MOLECULAR AND CELLULAR BIOLOGY, Aug. 1988, p. 3382–3390
0270-7306/88/083382-09$02.00/0

Vol. 8, No. 8

Requirement for the Simian Virus 40 Small Tumor Antigen in Tumorigenesis in Transgenic Mice

YONGWON CHOI,[1] INCHUL LEE,[2]† AND SUSAN R. ROSS[1]*

Department of Biological Chemistry, University of Illinois,[1] and Department of Surgery and Pathology, St. Luke's-Rush Presbyterian School of Medicine,[2] Chicago, Illinois 60612

Received 11 March 1988/Accepted 29 April 1988

To examine the role of simian virus 40 (SV40) large T and small t antigens in tumorigenesis in animals, we generated transgenic mice which expressed either both the SV40 large T and small t antigens or the SV40 large T antigen alone under the control of the mouse mammary tumor virus long terminal repeat. The mouse mammary tumor virus long terminal repeat directs the expression of transgenes in ductal epithelial cells of several organs, including the mammary gland, lung, and kidney, and in lymphoid cells. The mice which expressed both the T and t tumor antigens developed lung and kidney adenocarcinomas, while those which expressed large T alone did not. Both types of mice developed malignant lymphomas with similar frequencies and latency periods. Our results show that the SV40 small t antigen cooperates with the large T antigen in inducing tumors in slowly dividing epithelial cells in the lung and kidney.

There is substantial evidence that multiple events are required to achieve cellular transformation, including overexpression and mutation of cellular oncogenes. Several model systems have been used to study the cooperation between different oncogenes in cellular transformation by using both cultured cells and animals (4, 5, 20, 21, 23, 39, 49). Transgenic mice have also been used successfully to study tumor induction by cellular and viral oncogenes (33). The process of tumorigenesis in transgenic mice appears to require activation of cellular or humoral factors in addition to expression of the introduced oncogene(s) (1, 16, 32, 33, 43, 44).

Cooperation between transforming genes has also been extensively studied with simian virus 40 (SV40), a DNA tumor virus which encodes two genes expressed in the early stage of viral infection, large T antigen (94 kilodaltons) and small t antigen (17 kilodaltons) (47). These two proteins share common amino termini but differ at their carboxyl end because of differential splicing of a single transcript (2, 19). SV40 large T antigen is required for the normal virus lytic cycle, transformation of cells in tissue culture, or causing tumors in hamsters, since mutations in this region abolishes these properties (45). The role of small t antigen is not clear in either the transformation process or the viral replication cycle (45). Large T antigen alone seems to support efficient viral replication, but there has been a controversy about the role of small t antigen in transformation. Cells infected with wild-type or mutant SV40 or with recombinant retroviruses containing large T and small t, or large T alone, appear to be transformed with equal efficiency under certain experimental conditions (3, 8, 11, 12, 22, 41, 47), while under other conditions, both the large T and small t antigens have been shown to be necessary for this process (3, 6, 13, 14, 17, 18, 25, 38, 40, 43). In addition, when viruses with deletions in small t antigen are injected into newborn hamsters, the latency of fibrosarcomas is increased relative to wild-type viruses (27, 48).

To study the role of the SV40 early-region tumor antigens in tumorigenesis of different cell types and tissues, we introduced the large T and small t antigens into transgenic mice so that the antigens would be expressed in several different tissues. We made transgenic mice carrying both SV40 large T antigen and small t antigen under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and mice containing the same transgene in which the small-t-coding sequences were disrupted. MMTV is an endogenous retrovirus in mice which is causally associated with mammary carcinomas and is expressed at high levels in the lactating mammary gland (30). We and others have shown that genes placed under the control of the MMTV LTR are expressed in the lactating mammary gland and in several different tissues of transgenic mice (10, 24, 37, 44) and that this expression mimics that of the endogenous proviruses (16a). Expression of MMTV LTR hybrid genes appears to be limited to ductal epithelial cells in various organs, to lymphoid cells, and possibly to Leydig cells in the testes (10). We chose to use the MMTV LTR as a control element for expression of the SV40 early-region genes, because we could then direct their expression in several different cell types and tissues. In this way, we could compare, for example, the tumor-inducing ability of the SV40 genes in epithelial cells in lung, kidney, mammary gland, and salivary gland and in lymphoid cells, within the same and among different strains of mice. In an individual transgenic strain, the integration site and copy number of the transgene remains constant from tissue to tissue, and thus the ability of an oncogene to cause tumors is not dependent on viral infectivity or replication; a comparison of the tumor-inducing ability of a transforming gene(s) can then be made in the different tissues where it is expressed.

We found that four out of six independent transgenic strains of mice expressing both large T and small t antigen developed adenocarcinomas in the lung and/or kidney by 4 months of age, but none of the transgenic mice expressing only SV40 large T antigen developed adenocarcinomas in either of these tissues by the age of 6 months. All of the transgenic mice carrying either both large T and small t antigen or large T antigen alone developed malignant lym-

* Corresponding author.
† Present address: Institute of Cell and Tumor Biology, German Cancer Research Center, Heidelberg, Federal Republic of Germany.

phomas with the same latency. Our results indicate that SV40 small t antigen facilitates tumorigenesis by large T antigen in slowly dividing epithelial cells in the lung and kidney but is dispensable for the transformation of rapidly dividing lymphoid cells. These results also suggest that the transformation of cells in different tissues depends on their rate of division and requires the participation of other factors which act on those cells.

MATERIALS AND METHODS

DNA constructions. The construction of pLTag was previously described (10). To generate pLTM, pLTag was linearized at a unique *Bst*X1 site in the small t coding region, the 4-base-pair (bp) 3′ overhang was converted to a blunt end with T4 DNA polymerase, and *Bgl*II linkers were ligated on. A 1.8-kilobase-pair (kbp) *Bgl*II-*Bam*HI fragment of the MMTV proviral genome, isolated from plasmid p2.5 (a gift from J. Majors), was inserted into this site. Plasmid pGemSH was generated by inserting the 230-bp *Sac*I-to-*Hin*dIII fragment of pLTag (containing the MMTV transcription initiation site and extending into the SV40 region) into the pGEM-blue vector (Promega Biotec) in the antisense direction relative to the SP6 promoter.

Microinjection and DNA analysis. pLTM DNA was purified from *Escherichia coli* derivative strain DH1 by the alkaline extraction method followed by CsCl-ethidium bromide equilibrium density centrifugation. The DNA was digested with *Sfi*I and *Bam*HI, and the fragment to be injected was purified as previously described (10). The DNA was diluted to 0.5 μg/ml in phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 15 mM Na_2HPO_4) before injection. Fertilized zygotes were injected with about 500 copies of DNA. The presence of injected DNA was determined by preparing genomic DNA from tails by Southern blot analysis as previously described (10).

RNA analysis. RNA was isolated by guanidine thiocyanate extraction and CsCl gradient fractionation by the method of Chirgwin et al. (9). Northern (RNA) analysis and S1 nuclease protection analysis were carried out as described by Choi et al. (10). For RNase T1 protection experiments, a 250-bp antisense RNA probe was synthesized from pGemSH by using SP6 polymerase (28, 46, 51). An excess of uniformly labeled probe (5×10^5 cpm) was hybridized with 25 μg of total RNA. Hybridizations were carried out overnight at 54°C in 30 μl of hybridization buffer consisting of 80% formamide, 400 mM NaCl, 40 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4), and 1 mM EDTA. A 300-μl sample of RNase T1 solution (330 U of RNase T1 [Bethesda Research Laboratories, Gaithersburg, Md.] per ml in 10 mM Tris hydrochloride [pH 7.6]–300 mM NaCl–1 mM EDTA) was added, and digestions were carried out at 37°C for 1 h. The digestion was stopped by adding 20 μl of a 10% sodium dodecyl sulfate (SDS)–5 mg of proteinase K per ml solution followed by incubation for 15 min at 37°C, phenol-chloroform extraction, and ethanol precipitation. The protected fragments were analyzed on 5% sequencing gels with end-labeled *Hpa*II-digested pBR322 as markers.

Histology and immunofluorescence. Tissue samples were fixed in 10% Formalin and embedded in paraffin by standard procedures, and 6-μm sections were stained with hematoxylin and eosin. For immunofluorescence, frozen 4-μm sections were prepared and fixed in cold acetone for 10 min at −20°C and then air dried. Primary rabbit antibody against T antigen was a gift from R. Kucherlapati. Hamster anti-SV40 tumor serum was a gift from E. Gurney. The sections were incubated with the primary antibody for 30 min at room temperature and washed extensively with phosphate-buffered saline. They were incubated for an additional 30 min with a fluorescein-labeled goat anti-rabbit or anti-hamster immunoglobulin secondary antibody (Jackson ImmunoResearch, Avondale, Pa.). After being washed, the sections are dehydrated, mounted, and examined with a fluorescence microscope (Carl Zeiss, Inc., Thornwood, N.Y.).

Protein analysis. Preparation of tissue extracts, immunoprecipitation, and Western blotting (immunoblotting) were carried out as previously described (10). Immunoprecipitation from 1 mg of total protein from each tissue was carried out, and the samples were run on 7.5% polyacrylamide gels. After blotting, the nitrocellulose filters were incubated in hamster anti-adeno-SV40 tumor serum (ND4) (36) and then with ^{125}I-protein A (Amersham Corp. Arlington Heights, Ill.) and exposed to Kodak XAR-5 film.

RESULTS

Construction of transgenic mice carrying an MMTV LTR-SV40 large-T-antigen transgene and its expression. pLTag is a plasmid expressing both the SV40 large T and small t antigens under the control of the MMTV LTR (Fig. 1A); transgenic mice carrying this construct are called LT/t 1 to 6 (called LTag 1 to 6 in reference 10). Plasmid pLTM was derived from pLTag so that it would express only the SV40 large T antigen under the control of the MMTV LTR. To disrupt small-t-antigen expression, a *Bst*X1 site within the coding region of the small t antigen was changed into a *Bgl*II site and a 1.8-kbp *Bgl*II-*Bam*HI fragment of the MMTV *pol* gene was inserted (Fig. 1A). Northern blot analysis of RNA isolated from cells transfected with pLTM shows only a 2,300-base RNA corresponding to the hybrid MMTV-large T mRNA initiated from the MMTV promoter (Fig. 1B). Insertion of the 1.8-kb MMTV DNA into pLTag resulted in the loss of detectable small-t-antigen mRNA, probably by mRNA destabilization, since we detect stable transcripts for the large T antigen in which the 1.8-kb fragment has been spliced out. pLTM has been tested in tissue culture; the transcription initiating within the MMTV LTR is regulated by glucocorticoid hormones (data not shown).

A 5.8-kbp *Sfi*I-to-*Bam*HI fragment of pLTM was used for microinjection; all of the SV40 early control region elements and plasmid sequences are absent from this fragment. Around 500 copies of the fragment were injected into fertilized zygotes from Swiss Webster mice. Six independent transgenic mice were identified by analysis of tail DNA. RNA and proteins from various organs from each strain have been analyzed by S1 nuclease protection, by RNase T1 protection, by Northern analysis, and by immunostaining of tissue sections. For S1 nuclease or RNase T1 protection studies, probes were prepared either by end labeling a 250-bp junction fragment at a *Hin*dIII site within the SV40 region (Fig. 2A) or by making antisense RNA by using SP6 polymerase (Fig. 2B). The antisense RNA can detect both MMTV-SV40 hybrid transcripts (130 bases) and endogenous MMTV transcripts (100 bases). Prior to the onset of the malignant lymphomas (see below), MMTV-SV40 hybrid transcripts were detected only in the tissues where endogenous MMTV was expressed (Fig. 2A and 2B; 16a). Immunostaining was also done to determine which cell types expressed the large T antigen (see below). Results from the six different strains showed that the expression was limited to ductal epithelial cells in mammary glands, salivary glands, lung, kidney, epididymis, and seminal vesicle; lymphoid

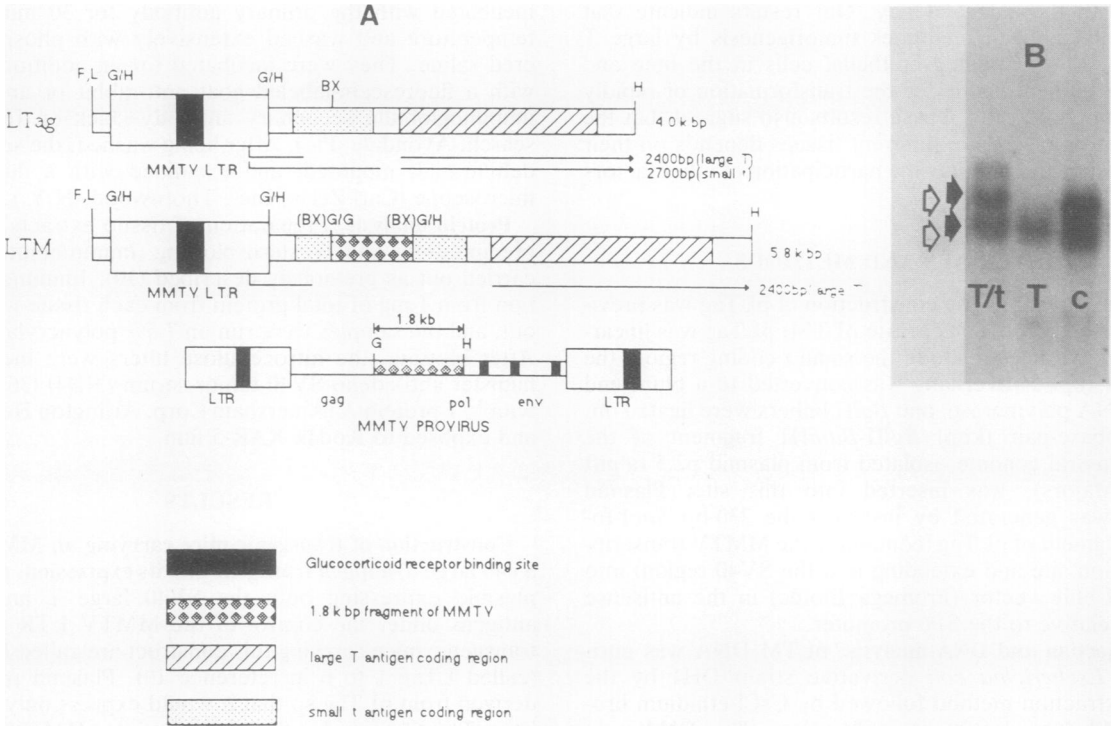

FIG. 1. Diagram of pLTag and pLTM used to make transgenic mice and their RNA transcripts. (A) Plasmid pLTag was described by Choi et al. (10). Abbreviations: F, *Sfi*I; L, *Bgl*I; G, *Bgl*II; H, *Bam*HI; Bx, *Bst*X1. (B) RNA transcripts corresponding to the MMTV-SV40 large T and small t antigens. Filled arrows indicate hybrid mRNAs. Open arrows indicate authentic SV40 early-region mRNAs. Each lane contains 20 μg of total RNA from cells transformed by pLTag (T/t) or pLTM (T) and 5 μg of total RNA from Cos-1 cells (c), and hybridized with SV40-specific probe derived from pMK16-2 (a gift from K. Subramanian).

cells in spleen and thymus; and Leydig cells in testes. Although the absolute levels of expression of the transgene among different mice varied, the ratio of expression among different organs was relatively constant and the lactating mammary glands had the highest level of expression (Y. Choi and S. R. Ross, unpublished results); this ratio mimics what is seen for endogenous MMTV expression, with lactating mammary gland expressing at the highest level and the other tissues expressing at about 50- to 100-fold-lower levels (10; Fig. 2A and 2B; 16a). The cell types and organs that expressed the transgene are exactly the same ones in which we detected expression in transgenic mice carrying pLTag (strains LT/t 1 to 6) (10).

Tumorigenesis in transgenic mice carrying the pLTM construct. We examined tumorigenesis in five independent strains (one founder animal died from unrelated causes and was only examined for expression). Two founder strains were expanded to give partial pedigrees, which are shown in Fig. 3. The founder mouse, LT57, was found to have a Y chromosome insertion of the transgene, since all and only male offspring from five independent litters of three generations of mice were transgenic (Fig. 3A). In this lineage, 100% of the male mice carrying the transgene showed signs of lymphoma at around 4 months of age, as judged by palpable lymph nodes and leukemic appearance. By 6 months of age, more than 90% of the transgenic males died; none of their nontransgenic female siblings showed symptoms of lymphoma. Upon autopsy, all the mice had greatly enlarged spleens and lymph nodes, and their livers were filled with nodules of lymphoma cells. Histopathological examination showed that all 14 of the autopsied transgenic animals from the LT57 lineage had malignant lymphomas (Fig. 4F), but none had any adenocarcinomas in the lung or kidney (Table 1). Histological examination of spleen and lymph node sections, analysis of the immunoglobulin heavy- and light-chain and T-cell receptor gamma-chain gene rearrangement, and fluorescence-activated cell sorter (FACS) analysis by using anti-Thy 1 antibody indicated that these lymphomas were either pre-B cells or pre-T cells (Y. Choi and I. Lee, unpublished observations). Individual mice appeared to have only one type of lymphoma; however, because we did not analyze individual clones from the mice, we cannot rule out the possibility that they had different types of tumors at the same time. We have analyzed expression from both concanavalin A- and lipopolysaccharide-stimulated primary spleen cultures and found that the transgene and endogenous viruses are transcribed in both populations (Y. Choi, unpublished observations); thus, it appears that both B and T cells can be transformed by the SV40 T antigen.

We also examined three 2-month-old animals from the LT57 lineage which looked healthy at the time of sacrifice. Microscopic and histopathological examination of the spleens of these animals revealed lymphocytes of abnormal size. FACS analysis of the spleen cells by using anti-Thy 1 antibody showed that greater than 70% were T cells; control Swiss Webster mice have around 30% T cells in their spleens at this age (data not shown). This indicates that the onset of T cell lymphomas in these mice occurred before 2 months of age. The average life span of these animals is about 6 to 7 months, and the lymphoma onset is about 2 months through the G1 and G2 generations. Since transgenic animals were mated with normal outbred Swiss Webster females, the fact

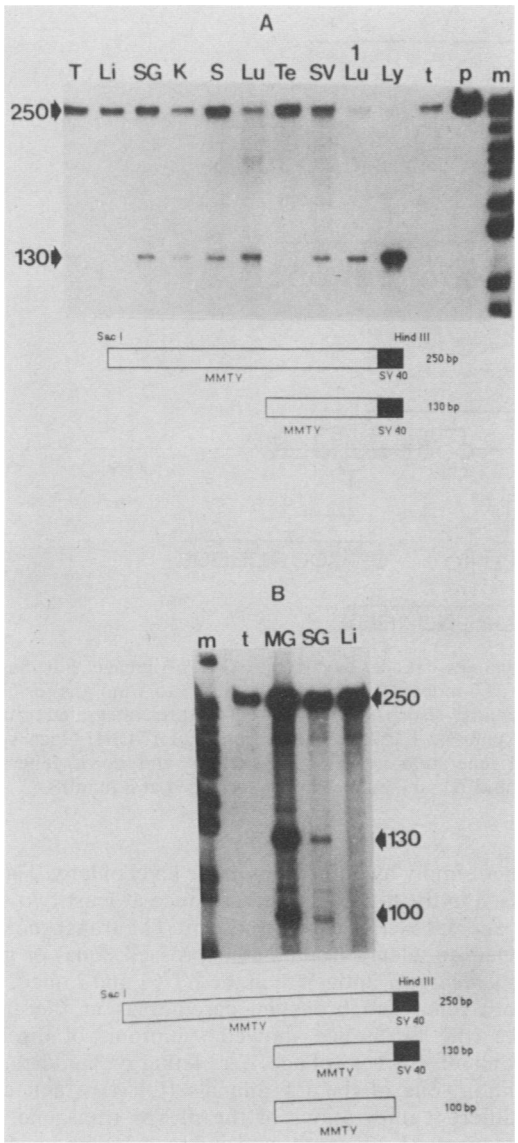

FIG. 2. RNA expression in LT transgenic mice. (A) RNA transcription from various organs of an LT21 mouse was determined by S1 nuclease protection. The end-labeled probe is diagrammed below the autoradiograph. RNA from the transgene protects 130 bp of a 250-bp probe. Symbols: filled portion, SV40 sequences; open portion, MMTV LTR sequences. (B) Detection of RNA transcribed from the transgene and endogenous MMTV in an LT54.III mouse. The antisense riboprobe is diagrammed below the autoradiograph. The transgene RNA protects 130 bases and endogenous MMTV RNA protects 100 bases of a 250-base probe. Symbols: filled portion, SV40 sequences; open portion, MMTV LTR. Abbreviations: MG, lactating mammary gland; SG, salivary gland; Li, liver; T, thymus; K, kidney; S, spleen; Lu, lung; Te, testes; SV, seminal vesicle; 1LU, lung adenocarcinoma from LT/t1; Ly, lymphoma from LT57; t, yeast tRNA; p, probe; m, marker.

that all the transgenic animals developed malignant lymphomas around the same time strongly suggests that the transformation of this cell type is not affected by the genetic background of these animals.

The LT54 founder mouse had three insertions of the transgene, as determined by the segregation of different junction fragments by Southern blot analysis (not shown), and was bred to give three independent strains (LT54.I, LT54.II, LT54.III) (Fig. 3B). The LT54 mouse was sacrificed at 4 months of age after producing two litters, because it showed symptoms of lymphoma. Histopathological examination of its organs showed that the LT54 mouse had also developed a malignant lymphoma, which had spread into all its organs. Focal adenocarcinomas were also seen in the mammary and Harderian glands, surrounded by normal epithelial cells. One hundred percent of the transgenic offspring in LT54 lineage developed malignant lymphomas by 5 months of age (Table 1), independent of which integration pattern of the transgene was inherited. We have also sacrificed LT54 offspring, which had the transgene integrated at different sites (I and II, III), at the ages of 2.5 and 3 months, respectively. Both had lymphomas that were not as widespread as those seen in LT54 or older animals from these same lineages, indicating that the onset of lymphoma is also around 2 months of age. We have analyzed 11 animals from these three strains, including the founder, but none of them developed any tumors in the lung or kidney by 180 days of age (Fig. 4B and D; Table 1).

Another founder mouse, LT21, also developed malignant lymphoma and focal adenocarcinomas in the mammary and Harderian glands at 4 months of age, but lung or kidney adenocarcinomas were not detected (Table 1). Thus, the LT mice differ from the LT/t mice, which frequently developed adenocarcinomas in the lung and kidney (10; Table 1).

Comparison of large-T-antigen expression and tumor formation in LT/t and LT mice. Although both the LT/t and LT strains expressed the SV40 early-region genes in the same cell types (see above), the lack of tumorigenesis in the lung and kidney in the LT strain mice could result from lower expression of large T antigen in the LT strains than in the LT/t strains. We therefore compared the levels of large T antigen among the different LT/t and LT mice. In Fig. 2A, for example, it can be seen that the LT/t 1 lung, which contained adenocarcinomas (Table 1), contains approximately the same amount of protected fragment as LT21, which had no tumor tissue. However, because tumor-containing organs have a proliferation of the transgene-expressing cells, the level of expression from these organs is always higher than that seen in normal transgene-expressing organs from the same strain. Therefore, to determine the relative level of expression between the strains, we analyzed large-T-antigen RNA and protein from salivary gland and epididymis from several animals by Northern and Western blot analyses. We chose salivary gland and epididymis for these analyses because these organs showed no histologically detectable tumor cells and both expressed detectable amounts of large-T-antigen RNA and protein. The level of large-T-antigen RNA among strains varied by, at most, 10-fold, and at least two LT strains, LT57 and LT54.III (lanes A and C, Fig. 5A), had greater amounts of T antigen RNA than the LT/t 2 strain did (lane E, Fig. 5A). Similar analysis of protein immunoprecipitated from equal amounts of extracts from the epididymides of LT54.III and LT/t 1 (lane T versus lane T/t, Fig. 5B) showed that almost equal amounts of large-T-antigen protein are expressed in these two strains.

A comparison of the tumorigenesis in different tissues between the LT/t and LT strains is shown in Table 1. Three of the six LT/t mice developed malignant lymphomas by 5 months. Lymphoma cells were seen in the lymph nodes, thymus, lung, liver, Peyer's patches, kidney, spleen, salivary gland, and skeletal muscle and within the dermis; all stained positive for large T antigen. Four independent LT/t mice also developed lung adenocarcinomas, and two dif-

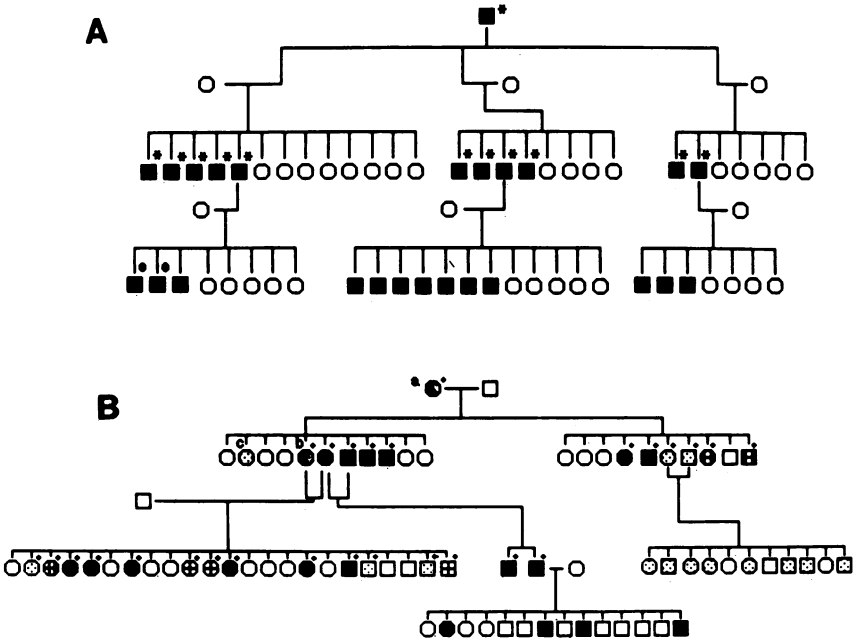

FIG. 3. Partial pedigrees of the LT57 and LT54 lineages. Males are shown as boxes, females as circles. (A) LT57 lineage. Filled symbols indicate the transgenic animals. *, Lymphoma-bearing animals. The life span was 6 months for the founder and 6 to 7 months for G1. The G2 generation is still alive, and only animals marked (●) were sacrificed for further analysis. (B) LT54 lineage. The three integrated transgenes were separated and are shown as follows: hatched symbols, LT54.I; dotted symbols, LT52.II; filled symbols, LT54.III. Open symbols indicate nontransgenic siblings. +, Tumor-bearing animals. The founder and G1 generation died by 4 to 5 months, and the G2 generation is still alive. Animal a has three insertions (I, II, and III), b has two insertions (I and II), and animal c was sacrificed at 3 months.

ferent strains of mice developed renal ductal cell adenocarcinoma. Both the lung and kidney tumors were multifocal, and many ducts in both the animals with and those without tumors had epithelial cells which showed in situ alterations and severe dysplasia (Fig. 4A and 4C). By immunostaining, the nuclei of the tumor and surrounding epithelial cells were positive for large T antigen (Fig. 4A and 4C).

As described above, all the animals carrying pLTM constructs developed malignant lymphomas. Lymphoma cells were also found in almost all organs examined, similar to the lymphomas found in LT/t animals. Analysis of RNA showed that all tumors contained large-T-antigen mRNA (Fig. 2A). However, unlike the LT/t mice, no LT mice developed adenocarcinomas in the lung or kidney by 6 to 7 months of age even though some LT animals expressed higher levels of large T antigen than the LT/t animals. Occasionally, single ducts which manifested mild dysplasia were seen; however, even in the oldest animals (7 months), these represented less than 5% of the total ducts observed (Fig. 4B). As with the LT/t mice, these ducts also showed characteristic nuclear immunostaining for large T antigen (Fig. 4B).

DISCUSSION

In this study, we show that in transgenic animals the SV40 small t antigen cooperates with the large T antigen in the transformation of ductal epithelial cells in the lung and kidney. We have shown that SV40 T/t transgenes caused tumors in epithelial cells in lung and kidney and in lymphoid cells. We could not identify any tumors in the lung and kidney of transgenic animals expressing only large T antigen, although these animals developed malignant lymphoma with the same frequency and latency as did animals expressing both large T and small t antigen. This difference cannot be explained simply by differences in the level of large T antigen expressed in the different animals, since at least two strains (LT57 and LT54.III) carrying the pLTM transgene which developed no adenocarcinomas expressed equal or greater amounts of large T antigen than the LT/t 1 and 2 mice, which had both renal and bronchial carcinomas at the time of sacrifice (the LT/t mice showed symptoms of disease at approximately 5 weeks of age, e.g., lethargy, jaundice, etc.). In addition, one of the LT animals (LT54) which carried three different integrations of the pLTM transgene, all of which expressed efficiently and induced lymphomas when carried in separate animals, did not develop any lung or kidney tumors. In fact, all of the LT mice developed lymphomas with approximately the same latency, although the level of T-antigen expression varied among the different strains. Thus it is unlikely that an increase in large-T-antigen expression can compensate for the absence of small t antigen, at least within the range of expression that occurred in these animals.

Ductal epithelial cells in the lung and kidney where MMTV-SV40 transgenes are expressed are slowly dividing in the adult, while lymphoid stem cells are relatively rapidly proliferating at all stages of life (29, 50). This is particularly interesting because it has been reported that SV40 mutants deleted for small t antigen which showed reduced ability to transform growth-arrested fibroblasts could be partially complemented by the addition of mitogens such as the phorbol ester (25, 40, 43). Our results strongly suggest that small t antigen is necessary for rapid transformation of slowly dividing ductal epithelial cells in lung and kidney. We cannot determine whether the deletion of small t antigen abolishes the transforming ability of large T antigen or increases the latency of tumorigenesis in these cells, since all

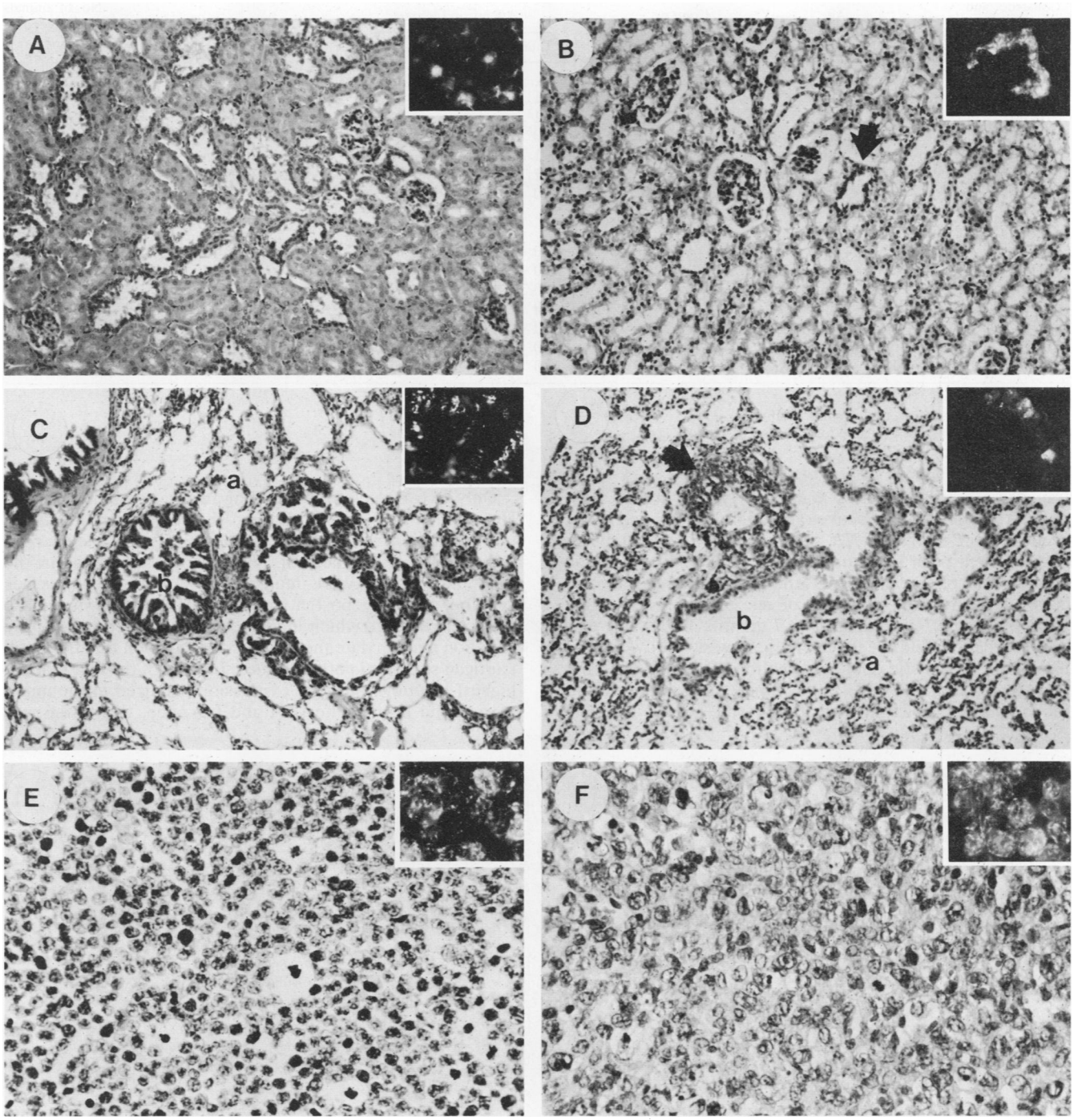

FIG. 4. Histology and immunostaining of tissues from LT/t and LT mice. Magnifications of hematoxylin-and-eosin-stained sections are ×104 and anti-SV40 T-antigen-stained sections (insets) are ×345, unless otherwise noted. (A) Tubules of LT/t 1 kidney show epithelial proliferation and cytological atypia in neoplastic ducts. The nuclei of the tubular epithelial cells are immunostained for T antigen. (B) Tubules of LT54.III kidney are normal, with atypical proliferation of epithelial cells in a single duct (arrow) and immunostain for T antigen. (C) LT/t 2 lung showing malignant proliferation of bronchial epithelial cells (b). Alveoli (a) are normal. The same LT/t 2 lungs shows nuclear immunostaining of the bronchial epithelial cells. (D) LT54.III lung with perivascular lymphoma (arrow). The lung is otherwise normal (a, alveoli; b, bronchia). Bronchial epithelial cells and inflammatory (lymphoid) cells show nuclear immunostaining. (E and F) LT/t 3 and LT57 lymphoma cells (respectively) have large irregular vesicular nuclei with abundant cytoplasm and frequent mitotic figures (hematoxylin and eosin; magnification, ×276). All cells are immunostained for large T antigen (magnification, ×345).

TABLE 1. Comparison of tumorigenesis between LT/t transgenic animals and LT transgenic animals

Construct and strain	Age	Copy no.	No. of animals with:			No. of animals analyzed
			Adenocarcinoma		Lymphoma	
			Lung	Kidney		
pLTag (T/t)						
LT/t 1[a]	3.5 mo	4	1	1	0	1
LT/t 2[a]	3.5 mo	2	1	1	0	1
LT/t 3[a]	5 mo	1–2	0[b]	0	1	1
LT/t 4[a]	<1 day	>50	1	NT[c]	1	1
LT/t 5[a]	4 mo	2	1	0[b]	1	1
LT/t 6[a]	<1 day	6	0	0	0	1
pLTM (T)						
LT21[a]	4 mo	4	0	0	1	1
LT57	6–7 mo	5	0	0	14	14
LT54.I	5 mo	2	0	0	2	2
LT54.II	5 mo	5	0	0	2	2
LT54.III	4–6 mo	5	0	0	5	5
LT54.I + II	2.5 mo	7	0	0	1	1
LT54[a]	4 mo	12	0	0	1	1

[a] Founder animal.
[b] Many ducts were severely dysplastic (10).
[c] NT, Kidney was not available for histological analysis.

transgenic animals died of malignant lymphoma by 6 to 7 months of age. Histological examination of lung and kidney sections from neonatal LT/t mice revealed no ductal dysplasia, although both antigens were expressed (10), indicating that the morphological transformation of these tissues occurs between 1 and 5 weeks of age. In lung and kidney sections from LT mice from 2 to 7 months of age, however, no significant difference is seen in the severity of dysplasia or number of dysplastic ducts per section. These results imply that the function supplied by the small t antigen is required for the initiation of transformation by the large T antigen.

Only two tissues—the lactating mammary gland and the Harderian gland—in the LT mice showed any epithelial cell transformation, although in both of these cases, the changes were focal, unlike the lung and kidney carcinomas in the LT/t mice which were multifocal. Both of these tissues develop primarily postnatally. At least for the mammary gland, the factors which induce cellular proliferation during lactation may obviate the need for the function that the small t antigen supplies in other tissues. In spite of the fact that the highest level of transgene expression occurred in the mammary gland in both the LT/t and LT mice, this tissue was

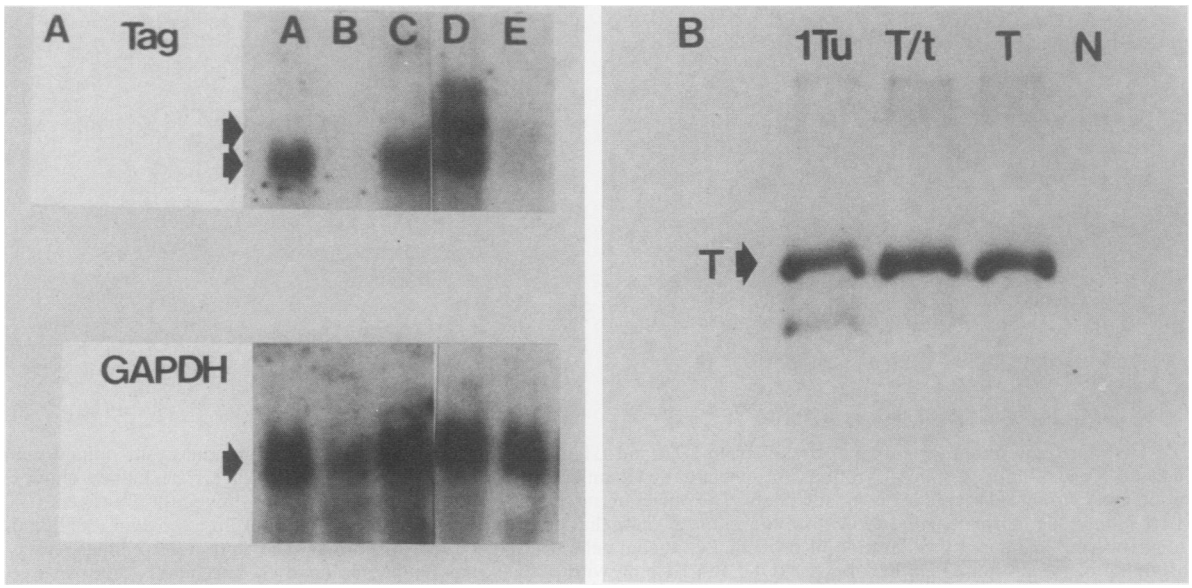

FIG. 5. Comparison of the levels of SV40 large T antigen (Tag) in various strains. (A) Each lane contained 20 μg of total RNA prepared from salivary gland of LT57 (lane A), LT54.III (lane C), and LT/t 2 (lane E,), liver (negative control) (lane B), and lymphoma from LT/t 3 (lane D), and the blot was hybridized with pMK16-2. The same blot was melted and rehybridized with GAPDH probe (26). (B) Each lane contained protein immunoprecipitated from 1 mg of total protein extract from seminal vesicle tumor of LT/t 1 (lane 1 Tu), epididymis from LT/t 1 (lane T/t), LT54.III (lane T), and a nontransgenic control (lane N).

relatively resistant to tumorigenesis by the SV40 genes, as was the salivary gland (10). The ability to transform only specific cell types is not unique to the SV40 T/t antigens; several other oncogenes have been shown to exhibit cell type specificity in transgenic animals (1, 24, 35, 42, 44).

Our results are in contrast to those of Palmiter et al. (34), which showed that the deletion of small t antigen did not affect the development of tumors in the choroid plexus, a tissue which also slowly divides in adult animals (29, 31). Possibly, cellular factors complementing the action of small t antigen are expressed during the differentiation of the choroid plexus epithelia, or perhaps the microenvironment of this tissue provides humoral factors which are lacking in the lung and kidney. This would be in agreement with the hypothesis that the transformation of cells in different tissues requires the participation of growth factors which act on those cells (15). Alternatively, transgenic animals carrying pSV11 (expressing only large T antigen) may have expressed very high levels of large T antigen because of the SV40 enhancer present in the transgene (7, 34); it has been suggested that small t antigen is dispensable for the transformation process when high levels of T antigen are expressed (3). Interestingly, transgenic mice carrying pSV11 showed a decreased incidence of kidney glomerular lesions relative to those carrying the SV40 wild-type early region (34).

Our identification of the ductal epithelial cells of the lung and kidney as two tissues where SV40 small t antigen plays a role in tumorigenesis will facilitate the understanding of the role of this protein in the process of transformation. We have examined the effect of small-t-antigen deletion on tumorigenesis in vivo, where the target cells are diploid and are present in the different environments in which they normally develop. Our results show that small t antigen is indeed necessary for rapid SV40-induced tumorigenesis in the lung and kidney. In this experimental system, we can now address the question as to how the small t antigen affects the action of the large T antigen. It has been shown that the synergistic action of two oncogenes can be studied by crossing transgenic mice carrying different oncogenes (42). We are currently producing transgenic animals carrying small t antigen alone under control of the MMTV LTR. The analysis of the biochemical changes in the lung and kidney epithelial cells expressing only small t, only large T, and both large T and small t antigens will provide insight into the molecular mechanisms of small-t-antigen involvement in tumorigenesis.

ACKNOWLEDGMENTS

We thank E. Gurney for supplying us with hamster anti-SV40 tumor serum. We also thank J. Bluestone for FACS analysis. We are grateful for R. Kucherlapati for critically reviewing the manuscript.

This work was supported by a Public Health Service grant from the National Institutes of Health (PHS CA 45954). S.R.R. is a Leukemia Society of America Scholar.

LITERATURE CITED

1. **Adams, J. M., A. W. Harris, C. A. Pinkert, L. M. Corcoran, W. S. Alexander, S. Cory, R. D. Palmiter, and R. L. Brinster.** 1985. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature (London) **318**:533–538.
2. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. USA **75**:1274–1278.
3. **Bikel, I., X. Montano, M. E. Agha, M. Brown, M. McCormack, J. Boltax, and D. M. Livingston.** 1987. SV40 small t antigen enhances the transformation activity of limiting concentrations of SV40 large T antigen. Cell **48**:321–330.
4. **Bishop, J. M.** 1985. Viral oncogenes. Cell **42**:23–38.
5. **Bishop, J. M.** 1987. The molecular genetics of cancer. Science **235**:305–311.
6. **Bouck, N., N. Beales, T. Shenk, P. Berg, and G. DiMayorca.** 1978. New region of simian virus 40 genome required for efficient viral transformation. Proc. Natl. Acad. Sci. USA **75**: 2473–2477.
7. **Brinster, R. L., H. Y. Chen, A. Messing, T. Van Dyke, A. J. Levine, and R. D. Palmiter.** 1984. Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. Cell **37**:367–379.
8. **Brown, M., M. McCormack, K. G. Zinn, M. P. Farrell, I. Bikel, and D. M. Livingston.** 1986. A recombinant murine retrovirus for simian virus 40 large T cDNA transforms mouse fibroblasts to anchorage-independent growth. J. Virol. **60**:290–293.
9. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
10. **Choi, Y., D. Henrard, I. Lee, and S. R. Ross.** 1987. The mouse mammary tumor virus long terminal repeat directs expression in epithelial and lymphoid cells of different tissues in transgenic mice. J. Virol. **61**:3013–3019.
11. **Clayton, C. E., D. Murphy, D. Lovett, and P. W. J. Rigby.** 1982. A fragment of the SV40 large T antigen transforms. Nature (London) **299**:59–61.
12. **Colby, W. A., and T. Shenk.** 1982. Fragments of simian virus 40 transforming gene facilitate transformation of rat embryo cells. Proc. Natl. Acad. Sci. USA **79**:5189–5193.
13. **Fluck, M., and T. Benjamin.** 1979. Comparisons of two early gene functions essential for transformation in polyoma virus and SV40. Virology **96**:205–225.
14. **Frisque, R. J., D. B. Rifkin, and W. C. Topp.** 1979. Requirement for the large T and small t proteins of SV40 in maintenance of the transformed state. Cold Spring Harbor Symp. Quant. Biol. **44**:325–331.
15. **Goustin, A. S., E. B. Leof, G. D. Shipley, and H. L. Moses.** 1986. Growth factors and cancer. Cancer Res. **46**:1015–1029.
16. **Hanahan, D.** 1985. Heritable formation of pancreatic b-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature (London) **315**:115–122.

16a. **Henrard, D., and S. R. Ross.** 1988. Endogenous mouse mammary tumor virus is expressed in several organs in addition to the lactating mammary gland. J. Virol. **62**:3046–3049.

17. **Jat, P. S., C. L. Cepko, R. C. Mulligan, and P. A. Sharp.** 1986. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. Mol. Cell. Biol. **6**:1204–1217.
18. **Jat, P. S., and P. A. Sharp.** 1986. Large T antigens of simian virus 40 and polyomavirus efficiently establish primary fibroblasts. J. Virol . **59**:746–750.
19. **Khoury, G., J. C. Alwine, R. Dhar, P. Gruss, C.-J. Lai, S. Segal, and I. Seif.** 1980. Regulation of SV40 gene expression through RNA splicing. Cold Spring Harbor Symp. Quant. Biol. **44**:41–54.
20. **Klein, G., and E. Klein.** 1985. Evolution of tumours and the impact of molecular oncology. Nature (London) **315**:190–195.
21. **Knudson, A. F., Jr.** 1986. Genetics of human cancer. Annu. Rev. Genet. **20**:231–251.
22. **Kriegler, M., C. F. Perez, C. Hardy, and M. Botchan.** 1984. Transformation mediated by the SV40 T antigens: separation of the overlapping SV40 early genes with a retroviral vector. Cell **38**:483–491.
23. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:602–607.
24. **Leder, A., P. K. Pattengale, A. Kuo, T. A. Stewart, and P. Leder.** 1986. Consequences of widespread deregulation of c-myc gene in transgenic mice: multiple neoplasms and normal development. Cell **45**:485–495.
25. **Martin, R. G., V. P. Setlow, C. A. F. Edwards, and D. Vembu.** 1979. The roles of the simian virus 40 tumor antigens in

transformation of Chinese hamster lung cells. Cell **17**:635–643.
26. **Marty, P. H. L., M. Piechaczyk, S. E. Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard.** 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. **13**:1431–1442.
27. **Matthews, B.-J., A. S. Levine, and K. Dixon.** 1987. Deletion mutations in the small t antigen gene alter the tissue specificity of tumors induced by simian virus 40. J. Virol. **61**:1282–1285.
28. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
29. **Messier, B., and C. P. Leblond.** 1960. Cell proliferation and migration as revealed by autoradiography after injection of thymidine-H^3 into male rats and mice. Am. J. Anat. **106**:247–285.
30. **Nandi, S., and C. M. McGrath.** 1973. Mammary neoplasia in mice. Adv. Cancer Res. **17**:353–414.
31. **Netsky, M. G., and S. Shuangshoti. (ed.).** 1975. The choroid plexus in health and disease. University Press of Virginia, Charlottesville.
32. **Ornitz, D. M., R. D. Palmiter, A. Messing, R. E. Hammer, C. A. Pinkert, and R. L. Brinster.** 1985. Elastase I promoter directs expression of human growth hormone and SV40 T antigen genes to pancreatic acinar cells in transgenic mice. Cold Spring Harbor Symp. Quant. Biol. **50**:399–409.
33. **Palmiter, R. D., and R. L. Brinster.** 1986. Germ-line transformation of mice. Annu. Rev. Genet. **20**:465–499.
34. **Palmiter, R. D., H. Y. Chen, A. Messing, and R. L. Brinster.** 1985. SV40 enhancer and large-T antigen are instrumental in development of choroid plexus tumours in transgenic mice. Nature (London) **316**:457–460.
35. **Quaife, C. J., C. A. Pinkert, D. M. Ornitz, R. D. Palmiter, and R. L. Brinster.** 1987. Pancreatic neoplasia induced by ras expression in acinar cells of transgenic mice. Cell **48**:1023–1034.
36. **Ross, S. R., D. I. H. Linzer, S. J. Flint, and A. J. Levine.** 1978. The adenovirus and SV40 tumor antigens detected in adenovirus and adenovirus-SV40 hybrid virus transformed cells, p. 469–484. *In* J. Stevens and G. Todaro (ed.), Persistent viruses. Academic Press, Inc., New York.
37. **Ross, S. R., and D. Solter.** 1985. Glucocorticoid regulation of mouse mammary tumor virus sequences in transgenic mice. Proc. Natl. Acad. Sci. USA **82**:5880–5884.
38. **Rubin, H., J. Figge, M. T. Bladon, L. B. Chen, M. Ellman, I. Bikel, M. P. Farrell, and D. M. Livingston.** 1982. Role of small t antigen in the acute transforming activity of SV40. Cell **30**:469–480.
39. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304**:602–607.
40. **Seif, R., and R. G. Martin.** 1979. Simian virus 40 small t antigen is not required for the maintenance of transformation but may act as a promoter (cocarcinogen) during establishment of transformation in resting rat cells. J. Virol. **32**:979–988.
41. **Shenk, T. E., J. Carbon, and P. Berg.** 1976. Construction and analysis of viable deletion mutants of simian virus 40. J. Virol. **18**:664–671.
42. **Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace, and P. Leder.** 1987. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell **49**:465–475.
43. **Sleigh, N. J., W. C. Topp, R. Hanich, and J. Sambrook.** 1978. Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. Cell **14**:79–88.
44. **Stewart, T. A., P. K. Pattengale, and P. Leder.** 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell **38**:627–637.
45. **Tegtmeyer, P.** 1980. Genetics of SV40 and polyoma virus, p. 297–338. *In* J. Tooze (ed.), Molecular biology of tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Toohey, M. G., K. L. Morley, and D. O. Peterson.** 1986. Multiple hormone-inducible enhancers as mediators of differential transcription. Mol. Cell. Biol. **6**:4526–4538.
47. **Tooze, J. (ed.).** 1980. Molecular biology of tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Topp, W. C., D. B. Rifkin, and M. J. Sleigh.** 1981. SV40 mutants with an altered small-t protein are tumorigenic in newborn hamsters. Virology **111**:341–350.
49. **Weinberg, R.** 1985. The action of oncogenes in the cytoplasm and nucleus. Science **230**:770–776.
50. **Wright, N., and M. Alison (ed.).** 1984. The biology of epithelial cell populations. Clarendon Press, Oxford.
51. **Zinn, K., D. DiMaio, and T. Maniatis.** 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell **34**:865–869.