Lymphotropic Papovavirus Early Region Is Specifically Regulated in Transgenic Mice and Efficiently Induces Neoplasia

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Transgenic mice have been generated which carry the early region of lymphotropic papovavirus (LPV). Eight of eleven founder animals died before 3 months of age after developing one or both of two distinct proliferative disorders. Of the three surviving animals, two are known to have rearranged or partial copies of the LPV genes. The majority of the founder animals (six) developed debilitating choroid plexus tumors by 26 to 42 days. Although this is the same tumor type induced by the simian virus 40 T-antigen gene, those induced by LPV appeared at a much younger age. The LPV early region was expressed in the brain tumors of these mice, as well as in the thymus and spleen. Expression in the latter two tissues reflects the cell-type specificity of the LPV enhancer demonstrated in cultured cells (i.e., lymphoid cells). Two founder animals (LP41 and LP50) gave rise to lines of mice that routinely develop lymphoproliferative disorders. LP50 and its LPV-positive offspring developed aggressive lymphomas and choroid plexus tumors. The transgenic offspring of LP41 also developed lymphomas. High levels of LPV RNA were expressed in the lymphomas of these mice as well as in the spleens and thymuses. The origin of the lymphomas from B- and T-cell lineages suggests that the LPV early genes are expressed in and can transform both of these cell types *in vivo*.

The early regions of a number of primate polyomaviruses have been introduced into the mouse germ line with the result that they elicit cell-type specific neoplasms. Animals carrying the simian virus 40 (SV40) T-antigen gene develop tumors in the choroid plexus (7, 39, 45), a tissue that plays a major role in regulating the composition of the cerebral spinal fluid. The early regions of two related human viruses, BK and JC, induced tumors in cell types that reflect their natural viral tropisms; BK virus induced hepatocellular carcinoma and renal tumors, and JC virus caused neuroblastomas (40).

Expression of SV40 T antigen in the transgenic mouse is controlled at the level of transcription in a cell-type specific fashion. Replacement of part or all of the SV40 transcriptional signals with those of other cell-type-regulated genes changes the target for transformation in the mouse (17, 15, 27, 22, 34). It is likely that transcriptional control also plays an important role in the cell-type specificity of other primate polyomaviruses such as BK and JC viruses.

Transgenic mouse models can potentially be used to analyze the mechanisms of transcriptional regulation of these viral genes in the whole animal, the determinants of tumor tropism, and the similarities and differences among T antigens from various viruses with respect to *in vivo* transformation. With this in mind, we have begun to analyze the expression and tumor-inducing capabilities of T antigen from a simian B-lymphotropic papovavirus (LPV).

LPV was originally isolated from African green monkey B lymphoblasts (47) and was shown to have a very limited host range and cell-type specificity. Growth of the wild-type virus is restricted to certain human and monkey B cells (5, 6, 43, 48), and mutations in the VP1 gene enable the virus to grow in T-lymphoblastoid cell lines (20, 21). This suggests that the early genes in the wild-type virus can be expressed in both B

and T cells. Indeed, when a variety of cells in culture were transfected with constructs in which part of the LPV control region was linked to a chloramphenicol acetyltransferase (CAT) reporter gene, CAT activity was detected in lymphoid cells (including B and T cells) but not in the cells of epithelial or fibroblast origin that were examined (26, 31). Mosthaf et al. (26) demonstrated that the LPV sequence functioned as an enhancer in either orientation and at a distance. Therefore it appears that LPV early gene expression is regulated in a cell-type-specific manner by a transcriptional enhancer(s).

To test this notion in the whole animal and to determine the transforming capabilities of LPV T antigen, we inserted into the mouse genome the LPV early region with its own transcriptional signals. Here we report that the majority of founder transgenic animals developed choroid plexus tumors and expressed the LPV early region in those tumors as well as in the spleen and thymus. Two founder mice gave rise to lines of mice which develop lymphoproliferative disorders (leukemia and lymphoma) arising in cells of both the B and T lineage, and those neoplasms also contained high levels of LPV T-antigen RNA.

MATERIALS AND METHODS

Plasmids. Plasmid pLO2 (kindly provided by J. Pipas) carries an infectious copy of LPV (LO2 strain) viral DNA linked at the *Bam*HI site in the early region to pBR322 (43). To reconstruct the early region, pLO2 DNA was digested with *Bam*HI and the LPV insert was separated from the pBR322 vector by electrophoresis in low-melting-point (LMP) agarose (Bethesda Research Laboratories, Inc.). The fragment was purified by NACS (Bethesda Research Laboratories) column chromatography as suggested by the supplier. The LPV fragment was concatemerized by using T4 DNA ligase and was then digested with *Stu*I. The reconstructed early region *Stu*I fragment was isolated by using LMP agarose and NACS chromatography and was cloned into the *Sma*I site of pGEM-3 blue (Promega Biotec) to generate pELPV.

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Plasmid pLPV5' was constructed for the purpose of generating an antisense RNA probe. pELPV DNA was digested to completion with *ScaI* and *BamHI*, and the 510-base-pair (bp) fragment encoding the 5' end of LPV early RNA was isolated by using LMP agarose and NACS chromatography. This fragment was ligated to *SmaI*- and *BamHI*-digested pGEM-3 blue. Plasmid pK3' carries a fragment which encodes the 3' end of the mouse immunoglobulin kappa light chain RNA. To generate pK3', the 1-kilobase-pair *BclI* fragment from pDM-1 (kindly supplied by C. Milcarek) was subcloned into the *BamHI* site of pGEM-3 blue. Standard techniques were used for subcloning DNA fragments and are described by Maniatis et al. (23).

Production and screening of transgenic mice. Plasmid pELPV was digested with *Sall* and *EcoRI*, and the 3.8-kilobase-pair LPV fragment was isolated by electrophoresis in LMP agarose and NACS chromatography. Before microinjection, the fragment was dialyzed against injection buffer (5 mM Tris hydrochloride [pH 7.4], 5 mM NaCl, 0.1 mM EDTA), using a Millipore VM 0.05- μ m (pore size) filter. The DNA concentration was estimated by comparison with standards of known concentration after ethidium bromide staining of agarose gels and was then diluted with injection buffer to an approximate concentration of 2 ng/ μ l. About 2 μ l of the DNA solution was microinjected into one of the two pronuclei of fertilized mouse eggs that were obtained from a cross between B6D2F₁ (C57B16/J $\times$ DBA2; The Jackson Laboratory) parents. The same-day embryos that survived injection were implanted into the oviducts of Swiss Webster (Taconic) pseudopregnant recipients as described by Hogan et al. (18). Transgenic pups were identified after 5 μ g of tail DNA was spotted onto GeneScreen Plus filters (New England Nuclear Corp.) and hybridized with an LPV-specific probe as described below.

DNA isolation and Southern hybridization. To obtain DNA from the tail, approximately 0.25 in. (0.64 cm) of tail was incubated in 0.5 ml of 50 mM Tris hydrochloride (pH 8.0)–100 mM EDTA–100 mM NaCl–0.5 mg of proteinase K per ml at 55°C overnight. After the samples were vortexed, they were extracted twice with phenol and once with a mixture of chloroform and isoamyl alcohol (24:1) and the DNA was precipitated with 70% ethanol. DNA from other tissues was isolated in a similar fashion after 0.5 g of tissue was homogenized in 2 ml of isotonic buffer (150 mM NaCl, 10 mM Tris hydrochloride [pH 7.5], 1.5 mM MgCl₂) and after the sample was diluted with 3 ml 10 mM EDTA–2% sodium dodecyl sulfate. Proteinase K was added to a final concentration of 0.2 mg/ml.

DNA was analyzed for the presence of a normal transgene by the method of Southern (41), with the following modifications. Tissue DNA (5 μ g) was digested to completion with the appropriate restriction enzyme, and the samples were electrophoresed in 1% agarose gels in TAE (0.04 M Tris-acetate [pH 8.5], 0.001 M EDTA). After being stained with ethidium bromide, the gel was soaked for 30 min in 0.4 M NaOH–0.6 M NaCl and blotted overnight onto GeneScreen Plus filters (New England Nuclear) by capillary action, using 0.4 M NaOH–0.6 M NaCl. The filter was neutralized by soaking it for 20 min in 0.5 M Tris hydrochloride (pH 7.0)–1 M NaCl and was then air dried. Filters were prehybridized in 1% sodium dodecyl sulfate–1 M NaCl–10% dextran sulfate at 68°C for 30 min. The purified *Sall*-*EcoRI* LPV fragment, labeled with ³²P by the random priming method (14), was denatured, and 1 $\times$ 10⁵ to 5 $\times$ 10⁵ cpm/ml along with 0.1 mg of denatured salmon sperm DNA per ml was added to the prehybridization mixture. Hybridization was carried out at

68°C overnight followed by washing twice with 2 $\times$ SSPE (1 $\times$ SSPE = 0.15 M NaCl, 0.01 M NaH₂PO₄ [pH 7.4], 0.001 M EDTA) at room temperature, twice with 2 $\times$ SSPE–0.1% sodium dodecyl sulfate at 70°C, and once with 0.5 $\times$ SSPE at 70°C. All washes were for 15 to 30 min. The filter was exposed to Kodak XAR5 film for 1 to 4 days without an intensifying screen. Size markers were generated by end labeling *HindIII* fragments of lambda DNA, using Klenow polymerase and [α -³⁵S]dATP as described by Ausubel et al. (3).

RNA isolation and analysis. Total cellular RNA was isolated from tissues by using the guanidinium-cesium chloride method of Chirgwin et al. (9) as described by Maniatis et al. (23). ³²P-labeled RNA probes were synthesized by using the Riboprobe II system (Promega Biotec), following the method of Melton et al. (25). The pLPV5' plasmid was linearized with *SmaI*, and pK3' was linearized with *FokI*. Probes were generated by using the SP6 and T7 RNA polymerases, respectively.

For nuclease protection analysis, 4 $\times$ 10⁵ cpm of LPV and 4 $\times$ 10⁵ cpm of the control kappa probe were coprecipitated in 70% ethanol with 20 μ g of RNA from various tissues. The RNA pellet was suspended in 30 μ l of hybridization buffer {80% formamide, 40 mM PIPES [piperazine-*N*, *N'*-bis(2-ethanesulfonic acid)] [pH 6.4], 400 mM NaCl, 1 mM EDTA}, heated to 85°C for 10 min, and then incubated overnight at 60°C. Samples were chilled on ice, and 330 μ l of a solution containing 50 mM sodium acetate (pH 5.0), 100 mM NaCl, 2 mM EDTA, and 10 U of T2 RNase (Bethesda Research Laboratories) was added. After incubation at 35°C for 2 h, 10 μ g of carrier tRNA was added; the RNA was extracted once with phenol-chloroform and then precipitated in 70% ethanol. The pellet was suspended in 6 μ l of 98% formamide–1% bromophenol blue–1% xylene cyanol, heated at 100°C for 2 min, chilled on ice, and electrophoresed in a 5% polyacrylamide gel containing 7 M urea and TBE (0.1 M Tris hydrochloride, 0.083 M borate [pH 8.3], 0.001 M EDTA). The M13 DNA sequence ladder which served as a marker was synthesized as described by Sanger et al. (35). Autoradiography was as described above.

Histology and immunohistochemistry. Tissue submitted for routine microscopic examination was fixed in 10% formaldehyde–0.1% phosphate buffer and postfixed for 1 h in alcohol-zinc-Formalin. Blocks were embedded in paraffin (Tissue Prep; Fisher Scientific Co.), and 4- μ m sections were stained with hematoxylin and eosin.

For immunohistochemistry, the following antisera were diluted as indicated in parentheses with 0.1 M Tris hydrochloride (pH 7.6)–2% albumin: rat anti-mouse Thy-1 monoclonal antibody (1:30) (Hybritech); rat anti-mouse Lyt2 (1:20) (Becton Dickinson and Co.); goat anti-mouse immunoglobulin G (IgG), IgM, and IgA (1:50) (Cappel Laboratories); tissue culture supernatant containing rat anti-mouse B220 (1:10) or rat anti-mouse L3T4 (1:10); mouse anti-rat IgG (1:20) (Pel-Freez); peroxidase-anti-peroxidase rat antibody (1:50) (Pel-Freez); biotinylated rabbit anti-goat IgG (1:250) (Vector); and vectastain avidin-biotin complex (Vector). Hybridoma cell lines producing the monoclonal antibodies to B220 (RA3-3A1) (10) and L3T4 (GK1.5) (13) markers were obtained from the American Type Culture Collection, Rockville, Md.

Frozen sections (approximately 4 μ m thick) were prepared from tissue that was snap frozen in liquid nitrogen and then dipped in acetone and air dried. After overnight refrigeration, sections were fixed in acetone for 5 min, air dried, and placed in hypertonic Tris-saline for 5 min. Sections were

FIG. 1. The LPV early region in pELPV. Plasmid pELPV contains an intact early region from the LPV isolate, LO2. The transcript for large T antigen (T) and the putative transcript for small T antigen (t) are indicated above the linear map. Open boxes represent protein-coding regions based on the DNA sequence (16). LPV DNA is denoted as a hatched box, and pGEM-3 blue vector DNA (only partially shown) is represented as a line. The *SmaI* sites indicated in parenthesis denote the boundaries of the LPV sequence but are no longer present in the plasmid. The *SmaI*-to-*EcoRI* fragment was used for microinjection. The LPV control region is enlarged at the bottom to show the 60-bp repeated sequence present in LO2 and a single copy of the 63-bp unit which is repeated in another LPV strain, K38. Within these areas lie sequence elements that share homology with other known transcriptional signals. They are the viral enhancer core sequence (stippled box), G+C-rich boxes (triangles), and the purine-rich sequence with homology to the IgH enhancer (the Pu box; solid box). The indicated transcription start site is the major one used in transgenic mice in this study. Numbers above and below the diagram indicate the sizes (in base pairs) of the LPV DNAs.

incubated at room temperature for 1 h with antibodies Thy-1, Lyt2, IgG, IgM, and IgA, or L3T4. They were then washed with hypertonic Tris-saline followed by Tris-saline and were incubated for 20 min at room temperature with mouse anti-rat IgG or biotinylated rabbit anti-goat immunoglobulin. Washes were repeated, and sections were then incubated with rat peroxidase-anti-peroxidase complex or avidin-biotin complex for 20 min at room temperature. After additional washes, peroxidase was localized by incubation with 3,3'-diamino-benzidine solution activated by 0.03% hydrogen peroxide. Sections were washed once more and were counterstained with ethyl green before they were mounted or they were mounted without being counterstained, depending on the intensity of the immunostaining. Immunostaining with the anti-B220 antibody required repetition of the second and third antibody steps before application of activated diaminobenzidine solution. Tissue controls included spleen, thymus, and lymph node specimens from nontransgenic mice. Negative staining controls were obtained by omitting the primary antibody.

RESULTS

Generation of transgenic mice carrying the LPV early genes. The plasmid pELPV contains a *SmaI*-to-*EcoRI* fragment with the early genes and the control region of LPV and is missing most of the late region (Fig. 1). This fragment of pELPV was injected into fertilized mouse eggs. The 349 embryos were implanted into pseudopregnant foster mothers, and of the resulting 67 pups that were screened, 11 (16.4%) were transgenic.

To determine the integrity of the transgene in founder animals, Southern blotting analysis was performed on genomic DNA isolated from liver tissue (Fig. 2). Digestion of the DNA with a number of enzymes indicated that two mice, LP49 and LP51, carried only partial or rearranged copies of the LPV sequences (Fig. 2; data not shown). As expected, these animals did not develop a phenotype (Table 1) and were not examined further. The remaining transgenic mice carried apparently normal integrated copies of the LPV fragment. The LPV DNA was integrated at or near the ends

FIG. 2. Southern hybridization analysis of founder mice. DNA from liver was digested with *HindIII* (top) or with *HindIII* and *BlnI* (bottom) and analyzed with an LPV-specific probe. A map at the bottom shows the positions at which these enzymes cut and depicts a tandem head-to-tail arrangement of the LPV fragment integrated via the ends into cellular DNA. The sizes of expected LPV-specific fragments are indicated. End-labeled lambda *HindIII* fragments serve as markers (M).

TABLE 1. Summary of transgenic mice produced with the LPV early region

Founder mouse (sex) ^a	Transgene arrangement ^b	Copy no. ^c	F ₀		F ₁ and F ₂	
			Phenotype ^d	Age at death (days)	Phenotype ^e	Age at death (days)
LP43 (F)	Head to tail	5	CP	26		
LP26 (M)	Head to tail	10	CP, Sp, Th	27		
LP39 (M)	Colinear	0.4	CP	36		
LP55 (M)	Colinear	1	CP	36		
LP42 (M)	Head to tail	11	CP, Sp, Th	39		
LP44 (F)	Head to tail	45	CP, Sp	42		
LP41 (M)	Colinear	1 ^f	Kidney failure	85	CP (2/25)	125, 143
					Lym (22/25)	102-143
LP50 (F)	Colinear	1	CP, Lym	87	CP (13/13)	51-98
					Lym (4/13)	81-98
LP49 (M)	Abnormal	<1	Normal at 7 months		Normal	
LP51 (F)	Abnormal	<1	Normal at 7 months		Normal	
LP56 (F)	Colinear	1	Normal at 7 months			

^a F, Female; M, male.^b Determined by Southern blotting analysis (e.g., Fig. 2).^c Determined by densitometric tracing of an autoradiogram similar to the one shown in Fig. 2 (top).^d CP, choroid plexus tumor; Sp and Th, abnormalities detected in the spleen and thymus, respectively; Lym, lymphoma.^e Numbers in parenthesis indicate the number of mice with a particular neoplasm of the total examined. In the LP50 F₂ generation, five mice that were sacrificed before 2 months of age were not included.^f On the basis of Southern blotting analysis, LP41 contains a full copy of the LPV fragment in tandem with a small portion of a second copy.

in each case, since both the *Bam*HI site 12 bp from one terminus (data not shown) and the *Ban*I site 60 bp from the other end (Fig. 2) were retained. Four mice (LP26, LP42, LP43, and LP44) carried multiple copies of the LPV fragment, and in each case integration was in a tandem head-to-tail arrangement, as evidenced by the 2,670-bp junction fragment in the *Hind*III-digested samples (Fig. 2).

Five mice (LP39, LP41, LP50, LP55, and LP56) carried only a single complete copy of the LPV fragment (Fig. 2), as is indicated by the consistent presence of the LPV-specific internal fragments (1,120 bp from a *Hind*III digest and 1,120 bp plus 2,440 bp from digestion with *Hind*III and *Ban*I, Fig. 2) and the absence of fragments which would be produced by a tandem arrangement of multiple copies. Densitometric tracing and quantitation of the bands in an autoradiogram similar to the upper one in Fig. 2 confirmed this interpretation (Table 1). Mouse LP39 was apparently mosaic, because quantitation indicated the presence of only 0.4 copies (Table 1). Information on the chromosomal location of a transgene was obtained for founder mouse LP50. Upon breeding, analysis of the segregation of the single transgene in LP50 indicated that it is linked to the X chromosome (Fig. 3A). Table 1 presents a summary of the above information.

Pathological abnormalities in LPV founder mice. Eight of eleven transgenic mice that were created with the LPV fragment developed disease and were sacrificed for analysis. Six mice developed bulging crania within 26 to 42 days of life (Table 1), and tumors of the choroid plexus were identified grossly and histologically in each of them. Microscopically, there were malignant changes and an overall increase in the amount of choroid plexus tissue (Fig. 4A). Malignant areas were recognized as papillae lined by crowded cuboidal to low columnar cells with large, pleomorphic, hyperchromatic nuclei (Fig. 4B). Locally, the papillary architecture was replaced by solid proliferations of similar cells and frequent mitoses were observed.

Routine light microscopic examination of multiple organs from five of these mice revealed that additional abnormal findings were limited to spleen and thymus. Splenomegaly was present in three mice (LP26, LP42, and LP44). Histologically, expansion of the lymphocyte population, particu-

larly involving the red pulp but without clear lymphomatous transformation, was observed in two mice (LP26 and LP42). The thymus in LP42 was enlarged, with medullary expansion by a polymorphous population of cells.

The above-mentioned six mice died before sexual maturation, precluding the establishment of lines. Two other mice, LP50 and LP41, lived for 87 and 85 days, respectively, and lineages were established from each of them (Fig. 3). At autopsy, mouse LP50 had massive lymphadenopathy involving the abdominal and peripheral lymph nodes. The thymus and spleen were enlarged, the latter being 20 times the mass of a spleen from a nontransgenic littermate. Microscopic examination showed malignant lymphoma involving lymph nodes (Fig. 4D), spleen (Fig. 4C), and thymus, with infiltration of the lungs (Fig. 4E), liver (Fig. 4F), and kidneys. Gross examination also showed the presence of a choroid plexus tumor.

LP41 died from renal failure, and findings at autopsy included anasarca and renal and thymic atrophy. Histological examination of the kidneys revealed diffuse glomerulopathy, consisting of nodular hyalinization of glomeruli. Although the thymus was small, secondary to complete cortical atrophy, the medulla was expanded. Increased numbers of lymphoid cells were present in the splenic red pulp, although splenic follicles appeared small. A choroid plexus tumor did not develop in this mouse.

Lymphoproliferative disorders in the LP50 and LP41 lineages. LP41 and LP50 were backcrossed to nontransgenic B6D2F₁ mice to produce lines. LP50, in which the transgene was X linked, gave rise to a line of animals with two phenotypes (Fig. 3A). Of the 13 transgenic offspring analyzed, approximately two-thirds (9) developed debilitating choroid plexus tumors by the age of 2 months. Splenomegaly was also present in some of these mice. The remaining third (4) developed a phenotype similar to that of the founder mouse; that is, lymphoma and choroid plexus neoplasia and death at 3 to 4 months. All of the LP41 transgenic offspring developed lymphomas, with the exception of three mice that died without autopsy (Fig. 3B). Unlike the LP50 line, only a few of the LP41 mice also developed choroid plexus tumors. Generally, these mice lived to about 4 months of age.

FIG. 3. Early pedigrees of the LP50 (A) and LP41 (B) lineages. Females are indicated by circles, males by squares, and transgenic mice by solid circles and squares. In each cross, an LPV-positive transgenic mouse was bred with a nontransgenic B6D2F₁ mouse. The first generation of LP50 offspring was produced after superovulation of LP50, which was then mated with a B6D2F₁ male, and the embryos were transplanted into a foster recipient. The neoplasm present at death is indicated under each transgenic mouse: lym, lymphoma; cp, choroid plexus tumor. Some mice were sacrificed before developing disease (s) or were found dead and not autopsied (d). *, LP41 died of renal failure.

The lymphoma cells of both LP41 and LP50 mice showed small differences in morphology. One group was composed of moderately large cells with predominantly round nuclei, little cytoplasm, fine chromatin, small nucleoli, and numerous mitoses. Generally, this cell type was seen as a mass at the periphery of the thymus, in splenic blood vessels and red pulp, and in lymph nodes, sometimes as the predominant cell type and sometimes in vascular channels. The second morphological group was composed of large cells with predominantly irregular nuclei, visible cytoplasm, coarse chromatin with parachromatin clearing, nucleoli, and numerous mitoses. This cell type was seen most often in thymic medulla, lymph nodes, and splenic white pulp. Usually, the various lymphoid organs contained a mixture of these cell types.

Although bone marrow was not examined, peripheral blood lymphocyte (PBL) counts performed on many mice indicated the presence of leukemia. Six mice in the LP41 line and one mouse in the LP50 line were examined at 3 to 4 months of age when lymphomas were apparent. All of these mice were leukemic, with PBL counts ranging from 4×10^4

to 2.5×10^6 cells per mm^3 . These values contrast with a value of 3×10^3 cells per mm^3 obtained for a normal nontransgenic mouse tested at the same time and with published normal values of 5×10^3 to 11×10^3 cells per mm^3 (summarized in reference 4). In one mouse (LP50-2-2), a leukemic process was clearly identified in tissue sections from nonlymphoid (not shown) and lymphoid (Fig. 5C and D) organs.

Immunohistochemical analysis of lymphoid tissue. Immunohistochemical studies were performed on splenic, lymph node, and thymic tissues from two mice each of the LP50 and LP41 lineages (Table 2, LP50-1-9, LP50-2-2, LP41-1-23, and LP41-2-8). B-cell lineage was assumed if positive staining occurred with anti-IgG, anti-IgM, anti-IgA (mature B cells), and/or anti-B220 (pre-B and mature B cells). Cells showing positive staining with anti-Thy-1 (thymocytes and T cells), anti-Lyt2 (suppressor and cytotoxic T cells), and/or anti-L3T4 (helper T cells) were assumed to be of T-cell lineage. In addition to immunohistochemical studies, routine histology was performed on Formalin-fixed lymphoid tissues

FIG. 4. Morphological appearance of neoplasia in transgenic mice harboring the LPV early region. Light micrographs of malignant choroid plexus tissue are shown in panels A and B. Magnification, $\times 2.5$ and $\times 50$, respectively. A lymphomatous spleen (C) and lymph node (D) are shown. Magnification, $\times 2.5$ and $\times 50$, respectively. Invasion of lymphoma into lung (E) and liver (F) tissues is shown. Magnification, $\times 16$.

from two mice (LP41-2-8 and LP50-2-2) and confirmed the impression of lymphoma.

In lymph node tissues from three mice (LP50-1-9, LP41-1-23, and LP41-2-8), the vast majority of cells stained with antibodies to B-cell markers, the intensity of staining being greatest with the anti-B220 antibody (Fig. 5A). The lymph node tissue from mouse LP50-2-2 showed scattered cells which stained positively for B-cell markers, but the majority of cells carried T-cell markers, consistent with a T-cell lymphoma (Fig. 5C). Thymic tissues were examined from three mice. All of the specimens showed a band of B220 and immunoglobulin-positive, Thy-1-negative cells at the periphery of the tissue (Fig. 5B). Interior to this, the cells were Thy-1 positive and carried either Lyt2 or L3T4 markers (data not shown). In combination with the morphological data, this finding suggested a peripheral B-cell lymphoma and a T-cell lymphoma in the interior. Large nodules of histologically malignant cells carrying T-cell markers were demonstrated in splenic tissues from two of the four mice (LP41-2-8 and LP50-2-2), consistent with T-cell lymphoma. The intervening cells carried B-cell markers (data not shown). Generally, the T cells in each tissue were identified as such with antibody to Thy-1 and within that group many more cells carried L3T4 than Lyt2. However, a subpopulation of Thy-1-positive cells carried neither of these markers. In LP50-1-9 and LP41-1-23 the majority of cells in the spleen carried B-cell markers, although small nodules of cells staining with T-cell markers were present (data not shown).

Finally, one mouse (LP50-2-2) had a leukemic process

which was identified by routine histology in sections from brain, lungs, and liver, as well as in lymphoid organs. Immunohistochemical examination of the lymphoid organs showed staining of these intravascular malignant cells with anti-IgG, anti-IgM, and anti-IgA antibody (Fig. 5C), consistent with a B-cell leukemia. However, the lymphoma in this mouse was of T-cell origin (B220 and immunoglobulin negative, Thy-1 positive) (Fig. 5D).

Tissue distribution of LPV early RNA. RNA extracted from tissues was analyzed for the presence of LPV-specific transcripts by a T2 ribonuclease protection assay. A 510-nucleotide antisense RNA probe specific for the 5' end of the LPV early message (Fig. 6C) was used to detect transgene expression. This probe should protect fragments of approximately 230 or 350 nucleotides, depending on which of the two TATAA boxes upstream of T antigen is utilized (Fig. 1). To identify LPV expression in nonlymphoid tissues that was merely due to contamination or infiltration by B cells, the RNA preparations were also screened with a probe for the 3' end of the kappa light chain transcript. The appearance of a 180-nucleotide fragment is indicative of the presence of B cells in a particular tissue.

Figure 6 shows the results of a T2 nuclease protection experiment performed on two founder mice that developed choroid plexus tumors at a young age, LP39 (Fig. 6A) at 36 days and LP26 (Fig. 6B) at 27 days. The major protected LPV fragment in each case was about 230 nucleotides long, consistent with the use of the TATAA box proximal to the start codon of T antigen (Fig. 1). This was also the major

FIG. 5. Immunohistochemical staining of lymphomatous tissue from LP41 and LP50 mice. (A) Lymph node stained with anti-B220. Magnification, $\times 50$. (B) Thymus stained with anti-IgM, anti-IgG, and anti-IgA. Magnification, $\times 16$. (C and D) LP50-2-2 spleen with intravascular malignant B220+ (C) lymphocytes and Thy-1+ (D) lymphoma. Magnification, $\times 50$.

early start site observed in cultured B cells (1). Minor LPV-specific species of larger size were sometimes present and may include transcripts initiating from the upstream TATAA box (for instance, compare the nonspecific fragments in the tRNA lane to the LP39 thymus).

Expression of the transgene was detected in the choroid plexus tumor, the spleen, and the thymus of each mouse (Fig. 6). In both mice, the level of expression was highest in the thymus and lowest in the spleen. A low level of expression was also detected in the brain of each mouse, possibly due to expression in the remaining choroid plexus. The kidneys of LP39 and the testes of LP26 also contained some LPV transcripts, but expression in these tissues was not consistently observed in all LPV mice (for example, mouse

LP41, Fig. 7). No transcripts were detected in heart, liver, or muscle tissue (Fig. 6). The presence of LPV transcripts in the lungs of these mice probably resulted from B-cell contamination, since immunoglobulin transcripts were also detected with the control kappa probe (Fig. 6).

A pattern of expression similar to that of LP39 and LP26 was found in LP50-1-5, an LP50 offspring which also developed a choroid plexus tumor at a young age (72 days). As shown in Fig. 7A, expression of LPV in LP50-1-5 was predominantly in the choroid plexus tumor, the thymus, the spleen, and the brain. The LPV-specific transcripts in the kidneys and lungs of this mouse may have resulted from contamination by B cells, since the relative intensities of kappa- and LPV-specific signals in these tissues are similar to those in spleen. Note that the thymus contained a significant level of kappa RNA (Fig. 7A). As mentioned above, immunohistochemical analysis of several mice revealed the presence of B220-positive, immunoglobulin-positive cells in the thymic cortex (Fig. 5B), indicating the presence of an abnormal population of B cells.

In LP50-1-9, and other LP50 offspring which developed lymphoma, both kappa and LPV transcripts were present in a multitude of tissues (Fig. 7B). The widespread presence of both of these transcripts is consistent with the notion that the lymphoma, which expressed high levels of LPV RNA (Fig. 7B, lymph node), was aggressive and consisted of B cells. As described above, histological examination of this and similar mice revealed the presence of malignant lymphocytes in a variety of tissues (Fig. 4E and F). In LP50-1-9, LPV tran-

TABLE 2. Predominant cell type identified by immunohistochemistry

Mouse no.	Lymph node	Thymus		Spleen
		Peripheral	Central	
LP50-1-9 ^a	B	B	T	B
LP50-2-2	T	B	T	T
LP41-1-23 ^a	B	NE ^b	NE	B
LP41-2-8	B	B	T	T

^a PBL counts of these mice indicated the presence of leukemia. The PBL counts were 7×10^4 cells per mm^3 for LP50-1-9 and 2.5×10^6 cells per mm^3 for LP41-1-23.

^b NE, Not examined.

FIG. 6. Transgene expression in young founder animals with choroid plexus tumors. Total RNA from indicated LP39 (A) and LP26 (B) tissues was analyzed, using an RNase T2 protection assay as described in the text. The LPV and kappa probes were run separately in the gel in panel A. Each RNA sample was hybridized with both probes. Hybridization to separate probes (not shown) was performed to determine the specificity of each band. The positions of the major protected LPV (230 nucleotides) and kappa (180 nucleotides) fragments are indicated by arrows. Nonspecific fragments are present in the lane marked tRNA (A). An M13 sequencing ladder was run in each gel and served as a marker. A map indicating the position of the LPV specific probe (hatched box) relative to the T-antigen transcript is shown in panel C.

scripts also appeared to originate in the choroid plexus tumor, brain, thymus, testes, and kidneys.

Although LP41 died of renal failure, no LPV-specific transcripts were detected in the kidney tissue (Fig. 7C). As in the other mice, transgene expression was detected in the spleen, and the lungs contained both kappa and LPV transcripts. Thymus RNA was not analyzed due to the limited amount of tissue available from this mouse. LP41 showed no evidence of LPV expression in other tissues examined, including the brain (Fig. 7C). Offspring of this mouse that developed lymphoma expressed LPV to high levels in the thymus, spleen (Fig. 7C), and lymphomatous tissues (data not shown). Some LP41 offspring expressed the transgene in

the brain, but the majority did not (Fig. 7C. LP41-1-2 and LP41-1-23; data not shown).

DISCUSSION

LPV-induced neoplasia. Information on the transforming activity of LPV T antigen is limited. In part, this could be due to the fact that a limited number of cell lines support transcription of the early region. Takemoto and Kanda (44) isolated transformed cells only after a 10-week delay after the infection of hamster embryo cells with LPV. Attempts to transform human and rodent lymphocytes in vitro with the intact virus were not successful (44). We have demonstrated that the isolated early region of LPV, when present in the mouse germ line, efficiently induces neoplastic transformation of certain B- and T-lymphoid cells as well as of the choroid plexus. These experiments show directly the ability

FIG. 7. Transgene expression in mice of the LP50 and LP41 lineages. Tissue RNA was analyzed for the presence of LPV and kappa light chain transcripts. Two first-generation mice from the LP50 lineage were analyzed. LP50-1-5 (A) died at 72 days with a choroid plexus (CP) tumor. LP50-1-9 (B) had lymphoma and a choroid plexus tumor and died at 87 days. The lymph node (l. node) was lymphomatous tissue. A similar analysis of the LP41 founder mouse and two of its offspring (LP41-1-23 and LP41-1-2) is shown in panel C. LP41 died of renal failure, and its offspring developed lymphomas.

of LPV T antigen to induce tumors and provide a system for further characterization of its transforming properties.

The generation of choroid plexus tumors in animals harboring the LPV T-antigen gene was unexpected since the cell-type preference of the virus is lymphoid cells. Neoplasia of the choroid plexus occurs in transgenic mice carrying the wild-type SV40 large T-antigen gene and regulatory region (7, 29, 39, 45, 46). However, there is a difference in the timing of choroid plexus tumorigenesis induced by the LPV and SV40 T-antigen genes. In the case of SV40, choroid plexus tumors arise at an adult age (from about 69 to 155 days) (7, 33, 46; unpublished results). With LPV, the majority of choroid plexus tumors (six of seven) occurred in founder animals before 42 days of age. Morphologically, an overall expansion of the choroid plexus tissue that lay outside of the solid tumor was seen with LPV (Fig. 4A) and not with SV40 (46). These observations suggest that transformation of the choroid plexus by LPV may be more efficient than by SV40. This could be a result of differences in the T antigens themselves or in the level or timing of their expression. Studies in transgenic mice with hybrid gene constructs between the regulatory regions and T antigens of these viruses are in progress to distinguish between these possibilities.

Although most of the founder LPV mice died before 42 days of age due to neoplasia of the choroid plexus, two mice survived for about 3 months. LP50 had a choroid plexus tumor at the time of death, whereas LP41 did not. The delayed onset or absence of choroid plexus tumors in these mice may have resulted from an influence of the integration site on transgene expression in the choroid plexus. LP41 and many of its offspring had no detectable LPV expression in the brain. Lymphoproliferative disorders (lymphoma and leukemia) consistently arise in mice of this lineage and may have arisen in mice which succumbed to choroid plexus neoplasia had they lived longer. High levels of LPV expression in the thymus and spleen of these animals may indicate that LPV-induced neoplasia of lymphoid cells compared with that of the choroid plexus is less efficient. Further experiments on the timing of LPV T-antigen expression and development of pathology in the two cell types are required to test this notion.

The members of the LP50 line of mice display one of two phenotypes with regard to the timing of choroid plexus tumors. Like the founder animal, some mice survive to about 3 months of age before developing choroid plexus tumors. These mice develop malignant lymphomas. The remaining mice succumb to choroid plexus tumors by about 2 months of age. Splenomegaly is often apparent in these animals, but overt lymphoma is not. The difference in timing of choroid plexus tumors within the line is not due to segregation of independent transgenes, because the founder animal contained only a single copy of the LPV early region. It is conceivable that differences among mice of the LP50 lineage are due to the fact that the transgene is X linked (Fig. 3A). One possibility is that expression of the transgene is influenced by X inactivation. It is clear, however, that the transgene is never completely inactivated in this line.

As mentioned, neoplasia of lymphoid cells was apparent in many mice of the LP50 lineage and in all LP41 offspring. Preliminary analyses of lymphomatous tissues from these mice for the presence of B- and T-cell markers suggest that LPV can transform both cell types *in vivo*. The B-cell lymphomas that were analyzed appeared to be a mature class of B cells on the basis of the presence of surface immunoglobulin as well as the B220 marker. Of the T-cell

lymphomas that were analyzed, the major expanded population was of the T-helper subclass (L3T4+, Lyt2-). A Thy-1+, L3T4-, Lyt2- class of cells which has not been identified was sometimes present.

In at least one case (LP50-2-2), it was clear that both B and T cells were transformed in a single animal. This mouse had a T-cell lymphoma concomitant with a B-cell leukemia. Transformation of multiple cells may also have occurred in animals in which the thymus contained a T-cell lymphoma surrounded by an abnormal band of B cells. Whether individual lymphomas are actually oligoclonal has not yet been determined. Studies are currently in progress to analyze the nature of T-cell receptor and immunoglobulin gene rearrangements in the lymphomas to obtain an answer to this question.

The LP41 line of mice should be a useful model for further work on the ontogeny of B- and T-cell lymphomas and leukemias. They may also provide a useful resource for the study of normal lymphocyte differentiation, particularly that of the T cells. Transgenic lines of mice which develop B-cell lymphomas have been generated with the *c-myc* (2) and the SV40T-antigen (42) genes linked to an immunoglobulin enhancer and by the *v-Ha-ras* gene linked to the mouse mammary tumor virus long terminal repeat (38). The LPV transgenic mice should supplement, and may provide a useful alternative to, existing mouse models that utilize virus infection (11 and references therein) or carcinogen treatment (8, 12 and references therein) to induce T-cell lymphoma and leukemia. Transgenic mice provide a logistic advantage in that special facilities and precautions for handling hazardous materials are not required.

Specificity of LPV transcription. In the transgenic mice examined in this study, LPV RNA expression was consistently high only in choroid plexus tumors and in lymphoid organs. The specificity of transformation by SV40 for the choroid plexus occurs at the level of transcription. Deletion of the two 72-bp motifs reduces the frequency of choroid plexus tumors (29), and changing the transcriptional signal alters the cell type which becomes transformed in transgenic mice (reviewed in references 19 and 28). In view of the present study, it is likely that LPV shares with SV40 some aspects of transcriptional regulation that result in expression in choroid plexus cells. Some sequence elements known to affect transcription in cultured cells are common between the two viruses. These include the viral enhancer core sequence (reviewed in reference 36), the G+C-rich boxes (reviewed in reference 24), and a purine-rich sequence referred to as the Pu box (32).

Transcription of the LPV early region in the lymphoid organs, including lymphomas of both T- and B-cell origin, reflects the natural tropism of wild-type LPV (5, 6, 43) and certain of its mutants (20, 21). This result also supports conclusions of experiments in cultured cells which indicate that the LPV control region is primarily specific for cells of lymphoid origin (26, 31). Mosthaf et al. (26) showed that in cultured cells the LPV enhancer was functional in non-B-, non-T-lymphoid cells in addition to B- and T-cell lines. Our experiments do not rule out the fact that this also occurs in transgenic mice, but the lymphomas of the four mice examined by immunohistochemical staining were either of B- or T-cell origin (Table 2). Further experiments involving cell fractionations will be required to determine whether LPV is actually expressed in other hematopoietic cells *in vivo*.

The fragment used for microinjection in this study was derived from the LO2 strain of LPV (Fig. 1). Most of the earlier experiments concerning transcriptional regulation of

this virus have used an LPV variant (K38) which contains a duplication of sequences surrounding the origin of DNA replication (16, 30). As a result of the duplication, a 63-bp sequence present only once in LO2 is present three times in K38. This 63-bp stretch contains the SV40 enhancer core homology which is present in many immunoglobulin genes as well as an adjacent purine-rich sequence (the Pu box [32], the IgPE-2 binding site [37]) that also has homology to the IgH enhancer (Fig. 1). Pettersson and Schaffner (32) have shown that this sequence activates transcription in lymphoid cells in culture. It also binds to a lymphoid cell-specific factor(s) (32, 37). If this sequence is responsible for lymphoid specificity in transgenic mice, our studies with the LO2 strain of LPV indicate that a single copy is sufficient. Another difference between the two control regions is that a separate sequence (60 bp in length) present only once in K38 is duplicated in LO2 (Fig. 1) (16). The contribution of these various sequences to lymphoid and choroid plexus epithelial cell specificity can be tested in vivo, using transgenic animals.

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