

p53 Mutations Are Not Selected for in Simian Virus 40 T-Antigen-Induced Tumors from Transgenic Mice

M. MOORE, A. K. TERESKY, A. J. LEVINE,* AND M. SEIBERG

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014

Received 22 August 1991/Accepted 16 October 1991

Many diverse tumors contain cells that select for mutations at the p53 gene locus. This appears to be the case because the p53 gene product can act as a negative regulator of cell division or a tumor suppressor. These mutations then eliminate this activity of the p53 gene product. The simian virus 40 (SV40) large T antigen binds to p53 and acts as an oncogene to promote cellular transformation and initiate tumors. If the binding of T antigen to the p53 protein inactivated its tumor suppressor activity, there would be no selection pressure for p53 mutants to appear in tumors. To test this idea, transgenic mice that carried and expressed the SV40 large T-antigen gene were created. Expression of the T antigen was directed to the liver, using the albumin promoter, and the choroid plexus, using the SV40 enhancer-promoter. A large number of papillomas (indicated in parentheses) of the choroid plexus (14), hepatocellular carcinomas (5), liver adenomas (10), and tumors of clear-cell foci (5) were examined for mutant and wild-type p53 genes and gene products. In all cases, the tumor extracts contained readily detectable T-antigen-p53 protein complexes. A monoclonal antibody specifically recognizing the wild-type p53 protein (PAb246) reacted with p53 in every tumor extract. A monoclonal antibody specifically recognizing mutant forms of the p53 protein (PAb240) failed to detect p53 antigens in these extracts. Finally, p53 partial cDNAs were sequenced across the regions of common mutations in this gene, and in every case only the wild-type sequence was detected. These results strongly support the hypothesis that T antigen inactivates the wild-type p53 tumor-suppressing activity and there is no need to select for mutations at the p53 locus.

Tumor formation is a complex process that appears to involve the activation and expression of oncogenes and the inactivation of tumor suppressor genes, in each case via mutation. Several diverse cancers appear to select for mutations in both alleles at the retinoblastoma susceptibility (Rb) locus (19) and the p53 gene (25). Both the Rb and p53 genes will suppress the ability of a tumorigenic cell line to produce tumors in nude mice (6, 22). The wild-type p53 gene has also been shown to inhibit the transformation of primary cells in culture by other oncogenes (15). Indeed, returning the wild-type p53 gene into transformed cell inhibits its replication (1, 11, 33). A transformed cell line with a mutant temperature-sensitive p53 protein, which was phenotypically wild type at 32°C and mutant at 39.5°C, stopped cell division at 32°C in the G₁ phase of the cell cycle (32, 34). All of these experimental observations indicate that the wild-type Rb and p53 gene products negatively regulate cell growth or division and that the mutations observed in these genes eliminate their ability to suppress cell division.

The oncogene products of several of the DNA tumor viruses also appear to target the Rb and p53 proteins. The simian virus 40 (SV40) large tumor antigen (T antigen) contains specific domains for binding to the Rb protein (9) and the p53 protein (24, 28). These domains of T antigen correspond to the regions of this protein critical for transforming cells in culture (8, 37). Similarly, the adenovirus E1A protein binds to Rb (46), and the E1B 55-kDa protein binds to p53 (39). Finally, the human papillomavirus type 16 or 18 oncogenes E6 and E7 bind to p53 and Rb, respectively (12, 45). In this case, the E6-p53 complex is targeted for destruction of p53 via proteolytic breakdown employing the ubiquitin system of degradation (41). Thus, it is clear in the

case of E6-p53 that the function of the E6 oncogene is to eliminate or inactivate p53 function. This view is supported by the observation that cell lines in culture derived from cervical carcinomas and containing the human papillomavirus type 16 or 18 E6 gene product have a wild-type p53 gene (40). There is apparently no need to select against the p53 wild-type function. Strikingly, cell lines derived from cervical carcinomas with no human papillomavirus E6 gene contain mutant p53 genes (40).

If the function of the binding of SV40 large T antigen to p53 is to eliminate the ability of the p53 protein to suppress tumor cell division, then SV40-induced tumors expressing T antigen should contain a wild-type p53 gene and protein. This would reflect the absence of a need to select for mutant p53 forms in such tumors. This clear prediction could be tested in transgenic mice harboring the SV40 gene for the large T antigen.

In this study, transgenic mice expressing the SV40 large T antigen in two different tissues, the choroid plexus and liver, were created. The p53 gene and gene product were analyzed in papillomas of the choroid plexus, adenomas of the liver, hepatocellular carcinomas, and tumors of clear cell foci. In all cases, a T-antigen-p53 complex was detected in tumor extracts. Monoclonal antibodies specific for the wild-type form of p53 protein (PAb246) reacted with the p53 protein in these extracts, whereas PAb240, specific for mutant forms of p53, failed to react with the p53 antigen in these tumor extracts. Direct nucleotide sequencing of the p53 cDNAs derived from these tumors (the cDNAs covered codons 120 to 290) detected only wild-type p53 sequences. Despite the fact that many diverse tumors, including liver tumors (3, 21), commonly contain p53 mutations, no evidence for such mutations could be found when the SV40 large T antigen was the initial or primary oncogene used to originate these

* Corresponding author.

tumors. This is the expected result if indeed the SV40 large T antigen inactivates the tumor suppressor functions of the p53 protein. This result is consistent with the observation that the great majority (93%) of the SV40 T-antigen-transformed primary cells in culture contain the wild-type form of the p53 protein (27).

MATERIALS AND METHODS

Construction of the ALT fusion gene and production of transgenic mice. The hybrid albumin promoter/enhancer driving the SV40 large T antigen (ALT) was cloned by initially subcloning the *Bam*HI fragment containing the albumin enhancer and promoter from RB6 (38) into a pGem 3 vector. T antigen was obtained from pSV11. pSV11, a plasmid containing the SV40 T antigen, contains a deletion of positions 4854 to 4586 in the small-t splice site such that small t is not expressed (8). pSV11 was restriction digested with *Stu*I and *Bam*HI, and the T-antigen sequence was cloned into AIGem at the *Sma*I site. The plasmid was excised from the vector sequences for microinjection with *Sal*I and *Sac*I and injected into the pronuclei of fertilized DBA × Blk6 hybrid mouse eggs at a concentration of 1 µg/ml. Eggs that survived injection were implanted at the two-cell stage into pseudopregnant CD.1 recipients. Founder transgenic mice were identified by Southern analysis of tail DNA, using a random-primed *Stu*I-*Bam*HI fragment from pSV11.

A C57BL/6J mouse strain was used to mate a male transgenic mouse with a female that did not carry the transgene. All mice were purchased from the Jackson Mouse Laboratory, Bar Harbor, Maine.

The SV11 transgenic line has been previously described (4).

DNA and RNA analysis. RNA was isolated from various mouse tissues by the guanidine isothiocyanate procedure (7). Northern (RNA) blots, RNA dot blots, and Southern blots were performed as previously described (30). The RNA was run out on an ethidium-stained agarose gel; 18S and 16S RNAs were visualized intact.

Western immunoblot analysis. Liver tumor extracts (2 mg/ml) or brain tumor extracts (300 µg/ml) were initially immunoprecipitated with p53 monoclonal antibodies PAb421 and PAb246 and T-antigen monoclonal antibody PAb419. PAb421 recognizes both mutant and wild-type forms of p53; PAb246 recognizes only the native conformation. Immunoprecipitates were electrophoresed on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to Immobilon, probed with PAb421, and visualized with ¹²⁵I conjugated to protein A (5). In some cases, blots were reprobed directly with PAb419. The A₁ cell line lysate was used as a qualitative and quantitative control for p53 protein level and conformation. The transformed cell line, A₁, expresses copious amounts of a temperature-sensitive murine p53 protein; alanine is altered to valine at amino acid residue 135. At 32°C, the p53 protein is predominantly wild type and reactive to PAb246; at 39°C, the protein is mutant and reacts with PAb240. Immunoprecipitation carried out at liver tumor protein concentrations greater than 2 mg/ml led to reduced binding of T antigen to p53 as seen with PAb419 as well as reduced reactivity to PAb246. We estimate that the tumor tissue used in our analysis contains less than 1% contaminating normal tissue by pathological sectioning.

p53 gene analysis. The 1 µg of RNA derived from the various tumors was reverse transcribed and subsequently amplified by the polymerase chain reaction (PCR). The

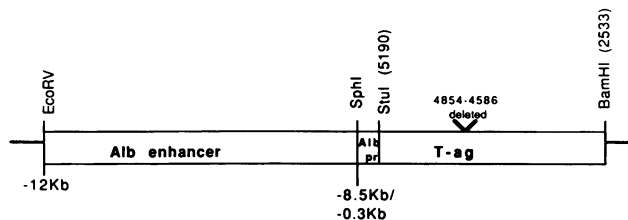


FIG. 1. The ALT construct. The 3.5-kb albumin (Alb) enhancer and 300-bp albumin promoter are fused to the *Stu*I-*Bam*HI fragment from pSV11 encoding the large T antigen (T-ag). The deletion from pSV11 eliminating the expression small t is indicated. The hybrid gene ALT was excised from the plasmid by restriction digestion with *Sal*I and *Sac*I, which are in the vector's polylinker.

cDNA reaction was incubated in a total volume of 100 µl of solution containing 50 mM KCl, 20 mM Tris buffer (pH 8), 10 mM MgCl₂, 20 pmol of oligonucleotide primers, and 1 U of *Taq* polymerase (Perkin-Elmer Cetus). The PCR procedure consisted of 35 cycles of 95°C (1 min), 58°C (1 min), and 70°C (2 min). The primers were used 5'-GCGAATTCCCGGCTC TGAGTATACCAC-3' and 5'-GCGAATTCCCATGGCGCG GACACGGCTC-3'. The amplified product spanning the four conserved domains in p53 was cloned into Bluescript KS⁻ and sequenced by using the T3 and T7 primers. Only data for clones repeated in separate PCRs are given in Table 2.

RESULTS

Transgenic mice containing SV40 large T antigen (ALT). A hybrid gene consisting of the albumin regulatory elements and the SV40 large T antigen was constructed (Fig. 1). A total of eight founder animals were produced after microinjection of this construct into fertilized mouse eggs (Table 1). Among these animals, three founders developed liver tumors, hepatocellular carcinomas appearing between 2 and 5 months of age. Microinjection of this construct into fertilized mouse eggs also led to transgenic animals which were stillborn as well as fetuses which appeared to be absorbed in utero. A total of five animals were stillborn, and three of these animals were analyzed for the presence of T-antigen DNA by Southern analysis (data not shown) and for RNA expression in the liver. Figure 2A shows a slot blot of liver RNA probed with T-antigen sequences. All three stillborn animals examined were transgenic and expressed low levels of the transgene RNA in the liver. The livers from these animals were examined at the histological level, and all showed increased hemopoiesis. It was not determined

TABLE 1. Pathological findings in transgenic mice

Transgenic line		Pathological findings
Founder	Copy no.	
ALT-1	1-2	Stillborn, no pathology, increased hemopoiesis observed in liver
ALT-2	Mosaic	No pathology at 4 months, sacrificed
ALT-3	Mosaic	Liver nodules at 4 months, sacrificed
ALT-4	2-4	Stillborn, no pathology, increased hemopoiesis observed in liver
ALT-5	<1	Not intact, no pathology
ALT-6	5	Premalignant cyst, sacrificed at 4 months
ALT-7	2-3	Stillborn, no pathology in liver
ALT-8	1-2	Multiple hepatocellular carcinoma

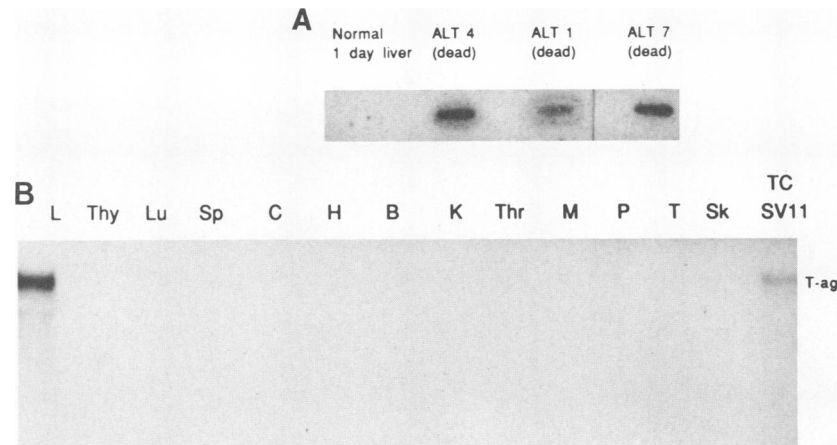


FIG. 2. (A) RNA slot blot analysis of the stillborn transgenic mice. Total RNA (10 μ g) isolated from the livers of the stillborn transgenic animals was bound to a nitrocellulose filter by using a slot blot apparatus. The filter was baked and subsequently probed with a random-primed *StuI-BamHI* fragment from pSV11. RNA isolated from a 1-day-old normal mouse liver was used as a control. (B) Qualitative Northern analysis from founder ALT-8. Total RNAs (20 μ g) from tumorigenic liver (L) and normal tissues (thymus [Thy], lung [Lu], spleen [Sp], colon [C], heart [H], brain [B], kidney [K], thyroid [Thr], muscle [M], pancreas [P], testes [T], and skin [Sk]) were electrophoresed in 1.5% agarose formaldehyde gel, transferred to nitrocellulose, and probed with a random-primed *StuI-BamHI* fragment from pSV11. RNA from the tissue culture line pSV11 (TC SV11) was used as a size marker. T-ag, T antigen.

whether this phenotype was a direct consequence of large T-antigen expression in the liver. However, when stop codons were inserted into the T-antigen sequences of an isogenic construct, the litter sizes were normal and no stillbirths were observed (28a).

One mouse line, ALT-8, was bred for further characterization. The transgene was carried in this line with 100% penetrance. To study the expression of the T-antigen gene, RNA was extracted from various tissues of the ALT-8 founder. A qualitative Northern analysis was performed, using the *BamHI-StuI* fragment of the SV40 large T antigen as a probe. As shown in Fig. 2B, the T-antigen RNA was detected only in the tumorigenic liver and not in 12 other tissues examined.

Pathology of ALT-8 livers. In mice from the ALT-8 transgenic line, enlarged livers were observed at or before 2 months of age. On gross inspection, such livers often had a granular appearance. Histological examination of these livers (stage 1) from 2-month-old mice showed many altered or dysplastic cells as well as cells classified as adenomas (benign tumors) (Fig. 3A2, A3, and B1). With increasing age, the livers enlarged and small tumor nodules appeared. This was termed a stage 2 tumor. Upon histological examination, these small nodules were neoplastic and some contained foci of hepatocellular carcinomas (Fig. 3A4). At approximately 3 months (termed stage 3), the small nodules increased in size and possibly fused to form multiple tumor masses which appeared on the background of granular cells or smaller liver nodules. By 4 to 5 months of age, large tumors that bore little resemblance to normal liver were present. At this point, the liver was 10 to 20 times the weight of a normal liver and several types of pathology were observed (Fig. 3B).

T-antigen RNA expression in the liver of ALT-8 transgenic mouse line. To quantitate and map the T-antigen transcripts, a riboprobe protection analysis was performed. Livers were isolated from the transgenic animals at different ages, and RNA was extracted. The RNAs were hybridized to a T-antigen antisense probe made from the *PvuII-BamHI* DNA fragment of the SV40 T antigen. The hybrids were treated with RNase, and the protected fragments were analyzed on

a 7 M urea-6% acrylamide gel. With this assay, the level of steady-state T-antigen transcripts was found to be lower than in the SV11 line of mice, which express the SV40 large T antigen in the choroid plexus (44).

RNAs from dysplastic regions and tumor or nodular regions from the same liver were isolated and compared for T-antigen expression. Analysis of these data shows that the RNA level varied with the progression of the tumor. The dysplastic liver from stage 1 mice showed low or undetectable levels of the T-antigen mRNA by RNase protection. The highest level of T-antigen RNA was detected in the tumor nodules in stages 2 to 3. T-antigen RNA levels were observed to decrease in some of the severe late-stage tumors older than 4 months (data not shown). In all animals examined, expression of the transgene was low and never approached the level expected for endogenous albumin mRNA.

Analysis of p53 antigens in liver tumors. To examine p53 protein, liver tumor extracts (Table 2) were initially immunoprecipitated with p53 monoclonal antibodies PAb421 and 246 and T-antigen-specific monoclonal antibody PAb419. PAb421 recognizes both mutant and wild-type forms of p53 protein; PAb246 recognizes only the wild-type configuration of the p53 protein (47). The immunoprecipitates were electrophoresed on SDS-polyacrylamide gels, and Western analysis was performed with using monoclonal antibody PAb421. Table 2 shows the results obtained for liver tumors. As shown, normal liver p53 protein was not detectable at this protein concentration of the extracts used (2 mg). There was correlation between the observed pathology and the absolute levels of p53 and T antigen. In general, normal tissue or hyperplastic tissues had undetectable levels of p53 and T antigen; adenomas or carcinomas contained p53-T-antigen complexes that were readily detected and reacted with PAb246 (Fig. 4A). PAb240 recognizes many mutant p53 proteins but not wild-type p53 protein (17). None of the liver tumor extracts examined (36 liver tumors at various stages) reacted with PAb240 (Fig. 5); adenomas and carcinomas contained elevated levels of p53, whereas hyperplastic nodules did not.

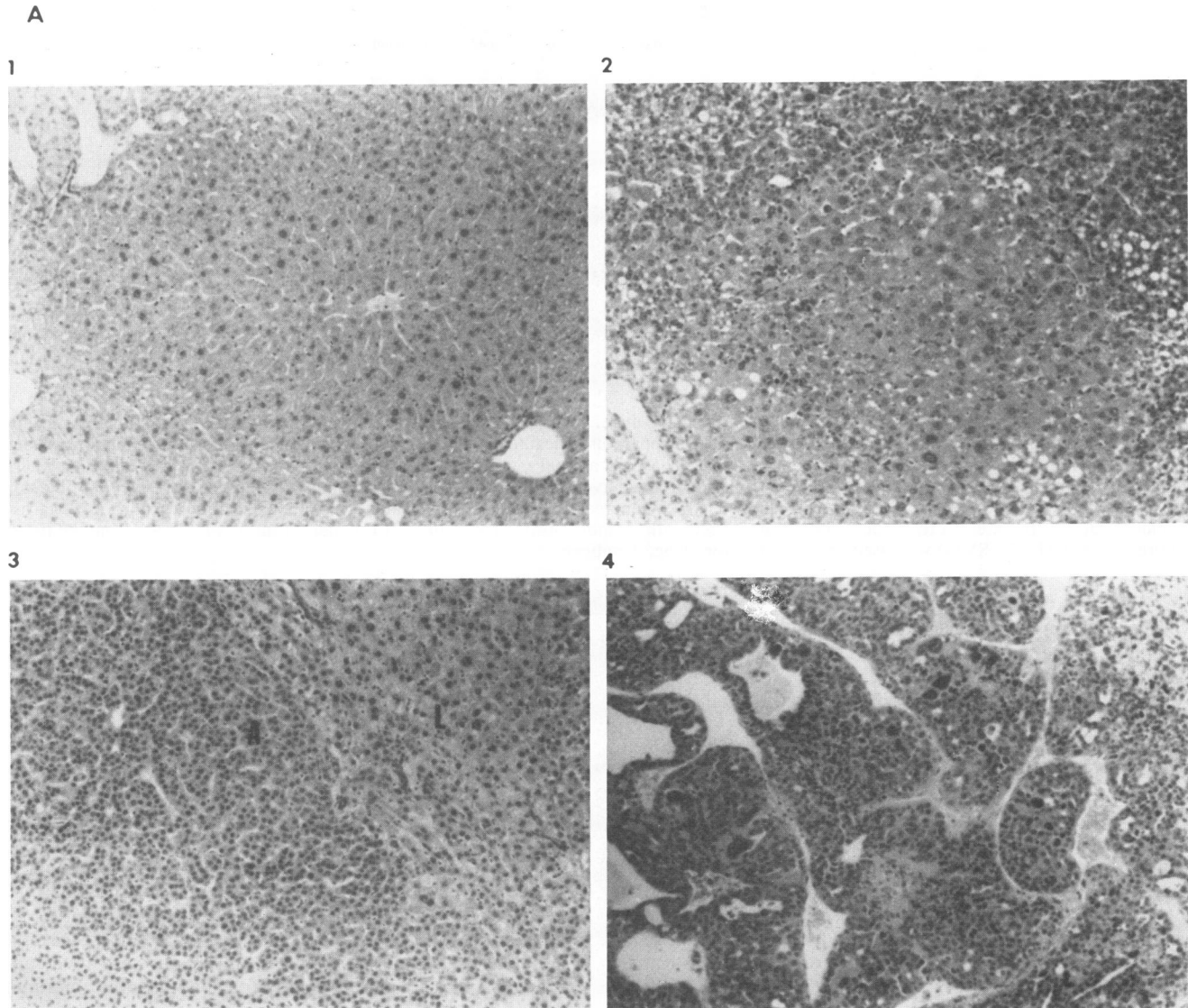


FIG. 3. Histological appearance of ALT-8 livers. (A) Development of tumor (hematoxylin-and-eosin stain; magnification, $\times 84$). 1, normal mouse liver; 2, hyperplastic nodule; 3, hepatocellular adenoma (A) compressing preexisting liver (L); 4, hepatocellular carcinoma, poorly differentiated. (B) Individual tumors examined in detail (hematoxylin-and-eosin stain; magnification, $\times 400$). 1, hyperplastic nodule (note anisokarya and intranuclear inclusions [I]); 2, hepatocellular adenoma (note loss of lobular architecture and anisokarya); 3, hepatocellular adenoma (note clear cells [C], individual necrotic cells [N], karyomegalic cells [K], and mitotic figure [M]); 4, bile duct hyperplasia; 5, hepatocellular carcinoma, poorly differentiated (note anisokarya and numerous mitotic figures [M], including atypical forms); 6, hepatocellular carcinoma, well differentiated (note lack of lobular architecture).

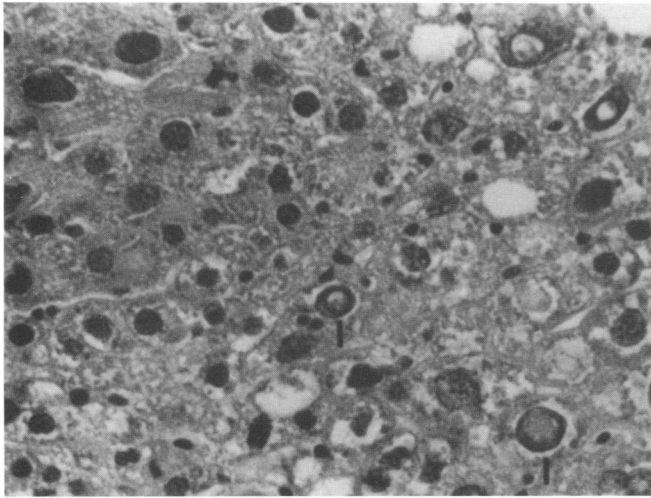
Nucleotide sequence analysis of p53 cDNAs from tumors. RNA from some liver tumors, unless otherwise indicated, was reverse transcribed, and p53 cDNA was amplified by PCR, cloned, and subsequently sequenced. Only data that were repeatedly obtained in independent PCR reactions are contained in Table 2. The PCR-amplified sequenced cDNA clones span codons 120 to 290 in the p53 gene. This covers the conserved domains II, III, IV, and V of the p53 gene. The p53 clones were wild type over this region, and Southern blot analysis revealed no gross deletions or rearrangements of the p53 gene (data not shown).

Papillomas of the choroid plexus. The results of the Western blot analysis and cDNA sequence information suggested that T-antigen-induced liver tumors did not appear to select

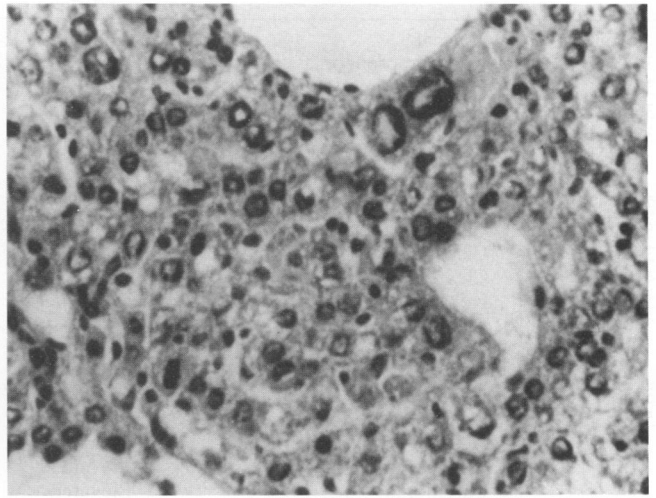
for mutations in the p53 gene. To determine whether p53 mutation occurred in T-antigen-induced tumors of another tissue origin, the SV11 transgenic line of mice was used. This line carries T antigen under the control of the SV40 promoter expressed solely in the choroid plexus (4, 31, 43, 44) and develops papillomas of the choroid plexus. Expression of the p53 gene and product was examined in 10 independent rapidly growing late-stage tumors (older than 90 days). The 10 dissected papillomas were analyzed by immunoprecipitation of p53 protein with PAb421, PAb246, and PAb419. The immunoprecipitates were collected, electrophoresed in SDS-polyacrylamide gels, transferred to Immobilon, and probed with PAb421. As depicted in Fig. 4B, p53 protein from normal tissue cannot be detected by such an analysis.

B

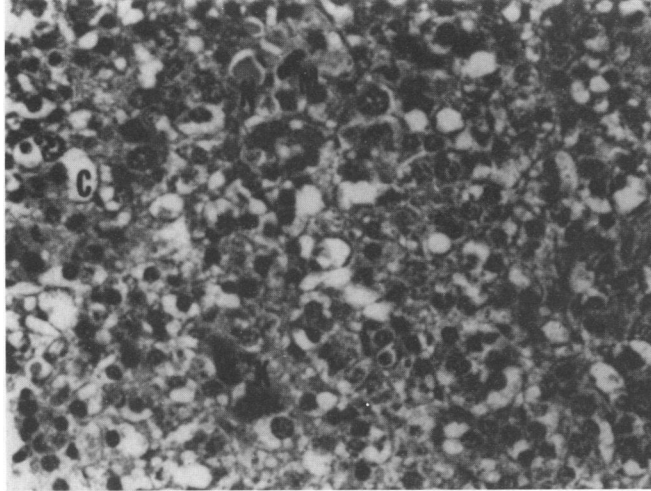
1



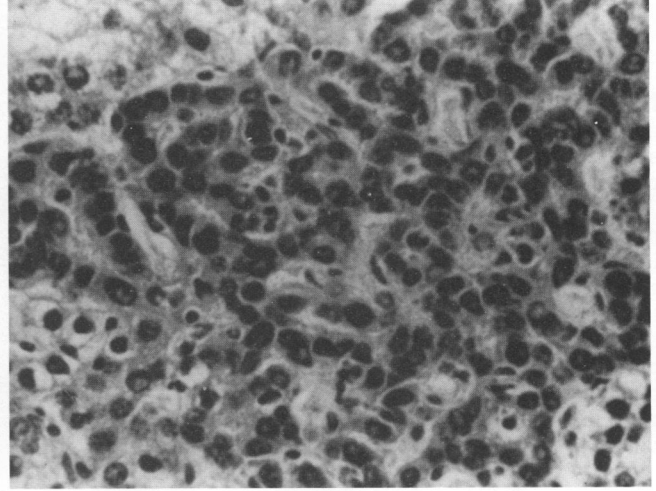
2



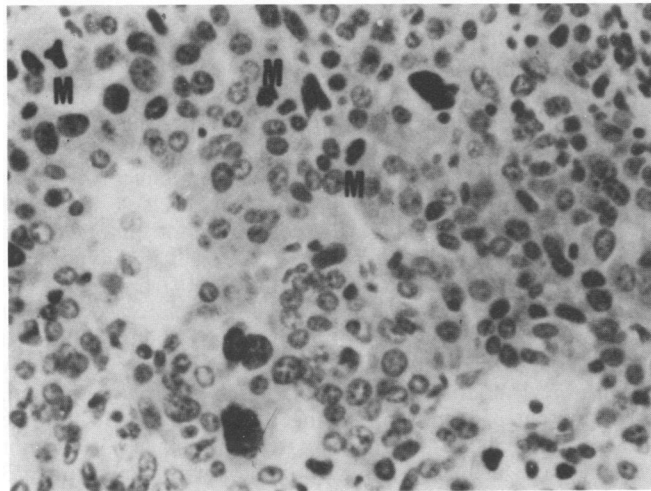
3



4



5



6

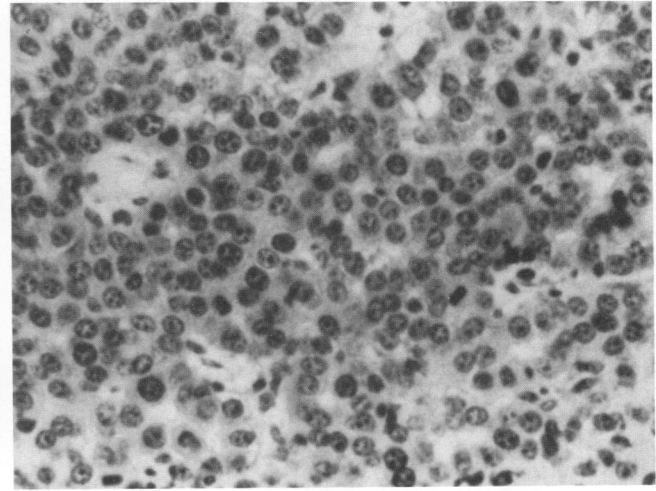


FIG. 3—Continued.

TABLE 2. State of p53 in liver tumors in ALT-8 mice^a

Liver specimen	Pathology	p53-T-antigen binding				PCR cDNA
		PAb421	PAb246	PAb240	PAb419	
Normal	Normal	ND	ND	ND	ND	Wild type
3828	Necrotic preneoplastic	Low level	+	-	-	UND
3817	Hyperplastic nodule	ND	ND	ND	ND	UND
3685	Hyperplastic nodule	Low level	+	-	+	UND
3788	Hyperplastic with adenoma	ND	ND	ND	ND	
		Low level	±	-	±	Wild type DNA wild type
3601	Hyperplastic with adenoma	ND	ND	ND	ND	UND
3521	Clear-cell foci	Elevated	+	-	+	UND
3999	Clear-cell foci	Low level	+	-	+	Wild type
4605	Clear-cell foci	Low level	+	-	+	Wild type
5005	Hepatocellular adenoma	Low level	+	-	+	UND
5006						
B	Adenoma	Elevated	+	-	+	UND
C	Adenoma	Elevated				
3328	Hepatocellular adenoma	Elevated	+	-	+	Wild type
5188						
A	Hepatocellular carcinoma	Elevated	+	-	+	Wild type
B	Carcinoma	Elevated				
C	Carcinoma	Elevated				
3523	Hepatocellular carcinoma	Elevated	+	-	+	Wild type DNA wild type

^a DNA wild type is a genomic PCR product; the remainder are cDNA PCR product (codons 120 to 290). ND, not detectable; UND, undetermined.

However, the tumor tissue contained readily detectable p53 antigen, and all of the p53 protein was complexed to large T antigen (Fig. 4B). The T-antigen-induced tumors of the choroid plexus all had a p53 protein that reacts with PAb246 (Table 3), which detects only the wild-type form of the p53 protein. p53 levels, as well as T-antigen levels, seen by immunoprecipitation and Western analysis were much greater in the choroid plexus papillomas than in the liver tumors (five times more protein was immunoprecipitated in the latter case [Fig. 4]). Three of the brain tumors analyzed by immunological criteria were also examined by PCR and nucleotide sequencing of the amplified p53 cDNA. The p53 gene in these T-antigen tumors of the choroid plexus contained a wild-type nucleotide sequence.

DISCUSSION

In the analysis of the hepatocellular tumor model reported here, the detection of the p53 protein correlates well with the expression of T antigen. Both the steady-state RNA analysis and steady-state levels of protein (Western analysis) indicate that T-antigen levels are initially very low in hyperplastic tissue (at the earliest stages of tumor formation) but are much increased in the tumor nodules. These data indicate that tumor formation occurs in stages at which additional events that select for increased T-antigen mRNA levels are occurring. However, even in the liver tumor nodule, the level of T-antigen expression was lower than in the SV11 choroid plexus papillomas. In this regard, it is interesting that the livers of the transgenic stillborn animals expressed T-antigen mRNA levels comparable to those found in the tumor nodules of the adult transgenic lines. The phenomenon is consistent with the notion that this level of expression of T antigen may be lethal at early times in development and may also explain why T-antigen expression in the established transgenic lines was low.

Many transgenic animal models of T-antigen-induced tumor formation, including the hepatocellular model reported

here, suggest that second events are required for tumor formation (4, 18, 20, 29, 35, 43, 44). This mouse model system was used to examine whether mutations in p53 would be such a second event selected for during tumor development. In the SV40 T-antigen-transformed cells, T antigen is physically complexed to the wild-type form of p53 and the protein half-life is extended from 20 min to 24 h (27, 28).

On the other hand, many different spontaneous transformed cell lines (16) and tumors (2, 3, 21) contain cells with mutations at the p53 locus. The most common mutations in the p53 gene are missense point mutations that create faulty or altered p53 proteins in one mutant allele. There is then a loss of the other (wild-type) allele via deletion or gene conversion, resulting in a reduction to homozygosity (25, 26). Such mutations could result in a loss of p53 function in these cells, which would be similar to what has been found for the Rb gene (25, 26). Alternatively, the mutant p53 gene and the resultant altered protein might gain a new function to promote cell growth. This view may be correct because the most common mutation selected for in a tumor is a missense mutation. It is rare to find a nonsense or frameshift mutation or a deletion that would each eliminate the protein. Thus, faulty or altered proteins are selected for in this case. Second, the p53 gene with a missense mutant protein can cooperate with the *ras* oncogene to transform cells in culture (unlike the Rb mutant gene), possibly contributing to the gain of a transformed phenotype (13, 36). If this hypothesis were correct, then the binding of the SV40 T antigen to p53 might result in only the loss of p53 function as a tumor suppressor, but now a mutation at the p53 locus would still be selected for, because an altered p53 protein could now gain a new function and promote cell growth and division. The mutant p53 protein no longer binds to the SV40 large T-antigen protein (42). In this case, early stages of a tumor might have a T-antigen-p53 complex with wild-type p53 protein, and late-stage (more aggressive) tumors might have a mutant p53 protein.

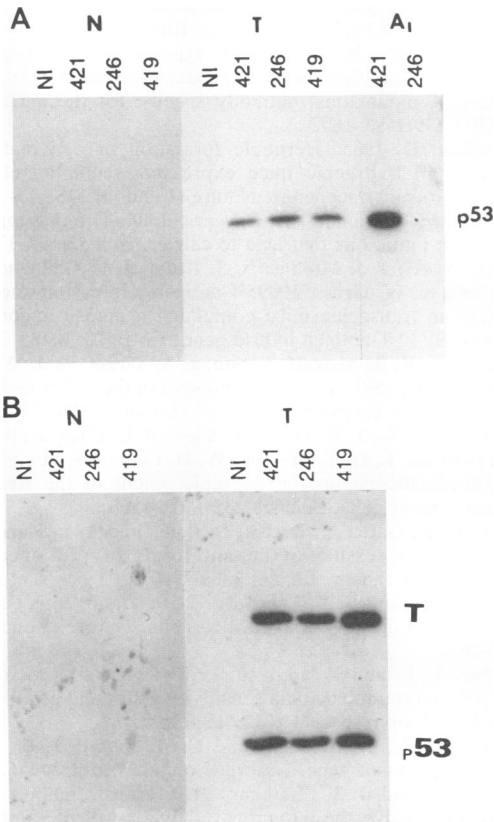


FIG. 4. Immunoprecipitation and Western blot analysis of tumor tissues. Tumors were chopped and lysed in CLB buffer (34) on ice. Cell extracts were normalized for protein by the Bio-Rad assay. Normal liver (N) and liver tumor (T) (2 mg/ml each; A) or normal brain (N) or brain tumor extract (T) (500 mg/ml each; B) were subjected to immunoprecipitation with PAb421, PAb246, PAb419, and an E1B monoclonal antibody (NI) as a nonimmune control. A₁ represents 25 μg of protein extract from a temperature-sensitive cell line harboring a mutant p53 gene. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gel, transferred to nylon, incubated with PAb421 (A) or PAb421 and PAb419 (B), probed with ¹²⁵I-labeled protein A, and exposed to X-ray film (16).

TABLE 3. State of p53 in SV11 brain tumors^a

Brain tumor specimen	p53 level			PCR cDNA ^b
	421	246	419	
1	Elevated	+	+	Wild type
2	Elevated	+	+	Wild-type
3	Elevated	+	+	Wild-type
4	Elevated	+	+	
5	Elevated	+	+	
6	Elevated	+	+	
7	Elevated	+	+	
8	Elevated	+	+	
9	Elevated	+	+	
10	Elevated	+	+	

^a All specimens exhibited choroid plexus papilloma. Approximately 300 μg of total protein was immunoprecipitated.

^b Codons 120 to 290.

These ideas were tested in two model systems employing transgenic mice expressing the SV40 large T antigen in the liver and the choroid plexus. Several independent criteria were used to determine whether p53 in such tumors was mutant or wild type. An antibody (PAb419) directed against the SV40 large T antigen was shown to coimmunoprecipitate p53 protein. Such a T-antigen-p53 complex (42) is indicative of a wild-type p53 protein. Second, monoclonal antibody PAb246, which recognizes the wild-type p53 conformation (42, 47), reacted with the p53 antigens in each liver and choroid plexus extract. Conversely, monoclonal antibody PAb240 (17, 23), which reacts only with certain mutant conformations of p53, failed to detect p53 in the liver or choroid plexus extracts. Finally, PCR-amplified probes of p53 cDNAs, or in some cases genomic DNAs, consistently provided nucleotide sequences (between codons 120 and 290) of the wild-type p53 gene.

Occasionally, PCR amplification followed by nucleotide sequencing did demonstrate point mutations in the p53 cDNA from a tumor RNA extract. These mutations were not reproducible in an independent experiment, and so it is assumed that they were artifacts or mistakes of the PCR procedure. Alternatively, it is not possible to rule out the possibility that a small percentage of T-antigen-containing

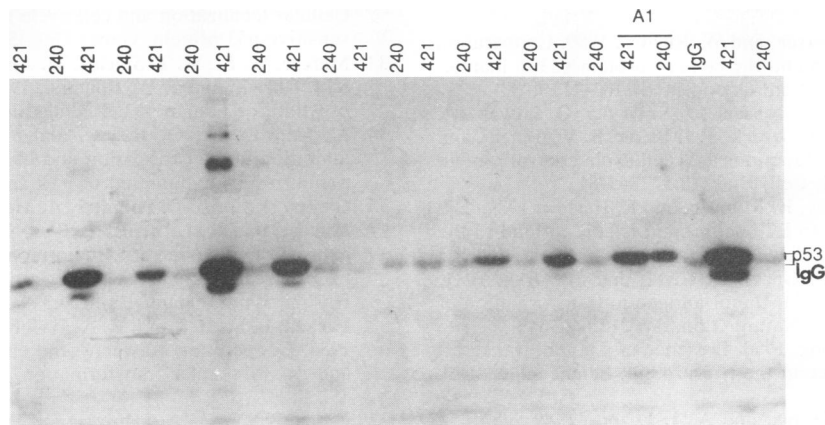


FIG. 5. Immunoprecipitation and Western blot analysis of tumor tissues. Independent liver tumors from various stages of tumor progression were chopped and lysed in CLB buffer on ice. Lysates (1 mg/ml) were subjected to immunoprecipitation with PAb421 and PAb240; 25 μg of lysate from an A₁ cell line maintained at 39°C containing mutant p53 with a 240 epitope was immunoprecipitated as a control. Note that at the exposure represented, the immunoglobulin G (IgG) band is visible.

tumor cells had selected for a p53 mutant late in tumor development. This would result in a tumor mixed with a majority of wild-type p53 mRNA and rare (observed one time only) mutant p53 mRNA. Neither Western blots nor PCR would be expected to detect less than 5 to 10% mutant p53 mRNA (cDNA) or protein in a mixed tumor. Even if this were the case, the tumor clearly arose with a T-antigen-p53 complex in which the wild-type p53 function was eliminated by complexing with T antigen.

ACKNOWLEDGMENTS

This research was supported by NJCCR postdoctoral grant 88-2044 (M. Moore) and by grant R37 CA38757 from the National Cancer Institute.

We thank Kate James for help in preparation of the manuscript.

REFERENCES

- Baker, S. J., S. Markowitz, E. R. Fearon, J. K. U. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* **249**:912-915.
- Bressac, B., K. M. Galvin, T. J. Liang, K. J. Isselbacher, J. R. Wands, and M. Ozturk. 1990. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **87**:1973-1977.
- Bressac, B., M. Kew, J. Wands, and M. Ozturk. 1991. Selective G to T mutation of p53 gene in hepatocellular carcinoma from southern Africa. *Nature (London)* **350**:429-431.
- Brinster, R. L., H. Y. Chen, A. Messing, T. van Dyke, A. J. Levine, and R. Palmiter. 1984. Transgenic mice harboring SV40 T antigen genes develop characteristic brain tumors. *Cell* **37**:367-379.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**:195-203.
- Chen, P.-L., Y. Chen, R. Bookstein, and W.-H. Lee. 1990. Genetic mechanisms of tumor suppression by the human p53 gene. *Science* **250**:1576-1579.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.
- Colby, W. W., and T. Shenk. 1982. Fragments of the simian virus 40 transforming gene facilitate transformation of rat embryo cells. *Proc. Natl. Acad. Sci. USA* **79**:5189-5193.
- DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**:275-283.
- Deppert, W., T. Steinmayer, and W. Richter. 1989. Cooperation of SV40 large T-antigen and the cellular protein p53 in maintenance of cell transformation. *Oncogene* **4**:1103-1110.
- Diller, L., J. Kassel, C. E. Nelson, M. A. Gryka, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and S. H. Friend. 1990. p53 functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.* **10**:5772-5781.
- Dyson, N., P. M. Howley, K. Munger, and E. Harlow. 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**:934-936.
- Eliyahu, D., A. Raz, P. Gruss, D. Givol, and M. Oren. 1984. Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. *Nature (London)* **312**:646-649.
- Fields, S., and S. K. Jong. 1990. Presence of a potent transcription activating sequence in the p53 protein. *Science* **249**:1046-1049.
- Finlay, C. A., P. W. Hinds, and A. J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell* **57**:1083-1093.
- Finlay, C. A., P. W. Hinds, T.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine. 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell Biol.* **8**:531-539.
- Gannon, J. V., R. Greaves, R. Iggo, and D. P. Lane. 1990. Activating mutations in p53 produce common conformation effects. A monoclonal antibody specific for the mutant form. *EMBO J.* **9**:1595-1602.
- Hanahan, D. 1985. Heritable formation of pancreatic β -cell tumours in transgenic mice expressing recombinant insulin-simian virus 40 oncogenes. *Nature (London)* **315**:115-122.
- Hansen, M. F., and W. K. Cavenee. 1988. Tumor suppressors: recessive mutations that lead to cancer. *Cell* **53**:172-173.
- Held, W. A., J. J. Mullins, N. J. Kuhn, J. F. Gallagher, G. D. Gu, and K. W. Gross. 1989. T-antigen expression and tumorigenesis in transgenic mice containing a mouse major urinary protein/SV40 T-antigen hybrid gene. *EMBO J.* **8**:183-191.
- Hsu, I. C., R. A. Metcalf, T. Sun, J. A. Welsh, N. J. Wang, and C. C. Harris. 1991. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature (London)* **350**:427-429.
- Huang, H. J. S., J. K. Yee, J. Y. Shew, P. L. Chen, R. Bookstein, T. Friedman, E. H. Y. Lee, and W.-H. Lee. 1988. Suppression of the neoplastic phenotype by replacement of the Rb gene in human cancer cells. *Science* **242**:1563-1566.
- Iggo, R., K. Gatter, J. Bartek, D. Lane, and A. L. Harris. 1990. Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* **335**:675-679.
- Lane, D. P., and L. V. Crawford. 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature (London)* **278**:261-263.
- Levine, A. J., and J. Momand. 1990. Tumor suppressor genes: the p53 and retinoblastoma sensitivity genes and gene products. *Biochim. Biophys. Acta* **1032**:119-136.
- Levine, A. J., J. Momand, and C. A. Finlay. 1991. The p53 tumor suppressor gene. *Nature (London)* **351**:453-456.
- Lin, J.-Y., and D. T. Simmons. 1990. Transformation by SV40 does not involve the mutational activation of p53 to an oncogenic form. *Virology* **176**:302-305.
- Linzer, D. I. H., and A. J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40 transformed cells. *Cell* **17**:43-52.
- Logan, J. S. Personal communication.
- Mahon, K. A., A. B. Chepelinsky, J. S. Khilian, P. A. Overbeek, J. Piatigorsky, and H. Westphal. 1987. Oncogenesis of the lens in transgenic mice. *Science* **235**:1622-1628.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Marks, J. R., J. Lin, P. Hinds, D. Miller, and A. J. Levine. 1988. Cellular gene expression in papillomas of the choroid plexus from transgenic mice that express the simian virus 40 large T antigen. *J. Virol.* **63**:790-797.
- Martinez, J., I. Georgoff, J. Martinez, and A. J. Levine. 1991. Cellular localization and cell cycle regulation by a temperature sensitive p53 protein. *Genes Dev.* **5**:151-159.
- Mercer, W. E., M. T. Shields, M. Amin, G. J. Suave, E. Appella, S. J. Ullrich, and J. W. Romano. 1990. Antiproliferative effects of wild-type human p53. *J. Cell. Biochem.* **14C**:285.
- Michalovitz, D., O. Halevy, and M. Oren. 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* **62**:671-680.
- 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.
- Parada, L. F., H. Land, R. A. Weinberg, D. Wolf, and V. Rotter. 1984. Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation. *Nature (London)* **312**:649-651.
- Peden, K. W. C., A. Srinivasan, J. M. Farber, and J. M. Pipas. 1989. Mutants with changes within or near a hydrophobic region of simian virus 40 large tumor antigen are defective for binding cellular protein p53. *Virology* **168**:13-21.
- Pinkert, C. A., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter.

1987. An albumin enhancer located between 10kb upstream functions along with its promoter to direct efficient, liver specific expression in transgenic mice. *Genes Dev.* 1:268-276.
39. Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine. 1982. Adenovirus E1B-58Kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54Kd cellular protein in transformed cells. *Cell* 28:387-394.
40. Scheffner, M., K. Münger, J. C. Byrne, and P. M. Howley. 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Natl. Acad. Sci. USA* 88:5523-5527.
41. Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus 16 or 18 promotes the degradation of p53. *Cell* 63:1129-1136.
42. Tan, T.-H., J. Wallis, and A. J. Levine. 1986. Identification of the p53 protein domain involved in formation of the simian virus 40 large T antigen-p53 protein complex. *J. Virol.* 59:574-583.
43. van Dyke, T., C. Finlay, and A. J. Levine. 1986. A comparison of several lines of transgenic mice containing SV40 early genes. *Cold Spring Harbor Symp. Quant. Biol.* 50:671-678.
44. van Dyke, T. A., C. Finlay, D. Miller, J. Marks, G. Lozano, and A. J. Levine. 1987. Relationship between simian virus 40 large tumor antigen expression and tumor formation in transgenic mice. *J. Virol.* 61:2029-2032.
45. Werness, B. A., A. J. Levine, and P. M. Howley. 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 248:76-79.
46. Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an anti-oncogene; the adenovirus E1a proteins bind to the retinoblastoma gene product. *Nature (London)* 334:124-129.
47. Yewdell, J. W., J. V. Gannon, and D. P. Lane. 1986. Monoclonal antibody analysis of p53 expression in normal and transformed cells. *J. Virol.* 59:444-452.