Uniform Cell-Autonomous Tumorigenesis of the Choroid Plexus by Papovavirus Large T Antigens

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The simian virus 40 (SV40) large tumor antigen (T antigen) under its natural regulatory elements induces choroid plexus papillomas in transgenic mice. Because these tumors develop focally after several months, it has been suggested that secondary cellular alterations are required to induce a tumor in this tissue. In contrast to SV40, the related lymphotropic papovavirus early region induces rapid nonfocal choroid plexus neoplasia in transgenic mice. Here, using hybrid gene constructs, we showed that T antigen from either virus is in fact sufficient to induce these tumors. Their abilities to induce proliferative abnormalities in other tissues, such as kidney and thymus, were also indistinguishable. Differences in the rate of choroid plexus tumorigenesis reflected differences in the control regions of the two viruses, rather than differences in T antigen per se. Under SV40 regulation, expression was limited to a fraction of the choroid plexus cells prior to the formation of focal tumors. When SV40 T antigen was placed under lymphotropic papovavirus control, in contrast, expression was generally uniform in the choroid plexus and rapid expansion of the tissue ensued. We found a direct relationship between T-antigen expression, morphological transformation, and proliferation of the choroid plexus epithelial cells. Analysis of mosaic transgenic mice indicated further that T antigen exerts its mitogenic effect cell autonomously. These studies form the foundation for elucidating the role of various T-antigen subactivities in tumorigenesis.

The natural induction of neoplasia appears to involve multiple genetic events (28), including alteration of cellular proto-oncogenes (dominant events) and of tumor suppressor genes (recessive events) (2, 8, 15, 28, 34, 40). In transgenic mice, simian virus 40 (SV40) large T antigen induces a variety of neoplasms depending on the transcriptional signals used for its expression (for reviews, see references 25 and 26); included are eye lens (37), acinar (47, 49) and beta (24) cell pancreatic tumors, B-cell and histiocytic lymphomas (58), rhabdomyosarcoma (1, 19), stomach carcinoma (9, 49), osteosarcoma (1, 31), hepatoma (41, 52, 54), hibernoma (21), melanoma (3), retinoblastoma (64), and choroid plexus papilloma (7, 55). The wide spectrum of susceptible cell types could mean that T antigen acts through targeting proteins common to all these cell types. Alternatively, the subset of T-antigen functions, and the corresponding target proteins, could differ depending on the cell type. T antigen encodes multiple transformation-related activities (43), including complex formation with at least two cellular tumor suppressor proteins, pRB (16) and p53 (33, 35). Our ultimate goal using transgenic mice is to determine the role of each T-antigen subactivity in the tumorigenesis of a variety of cell types. A necessary first step, however, is to establish the effects of the complete protein on a particular tissue. Because previous studies raised questions about whether wild-type T antigen is sufficient to induce proliferative abnormalities (see references 25 and 26 for a review), this report begins by addressing that issue.

We focused here on the response of the choroid plexus epithelium (CPE) to large T antigen. The CPE, located in the ventricles of the mammalian brain, forms the major component of the blood-cerebrospinal fluid barrier (45, 56). Normally, this tissue is differentiated by embryonic day 15 in the mouse (57), and autoradiographic studies in rats indicate that

most CPE cells stop dividing by 2 weeks after birth (38). When the T-antigen gene is present in the mouse germ line, tumors of the CPE develop in most founder animals at 2 to 6 months of age (7, 55). Within lines, penetrance of this phenotype is 100%, and the timing of tumor development is remarkably consistent (7, 62, 63). Since the CPE tumors develop focally and low levels of T antigen can be detected in apparently normal CPE (42, 63), previous reports suggested that T antigen is not sufficient to produce the tumors (42, 63).

Our studies in transgenic mice with the early region of a related simian virus, the lymphotropic papovavirus (LPV), provided an approach for further examining the mechanism of T-antigen-induced tumorigenesis. Consistent with the tissue tropism of LPV (22, 30), the LPV early region transgene is expressed in lymphoid tissues where it induces B- and T-cell lymphomas and leukemias (11). In addition, these mice also develop neoplasia of the CPE (11). Unlike the tumors induced by SV40 T antigen, the LPV-induced tumors appear to involve overall expansion of CPE and arise early in life (usually by 26 to 42 days [11]). In the present study, we determined the basis for this difference in CPE tumor development by examining the activity of hybrid transgenes that carry the transcriptional signals from one virus and the T-antigen-coding region from the other.

MATERIALS AND METHODS

Plasmid constructions. To construct pLST (containing the LPV control region and the SV40 T-antigen gene), we isolated the LPV (LO2 strain) *ScaI*-to-*NcoI* control region fragment from low-melting-point agarose (SeaKem) as described previously (11). The start codon contained within the *NcoI* site was removed with mung bean nuclease (New England BioLabs), and the fragment was inserted into the pGEM-3 blue vector (Promega) at the repaired *SacI* site. The junction between the modified *NcoI* terminus and the vector

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was sequenced by the method of Sanger et al. (53), using a primer complementary to the Sp6 promoter in pGEM-3 blue. The *EcoRI*-to-*KpnI* fragment was then removed and inserted into a plasmid (pSVT) which contained the T-antigen-coding region of pSV11 (14). This gene carries a deletion of the splice donor site for the small-t-antigen transcript and thus cannot produce a small t antigen. Plasmid pSLT (containing the SV40 control region and the LPV T antigen) was constructed from plasmids pSVE and pELPV. Plasmid pSVE was generated by inserting the *HindIII*-to-*Asp718* fragment of SV40, which harbors the control region, into *HindIII*- and *Asp718*-digested pGEM-3 blue. The *NcoI*-to-*EcoRI* LPV T-antigen-coding region fragment was isolated from pELPV (11). The termini were converted to blunt ends with Klenow enzyme, and the fragment was cloned into the *StuI* site of pSVE. The *StuI*-*NcoI* junction was sequenced by using a primer which hybridized in the SV40 control region to ensure retention of the start codon for LPV T antigen.

Production and screening of transgenic mice. To generate the LST, SLT, and SV40 transgenic mice, we isolated the *BamHI*-to-*EcoRI* fragment, the *EcoRI* fragment, and the *BamHI*-to-*KpnI* fragment of pLST, pSLT, and pSVE1, respectively, as described previously (11). The final DNA concentrations were adjusted to 4 ng/ μ l. Single-cell embryos were obtained from a cross between B6D2F1 (C57B16/J $\times$ DBA2; Jackson Laboratory) mice, and transgenic mice were generated as described previously (27). Positive animals were detected by dot hybridization analyses as previously described (11). Southern blotting analysis was performed to assess the configuration and integrity of the transgenes.

Immunohistochemistry. Tissues used for immunohistochemistry were frozen in liquid nitrogen and stored at -70°C . Sections (8 μ m) were thaw mounted on polylysine-treated slides, air dried for 20 min at 25°C , fixed in 2% paraformaldehyde in TS buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl) for 40 min, and treated with 0.1% H_2SO_4 (vol/vol)-0.2% KMnO_4 (wt/vol) for 15 to 20 s. After being washed for 2 min in H_2O and for 10 min in TS, the sections were stained by the Vectastain ABC method according to instructions supplied by Vector Laboratories. The primary antibody was a tissue culture supernatant containing PAb101, a monoclonal antibody specific to SV40 T antigen (23). The secondary antibody was biotinylated horse anti-mouse immunoglobulin G (IgG). Immune complexes were detected by incubation with horseradish peroxidase-avidin complex (Vector) and diaminobenzidine substrate.

Immunofluorescence. Frozen sections (8 μ m) were thawed and fixed in 1% paraformaldehyde for 1.5 min and treated with 100% methanol at 0°C for 10 min and 0.1% Nonidet P-40 at 0°C for 5 min. Sections were treated with 10% normal goat serum for 20 min and then incubated for 1 h with a 1:100 dilution of mouse anti-rabbit proliferating cell nuclear antigen (PCNA) monoclonal antibody 19A2 (46) (Coulta). Anti-PCNA antibody was detected by incubation with a 1:100 dilution of goat anti-mouse IgM and IgG antibody (Boehringer Mannheim Biochemicals). T antigen was then detected with PAb101 hybridoma supernatant and rhodamine-conjugated goat anti-mouse IgG antibody. Sections were washed three times for 3 min each with TS buffer between each incubation. All solutions were made in TS, and antibodies were diluted in 10% normal goat serum. Sections were photographed with fluorescein isothiocyanate and rhodamine filters. Control experiments which detected PCNA and T antigen separately indicated that the double-labeling procedure accurately identified cells expressing each antigen.

In situ hybridization. The procedure for in situ hybridization to transgenes was modified from that of Lo (36). Probe specific for the LST transgene was synthesized at 37°C for 4 h in a 30- μ l reaction mixture containing 200 ng of template, 2 μ g of random hexanucleotide primer, 0.1 mM each dCTP, dATP, and dGTP, 0.07 mM dTTP, 0.03 mM digoxigenin-11-dUTP (Boehringer Mannheim), 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 1 mM dithiothreitol, and 50 μ g of bovine serum albumin per ml. The LST fragment used for microinjection was the template. Frozen brain sections were fixed for 10 min with 5% paraformaldehyde, treated with 0.2 N HCl for 20 min, and incubated with $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min at 70°C , with 10 μ g of proteinase K per ml in 0.5 M EDTA (pH 8.0) for 15 min at 37°C , and then with 0.2% glycine for 10 min at 25°C . Sections were incubated in 0.5 mg of RNase A per ml for 30 min at 37°C and then in 5% paraformaldehyde for 2 h at 25°C . They were then dehydrated through graded ethanols and air dried. Sections were treated with 95% deionized formamide in $0.1\times$ SSC for 15 min at 70°C and then rinsed in $0.1\times$ SSC at 0°C for 2 min and then with H_2O . After dehydrating and air drying, 30 μ l of a solution containing denatured DNA probe (0.05 ng/ μ l) in 45% formamide- $2\times$ SSC-5 $\times$ Denhardt's solution-40 μ g of salmon sperm DNA per ml-10% dextran sulfate was added to each slide, covered with a coverslip, and sealed with rubber cement. Hybridization was done for 16 h in a 37 to 40°C humidified chamber. Sections were washed three times for 10 min each time with 45% formamide in $2\times$ SSC at 42°C and twice for 15 min each time with $0.2\times$ SSC at 50°C . They were then treated with 10% normal goat serum in $4\times$ SSC for 20 min and incubated for 1 h with a 1:10,000 dilution of mouse antidigoxin antibody (Sigma) in $4\times$ SSC-0.1% Tween 20. The antibody-probe complexes were detected after incubating for 1 h with a 1:100 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody (Boehringer Mannheim). Nuclei were counterstained by incubating for 3 min with 0.1 μ g of propidium iodide per ml. All incubations were performed at 25°C unless otherwise specified. Photography was performed with a fluorescein isothiocyanate filter.

RESULTS

Transgenic founder mice. The hybrid genes, LST and SLT (Fig. 1), were introduced into B6D2F2 mice by pronuclear microinjection. LST contains the enhancers and early promoter of LPV driving the coding sequence for SV40 T antigen. This construct cannot express small t antigen because of a deletion of the splice donor site (Fig. 1; see Materials and Methods). Conversely, SLT contains the SV40 enhancers and early promoter driving the LPV T-antigen-coding region (Fig. 1). Eleven founder mice (15% of the mice born) were produced with LST, and 12 (12%) were produced with SLT. Because the transgenes used in previous studies on the SV40 early region were linked to prokaryotic vector sequences (7, 55) which sometimes interfere with expression in transgenic mice (29, 50), 13 additional founder mice were produced using a vector-free fragment (*KpnI* to *BamHI*) of the SV40 early region. Eleven founder mice which harbored the intact LPV early region were generated in a previous study (11). The integrated transgenes yielded predicted restriction fragments in Southern analyses, with the exception of two LPV mice in which rearrangements were detected (11). Copy numbers ranged from 1 to 50, and multiple copies were integrated in a head-to-tail configuration (data not shown).

FIG. 1. Constructs used to generate transgenic mice. The early regions of LPV (open boxes) and SV40 (stippled boxes) are diagrammed, with the control regions designated by the larger boxes and the coding regions designated by narrow boxes. The deletion of the SV40 small-t-antigen splice donor site is indicated. The origin of sequences in the hybrid genes LST (LPV enhancer-promoter, SV40 T-antigen-coding region) and SLT (SV40 enhancer-promoter, LPV T-antigen-coding region) is indicated. Restriction sites in parentheses were used to generate the hybrid constructs and are no longer present in the transgenes. nt, nucleotides.

SV40 and LPV T antigens, linked to the same regulatory elements, induce similar phenotypes. The abnormalities which developed in founder LPV, SV40, LST, and SLT mice are summarized in Fig. 2. Ten of the 13 SV40 mice developed proliferative disorders. As expected, most (nine) developed CPE tumors. Thymic hyperplasia and renal lesions, which were also observed in earlier studies (7), were present in some of these mice (Fig. 2). Thus, the removal of

plasmid sequences did not cause obvious changes in the types of abnormalities produced. One animal developed histiocytic lymphoma, a phenotype which also appeared in two SLT mice (Fig. 2). The three SV40 mice that did not develop abnormalities did not express detectable levels of the transgene in several tissues examined (data not shown). As with the SV40 mice, the majority of mice harboring the LPV early region developed CPE tumors (Fig. 2) (11). Unlike in the SV40 mice, B- and T-cell proliferative disorders (including lymphoma and leukemia) developed in several LPV mice (11). Likewise, the thymic hyperplasia and kidney pathologies common to SV40 mice were not observed in LPV mice (Fig. 2).

In general, founder mice harboring the hybrid transgenes developed abnormalities consistent with the control region of each virus. Nine of the 12 LST founders developed CPE tumors, and the timing of these tumors (discussed further below) was characteristic of that observed with LPV (Fig. 2). Although overt lymphomas were not observed in the LST mice, lymphoid tissues densely populated with T-antigen-positive lymphocytes were often enlarged (data not shown), as was observed in many LPV mice (11). Analysis of LST expression by immunoblotting and immunohistochemical staining demonstrated the same tissue restriction observed with LPV (11); i.e., expression was generally limited to the CPE tumors and lymphoid tissues (data not shown). Immunohistochemical staining showed strong nuclear staining of all CPE tumor cells (see below) and of a subpopulation of lymphoid cells. The density of T-antigen-positive cells in spleens and thymuses varied from a few percent in young mice to dense staining in older mice (data not shown).

All nine SLT founder mice developed proliferative disorders (Fig. 2). Of these, eight mice developed abnormalities of the types observed in SV40 mice. Four mice developed CPE tumors. As with SV40, SLT mice often developed thymic hyperplasia and polycystic kidneys. Two mice developed histiocytic lymphomas. In contrast with the other mice in this study, two SLT mice developed osteosarcoma (Fig. 2), and liver tumors were observed in the two mice that survived to old age (>300 days). SLT transcripts, detected by RNase protection analyses (11), were abundant in affected tissues, and low transcript levels were often detected in tissues with no apparent pathological abnormalities such as

Transgene		LPV											LST											SV40											SLT											
Founder #		1	2	3	4	5	6	7	8	9	10	11	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	13	1	2	3	4	5	6	7	8	9
Age at Death(days)		26	27	36	36	39	42	85	87	200	210	210	24	42	46	48	51	53	82	85	120	210	420	420	65	70	70	75	90	120	130	140	200	210	420	420	70	120	130	130	150	180	190	380	390	
Affected tissues	CP	+	+	+	+	+	+	+	+				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	Lym								++																																					
	Hist																																													
	Kid																									++	+	+	+																	
	Thy																									+	+	+																		
	Bone																																													
	Liv																																												++	

FIG. 2. Pathological changes induced by LPV, SV40, and hybrid transgenes LST and SLT. Mice were observed for up to 18 months; abnormalities (indicated by +) were detected grossly and analyzed by histological methods. A blank space indicates apparently normal tissue. CP, choroid plexus tumor; Lym, B- or T-cell lymphoma; Hist, histiocytic lymphoma; Kid, polycystic kidney; Thy, thymic hyperplasia; Bone, osteosarcoma; Liv, liver tumor.

FIG. 3. Timing of choroid plexus tumor development in transgenic mice. The percentage of choroid plexus tumor-free founder mice harboring the transgenes diagrammed in Fig. 1 is plotted as a function of age. Each point represents one animal that developed choroid plexus tumors and was sacrificed near death. An enlarged cranium was observed several days before this time. Mice with other types of abnormalities which did not develop choroid plexus tumors are considered part of the tumor-free group.

spleen, lymph nodes, lung, testes, and heart (data not shown).

Timing of choroid plexus tumor development depends on the regulatory element. The primary goal of this study was to determine why LPV and SV40 differ in the efficiency with which they induce CPE tumors. The question is whether the T antigens are different per se or whether the regulatory regions of the two viruses dictate differences in the levels, the kinetics, or the distribution of expression. The LPV early region induced CPE tumors at a young age, with most mice dying at 26 to 42 days of age, and tumors induced by SV40 appeared later, at 65 to 210 days (Fig. 3). When the SV40 T antigen was driven by the LPV control region (LST mice), however, the tumors arose in most mice at the early times (24 to 53 days) characteristic of the LPV phenotype (Fig. 3). Likewise, SLT animals (SV40 control, LPV T antigen) developed CPE tumors at later times, with the earliest tumor arising at 70 days. The timing of these tumors varied to a greater extent, and fewer of these mice (four of nine) developed CPE tumors.

As a first step in correlating expression of the SV40 transgene with pathogenesis, we analyzed the established SV11 transgenic line, in which focal CPE tumors develop predictably around 100 days of age (62, 63). SV40 T antigen was measured *in situ* in the brains of SV11 mice at different times from 1 to 100 days after birth (Fig. 4I). T antigen was first detected at postnatal days 12 (data not shown) and 20 (Fig. 4I-b) in scattered, morphologically normal CPE cells. By day 36, small abnormal foci of T-antigen-expressing cells were observed (data not shown); these subsequently increased in size (day 50, Fig. 4I-c). At 80 days, small, grossly visible tumors were observed that uniformly expressed T antigen (Fig. 4I-d), and by day 100 (data not shown), the tumors distorted the ventricles, resulting in visible enlargement of the cranium. At these later times, CPE with normal morphology showed the same scattered expression of T antigen detected at earlier times.

To determine whether sporadic expression of T antigen in the CPE was an invariant characteristic of SV40 regulation, eight additional founder SV40 mice were generated and analyzed (Fig. 4II). Expression was again detected in scattered CPE cells at days 1, 20, and 40, although the number of cells expressing T antigen was higher in the founder animals killed at birth than in comparable SV11 mice (Fig. 4II-a). (In

one newborn mouse, the density of T-antigen-positive cells in the CPE was high [30 to 60%; data not shown].) Neither mouse killed at 60 days showed expression of T antigen and presumably represent animals in which the integration site or transgene rearrangement precluded expression. Since each SV40 founder mouse in this experiment was sacrificed prior to tumor development and was independently derived, the exact timing of tumorigenesis could not be deduced. The experiment, however, does confirm the usually focal character of T-antigen expression induced by SV40.

The LST mice that developed tumors early died before reproductive age, and a line representative of that phenotype was not established. Instead, 32 additional LST founder mice were generated, and 2 or 3 mice were analyzed at specific ages between 1 and 60 days (Fig. 4III). In the majority of animals (19 [59%]), high levels of T antigen were detected in all cells of the CPE examined. Uniform expression was observed even on the day of birth (Fig. 4III-a). From day 9 to day 13 after birth, the nuclei of the CPE appeared enlarged and dense with little cytoplasm compared with normal CPE, and strong staining for T antigen appeared in every nucleus (Fig. 4III-b). From day 17 to day 21, obvious expansion of the CPE was observed, with strong uniform staining of T antigen in all nuclei (Fig. 4III-c). At these times, the ventricles were visibly filled with CPE tissue and were distended. At day 24 to day 30, the mice were overtly ill, the cranium was enlarged, and sections of the brain showed massive expansion of the CPE with uniform strong staining of T antigen (Fig. 4III-d). Of the 32 mice analyzed, only 5 showed a restricted pattern of expression in which T antigen was evident in only 10 to 90% of the CPE cells (2 of these mice were mosaic, see below); 8 had no detectable expression in the CPE; the rest were as shown in Fig. 4.

In contrast with the aforementioned group, a few LST mice from the initial experiment (Fig. 3) died at times more consistent with the SV40 phenotype. When the offspring from one such mouse were examined for CPE expression of T antigen, the results were similar to those obtained with the SV11 line (see above). Thus, the timing of CPE tumor development appears to correlate with the number of cells expressing high levels of T antigen and/or the timing of expression onset. Most of the mice with SV40 T antigen under LPV control (LST) expressed T antigen in all cells from birth and developed tumors rapidly, while most of the SV40 mice expressed T antigen inefficiently and developed tumors at later times.

SV40 T-antigen expression correlates with cell autonomous proliferation. If T antigen is sufficient to induce CPE tumors, one would expect a large fraction of T-antigen-expressing cells to also express S-phase markers. Thus, we analyzed the CPE of mosaic LST mice for the simultaneous expression of PCNA and T antigen. PCNA is a processivity factor for polymerase delta (6) and, under the conditions used, is only detectable in the cell nucleus during G₁ to the late S phase; it is not detectable in nonproliferating cells (5, 32, 44, 61). In an unsynchronized population of proliferating cultured cells, approximately 45% express PCNA (4). Figure 5 shows the correlation between the presence of T antigen, the morphological changes associated with its presence, and the expression of PCNA. The mosaicism of these mice permitted the analysis of nontransgenic CPE cells in the same tissue sections.

Genetic mosaicism for the transgene was demonstrated by *in situ* hybridization (Fig. 5A). Two populations of morphologically different cells were present in the CPE, one which

FIG. 4. T-antigen expression and tumor development in the choroid plexus of SV40 and LST mice. SV40 T antigen was detected in frozen sections with PAb101 and immunoperoxidase staining (brown); sections were counterstained with Giemsa (blue). Column I, T-antigen expression in offspring of the SV11 line; the data show scattered expression and focal development of tumors. Arrowheads indicate representative cells or regions expressing T antigen. Column II, T-antigen expression in founder SV40 mice. Obvious abnormalities were not observed in the choroid plexus of these mice. Column III, T-antigen expression and tumor development in founder LST mice. T antigen was expressed in all choroid plexus cells. Magnification was $\times 450$ except in panels I-d, III-c, and III-d, where it was $\times 50$. Numbers in parentheses indicate the age (days after birth) at which the animal was sacrificed. At least two animals were examined at each time.

appeared normal (arrowhead) and one in which the cells were densely crowded, having little cytoplasm compared with the normal cells (Fig. 5B). Immunohistochemical staining for SV40 T antigen detected expression only in the abnormal subset of cells (Fig. 5C). Moreover, approximately 30% of the T-antigen-positive cells, randomly distributed, also stained for PCNA (Fig. 5D). None of the cells with normal morphology showed expression of either protein. Analysis of late-stage LST tumors showed a similar percentage of randomly distributed PCNA-positive cells among the T-antigen-expressing cells (data not shown). In SV40 brain sections that display sporadic T-antigen expression, approximately 10% of the T-antigen-positive cells were also posi-

tive for PCNA, whereas it was absent from T-antigen-negative cells. PCNA-positive cells were not observed in CPE cells of normal mice at similar ages (data not shown).

DISCUSSION

Similarity of LPV and SV40 T antigens. Examination of each T antigen under the regulation of the heterologous control region showed that these proteins have a similar capacity to induce proliferative effects in several tissues. They are also indistinguishable in the timing and morphological character of CPE tumors they induce. In these experiments, it was the control region, dictating the place, level,

FIG. 5. Relationship between SV40 T-antigen expression and cell proliferation in the choroid plexus. (A) DNA in situ hybridization to the LST transgene (yellow dots) demonstrating the genetic mosaicism of this mouse. Nuclei were stained with propidium iodide (red; $\times 700$). (B) Hematoxylin and eosin staining of a paraffin-embedded section showing the hyperplastic morphological change in a distinct fraction of cells compared with adjacent normal choroid plexus ($\times 450$). (C) Immunoperoxidase staining of SV40 T antigen as described in the legend to Fig. 4 ($\times 450$). (D) Detection by immunofluorescence of T antigen (rhodamine; red) and PCNA (fluorescein; green). Yellow nuclei indicate the presence of both PCNA and T antigen ($\times 700$). Normal choroid plexus shows no staining for either protein. Arrowheads in all panels indicate the normal nontransgenic choroid plexus cells. Note the correlation between the presence of the transgene (A), hyperplastic morphology (B), T-antigen expression (C), and PCNA expression (D). Two mosaic mice were analyzed with similar results; only one is shown here.

and/or time of expression, that determined the pathological abnormalities produced. In the CPE, expression of T antigen from the SV40 signals was consistently low and scattered, while that from LPV was generally high and uniform. While the founder SV40 animals developed focal tumors and died between 65 and 210 days of age, most of the LST mice (harboring the same T-antigen gene but under LPV regulation) died between 24 and 53 days of age after uniform CPE enlargement (Fig. 4).

In addition to directing expression to the CPE, each control region also exhibited distinct specificities. Additional expression of either T antigen from the LPV control region was usually restricted to the lymphoid cells. While overt lymphoma did not develop in the LST (LPV control, SV40 T antigen) animals, expansion of the lymphoid cell population was observed in spleen and thymus as in young LPV mice (11). In other studies, the SV40 early genes induced B-cell lymphoma when regulated by Ig (58) or mouse mammary tumor virus (13) transcriptional signals. Lymphoma was probably not observed in the present study because these tumors generally appear at 3 to 4 months of age, after the time that most LST mice had already succumbed to CPE tumors.

Under SV40 control, both T antigens affected a wider range of tissues. In addition to CPE tumors, cortical cysts in the kidney and thymic hyperplasia developed frequently.

Histiocytic lymphoma was also induced in a small number of mice harboring either T antigen under SV40 control. Osteosarcoma and hepatocellular carcinoma developed occasionally when the LPV early genes, but not SV40 T antigen, were under SV40 control. It is unlikely that these tumor types reflect differences in T-antigen targets, however, because the SV40 early region genes induce hepatocellular carcinoma (41, 52, 54) and osteosarcoma (31) when under the control of the appropriate regulatory signals. Rare development of these two tumor types in SLT mice may indicate that novel expression was sometimes generated by the hybrid gene sequences or that the production of these tumor types depends on the activity of small t antigen or both. The capacity to express the SV40 small t gene was removed from SV40 and LST transgenes, while the putative LPV small t-coding sequences remained intact in LPV and SLT genes. SV40 small t antigen appears to be important for large-T-antigen-induced lung and kidney adenocarcinomas (13), but its effect in liver and bone has not been explored. Small t antigen is not required for CPE tumorigenesis (51 and this study).

A prediction of the observation that SV40 and LPV large T antigens elicit similar effects in many cell types is that they harbor common biochemical activities responsible for producing the phenotypes. Some of the known cellular protein-binding activities of SV40 T antigen have also been demon-

strated in the LPV protein. In cultured mouse cells, LPV T antigen complexes with and stabilizes p53 (59), and in vitro assays demonstrate the ability of LPV T antigen to form a complex with pRB (17). By comparison, then, both binding activities remain candidates for important steps in the production of phenotypes observed here. We have recently further examined the role of these activities in CPE tumorigenesis using mutant forms of SV40 T antigen in transgenic mice (10).

Sufficiency of T antigen for CPE neoplasia. Previous studies postulated that T antigen may not be sufficient to cause CPE tumors since SV40-induced tumors develop focally (42, 63). Messing et al. (42) also showed that, in several families of SV40 transgenic mice, T-antigen expression was limited to a subset of CPE cells (42). Since T antigen was present in many cells that appeared normal, it was possible that further cellular alterations were required before tumors could develop. Yet, the predictable timing and complete penetrance of tumors within a given line of mice (7, 63) argue against the participation of a random second event. Our present studies indicate that nonuniform expression and/or the times of expression onset in these SV40 animals is the reason for focal tumor development and that additional events unrelated to T-antigen expression are not involved. When expression was directed to the majority of CPE cells by the LPV control region, uniform nonfocal expansion of the CPE occurred. Under these conditions, the T-antigen-expressing cells in both end-stage tumors and CPE of young mice also appeared to be proliferating based on the detection of the S-phase marker PCNA.

These results could indicate that transformation of the CPE requires a critical level of T antigen which may not have been sustained in most cells by the SV40 regulatory signals. A requirement for threshold levels of T antigen has also been suggested in pancreatic beta-cell tumor induction in which families of mice expressing different levels of T antigen develop tumors at different times with different frequencies (18). A correlation between the overall amount of T-antigen expression and the time of CPE tumorigenesis was also suggested in a study in which mice of the SV11 family were backcrossed to different strains of nontransgenic mice (12). When the genetic background changed, both the time at which T antigen could be detected in brain extracts and the time of tumorigenesis were affected, with lower expression levels correlating to later tumor development (12).

Transgenic studies of other tumor types have also tentatively concluded that the SV40 genes are not sufficient to induce the tumors. As for SV40-induced CPE tumors, this suggestion has often stemmed from the observation of focal tumors. The expression of T antigen together with the onset of histopathological changes have been examined in some of these systems (24, 42, 47, 48, 54), and T-antigen expression was often shown to be nonuniform (42, 48, 54). Based on our results, further analysis is required to determine whether insufficiency resulted from inadequate expression of T antigen or from the inability of T antigen to elicit a response without additional events. In pancreatic beta cells, uniform expression of T antigen is not sufficient for the immediate induction of proliferation, but rather requires several weeks before abnormal proliferation begins (60). Compared with our study of the CPE, this result indicates an interesting difference in the ability of some distinct cell types to respond to T antigen. Other testable possibilities include differences in T-antigen levels or in the state of cellular differentiation at the onset of T-antigen expression. Additional detailed studies in a broader range of cell types and the determination of

the cellular targets for T antigen in each case should help to determine the reason for these potential differences.

Although T antigen appears to be sufficient to perturb the growth properties of CPE cells, initiating the early stages of tumor progression, it is not sufficient for progression to malignancy in the CPE or in other cell types so far examined (20, 25, 26). The CPE tumors induced by T antigen are benign and appear to be well differentiated. They express several normal CPE markers (39, 65) and maintain normal ploidy (42). Furthermore, rare metastasis of these tumors has been confined to the central nervous system (42, 63).

In summary, we characterized an in vivo system in which SV40 T antigen alone appears to induce abnormal proliferation in a normally nondividing cell type, the CPE. Our studies have also demonstrated that the action of T antigen on the CPE is completely cell autonomous, indicating that cellular targets of T antigen have intracellular activities. The studies described here provide a framework for the elucidation of T antigen subactivities that participate in tumorigenesis and are the first step in identifying these targets within a given cell type. Using the CPE system described here to analyze mutant forms of T antigen, we have begun such a study (10). In the future, it will be useful to probe the mechanisms of normal cellular growth control employed by many different cell types within the animal using a similar approach.

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