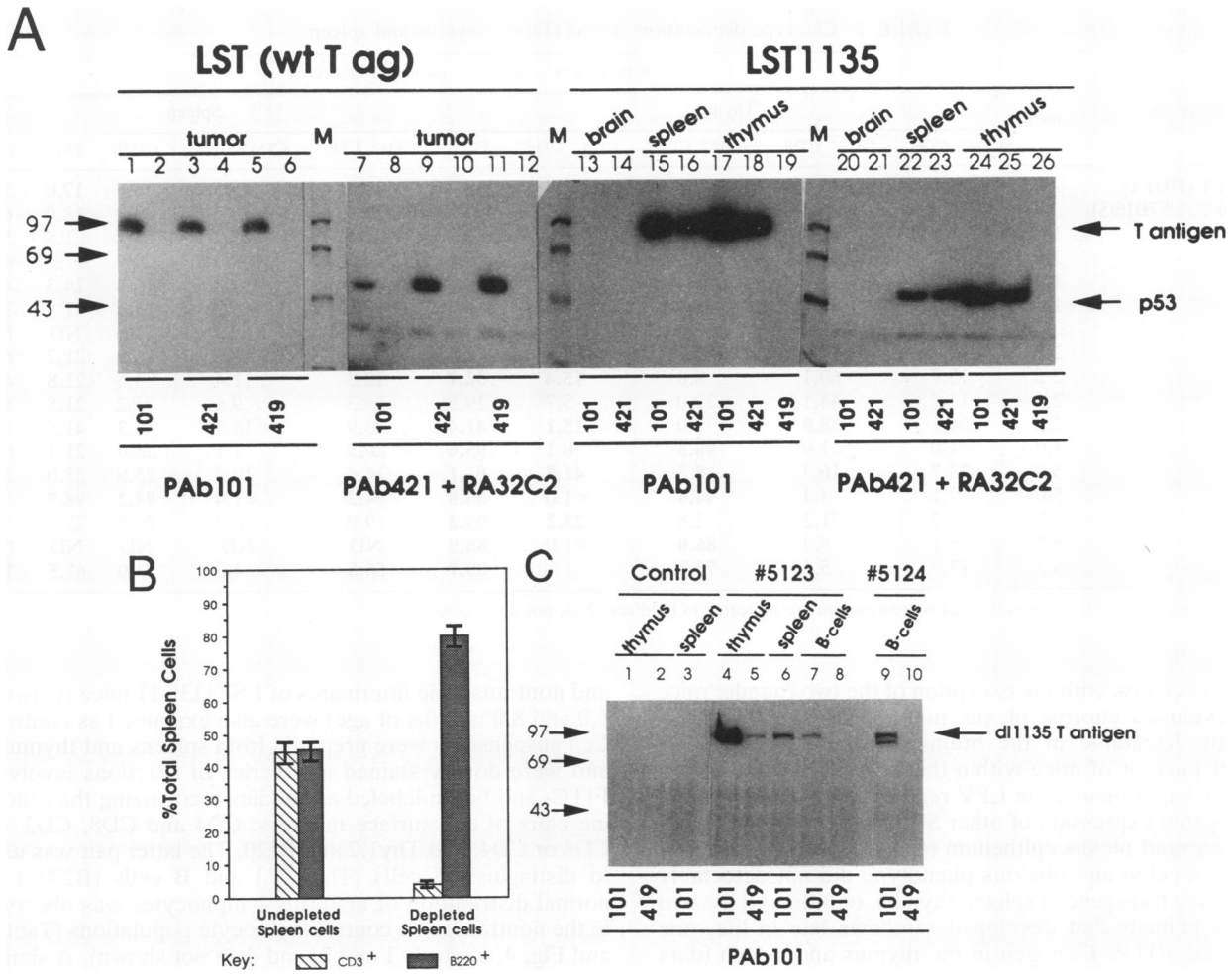

FIG. 3. Expression of T antigen in LST1135 transgenic mice. (A) Control choroid plexus tumor tissue of LST mice and brain, spleen, and thymus tissue from a mouse of the LST1135-11 lineage were examined for T-antigen expression by immunoblotting analysis. T antigen was immunoprecipitated with monoclonal antibodies recognizing the carboxy terminus of SV40 T antigen (PAb101), the amino terminus of T antigen (PAb419), or the cellular protein p53 (PAb421) prior to immunoblotting. Antibodies used for immunoprecipitation are listed vertically under each lane. The supernatants from control reactions were examined by a second round of immunoprecipitation (lanes 2, 4, 6, 8, 10, and 12) to demonstrate that the conditions for immunoprecipitation were optimal. Antibodies used for detection of immunoblots are listed horizontally at the bottom. The positions of size markers (in kilodaltons), T-antigen proteins, and p53 are indicated. (B and C) T cells were depleted from spleen cell suspensions of two mice from the LST1135-11 lineage, 5123 and 5124. The cell composition before and after depletion was verified by FACS analysis using anti-Thy1.2 to detect remaining T cells and anti-B220 to detect B cells (B). Extracts from the indicated cell suspensions were incubated with the indicated antibodies for immunoprecipitation as described for panel A and subjected to immunoblotting analysis, using the chemiluminescence for detection (see Materials and Methods). Sizes in panel C are indicated in kilodaltons.

well as T cells in LST1135-11 mice, T cells were depleted from spleen cell suspensions by treatment with anti-Thy1.2 and complement prior to immunoblotting. Flow cytometry confirmed enrichment of the population for B cells and removal of 90% or more of the T cells (Fig. 3B). Three mice were analyzed, and data from two representative mice are shown in Fig. 3C. All three mice expressed significant levels of *dl*1135 in the spleen cells after T-cell depletion (Fig. 3C, lanes 6 and 7, and data not shown). Two *dl*1135-specific bands were apparent in the B-cell samples from two mice (lane 9 and data not shown). The faster-migrating form was often expressed in the spleens, but not in thymuses, of LST1135 mice. Since this form was not always present (for example, mouse 5123; Fig. 3C), the significance of this observation, if any, is not clear. The *dl*1135 in all B-cell-enriched samples showed the characteristic reduction of reactivity with PAb419 (lanes 8 and 10).

Complex formation between p53 and *dl*1135 in spleens and thymuses was demonstrated by immunoprecipitation of T antigen with PAb421, a monoclonal antibody that recognizes p53 (Fig. 3A, lanes 16 and 18), and by coimmunoprecipitation of p53 with the T-antigen-specific antibody PAb101 (lanes 22 and 24). The ability of *dl*1135 to bind p53 was indistinguishable from that of wild-type T antigen (lanes 3 and 7). In two independent experiments, quantification of p53 and *dl*1135, both free and in complex, indicated that all of the detectable p53 in the thymus was complexed to 43 and 56% of the *dl*1135 T antigen (data not shown). In the spleen, 64 and 89% of the p53 was complexed to 43 and 52% of the total *dl*1135 protein (data not shown).

TABLE 2. Cell type distribution in LST1135-11 thymus and spleen

Mouse no.	Age (mo)	% of total cells positive for indicated marker[a]									
		Thymus					Spleen				
		$CD4^-$ $CD8^-$	$CD4^+$ $CD8^+$	$CD4^+$ $CD8^-$	$CD4^-$ $CD8^+$	$CD3^{hi}$	$CD4^+$ $CD8^-$	$CD4^-$ $CD8^+$	$CD3^{hi}$	Thy1	B220
Control 1 (BDF1)	2.0	4.6	87.8	5.3	2.3	9.4	12.1	5.5	16.6	17.0	75.4
Control 2 (C57BI6/5)	3.5	3.4	87.4	7.1	2.1	10.1	13.9	6.7	20.1	18.9	69.4
5003	1.0	2.5	90.0	6.3	1.2	10.0	12.5	8.7	21.7	21.5	55.1
5004	1.0	8.7	77.9	11.1	2.3	17.4	10.6	9.0	19.9	17.9	48.1
5010	1.0	3.0	85.4	9.6	2.0	14.5	6.8	9.5	16.4	14.3	46.6
5009	1.8	15.0	55.6	**25.1**	4.3	**36.2**	18.2	15.2	35.3	31.5	31.8
5005	2.0	4.6	85.8	7.6	1.9	15.2	ND	ND	ND	ND	ND
4979	2.5	3.1	27.2	2.1	**67.6**	15.4	11.9	9.6	20.9	21.2	65.1
4980	2.5	15.7	60.1	8.8	**15.4**	**32.7**	12.3	11.4	23.5	21.8	48.3
4981	2.5	18.2	64.1	12.0	5.7	19.5	15.3	9.6	25.2	21.8	51.6
4982	2.8	**38.1**	38.8	8.0	**15.1**	**41.4**	23.9	18.3	44.3	41.5	32.7
4593	4.0	<1.0	<1.0	**98.5**	8.1	**95.6**	20.3	7.1	23.3	21.1	61.3
4666	5.0	**33.7**	16.3	8.3	**41.8**	**81.1**	14.4	10.3	25.9	25.0	51.9
4527	5.0	2.1	3.4	**94.4**	<1.0	**86.8**	**94.2**	<1.0	**94.2**	**98.7**	<1.0
4528	5.0	2.7	71.2	2.8	**23.2**	**95.2**	19.0	8.4	28.9	27.2	57.1
4678	5.5	5.1	8.9	**86.0**	<1.0	**88.9**	ND	ND	ND	ND	ND
4589	6.5	17.7	5.4	**75.8**	1.1	**88.7**	**56.8**	4.3	**71.0**	**67.5**	11.8

[a] Cell populations with abnormally high representation are indicated in boldface. ND, not determined.

Unexpectedly, with the exception of the two founder mice that developed choroid plexus tumors, the *dl*1135 protein was not detectable in the brains of the other LST1135 founder mice or of mice within the LST1135-11 lineage. In previous experiments, the LPV regulatory region reproducibly targeted expression of other SV40 T-antigen transgenes to the choroid plexus epithelium (4, 5). The eight mice that did not develop any obvious phenotype did not detectably express the transgene in spleen, thymus, or brain. Of the two founder animals that developed sarcomas late in life, one expressed *dl*1135 T antigen in the thymus and spleen (data not shown). Both of these mice expressed *dl*1135 in the tumors (data not shown).

Characterization of *dl*1135-induced thymic lymphoma. In a normal mouse, thymocytes progress through several developmental stages that can be characterized by their expression of the CD4 and CD8 differentiation antigens as well as the TcR/CD3 complex (24). The most immature cells exhibit the $CD4^-$ $CD8^-$ phenotype and express no cell surface TcR/CD3. The majority of thymocytes, however, are $CD4^+$ $CD8^+$ and express either no cell surface TcR/CD3 or low levels of the complex. A small percentage of $CD4^+$ $CD8^+$ cells express higher, intermediate levels of the TcR/CD3 complex. Within the $CD4^+$ $CD8^+$ population, the progression of surface TcR/CD3 expression (from absent to low to intermediate) represents a developmental progression from less mature cells to more mature cells. Finally, the most mature thymocytes are $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ and express high levels of cell surface TcR/CD3. These latter populations emigrate from the thymus and are essentially equivalent to the mature functional T lymphocytes in the peripheral lymphoid organs such as the spleen. This distribution of cell types in the thymus remains constant throughout the life of a normal animal.

To determine which T-cell populations were affected by *dl*1135 in transgenic mice, thymic and splenic lymphoid cells from mice of the LST1135-11 lineage were analyzed by multiparameter flow cytometry (fluorescence-activated cell sorting [FACS] analysis). Three mice around 1 month of age, six mice between 2 and 2.8 months of age, and six mice between 4 to 6.5 months of age were analyzed. BDF1 mice and nontransgenic littermates of LST1135-11 mice (between 1.0 and 8.0 months of age) were also examined as controls. Cell suspensions were prepared from spleens and thymuses and were doubly stained in a series of reactions involving FITC- and biotin-labeled antibodies recognizing the following pairs of cell surface markers: CD4 and CD8, CD3 and CD8 or CD4, and Thy1.2 and B220. The latter pair was used to distinguish T cells ($Thy1.2^+$) and B cells ($B220^+$). A normal distribution of maturing lymphocytes was observed in the nontransgenic control thymocyte populations (Table 2 and Fig. 4, controls 1 and 2, and data not shown). A similar distribution was obtained with LST1135-11 transgenic mice which were around 1 month of age (Table 2 and Fig. 4, mice 5003, 5004, and 5010).

In contrast, thymocytes from older LST1135-11 mice exhibited a progressive divergence from the normal pattern with increasing age (Table 2 and Fig. 4B and C). By approximately 2 months of age, many transgenic mice had an excess of either $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ cells that expressed the $TcR/CD3^{hi}$ phenotype. By 4 to 5 months of age, most transgenic thymuses had expanded to fill the chest cavity and were nearly homogeneous for one of these mature T-cell subsets (for example, mice 4593, 4527, 4678, and 4589). In one lymphomic mouse (4528), the majority of T cells were of the $CD4^+$ $CD8^+$ type, which is usually indicative of an immature population. However, these cells were $TcR/CD3^{hi}$ and thus probably in transition to a mature phenotype. Two mice (4982 and 4666) had an excess of $CD4^-$ $CD8^-$ $TcR/CD3^{hi}$ cells, a mature T-cell subset that is normally rare in the thymus. The variation of predominant T-cell populations demonstrates that the thymic expansion found in LST1135-11 transgenic mice does not reflect a uniform excess proliferation of all cells but rather reflects the outgrowth of a particular T-cell subtype in each mouse. Although the most frequently affected cells had a mature T-cell phenotype (most predominantly $CD4^+$ $CD8^-$ $TcR/CD3^{hi}$), the major cell population affected could clearly vary from mouse to mouse.

Analyses of the spleens of LST1135-11 transgenic mice also revealed abnormalities in some, but not all, of the older mice (Table 2). Some mice (4527 and 4589) had the same

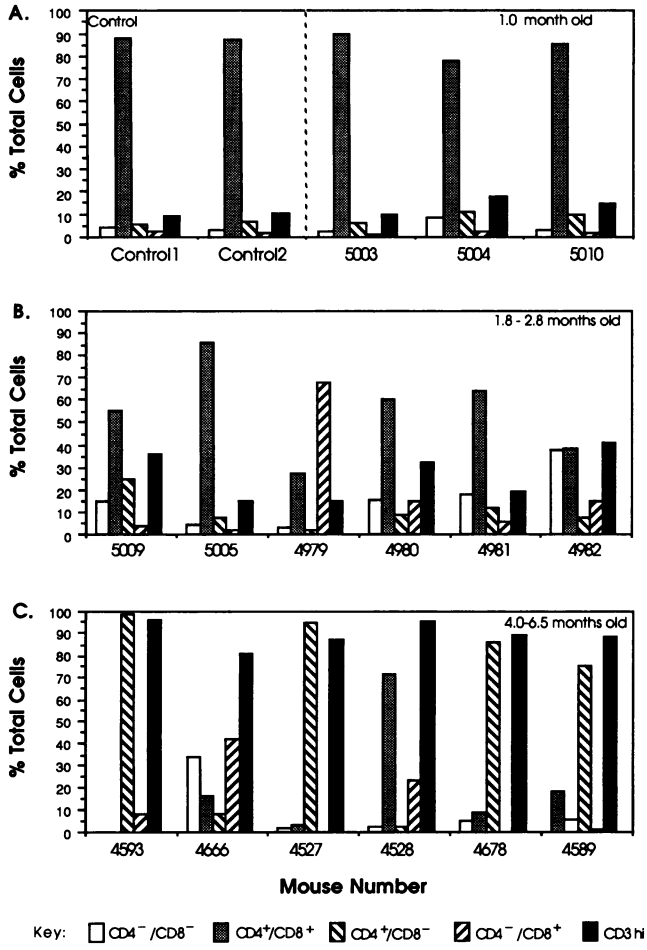

FIG. 4. Distribution of T cells in the thymuses of LST1135-11 transgenic mice. Thymocytes from LST1135-11 mice were analyzed by flow cytometry as described in Materials and Methods. The total cell population is characterized by the presence or absence of CD4 and CD8 antigens. The percentage of total cells which are also TcR/CD3^{hi} is given as a separate value. The cell populations shown in the histograms are indicated by the key at the bottom. Each number along the x axis refers to the mouse that was analyzed. The data have been divided according to age and phenotype development of each mouse. (A) Nontransgenic (control) mice (2 and 3.5 months of age) and 1.0-month-old LST1135-11 mice show a normal distribution of cells in the thymus. (B) LST1135-11 mice at 2.0 to 2.8 months of age show an abnormal distribution of T cells. (C) LST1135 mice beyond about 4 months of age show a predominant T-cell type in the thymus. Nontransgenic BDF1 mice of 5 and 8 months of age were found to have T-cell populations similar to those of younger control mice (data not shown).

excess of CD4$^+$ CD8$^-$ T cells in their spleens as in their thymuses, suggesting that disregulated thymocytes had emigrated to the periphery. Consequently, these mice had reduced frequencies of B lymphocytes in their spleens (as noted by the B220 staining results). However, in other mice with thymic overgrowth (4593, 4666, and 4528), the splenic populations were fairly normal, suggesting that massive thymic export of lymphomic T cells had not yet occurred.

TcR Vβ subtypes suggest clonality of tumors. The phenotypic homogeneity of thymocytes within older transgenic mice prompted us to examine whether these cells represented clonal lymphomic populations. We addressed this question by performing FACS analysis with antibodies specific for a variety of TcR subtypes. Tumors from eight LST1135-11 mice ranging in age from 3 to 6 months were analyzed for the presence of Vβ3, Vβ5 to -9, and Vβ11. Four of these tumors were also examined for Vβ2, -10, and -14. The heterogeneous T-cell populations from normal mice contain many different clones, each of which expresses a single variant of the TcR complex. In normal BDF1 mice Vβ3, -5, -6, -7, -9, and -11 together comprise about 5.5% of TcR-positive cells, while Vβ2, -8, -10, and -14 comprise 7.9, 16.3, 10.4, and 8%, respectively (Table 3). Additional TcR variants not assayed in these studies comprise the remaining 52% of the TcR-positive T cells in normal mice.

All transgenic thymuses examined contained an abnormal distribution of TcR subtypes (Table 3). The γ/δ population of T cells was significantly increased in two mice, 5274 and 4678, from 1.5% to 17.6 and 26.9%, respectively. These mice also demonstrated an abnormal distribution of Vβ subtypes. Although a single TcR subtype did not predominate in these two mice, the T-cell distribution does not reflect uniform expansion of all normal subsets. In mouse 5264, 87% of the T cells were of the Vβ8 class, although other populations were clearly still present at reduced levels. The lymphomic thymocytes from three mice (4593, 4528, and 5006) expressed none of the TcR variants assayed (including normally predominant classes) and presumably expressed one or more of the TcR variants not analyzed. The T cells from two mice, 4527 and 5119, exclusively expressed a single TcR subtype, Vβ8 and Vβ14, respectively. Although these data cannot exclude the existence of multiple clones of the same Vβ class within a tumor, they do indicate that the lymphomic T-cell population is often relatively homogeneous and suggest that a clonal transformation event has occurred in most mice.

Since thymomas generally consisted of a single class of T cells, we analyzed the expression of *dl*1135 within the T-cell population to determine whether clonality of the tumors resulted from clonal expression of the transgene. Single cell thymocyte suspensions from 5 nonlymphomic LST1135-11 mice were examined by immunohistochemistry. Data from a representative mouse (5120) are shown in Fig. 5. The normal distribution of T-cell subsets was confirmed by FACS analysis of each suspension (Fig. 5A and D). In each case, the majority (average, 98%), not a subset, of thymocytes expressed the *dl*1135 T antigen (compare Fig. E and F for a representative field). Immunofluorescence of tissue sections showed a similarly high percentage of cells expressing *dl*1135 (not shown). Thus, predominance of a single cell type in lymphomas was not merely the result of restricted T-antigen expression and probably reflects the involvement of secondary events (see Discussion).

DISCUSSION

We have used the multifunctional transforming protein, SV40 T antigen, to probe mechanisms of cell growth control in the intact organism. By targeting expression of mutant forms of T antigen to specific cell types in transgenic mice, we can test whether the cellular targets of T antigen have ubiquitous or cell-type-specific roles in growth regulation. In this report, we demonstrate that a mutant form of T antigen, *dl*1135, which is defective for transformation of certain cells in culture, induces thymic lymphoma in transgenic mice. Mutant *dl*1135 represents a class of mutants with deletions near the amino terminus that retain the ability to bind p53, pRB, and p107. Together with results of cultured cell exper-

TABLE 3. Characterization of receptor-positive T cells in lymphomic LST1135-11 mice[a]

Mouse no.	Age (mo)	% of total T cells						
		TCR subtype		Vβ subtype				
		γ/δ	α/β	Vβ2	Vβ3	Vβ8	Vβ10	Vβ14
Control	2.0–4.0	1.5 ± 0.2	98.5 ± 0.2	7.9 ± 1.1	0.5 ± 0.1	16.3 ± 1.0	10.4 ± 0.3	8.0 ± 1.1
5264	3.0	0.3	99.7	0.85	0.66	**87.1**	0.63	0.25
4593	4.0	<0.1	100.0	ND	<0.1	<0.1	ND	ND
5274	4.0	**17.6**	82.4	0.18	0.15	0.96	0.21	1.8
5119	4.5	<0.1	100.0	<0.1	<0.1	<0.1	<0.1	**100.0**
4528	5.0	<0.1	100.0	ND	<0.1	<0.1	ND	ND
4527	5.0	<0.1	100.0	ND	<0.1	**100.0**	ND	ND
4678	5.0	**26.9**	73.1	ND	**11.1**	4.4	ND	ND
5006	6.0	<0.1	100.0	<0.1	<0.1	<0.1	<0.1	<0.1

[a] For normal BDF1 mice (control), the Vβ subset distribution of $CD3^{hi}$ T cells was determined from lymph node, since $CD3^{hi}$ T cells are rare in normal thymus but represent the major lymph node population. Additional Vβ subtypes, Vβ5, -6, -7, -9, and -11, which together comprised 5.5% of the control T cells (see text), were each present on <0.1% of the cells from all transgenic mice tested. Overabundant populations of TcR subtypes appear in bold type, although the decreased representation of a particular subset (not in bold type) also indicates significant deviation from normal. ND, not determined.

iments, the study presented here suggests that the amino-terminal activity impaired in *dl*1135 is necessary to induce the transformation of certain cell types but is dispensable for transformation of others.

Mechanism of *dl*1135-induced lymphomagenesis. SV40 T antigen induces both B- and T-cell lymphomas in transgenic mice upon targeted expression with the LPV transcriptional signals (3, 5) or the immunoglobulin heavy-chain enhancer (43). Because of the high frequency of B-cell lymphomas in previous studies, complete analyses of T-cell tumor progression was not carried out. When directed by the lymphoid cell-specific tyrosine kinase (*lck*) gene regulatory region,

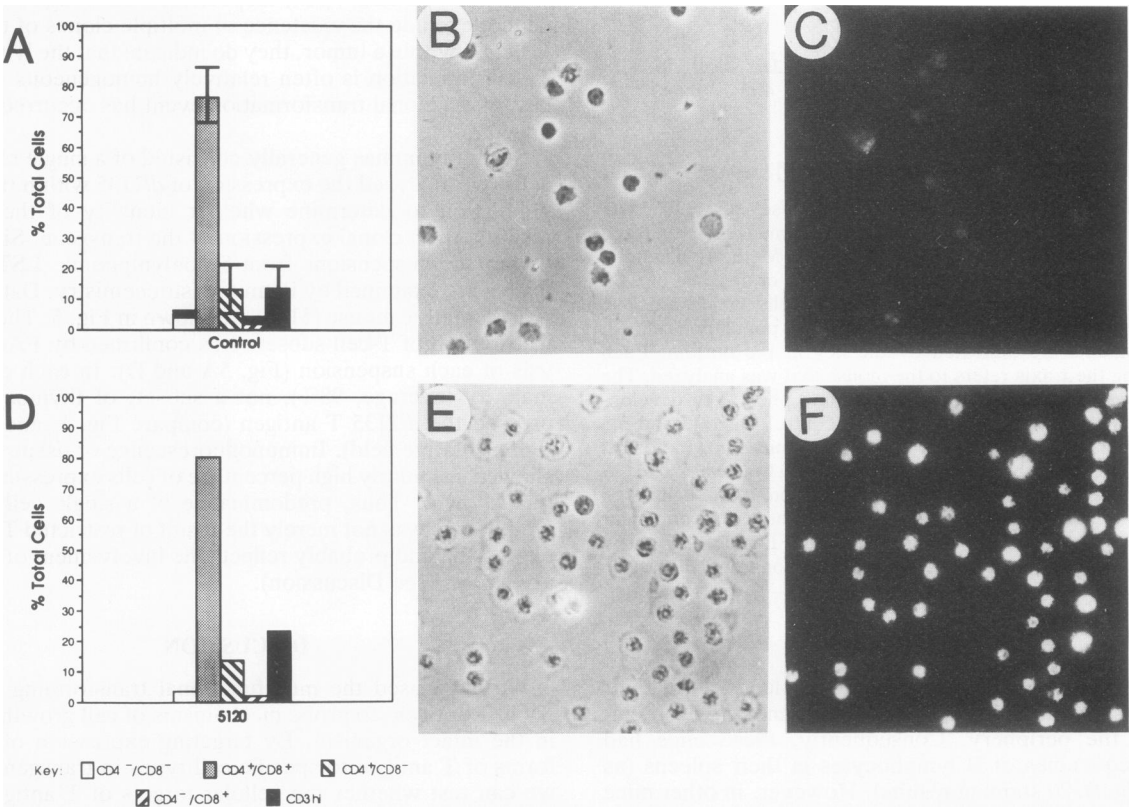

FIG. 5. Expression of *dl*1135 T antigen in thymocytes. Flow cytometry analysis (A and D), phase-contrast microscopy (B and E), and immunofluorescence detection of T antigen (C and F) of thymocytes from control (nontransgenic) mice and from a 2-month-old transgenic mouse of the LST1135-11 lineage are shown. T antigen was detected by using PAb101 and an FITC-conjugated secondary antibody. The normal distributions of T cells in six nontransgenic mice were determined and are represented in panel A. The T-cell preparation from one of these mice is shown in panels B and C. The transgenic thymocyte population shown in panel D reflects a normal distribution of T-cell subtypes. Nuclear staining characteristic of T antigen was detected in the majority of thymocytes from the LST1135-11 mouse (compare panels E and F). The data shown in panels D to F are representative of three LST1135-11 mice that were analyzed.

SV40 T antigen caused the development of thymoma which followed an initial reduction in the $CD4^+$ $CD8^+$ population. T-cell lines established from the tumors were $CD3^+$, with varied CD4 and CD8 profiles (19).

Consistent development of T-cell lymphoma in the LST1135 mice described here permitted the systematic analysis of disease progression. In the LST1135-11 line, the abnormal distribution of T cells first appears in the thymus after about 2 months of age. By 4 to 6 months, most mice show a predominance of a single class of T cell in the thymus; at late times, this same population of T cells also often predominates in the spleen. The affected T-cell type is not always the same, indicating that *dl*1135 can participate in the induction of tumors in a variety of T-cell types. Moreover, the *dl*1135-induced T-cell tumors appear to be clonal or oligoclonal, given the predominance of a single Vβ subtype in several lymphomic mice even though T antigen is expressed throughout the T-cell population at a time when the constitution of cell types is normal. Therefore, although the T-cell tumors arise with surprising consistency within an established mouse line, apparently random secondary changes seem to be required to produce the phenotype. Whether this pattern is characteristic of wild-type T antigen in T cells is not yet certain. However, the cell types involved in thymoma in this study do appear to be similar to those described by Garvin et al. for wild-type T antigen (19).

In all mice in this study, the affected T-cell population appears to be a mature cell, characterized by a high level of surface CD3. Mature cells may be the only targets for transformation by T antigen. Alternatively, an immature cell could be affected at any particular stage of T-cell differentiation but may proceed along the normal pathway with disturbed growth properties. In either case, T antigen could contribute to tumorigenesis by extending the survival of the T cells such that the probability of additional genetic lesions is increased. One way to achieve increased survival is by interference with the normal apoptosis, or programmed cell death, pathway. Since the apoptosis gateway for T cells occurs just prior to the final maturation step, this hypothesis would be consistent with the observation that mature T cells comprise T-antigen-induced thymomas. Interestingly, p53 was recently shown to induce apoptosis in a myeloid leukemia cell line (51) and in a colon cell line in culture (40). Moreover, thymocytes from p53 null mice fail to apoptose in response to irradiation (30). Hence, if p53 has a role in the induction of apoptosis, T antigen may interfere with this function. Another possibility is that T antigen generally increases the rate of T-cell proliferation, allowing more cells to become susceptible to secondary events. These hypotheses are currently being tested by in vitro assays using thymocytes from the LST1135-11 transgenic mice. Similar mechanisms have been proposed for the role of certain cellular oncogenes in B- and T-cell tumorigenesis. For example, c-*myc* may participate in B-cell tumorigenesis by increasing the number of proliferating cells (reviewed in reference 2). *bcl-2*, on the other hand, appears to increase the survival of these cells by interfering with apoptosis (reviewed in reference 26).

Is *dl*1135 defective in tumorigenesis of the choroid plexus epithelium and of B-lymphoid cells? Since the majority of LST1135 transgenic founder animals developed only T-lymphocyte tumors even though the LPV regulatory region usually targets expression to the choroid plexus and the B cells (3, 4), the ability of the *dl*1135 protein to transform cells within the mouse may be cell specific. In this study, immunoblotting analyses detected expression of the *dl*1135 protein in splenic B cells but not in nontumorous choroid plexus. Thus, we cannot yet conclude that the *dl*1135 protein is defective for tumorigenesis of the choroid plexus. One possible explanation may be that the protein is less stable than the wild type. Pulse-chase experiments in cultured cells have shown that the half-lives of certain amino-terminal T-antigen mutants are lower than that of wild-type T antigen (35). However, the *dl*1135 protein is readily detected in T and B cells, so if protein degradation explains the absence of *dl*1135 in choroid plexus, the effect is cell type specific.

In this study, 2 of 26 founder mice did develop choroid plexus tumors that expressed the *dl*1135 protein, suggesting that this mutant T antigen can at least participate in tumorigenesis of this cell type. However, we do not know whether the rare induction of these tumors reflects the requirement for secondary mutations in cellular genes to compensate for the loss of a T-antigen activity or whether the tumors arose from cells that somehow increased the steady-state level of the *dl*1135 protein or both. Thus, from these studies, we cannot conclude whether *dl*1135, like wild-type T antigen (5), is sufficient to induce choroid plexus tumors. We are currently attempting to increase the overall steady-state level of *dl*1135 by driving expression with stronger choroid plexus-specific transcriptional signals (50).

The observation that the *dl*1135 protein is expressed in B cells but fails to produce a phenotype there indicates that the ability of T antigen to complex pRB, p107, and p53 is not sufficient to perturb the growth of these cells. This observation further suggests that the amino-terminal transformation function(s) is required to elicit B-cell lymphoma even though it is dispensable for the production of T-cell tumors. Further studies will be required to determine which B-cell types express the protein and whether the level of protein per cell is comparable to that observed in T cells or in lymphomic B cells expressing wild-type T antigen. One unexplained observation from this study is that a faster-migrating form of *dl*1135 is often observed only in spleen and is enriched in purified B cells. Whether this form results from proteolytic cleavage or from modifications specific to B cells requires further characterization.

Do the cellular targets of T antigen have cell-type-specific roles in growth control? This report represents our first look at a mutant defective in the amino-terminal transformation activity of T antigen in the intact organism and suggests the possibility of a cell-specific role in growth control. The results obtained here constitute the first demonstration that this mutant can transform cells at all. Whether the T-cell population is the only permissive cell type for *dl*1135-induced transformation will require studies in which the expression of the protein is directed to many more cell types in transgenic mice. Since the amino acids deleted in *dl*1135 lie within the region also present in small t antigen, we emphasize that the present study of *dl*1135 and our previous studies of T antigen in transgenic mice (3, 4) were carried out in the absence of small t antigen, excluding the possibility that any effects measured in this study were due to changes in small t antigen.

The biochemical nature of the transforming activity disrupted by deletions near the amino terminus has not yet been fully characterized. Evidence suggests that in addition to binding the cellular proteins p53, pRB, and p107, T antigen may bind to p300, a protein identified by its interaction with the E1A protein of adenovirus (14, 48). This binding property is associated with the ability of E1A to transform cells in culture; T antigen can complement a p300-binding-defective mutant of E1A for transformation (49), suggesting that a

similar activity may be present. Recently a cDNA encoding p300 was isolated and a glutathione *S*-transferase/p300 fusion protein was shown to bind to SV40 T antigen in vitro. While this binding activity has not been fully characterized, preliminary evidence suggests that it maps near the amino terminus (13). Thus, p300 binding is a candidate for a biochemical activity affected in *dl*1135.

The results from this study combined with our previous analysis of T-antigen mutants suggest that different T-antigen transformation functions may have cell-specific effects. For example, a mutant which encodes only the amino-terminal fragment of 121 amino acids, *dl*1137, is unable to induce lymphoid tumors in both B and T cells but still induces choroid plexus tumors (reference 4 and our unpublished results). This mutant protein contains the pRB/p107-binding region and the amino-terminal region which is deleted in *dl*1135 but lacks the p53-binding region. Another mutant protein analyzed contains a single amino acid change that simultaneously disrupts pRB, p107, and p53 binding and presumably leaves the amino-terminal activity intact. It too is defective in lymphoid tumorigenesis and is further impaired in the induction of choroid plexus tumors, although it measurably perturbs the growth of choroid plexus cells (4). Mutant *dl*1135, which binds pRB, p107, and p53 but is defective in the amino-terminal function, readily induces T-cell lymphoma. Together, these studies suggest that T-antigen-induced lymphoma (at least in T cells) correlates with its p53-binding property. The present study suggests that additional T-antigen activities may be required for B-cell lymphomagenesis.

The generation of mice which are homozygous for a null mutation in p53 (11) provides additional evidence suggesting that p53 may play a role in controlling lymphoid cell growth. While loss of p53 still supports normal development to early adulthood, these mice develop tumors of certain cell types with increased frequency (11, 25). The tumors that arise with greatest frequency (approximately two-thirds) are of lymphoid cell origin (11). Interestingly, thymoma is the predominant lymphoma type (25). This observation supports the notion that T antigen interferes with the function of p53. Of interest, choroid plexus tumors have not been observed in the p53 null mice, consistent with our observation that the p53-binding property of T antigen is not required for the production of these tumors (4).

Further studies using T antigen in transgenic mice, together with gene disruption experiments, should help elucidate the mechanisms that different cell types utilize to maintain the growth control characteristic of their unique differentiated states. Ultimately a more direct approach would involve interfering with putative negative growth regulators in specific cell types without using transforming proteins such as T antigen. Until whole animal approaches utilizing antisense nucleic acids or cell-specific targeted gene mutation are further developed, the tumor virus proteins used in studies such as those described here provide a useful alternative.

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