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High Incidence of Lung, Bone, and Lymphoid Tumors in Transgenic Mice Overexpressing Mutant Alleles of the p53 Oncogene

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We have investigated the role of the p53 gene in oncogenesis in vivo by generating transgenic mice carrying murine p53 genomic fragments isolated from a mouse Friend erythroleukemia cell line or BALB/c mouse liver DNA. Elevated levels of p53 mRNA were detected in several tissues of two transgenic lines tested. Increased levels of p53 protein were also detected in most of the tissues analyzed by Western blotting (immunoblotting). Because both transgenes encoded p53 proteins that were antigenically distinct from wild-type p53, it was possible to demonstrate that overexpression of the p53 protein was mostly, if not entirely, due to the expression of the transgenes. Neoplasms developed in 20% of the transgenic mice, with a high incidence of lung adenocarcinomas, osteosarcomas, and lymphomas. Tissues such as ovaries that expressed the transgene at high levels were not at higher risk of malignant transformation than tissues expressing p53 protein at much lower levels. The long latent period and low penetrance suggest that overexpression of p53 alone is not sufficient to induce malignancies and that additional events are required. These observations provide direct evidence that mutant alleles of the p53 oncogene have oncogenic potential in vivo and that different cell types show intrinsic differences in susceptibility to malignant transformation by p53. Since recent data suggest that p53 may be a recessive oncogene, it is possible that the elevated tumor incidence results from functional inactivation of endogenous p53 by overexpression of the mutant transgene. The high incidence of lung and bone tumors suggests that p53 transgenic mice may provide a useful model to investigate the molecular events that underlie these malignancies in humans.

p53 is a cellular protein first detected in simian virus 40-transformed cells because of its physical association with the large T antigen (32, 34) and activity as a transplantation antigen in chemically induced sarcomas (11). p53 levels are frequently high in cells transformed by other biological or physical agents (8), in tumor cell lines, and in various primary tumors (9, 10, 30, 35, 37, 52).

Several in vitro studies have shown that the p53 gene has transforming potential. Mutated p53 can immortalize early-passage rodent cells (28, 49) and can cooperate with *ras* to transform primary cells in culture (14, 25, 28, 43, 49), whereas wild-type p53 is unable to cooperate with *ras* in this transformation assay (12, 16, 25, 26). Furthermore, p53 enhances the tumorigenic phenotype of established cell lines (13, 29, 58).

Aside from high levels of p53 protein frequently observed in various tumors and recent reports of p53 gene rearrangement in osteosarcomas and cell lines derived from lung tumors (37; J. D. Minna, personal communication), direct evidence that overexpression of the p53 gene or activation of its oncogenic potential by mutation can contribute to the oncogenic process in vivo is lacking. The p53 gene has not been detected as part of a naturally occurring acutely transforming retrovirus, nor has it been shown to be consistently involved in chromosomal translocations or gene amplification in naturally occurring tumors (5).

In an attempt to understand the role of the p53 gene in oncogenesis, we previously analyzed the structure and expression of this gene in tumorigenic cells induced by the murine Friend leukemia retrovirus (2, 6, 38, 50). These studies demonstrated that p53 gene rearrangements, including internal deletions or retroviral insertions, occur in about 20% of tumor cell clones isolated from the spleens of mice with Friend leukemia. Rearrangements of the p53 gene result either in the failure to synthesize p53 protein or in the production of aberrant or truncated p53 polypeptides (6, 24, 38, 39, 50). These observations, together with the finding that the normal p53 allele is commonly absent in these clones, suggested that p53 is a recessive oncogene whose inactivation or alteration may confer a selective growth advantage to erythroid target cells infected during the progression of Friend erythroleukemia in vivo.

The results of the experiments described above clearly suggest a role for the p53 gene in oncogenesis, although the precise contribution of this gene to the neoplastic process remains unclear. One experimental approach to determine whether p53 plays a causative role in malignancy and whether there are differences in susceptibility among different cell types is through the use of transgenic mice that overexpress the p53 protein. Several groups have shown that transgenic animals carrying a deregulated oncogene develop tumors (reviewed in references 7, 20, and 21) and that the transforming spectrum of some oncogenes seems to be limited to certain cell types (33, 57).

Here, we report that transgenic mice carrying mutated p53 genomic fragments under the control of their own promoters express the transgene in a wide variety of tissues. As a consequence of this deregulated expression, these animals have an increased tumor susceptibility, exhibiting a particularly high incidence of lung adenocarcinomas, osteosarcomas, and lymphoid tumors.

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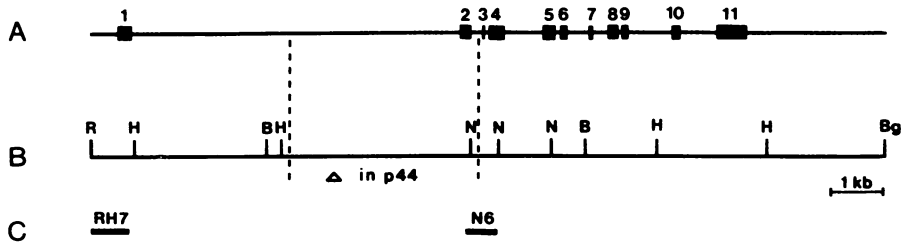

FIG. 1. Map of the p53 and p44 genomic fragments. (A) Diagrammatic representation of the murine p53 genomic fragment. The 11 exons are indicated as numbered boxes. This fragment contains the natural p53 promoter and poly(A) signal. (B) Restriction endonuclease map of p53. The deletion in p44 is shown between the dotted lines. Restriction sites: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; N, *Nco*I; Bg, *Bgl*II. (C) Probes used for the Southern and slot blot analyses. Restriction map of the p53 and p44 genes was modified from published data (3, 50). kb, Kilobases.

MATERIALS AND METHODS

DNA constructions. *Eco*RI genomic p53 fragments isolated from Friend cell lines DP15-2 (p44) and CB7 (p53) (6, 38, 50) were subcloned from bacteriophage lambda clones into the *Eco*RI site of pUC18 by previously described methods (36). The murine p53 *Eco*RI genomic fragment isolated from BALB/c liver DNA (pL53) (3) was obtained from plasmid pMSVp53G (14) and subcloned into the *Eco*RI site of pUC18.

Preparation of DNA fragments and microinjection. After digestion with *Eco*RI and *Bgl*II, the p53 genomic fragments of 13 (p44) or 16 (p53 and pL53) kilobases were purified from the pUC18 vector sequences by gel electrophoresis on a 0.6% agarose gel. The DNA was electroeluted into a dialysis bag, extracted once with phenol-chloroform (1:1), and then passed through an Elutip column (Schleicher & Schuell, Inc.). The DNA was ethanol precipitated and suspended in 10 mM Tris (pH 7.5)–0.1 mM EDTA. DNA fragments were microinjected at a concentration of 2 to 4 ng/μl into fertilized eggs of CD-1 mice (CD^R-1-Ha/Icr; Charles River Breeding Laboratories, Inc.), using standard procedures (27).

Identification of transgenic animals. DNA was extracted from tail biopsies by the proteinase K-sodium dodecyl sulfate (SDS) method (27). Southern blot analysis was performed on tail DNA of potential founder animals by using an *Eco*RI-*Hin*dIII 0.7-kilobase fragment (RH7; Fig. 1) labeled to high specific activity by nick translation (36). Progeny of the transgenic strains were screened by slot blot analysis, using an *Eco*RI-*Hin*dIII (RH7) or *Nco*I (N6) probe (Fig. 1).

Northern (RNA) blot analysis. All tissues were flash-frozen in liquid N_2 and stored at −70°C. Tissues were homogenized in 3 M LiCl–6 M urea–0.2% SDS in a Polytron (Brinkmann Instruments, Inc.), sonicated twice for 15 s each time, and incubated overnight at 4°C. RNA was pelleted by centrifugation at 3,000 rpm at 4°C for 30 min. The pellet was suspended in TE (1 mM Tris [pH 7.5]–EDTA) containing 0.5% SDS. RNA was sequentially extracted with phenol, phenol-chloroform (1:1), and chloroform, precipitated with ammonium acetate, and redissolved in TE containing 0.5% SDS (1, 18). Approximately 25 μg of RNA was run on a 1.2% agarose gel containing 0.66 M formaldehyde as previously described (17) and blotted onto GeneScreen Plus (Dupont, NEN Research Products) as suggested by the supplier. The filter was hybridized with a p53 exon I probe (*Eco*RI-*Hin*dIII 0.7-kilobase genomic fragment; RH7) labeled by random priming (15) as described by the supplier (Pharmacia, Inc.). The nylon membrane was washed in 10 mM Tris (pH 7.4)–0.2% SDS at 80°C for 1 h and then reprobed with a chicken β-actin cDNA.

Western blot (immunoblot) analysis. All tissues were flash-frozen in liquid N_2 and stored at −70°C. Tissues were disrupted in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml) in a Polytron and lysed for 30 min on ice. Lysates were cleared by centrifugation in an Eppendorf tube for 5 min at 4°C. Lysate volumes corresponding to 1 mg of protein were immunoprecipitated overnight, using the anti-p53 monoclonal antibody PAb421 (22) or the control monoclonal antibody PAb419 (22) in 400 μl of NET-gel buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.05% Nonidet P-40, 0.02% sodium azide, 0.25% gelatin). Immune complexes were collected on 50 μl of Formalin-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring), washed once with NET-gel buffer, and then eluted in 1× protein sample buffer (25% glycerol, 5% 2-mercaptoethanol, 1.0% SDS, 62.5 mM Tris [pH 6.8], 0.05% bromophenol blue) by heating at 70°C for 10 min. Samples were electrophoresed through a 10% polyacrylamide gel in the presence of SDS and transferred to nitrocellulose paper (Schleicher & Schuell). The nitrocellulose was reacted with the anti-p53 antibody PAb421, followed by incubation with ^{125}I-protein A (Amersham Corp.) as previously described (38).

For the experiment performed with monoclonal antibody PAb246 (see Fig. 4), Western blotting was done as described above except that the protein extracts were prepared in IP lysis buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) and immunoprecipitated in 400 μl of IP lysis buffer, and the immune complexes were washed twice with SNNTE (5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris [pH 7.4], 5 mM EDTA).

Histological analysis. Complete autopsies were performed, and tissues showing signs of abnormalities at the gross anatomical level were further analyzed. Tissues and tumors samples were fixed in 10% neutral buffered Formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

RESULTS

p53 constructs and transgenic strains. The constructs of the murine p53 genomic fragments used to generate transgenic mice are shown in Fig. 1. p44 is a naturally occurring deletion mutant of p53 cloned from the Friend cell line DP15-2; it encodes a protein of 44 kilodaltons with a very long half-life (>9.5 h) (50). The p53 genomic fragment used to generate the p53 transgenic strains was cloned from the Friend cell line CB7 (38, 50). It encodes a protein that is not recognized by the anti-p53 monoclonal antibody PAb246 and can, unlike the wild-type p53 gene product, complement *ras*

TABLE 1. Microinjection of p53 variants into CD-1 fertilized eggs

Construct[a]	No. of eggs injected	No. of eggs transferred	No. born	No. positive
p44	1,804	693	56	0
p53	1,157	542	142	5
pL53	993	232	41	8

[a] For all constructs, the DNA was microinjected as a linear *Eco*RI-*Bgl*I fragment. p44 is the rearranged p53 genomic fragment cloned from Friend cell line DP15-2. p53 is the p53 genomic fragment cloned from the Friend cell line CB7. pL53 is the p53 genomic fragment cloned from BALB/c liver DNA (kindly provided by M. Oren).

in the transformation of rat primary cells (14, 16, 25, 49). The third genomic fragment used to generate transgenic mice (pL53 strains) was cloned from BALB/c mouse liver (3). It contains a point mutation at amino acid 135, activating this allele in a *ras* complementation assay (14, 25, 49). This point mutation also affects the reactivity of the p53 protein encoded by this liver-derived allele to PAb246 (16). All three constructs were injected as linear 13 (p44)- or 16 (p53 and pL53)-kilobase *Eco*RI-*Bgl*II fragments that include regions exhibiting promoter activity (4) and polyadenylation signals (3).

The animals were screened by Southern blot analysis at 3 weeks of age for the presence of the transgene. As shown in Table 1, no animals carrying the p44 transgene were obtained after injection of over 1,800 fertilized eggs, suggesting that p44 might be incompatible with mouse development. Five transgenic strains carrying the CB7 p53 genomic fragment (p53) and eight strains bearing the liver p53 construct (pL53) were generated. These 13 strains carry between 2 and 20 copies of the transgene. In 10 of those strains, the Southern blot analysis was consistent with a head-to-tail tandem repeat arrangement. The three other strains showed a complex pattern of integration (data not shown).

Northern blot analysis of p53 transcripts in transgenic animals. To analyze the expression of the transgene, RNA was extracted from various organs of normal littermates and transgenic animals (strains p53-2 and pL53-3) and analyzed by Northern blotting. Control CD-1 animals expressed very low levels of p53 mRNA in the tissues tested (Fig. 2). An overexposure of this Northern blot (data not shown) clearly demonstrated the presence of p53 transcripts, as described previously (47). On the other hand, transgenic animals from the p53-2 and pL53-3 strains showed an elevated level of p53 mRNA in the tissues examined. Although we cannot distinguish the endogenous p53 transcripts from those encoded by the transgene, it is clear that the p53 transcripts were increased by at least 40- to 50-fold in transgenic tissues as compared with controls.

Steady-state levels of p53 protein in transgenic animals. It has been reported that the steady-state levels of p53 protein are very low in normal cells (8). Since the levels of p53 mRNA were higher in the transgenic tissues tested than in control tissues, the expression of p53 protein in various tissues was analyzed by the Western blot technique. The p53 protein was not detected in a variety of tissues from normal animals (Fig. 3B and data not shown). In contrast, high levels of p53 protein were observed in four of the Friend-derived p53 transgenic strains. The patterns of expression observed in the transgenic strain p53-2 are shown in Fig. 3A. High levels of p53 protein were observed in spleen, thymus, lymph nodes, and ovaries, and lesser amounts were found in lung and testis. Very low levels were detected in liver,

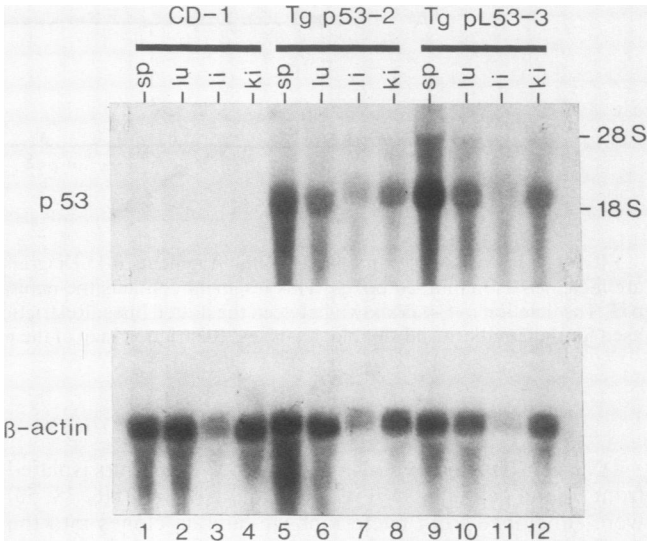

FIG. 2. Northern blot analysis of p53 mRNA in normal and transgenic animals. Steady-state levels of p53 transcripts were analyzed by using a p53 probe spanning the first exon (probe RH7). RNA was extracted from the indicated organs from control animals (CD-1) and transgenic animals from strains p53-2 and pL53-3. A 25-μg sample of total RNA was loaded on a 1.2% agarose gel containing 0.66 M formaldehyde. Tissues analyzed: sp, spleen; ki, kidney; lu, lung; li, liver.

kidney, and skeletal muscle, whereas p53 expression was undetectable in heart and blood. Similar analysis also showed elevated levels of p53 in five transgenic strains carrying the p53 gene isolated from BALB/c liver, as shown for strains pL53-2 and pL53-3 (Fig. 3B). The patterns of p53 expression in these transgenic animals were identical to each other and to those observed in transgenic mice carrying the Friend-derived p53 gene.

A difference of at least 50-fold was observed between transgenic lines expressing the highest and the lowest levels of p53. Although the levels of expression in different p53 transgenic lines varied, the patterns of expression in different tissues were the same in independently established strains expressing p53. These observations suggest that there are tissue-specific regulatory elements that regulate the expression of p53 independently of chromosomal position.

Epitope analysis of p53 protein in transgenic mice. To determine whether the p53 protein detected in the transgenic mice is encoded by the transgene or, alternatively, is derived from both the transgene and the endogenous p53 gene, we took advantage of the fact that most p53 mutant proteins are not recognized by the monoclonal antibody PAb246. This antibody seems to recognize a conformation-dependent epitope (16, 54, 55, 60). Consistent with this observation, the p53 protein encoded by the Friend p53 allele failed to react with this monoclonal antibody (Fig. 4). On the other hand, the liver-derived p53 genomic clone encodes two immunologically distinct populations of p53 protein, $PAb246^-$ and $PAb246^+$ (16). The p53 protein present in tissues analyzed from the p53 transgenic mice failed to react with PAb246, indicating that the high levels of p53 protein in these animals were encoded by the p53 transgene, not the endogenous p53 gene (Fig. 4A). Analysis of tumors derived from p53 transgenic strains (Fig. 4B) also indicated that the p53 protein detected in these tumors was encoded by the transgene. Interestingly, an overexposure of this Western blot showed

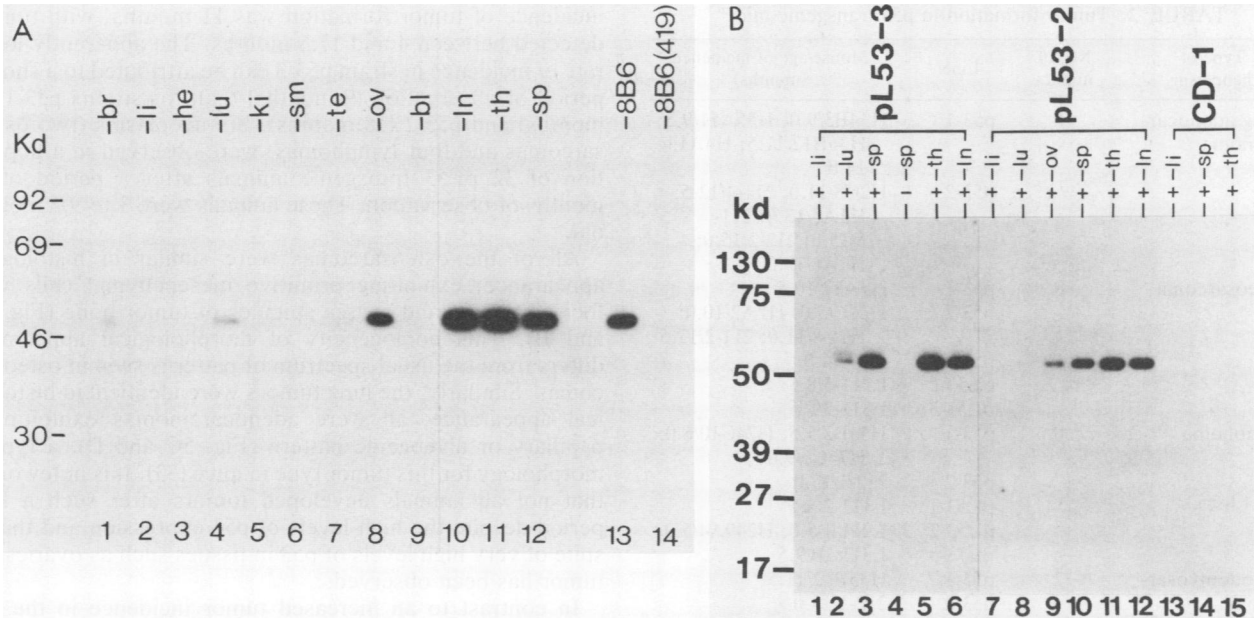

FIG. 3. Western blot analysis of p53 expression in various organs of CD-1 and transgenic strains. Western blot analysis was performed as described in the text. Immunoprecipitates were fractionated on a 10% acrylamide gel containing SDS. After electrophoresis, the proteins were transferred to nitrocellulose. Filters were blotted with antibody PAb421 and then reacted with ^{125}I-protein A. (A) F1 heterozygous animal from line p53-2. As a control, the Friend cell line DP15 8B6 was included (lanes 13 and 14). The anti-p53 monoclonal antibody PAb421 was used for all immunoprecipitations except that shown in lane 14, in which the control monoclonal antibody used was PAb419. (B) Founder animals of lines pL53-2 and pL53-3 and control CD-1 animal. PAb421 was used for all immunoprecipitations except that shown in lane 4, in which PAb419 was used. Organs are abbreviated as follows: br, brain; li, liver; he, heart; lu, lung; ki, kidney; sm, skeletal muscle; te, testis; ov, ovaries; bl, blood; ln, lymph nodes; th, thymus; sp, spleen.

the presence of a small amount of p53 immunoprecipitated by PAb246. This PAb246$^+$ p53 protein might reflect the low levels of p53 normally expressed in cells transformed in those tumors or, alternatively, might represent a more stable form of the endogenous protein, by virtue of complex formation with the transgene-encoded protein, as suggested by recently published work (12, 31).

Only a small amount of p53 protein was immunoprecipitated by PAb246 in the pL53 strains (Fig. 4A), indicating that the bulk of the p53 protein in these mice was also encoded by

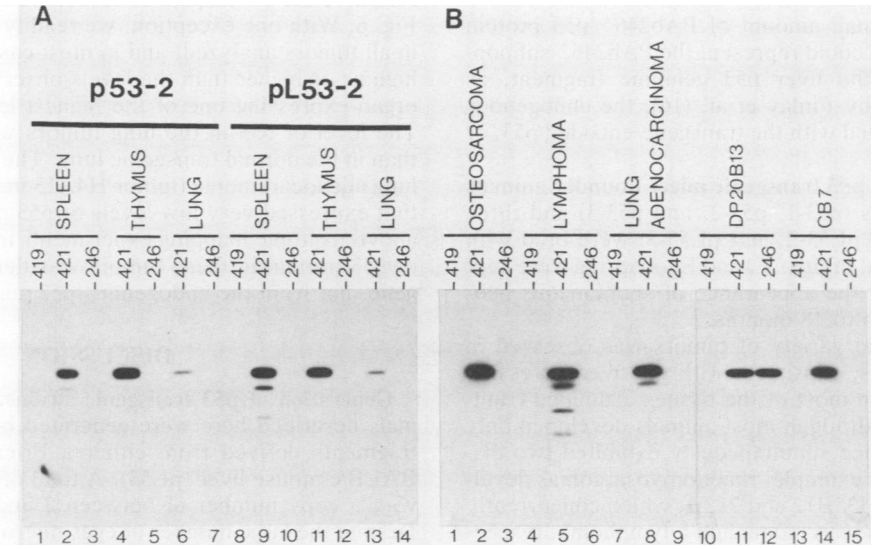

FIG. 4. Epitope analysis of the p53 protein from transgenic mice and tumors derived from transgenic animals. Western blot analysis was performed as described in the text. Immunoprecipitates were fractionated on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose. Filters were blotted with antibody PAb421 and then reacted with ^{125}I-protein A. (A) p53 epitope analysis of various tissues from transgenic strains p53-2 (lanes 1 to 7) and pL53-2 (lanes 8 to 14). (B) p53 epitope analysis of tumors derived from transgenic animals. Lanes: 1 to 3, osteosarcoma from mouse H264A8 (Tg p53-2); 4 to 6, lymphoma from mouse 212-1 (Tg p53-2); 7 to 9, lung adenocarcinoma from mouse H2C8 (Tg p53-2); 10 to 12, Friend cell line DP20B13 (PAb246$^+$); 13–15, Friend cell line CB7 (PAb246$^-$). The antibody used for each immunoprecipitation (PAb419 [419], PAb421 [421], or PAb246 [246]) is indicated above each lane.

TABLE 2. Tumor formation in p53 transgenic mice[a]

Type of neoplasm	No. of tumors	Line	Mouse/age of incidence (months)
Lung adenocarcinoma	10	p53-1	H34B3/9.0; H35A11/9.5; H35B12/11.5; H4A13/14.0
		p53-2	H2C8/11.0; 211-4/12.5; H1A5/13.0; 211-5/15.0; 212-2/15.0; H1A6/15.0
Osteosarcoma	8	p53-1	H4A12/10.5
		p53-2	H2C5/5.0; H2A2/10.0; H2C8/11.0; 211-2/17.5
		p53-3	311-2/4.0
		pL53-2	L211-5/8.5
		pL53-3	L311-3/5.5
Lymphoma	8	p53-1	H34B2/5.5; H74C10/6.0; H74C6/9.0
		p53-2	212-1/15.0
		pL53-1	L111-2/3.5
		pL53-2	L211-1/5.0; H249A4/5.0
		pL53-3	L311-10/9.5
Rhabdomyosarcoma	2	p53-1	H35B12/11.5
		p53-2	212-1/15.0
Skin carcinoma	2	p53-1	H35A11/9.5
		p53-2	H1A1/12.0
Fibrosarcoma	1	p53-2	211-13/10.0
Testicular carcinoma	1	p53-1	H35C17/6.5
Adrenal neuroblastoma	1	p53-2	211-5/15.0

[a] A total of 22 transgenic mice from three p53 strains developed a cumulative total of 27 neoplasms. One animal had both a lung adenocarcinoma and a skin carcinoma (H35A11); others had both a lung adenocarcinoma and an osteosarcoma (H2C8), a rhabdomyosarcoma and a lymphoma (212-1), a lung adenocarcinoma and a rhabdomyosarcoma (H35B12), and both a lung adenocarcinoma and an adrenal neuroblastoma (211-5). The mean age at onset of tumor formation was 11 months. The overall tumor incidence was 20% (23 of 112). Six transgenic mice from three pL53 strains developed tumors, with a mean age at the onset of tumor formation of 6 months. The overall tumor incidence was 19% (6 of 32).

the transgene. The small amount of PAb246$^+$ p53 protein detected in these mice could represent the PAb246$^+$ subpopulation encoded by the liver p53 genomic fragment, as described previously by Finlay et al. (16), the endogenous p53 protein oligomerized with the transgene-encoded p53, or both.

Tumor formation in p53 transgenic mice. Founder animals from three p53 strains (p53-1, p53-2, and p53-3) and three pL53 strains (pL53-1, pL53-2, and pL53-3) were bred with CD-1 mice, and several of their F_1 and F_2 offspring were kept under observation for the appearance of spontaneous neoplasms for a period up to 18 months.

Formation of a wide variety of tumors was observed in these three p53 strains, consistent with the overexpression of the p53 transgene in most of the tissues examined (summarized in Table 2). Although most animals developed only one tumor, several mice simultaneously exhibited two distinct tumor types. For example, rhabdomyosarcomas developed in two animals, H35B12 and 212-1, which concurrently developed a lung adenocarcinoma and a lymphoma, respectively.

A particularly high frequency of lung adenocarcinomas, osteosarcomas, and lymphomas was noted in p53 transgenic mice, these three types of tumors representing 74% of all tumors observed. In all, we observed 27 neoplasms in 22 animals in a population of 112 transgenic animals carrying the p53 gene derived from Friend cells. The average age of incidence of tumor formation was 11 months, with tumors detected between 4 and 17.5 months. The apparently lower rate of incidence in strain p53-3 can be attributed to a shorter period of observation (9 months) than for strains p53-1 (15 months) and p53-2 (18 months). Six neoplasms (two osteosarcomas and four lymphomas) were observed in a population of 32 pL53 transgenic animals after a period of 10 months of observation. These animals were 3 to 9.5 months old.

All of the osteosarcomas were similar in histological appearance, exhibiting primitive mesenchymal cells with foci of osteoid and sparce spicules of tumor bone (Fig. 5A and B). This homogeneity of morphological appearance differs from the usual spectrum of patterns seen in osteosarcomas. Similarly, the lung tumors were identical in histological appearance; all were adenocarcinomas exhibiting a papillary or alveogenic pattern (Fig. 5C and D), a typical morphology for this tumor type in mice (53). It is noteworthy that not all animals developed tumors after such a long period despite the high levels of p53 expression and that in spite of very high levels of p53 in tissues such as ovaries, no tumor has been observed.

In contrast to an increased tumor incidence in the p53 transgenic animals, only one malignancy (a thymoma) was observed during the same period in one of the negative littermates kept as controls (62 animals). In addition, the incidences of lung adenocarcinomas, osteosarcomas, and lymphomas (the three most common tumors observed in our transgenics) in normal CD-1 mice are reported to be 1.0, 0.6, and 1.5%, respectively, in animals aged 0 to 20 months (44). Thus, the tumor incidence observed in these transgenic animals is clearly above the spontaneous incidence in CD-1 mice.

Presence of the p53 protein in tumors. If p53 is directly involved in the formation of tumors in these transgenic mice, tumors from these animals should also contain high levels of p53 protein. Whenever possible, we analyzed the levels of p53 in fresh tumor samples by Western blotting. A representative set of samples of several tumor types is shown in Fig. 6. With one exception, we readily detected p53 protein in all tumors analyzed, and in most cases the levels were as high as or higher than the levels observed in the spleen, an organ expressing one of the highest levels of p53 (Fig. 6). The level of p53 in the lung tumors was also much higher than in the normal transgenic lung. The sole exception was a lung adenocarcinoma (tumor H4A13 in strain p53-1; Table 2) that expressed very low levels of p53 protein. As discussed above, epitopic mapping experiments indicated that the bulk of p53 protein in these tumors was derived from the transgene, not from the endogenous p53 gene (Fig. 4B).

DISCUSSION

Generation of p53 transgenic strains. The transgenic animals described here were generated by using p53 genomic fragments derived from either a Friend cell line (p53) or BALB/c mouse liver (pL53). A total of 13 transgenic strains with a copy number of between 2 and 20 were generated. Despite the large number of eggs microinjected (over 1,800), we were unable to obtain transgenic mice carrying the p44 genomic fragment. Furthermore, cotransfection of p44 with pSV2neo consistently reduced the number of G418^r colonies relative to the cotransfection of Friend CB7 or liver-derived p53 and pSV2neo (49). Taken together, these data suggest that overexpression of p44 may be toxic to certain cells and incompatible with normal mouse development. The reasons

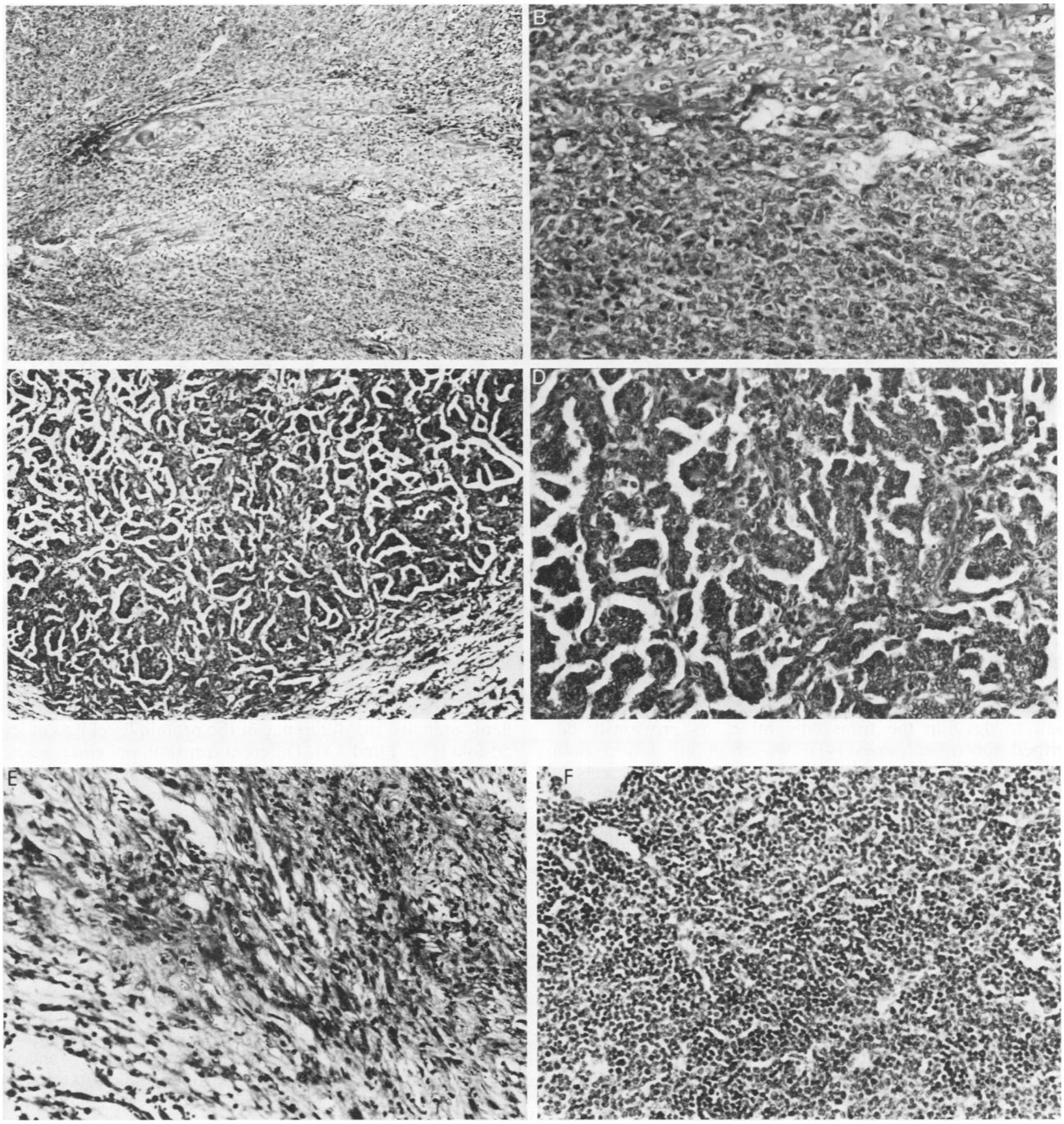

FIG. 5. Histology of tumors derived from p53 transgenic mice. (A) Low-power photomicrograph of an osteosarcoma from the rib. The tumor is cellular, with foci of atypical bone and osteoid (mouse H2C5) (magnification, ×60). (B) Higher-power photomicrograph of the same tumor, demonstrating marked cellular and nuclear pleomorphism. Mitotic figures are prominent, and atypical osteoid can be seen (magnification, ×150). (C) Adenocarcinoma of the lung showing typical morphology of these tumors found in the mouse line. The tumor has a papillary appearance and infiltrative pattern (mouse 211-4) (magnification, ×60). (D) Higher magnification of the adenocarcinoma shown in panel C. The papillary appearance is apparent, and the monomorphic cellular pattern can be seen. The tumor consists of oval to cuboidal cells with hyperchromatic nuclei (magnification, ×150). (E) Poorly differentiated tumor consisting of oval to spindle-shaped cells with marked nuclear pleomorphism and interspersed collagen. The poorly differentiated fibrosarcoma infiltrated adjacent tissue extensively (mouse 211-13) (magnification, ×150). (F) Lymphoma that arose in the mediastinum and infiltrated the lung and heart. The tumor consists of a sheet of small round cells with dark nuclei, exhibiting some pleomorphism (mouse H74C10) (magnification, ×150).

for the cytotoxicity of p44 are not clear. Cytotoxicity in cells transfected with v-*abl* (19, 56), v-*mos* (42), and c-*myc* (59) have been reported. Attempts to generate transgenic mice carrying the middle-T polyomavirus antigen by retroviral vector infection of preimplantation embryos also resulted in embryo lethality (57).

Analysis of p53 transcripts in control and transgenic tissues. Despite the absence of a strong heterologous promoter-

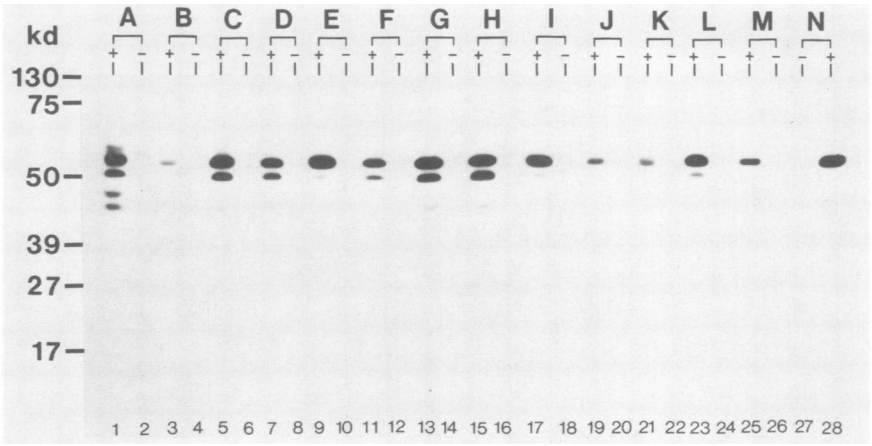

FIG. 6. Western blot analysis of p53 in tumors. Western blot analysis was performed as described in the text. Immunoprecipitates were fractionated on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose. Filters were blotted with antibody PAb421 and then reacted with ^{125}I-protein A. Lanes: A, Mouse 211-4, lung adenocarcinoma; B, mouse 211-13, fibrosarcoma; C, mouse H2C5, osteosarcoma; D, mouse H2A2, osteosarcoma; E, mouse H1A1, skin cell carcinoma; F, mouse H2C8, osteosarcoma; G, mouse H2C8, lung adenocarcinoma; H, mouse H2C5, spleen (control); I, mouse H35C17, testicular carcinoma; J, mouse H74C10, lymphoma; K, mouse H35A11, lung adenocarcinoma; L, mouse 311-2, osteosarcoma; M, mouse 311-2, spleen (control); N, Friend cell line CB7 (control); A to H, line p53-2; I to K, line p53-1; L to M, line p53-3. +, Monoclonal antibody PAb421; –, control antibody PAb419. kd, Kilodaltons.

enhancer sequence and the fact that normal mice express very low levels of p53 mRNA in most tissues, the transgenic animals expressed high levels of p53 transcripts. The elevated levels of p53 transcripts in our transgenic mice could be the result of transgene dosage, positive chromosomal position effects, mRNA stabilization, or the absence of *cis*-acting negative regulatory elements present in the native p53 gene but missing in the *Eco*RI-*Bgl*II genomic fragment used to develop the transgenic mice. As previously described for other genes (41), we observed only a poor correlation between transgene copy number and levels of p53 expression, making it unlikely that the markedly elevated levels of p53 were simply the result of an increase in gene dosage. Positive chromosomal position effects affecting transgene expression have been previously reported (41) and might account for the high levels of p53 transcripts in the two transgenic strains analyzed here. Alternatively, it is possible that a mutation has occurred in the p53 transgenes, resulting in mRNA stabilization; however, this explanation would have to account for the high levels of p53 transcripts observed with two different p53 alleles, including one derived from mouse liver. Finally, it is possible that *cis*-acting negative regulatory sequences are not included in the *Eco*RI-*Bgl*II p53 genomic fragment used to generate these transgenic animals. Indeed, there is evidence for a negative regulatory region upstream of the transcriptional start site of the murine p53 gene (4), although this region is within the p53 genomic fragment used to generate our transgenic mice. Nevertheless, it is possible that there is an additional regulatory region upstream of the one identified by Bienz-Tadmor et al. (4) or that there is a single large negative regulatory region that extends 5′ of the *Eco*RI site. Similar negative regulatory regions have been identified upstream of the c-*myc* gene (46). Further experiments are needed to distinguish between these possibilities.

Steady-state levels of p53 protein in transgenic animals. Western blot analysis showed that a large proportion of the transgenic strains expressed high levels of p53 in a wide variety of tissues. An identical pattern of expression of p53 protein was observed in independently derived transgenic animals. The highest p53 protein levels were consistently found in thymus, spleen, lymph nodes, and ovaries in all of the strains overexpressing p53. This observation suggests that tissue-specific regulatory mechanisms could be responsible for the control of p53 protein levels. It is evident from our results that an increase in the levels of p53 transcripts was not paralleled at the protein level. For instance, although there was a three- to fivefold difference in the levels of p53 transcripts between the spleen and kidney in transgenic animals, the difference at the protein level was at least 50-fold (Fig. 2 and 3A). Different translational efficiencies of p53 transcripts or different p53 protein stabilities among the tissues might have been responsible for the varying p53 protein levels observed in different tissues or cell types.

Epitope analysis of p53 protein expressed in transgenic tissues and tumors. Because both p53 transgenes have likely sustained mutations resulting in loss of the confirmation-sensitive epitope recognized by monoclonal antibody PAb246, we were able to ask whether the high levels of p53 protein in the transgenic animals were the result of overexpression from the transgenes or from the endogenous p53 gene. Immunoprecipitation analysis with monoclonal antibodies PAb246 and PAb421 indicated that most, if not all, of the p53 protein present in the transgenic strains overexpressing p53 and in tumors derived from these animals is encoded by the transgene. This result strongly suggests that the elevated levels of p53 protein in these mice are due to expression from the transgene rather than activation of the endogenous p53 gene (for example, as a result of the titration of a putative repressor protein).

Tumor formation in p53 transgenic mice. The widespread expression of p53 in these transgenic animals was associated with a significantly increased incidence of a broad spectrum of malignancies. Although a variety of different tumor types derived from different tissues were observed, lung adenocarcinomas, osteosarcomas, and lymphomas were particularly prevalent. The absence of lung tumors in pL53 strains likely resulted from a relatively high age of incidence of this type of tumor (an average of 12.5 months in p53 strains) compared with the length of the observation period (10 months). The high incidence of lung adenocarcinomas and osteosarcomas is particularly interesting in light of recent reports demon-

strating a high frequency of genomic rearrangements within the p53 gene in human osteosarcomas and in lung adenocarcinoma cell lines (37; Minna, personal communication). These observations suggest that the p53 gene plays an important role in both experimental and naturally occurring lung and bone tumors and therefore that p53 transgenic mice may provide a model for these human diseases.

Interestingly, there was no simple correlation between the levels of p53 protein and tumor incidence in different tissues. For instance, although the ovaries of p53 transgenic animals expressed high levels of p53 protein, no ovarian tumors have yet been observed. Conversely, although the lung expressed low levels of p53 protein, adenocarcinomas of the lung were the single most common neoplasia observed. These observations suggest that there are intrinsic differences in the susceptibility to malignant transformation in different cell types as a consequence of p53 overexpression, if it is assumed that the overall levels of p53 protein in total tissue extracts reflect the levels of p53 protein in cells that are the targets for malignant transformation by p53.

Our data demonstrate that the introduction into the mouse germ line of mutated p53 genes derived from either a leukemic Friend cell line or mouse liver results in markedly elevated tumor incidence. Thus, these experiments provide direct evidence that the p53 gene plays a causal role in tumor formation. However, the long latency period and the overall tumor incidence (20%) argue strongly that the deregulated expression of the p53 oncogene alone is not sufficient for tumor formation. As described previously for other transgenic animals carrying various oncogenes in their germ lines, further genetic or epigenetic changes in premalignant cells appear to be involved in tumorigenesis (7, 20, 21, 51).

Does p53 act as a dominant or recessive oncogene? We have previously suggested that inactivation of the p53 gene can contribute to the multistage malignancy induced by Friend leukemia virus (38). This hypothesis was based on the observation that a high proportion (approximately 20%) of independently isolated Friend cell clones fail to express any p53 protein as the result of large deletions or retroviral insertions within the p53 gene (2, 6, 38, 50). These observations have therefore raised the possibility that p53 is a recessive oncogene whose expression interferes with the neoplastic process. Consistent with this model, reports from another group have recently shown that mutation in the p53 gene is required for p53 to cooperate with the *ras* oncogene in in vitro transformation assays (12, 25) and that overexpression of the normal p53 allele can inhibit the ability of *ras* to transform rat embryo fibroblasts in cooperation with either E1A or mutated p53 (15a).

In light of these previous studies indicating that p53 can act as a recessive oncogene, one hypothesis to explain the elevated tumor incidence in the p53 transgenic mice described here is that overexpression of the protein product of the p53 transgene results in the functional inactivation of the wild-type endogenous p53 protein expressed in these mice. Two observations are consistent with this dominant-negative model (23) for p53 action. First, both alleles used to generate the transgenic mice appear to have sustained mutations in functionally important regions of the p53 molecule. The epitope analysis presented here indicates that the proteins encoded by both the Friend and liver p53 alleles lack an epitope defined by monoclonal antibody PAb246. Other workers have previously reported that the loss of this epitope is frequently associated with activation of the transforming ability of the p53 gene and its association with heat shock protein (16, 54, 55). Thus, both p53 alleles used in our study encode abnormal forms of the p53 protein whose expression might interfere with the functioning of the endogenous p53 gene in these mice. Second, the levels of protein encoded by the mutant p53 transgenes were in great excess of that expressed by the endogenous wild-type gene, making it quite possible that a functionally inactive protein would compete with the relatively low levels of a wild-type regulator of cell growth.

In summary, a number of recent observations suggest that p53 may be a recessive oncogene. If so, these mice represent the first animal model for tumorigenesis resulting from the functional loss of a negative growth regulator. One prediction of this hypothesis would be that inactivation of one or both alleles of the endogenous p53 gene should also render mice more susceptible to tumor formation. In addition, this model suggests that overexpression of functionally inactive alleles of other recessive oncogenes might result in animals with elevated tumor incidence.

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