Accelerated Development of Polyoma Tumors and Embryonic Lethality: Different Effects of p53 Loss on Related Mouse Backgrounds[1]

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Abstract

Molecular evidence linking polyoma virus to p53 inactivation is thus far lacking, setting this highly oncogenic virus apart from other DNA tumor viruses. As a biological test for interaction, we studied the effects of p53 loss on development of virus-induced tumors. The absence of p53 led to more rapid tumor development on two different mouse backgrounds, indicating synergism between p53 loss and oncogenic pathways controlled directly by the virus. No effects of p53 on tumor type or frequency were noted. Polyoma tumor-derived cells in culture retained p53, and most of these showed induction of $p21^{CIP1/WAF1}$ in response to DNA damage. These results indicate that p53 functions are not directly and fully impaired by the virus in the intact host. On one mouse background, it was discovered that loss of p53 resulted in complete embryonic lethality prior to 11 days of gestation. This lethality could be rescued by inclusion of gene(s) from a 129/SvJ background.

Introduction

Py[4] is among the most highly oncogenic of experimental cancer-inducing agents. When inoculated into newborn mice of certain genetic backgrounds, the virus rapidly induces a variety of solid tumors. Tumors appear grossly as early as 4–5 weeks. By 3 months of age, the combined tumor mass may account for 30% or more of total body weight. Several features of the virus contribute to this rapid induction of a wide tumor spectrum. The major viral capsid protein VP1 binds to terminal sialic acids as an essential component of virus receptor(s) (1, 2); sialo-glycoproteins of appropriate specificity are abundantly and broadly expressed in the mouse. The regulatory region of the viral genome carries multiple enhancer elements that allow efficient viral gene expression in a variety of cell types. Through interactions of the viral T antigens with cellular proteins having roles in regulating cell growth, the virus is well equipped to induce cell transformation (3, 4).

One notable function this virus appears to lack is a mechanism to inactivate p53. This is surprising in view of the prominent role p53 plays in maintaining genome integrity and as a tumor suppressor gene in naturally occurring and experimental cancers. It is also surprising in relation to other DNA tumor viruses of the polyoma, papilloma, and adenovirus groups. Proteins encoded by these viruses either bind and "stabilize" p53 while negating its functions or cause its rapid degradation (reviewed in Ref. 5). Activation of multiple signal transduction pathways by middle T is not sufficient to overcome p53-induced growth arrest (6, 7). Neither stabilization nor accelerated degradation of p53 has been reported after lytic infection or transformation by Py, and p53-dependent responses to irradiation have been shown to occur normally in at least some Py-transformed rat cells (8), suggesting that the virus does nothing to alter p53 function. On the other hand, Py large T can clearly override G_0 or G_1 checkpoint controls in both wild-type and ts mutant p53-expressing cells (7, 9), and large T can be replaced by mutant p53 in cooperating with middle T to transform primary rat fibroblasts (10). p53 is lost or mutated in some but not all Py tumor-derived cell lines (11), but it remains unclear whether changes occur in the primary tumors. These and other experiments in cell culture fail to provide evidence for direct interaction with p53 (12). However, p53 may be activated and deactivated by a variety of mechanisms (13–18), and regulation may well differ among different cell types (19). Experiments to date do not rule out any of a variety of mechanisms whereby the virus could affect p53 function(s) in the intact host.

In the current study, we turned directly to a biological test in the mouse to determine whether the absence of p53 in the host in any way affects the tumor profile induced by Py. The rationale is based in part on the known synergism between p53 loss or mutation and other oncogenic stimuli present either as activated transgenes or through infection with oncogenic viruses (20, 21). Thus, a finding of synergism in the form of more rapid, more frequent, or more aggressive tumor growth on a p53 −/− host background would signify cooperation between p53 loss and oncogenic functions initiated by the virus. Synergism would further imply that the virus fails on its own to fully inactivate p53 in the animal. A failure to find synergism would suggest either that the virus acts on p53 by yet undiscovered mechanisms, perhaps upstream or downstream of p53 itself, or simply that the virus is able to induce tumors without circumventing p53.

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[4] The abbreviation used is: Py, mouse polyoma virus.

To carry out this investigation, a polyoma-susceptible mouse with a p53 knockout had to be established. The original p53 knockouts (22, 23) were produced on a 129/SvJ or mixed 129/SvJ-C57BL6 background, both of which carry an MHC haplotype associated with resistance to Py tumors (24). In attempting to transfer the p53 *KO* allele from a 129/SvJ mouse on to the Py-susceptible CBA/J background, we found unexpectedly that no p53 −/− mice were born. This implied that absence of p53 in the CBA/J mouse is an embryonic lethal condition and that the 129/SvJ mouse carries one or more genes that effectively suppress the embryonic lethal effect of p53 loss. A subline of mice of mixed CBA/J-129/SvJ background capable of giving live births of p53 −/− pups at the expected frequency was sought and obtained, and this subline was used for studies with Py. Tumor development occurred significantly earlier in p53 −/− animals, although p53 loss had no discernible effects on the Py tumor profile in terms of tumor types or frequencies. Accelerated development of Py tumors was also seen on another mixed background involving AKR/J and 129/SvJ.

Table 1 Transfer of a p53 knockout allele on to a polyoma virus-susceptible background[a]

	Genotype		
	MHC	Mtv 7	p53
A. Parental strains			
CBA	k/k	+/+	+/+
129	b/b	−/−	−/−
B. Breeding scheme			
1. F_1 (CBA×129)	k/b	+/−	+/−
2. BC (F1×CBA)	k/k, k/b	+/+, +/−	+/−, +/+
• Genotype and select k/k, +/+, +/−			
3. Intercross of selected backcross mice. Breed together to generate test cross mice.			
C. Test cross mice			
1. Inoculate as newborns			
2. Determine tumor profile			
3. Determine p53 status (+/+, +/−, −/−)			

[a] Parental strains: CBA/J mice (CBA) from Jackson Laboratory were used as a standard polyoma-susceptible host. A p53 −/− 129/SvJ male mouse (46) was kindly provided by Dr. Tyler Jacks (MIT).

Results

Breeding Scheme for Transferring a p53 Null Allele on to a Polyoma-susceptible Mouse Background. Mice carrying the H2-b haplotype, such as C57BL6 and 129/SvJ, are known to be resistant to Py tumor induction. H2-k mice, on the other hand, are frequently found to be highly susceptible. We chose the CBA/J (H2-k) mouse for this study and began by confirming its susceptibility to tumor induction by Py. Of 30 newborn CBA/J mice inoculated with virus, 29 developed multiple tumors with an average latency of about 3 months. In contrast, no tumors were found among 41 C57BL6 mice similarly treated, even after 15 months of observation. Studies of Py susceptibility in MHC congenic strains have confirmed the important contributions of these haplotypes (24).

H2-k by itself is not sufficient to confer susceptibility, however. In crosses between susceptible and resistant H2-k mice, it was shown that Mtv-7, a mouse mammary tumor provirus carried in susceptible strains, was needed for susceptibility. The superantigen Mtv-7 sag, encoded by the endogenous virus, acts as a dominant susceptibility factor by deleting T-cell precursors of polyoma immunity (25, 26). H2-k and Mtv-7 sag thus act effectively as codeterminants of Py susceptibility. When present together, they would be expected to eliminate immune responses to Py tumors and confer susceptibility on any of a wide variety of different genetic backgrounds.

To study the effects of p53 loss on Py tumor development, it was necessary first to introduce a p53 null allele on to a mouse background that carried both H2-k and Mtv-7. Starting with a single homozygous p53 −/− male mouse of 129/SvJ background (23), this was accomplished in three generations as illustrated in Table 1. In the first generation, the 129/SvJ male was crossed with several CBA/J females to produce F1 mice triply heterozygous at the MHC, Mtv-7, and *p53* loci. F_1 mice were then backcrossed to CBA/J. Backcrossed progeny were genotyped by PCR at all three loci to select those that were homozygous at the MHC (*k/k*) and Mtv-7 (+/+) loci and heterozygous for p53 (+/−). Thirteen of 136 backcrossed animals had the desired genotype, a proportion consistent with the known separate chromosomal locations of the three genes. These selected backcrossed animals were then crossed to each other to produce mice in which MHC and Mtv-7 were "fixed," and p53 was expected to segregate as 1+/+: 2+/−: 1−/−. Test cross mice coming from this or subsequent generations from crosses of p53 +/− mice were used for studies with the virus. These mice are segregating for multiple allelic differences between 129/SvJ and CBA/J distributed across the genome. Any effects of genes other than MHC and Mtv-7 are assumed to distribute randomly among the p53 classes. In the original design of the experiment, test cross progeny were to be inoculated with Py, followed for tumor development without knowledge of p53 status, then retrospectively genotyped and analyzed to determine any effects of p53.

Loss of p53 on a CBA/J Background Is Lethal. Initially, six of the selected backcrossed mice (MHC k/k, Mtv-7 +/+, p53 +/−) were used to generate 81 test cross progeny. Thirty-five were inoculated with Py as newborns, and the remaining 46 were maintained as controls to monitor possible spontaneous tumor development in mice of this mixed background. All 35 of the Py-infected animals developed tumor(s) with an average age at necropsy of 79 days and with a typical Py spectrum of tumor types (27). None of the 46 control mice developed any gross tumor by 6 months. Surprisingly, genotyping results on these 81 mice showed a bimodal distribution of 27+/+: 54+/−: 0−/−. Because of the complete absence of −/− progeny, the results shed no light on the original question. This finding differs from earlier reports indicating that absence of p53 is compatible with normal embryonic development (22, 23).

An effort was made to determine how early in development embryos lacking p53 were lost in crosses between mice of mixed 129/SvJ-CBA/J background. Three intercrosses were carried out using [(129/SvJ × CBA/J)F1 × CBA/J] backcrossed animals. Parental mice were selected and genotyped as described above and in Table 1. Embryos were

Table 2 *p53* genotypes of 11–13-day embryos[a]

Cross	No. of embryos	Days of gestation	*p53* genotype		
			+/+	+/−	−/−
1	6	13	0	6	0
2	7	13	3	4	0
3	8	11	4	4	0
		Totals	7	14	0

[a] Matings were carried out with selected backcross mice (F_1×CBA/J) that were genotyped as MHC-k/k, Mtv-7 +/+, and p53 +/− (see Table 1 and text).

Table 3 Py tumor incidence in mice of mixed CBA/J-129/SvJ background segregating for loss of p53

p53	Fraction of animals with tumor(s)	Average age at necropsy (days)
+/+	12/12	90
+/−	29/29	77
−/−	11/11	60

taken at 11 or 13 days gestation and typed by PCR. Results are shown in Table 2. No p53 −/− embryos were found among a total of 21 that were recovered. We conclude that p53 −/− embryos are lost before 11 days.

Establishment of a Subline of Mixed CBA/J-129/SvJ Background That Gives p53 −/− Births. Initial results from the test cross suggested that 129/SvJ mice carry determinant(s) that allow p53 −/− mice to develop normally and that such determinants are missing from the CBA/J background. We therefore searched among the backcross progeny and test cross mice for individual animals that retained the putative suppressor gene(s) from 129/SvJ. In one cross between p53 +/− mice of the test cross generation that had been set up for embryo typing, a high proportion of p53 −/− embryos were found at 13 days (2 +/+: 3 +/−: 6 −/−). The male used in this cross was then bred to other +/− females of the same mixed generation and found to give rise to viable −/− progeny in the expected ratio. Of 22 offspring, 6 were +/+, 11 +/−, and 5 −/−. Brother-sister matings using the +/− progeny descended from this male were used to produce a new generation of test cross mice to evaluate the effects of p53 loss on the Py tumor profile.

Py Tumor Profile in Mice of Mixed CBA/J-129/SvJ Background Segregating for p53 Loss. Fifty-two test cross mice were inoculated as newborns and followed for tumor development. Gross and histological reports were compiled without knowledge of *p53* genotype. Results are shown in Table 3. The breakdown of genotypes was 12 +/+: 29 +/−: 11 −/−, confirming rescue of the embryonic lethal phenotype in this subline of mixed lineage. All mice developed multiple tumors. However, the average age at which p53 −/− animals came to necropsy showed a significant reduction compared with the +/+ and +/− groups. The time at which individual animals are taken for necropsy is necessarily a somewhat subjective decision, based on gross observations and criteria such as total tumor load, degree of morbidity, wasting, presence of paralysis, respiratory distress, or inability to feed and drink. These judgments were all made by a single individual in as consistent a manner as possible and without knowledge of p53 status. The reduction in average age at necropsy from 90 days in the +/+ animals to 60 days in the −/− group may therefore be taken as an indication of more rapid development of Py tumors attributable to the absence of p53.

Table 4 Polyoma tumor profiles in test cross mice[a]

	p53 genotype		
	+/+	+/−	−/−
Epithelial tumors			
Skin (hair follicle, tricoepithelioma)	11/12 (92)	27/29 (93)	10/11 (91)
Mammary gland (adenocarcinoma)	9/12 (75)	19/29 (66)	5/11 (45)
Salivary gland (adenocarcinoma)	9/12 (75)	12/29 (41)	0/11 (0)
Mesenchymal tumors			
s.c. connective tissue (fibrosarcoma)	3/12 (25)	7/29 (24)	3/11 (27)
Kidney (renal medulla)	9/12 (75)	14/29 (48)	8/11 (73)
Bone (osteosarcoma)	9/12 (75)	23/29 (79)	11/11 (100)

[a] Numbers given are fractions of mice (%) developing one or more tumors of the designated type.

Findings related to the six most common tumor types are summarized in Table 4. No significant differences among the p53 classes were noted with respect to the frequencies or histological properties of these tumors. Some of the rarer Py tumor types, including ameloblastomas, adrenal gland tumors, and spindle cell tumors of the heart, were also found in these animals, but with no obvious correlation with p53 status. The absence of salivary gland tumors in the −/− animals may be related to the shorter latency of tumor development in this group relative to the +/+ and +/− groups and to the fact that tumors of this type are typically not among the most rapid to develop. Two of the 11 −/− mice developed thymic lymphoma. This tumor type is not known to be induced by Py but is typical of those that arise spontaneously in p53 −/− or +/− mice of 129/SvJ or mixed 129/SvJ-BL6 background (22, 23). These two animals were taken for necropsy at 54 and 58 days, slightly earlier than the average of the group (60 days) but not sufficiently so as to alter the conclusion of an effect of p53 loss in decreasing the latency of Py tumors.

Py Tumor Profile in Mice of Mixed AKR/J-129/SvJ Background Segregating for p53 Loss. To extend the analysis, the AKR/J mouse was chosen as another Py-susceptible strain carrying H2-k and Mtv-7. A small group ($n = 7$) of newborn AKR/J mice animals were inoculated and followed for tumors to examine the general features of the tumor profile. All animals developed tumors but with an average age at necropsy of 129 days compared with 87 for CBA/J. Tumors were similar histologically to those seen in CBA/J mice, but their relative frequencies differed somewhat. In AKR/J mice, the profile was dominated by salivary gland tumors that arose in all seven mice, whereas other tumor types were seen less frequently than in CBA/J.

A breeding scheme was carried out using AKR/J mice in place of CBA/J but otherwise identical to that described in Table 1. No evidence of embryonic lethality attributable to p53 loss was seen in the mice of mixed AKR/J-129/SvJ lineage. Results are shown in Table 5. Segregation for p53 was normal with 10 +/+, 21 +/−, and 8 −/−. Four of the

Table 5 Tumor incidence in mice of mixed AKR/J-129/SvJ background segregating for loss of p53

p53	Fraction of animals with tumor(s)	Average age at necropsy (days)
+/+	6/10	187
+/−	20/21	142
−/−	8/8	87

+/+ animals inexplicably failed to develop any tumor, as did one of the 21 +/− mice. On the basis of those animals that did develop tumor(s), there again was a substantial reduction in overall latency of tumor development in the p53 −/− (87 days) compared with the +/− (142 days) and +/+ (187 days) groups. Four of the −/− mice showed disseminated thymic lymphoma attributable to loss of p53 (possibly also to the endogenous leukemogenic retroviruses carried in AKR/J mice; Ref. 28). Because these animals came to necropsy at roughly the same time as those that were lymphoma-free while carrying Py-type tumors, they do not affect the conclusion that loss of p53 on the AKR/J-129/SvJ mixed background leads to more rapid development of Py tumors.

Tests for Expression Stabilization and Functional Alterations of p53 in Polyoma Tumors. Despite the considerable homology between the SV40 and Py large T proteins, only the former has been shown to interact stably with mouse p53 (12). Levels of p53 are controlled by various mechanisms, some involving mdm-2 (13) and p19-ARF (14, 18). Changes in these or other pathways could alter p53 levels in Py tumors. Immunohistochemical staining was carried out on 10 primary Py-induced tumors using an antibody that recognizes mutant as well as wild-type murine p53 (29). The tumors arose from a variety of different tissues and in different strains of mice all carrying wild-type p53. All 10 proved negative. Fig. 1 shows a typical result with a Py-induced mammary adenocarcinoma along with a transplantable tumor from an SV40-transformed cell line as a positive control.

Negative results of immunostaining do not rule out possible small increases or decreases in p53 levels in Py tumors. Such results are also consistent with complete loss, mutation, or other changes in the functional state of p53. As a first step toward addressing these issues, a series of Py tumor-derived cell lines were tested by Western analysis for p53 levels and responses to DNA damage. Mouse embryo fibroblasts of known genotype were exposed to actinomycin D (20 nM) for 24 h. Under these conditions, the induction of $p21^{CIP1/WAF1}$ (p21) was clearly p53 dependent (Fig. 2*a*). Cell cultures from Py tumors from different organs and mouse strains (all p53 +/+) were similarly treated and tested. Six of these were primary cultures, *i.e.,* derived directly from virus-induced tumors, whereas five had prior histories of passage in culture. Using polyclonal rabbit antimouse p53 antibody, p53 was readily detected in each of the tumor cell lines, with some showing increased levels after exposure to actinomycin D (Fig. 2*b*). p21 was clearly induced in seven of the lines. Retention of wild-type p53 function was not linked to tumor type or passage number, although p21 levels were unaffected by actinomycin D in bone tumor cells (constitutively low) and hair follicle tumor cells (constitutively high) and showed only slight induction in one of the two thymic tumors tested. p53 responses to DNA damage are known to be cell type specific (19) and regulated at least partially through covalent modifications (16, 17, 30). The absence of an inducible p21 response in some Py tumor cell lines is therefore not necessarily an indication of mutationally altered p53.

Discussion

Polyoma virus is capable of rapidly inducing a broad array of tumors in mice. Here we have investigated the effects of absence of p53 in the host on tumor induction by the virus. p53 clearly imposes some constraints on Py tumor development because p53 −/− mice develop tumors leading to high morbidity more rapidly than either +/+ or +/− hosts. The "average age at necropsy" was used as a measure of the time required for tumors to develop into a life-threatening condition. On the basis of this parameter, substantial reductions of 33–50% were found in −/− compared with +/+ animals on two different Py-susceptible mouse backgrounds. Heterozygotes showed an intermediate response in both crosses, consistent with a dosage effect of p53 on tumor development (31). This synergism between p53 loss and viral oncogenesis supports the prevailing view that Py does not functionally inactivate p53, at least fully, on its own. The possibility remains, however, that Py could intervene selectively or partially in some p53 pathways.

Two findings reported here serve to underline the differences between Py and SV40 with respect to p53: (*a*) based on immunostaining, p53 is not detectably stabilized in polyoma tumors, whereas stabilization is readily seen in tumors induced by SV40; and (*b*) the effect of p53 loss in accelerating Py tumors contrasts with that seen in an SV40 large T transgenic model, where the absence of p53 reduces rather than enhances the development of β-cell tumors (32).

The effect of p53 loss in accelerating Py tumor development may be attributable to removal of cell cycle checkpoint controls or of an apoptotic response, either being expected to result in more rapid tumor growth. Py has p53-independent mechanisms for overriding G_1 checkpoint control and apoptosis, the former through large T binding the retinoblastoma tumor suppressor gene product pRb (7, 33), and the latter through middle T activation of phosphatidylinositol 3-kinase and downstream AKT (34, 35). The effects of these viral pathways might well be additive with those resulting from the absence of p53 in terms of their impact on tumor cell growth and survival. The present results do not rule out partial or indirect effects of Py on p53 or on cellular components of p53 responses. Py large T, for example, is able to bind and inactivate the CBP/p300 transcriptional coactivators (36); this interaction, which is essential for virus replication and tumor induction in the mouse,[4] could have effects on transcriptional activation by p53. Other interactions involving the Py T antigens and downstream targets of p53 may also occur.

[4] S. Cho and T. Benjamin, manuscript in preparation.

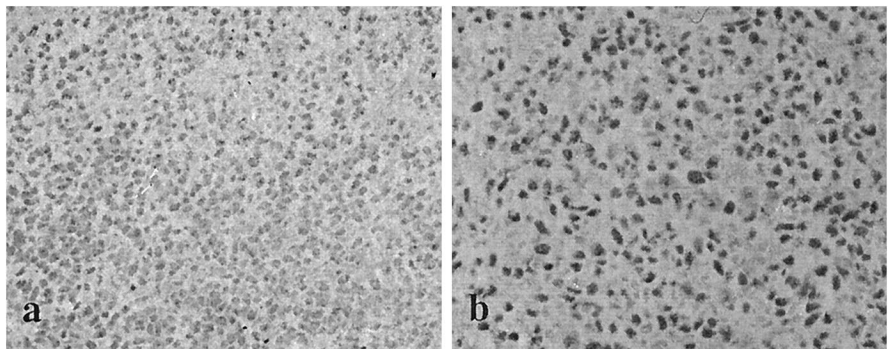

Fig. 1. Immunostaining for p53. *a,* Py-induced mammary tumor from a C3H/BiDa mouse is negative. *b,* a transplantable SV40 tumor in a BALB/c *nu/nu* mouse inoculated with 2×10^6 SVA31 cells is positive.

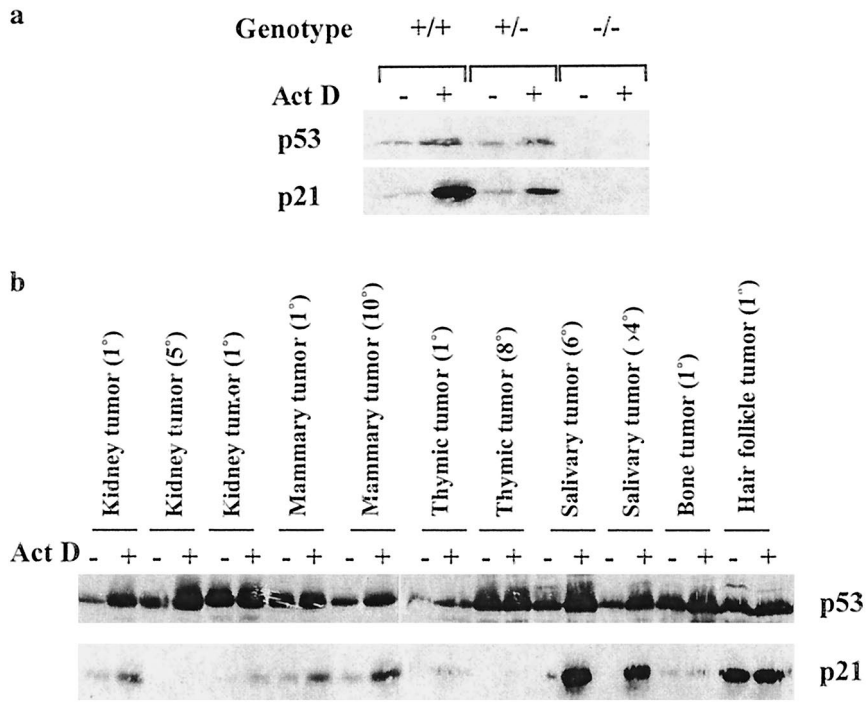

Fig. 2. p53 and p21 expression in mouse embryo fibroblasts and Py tumor-derived cell lines. Cells were treated ± actinomycin D (20 nM) for 24 h and tested for p53 and p21 levels as described in "Materials and Methods." *a,* mouse embryo fibroblast cultures from p53 +/+, +/−, −/− embryos. *b,* Py tumor-derived cell lines at the passage numbers given in *parentheses*.

No effects of p53 on the frequencies, types, or histological features of Py-induced tumors were noted. In this regard, the results may differ from those found in the Friend erythroleukemia virus system in which insertional or other mutational inactivation of p53 accompanies the progression from early- to late-stage disease (37), and infection of mice with a mutant *p53* transgene leads to accelerated development of end-stage disease (21). With Py, the effects appear to be quantitative, *i.e.,* increasing net growth rate of tumor cells, rather than qualitative, affecting biological aspects of tumor progression.

Previous studies of p53 in Py tumor-derived cell lines indicated that some but not all such cell lines have changes in p53 (11). Results reported here using primary or low-passage tumor cell cultures show the presence of immunoreactive p53 in 11 of 11 Py tumors examined, with 6 retaining the ability to induce $p21^{CIP1/WAF1}$ in response to DNA damage. These results are in agreement with some of those reported earlier (8, 11). Given the tendency of rodent cells in culture to lose p53 (38) and the rapidity with which p53 mutations and loss of heterozygosity can occur in the animal (22, 23, 37), an important follow-up to the present study would be to sequence the *p53* gene directly in a number of primary Py-induced tumors. Karyotype and comparative genomic hybridization analysis on Py tumors would also be important to establish to what extent genomic instability accompanies the development of tumors in this system and the role played by p53.

To carry out the present studies, it was necessary to have a p53 knockout on a mouse background that assured susceptibility to Py tumors. Previous studies suggested that two determinants would be sufficient, the "k" haplotype at the MHC locus and the superantigen encoded by the endogenous Mtv-7 provirus (25). A p53 null allele in a 129/SvJ background, which carries neither of the Py susceptibility determinants, was transferred on to two different backgrounds, CBA/J and AKR/J, both highly susceptible to Py tumors. In both instances, mice of mixed backgrounds selected solely for H2-k and Mtv-7 were found to be fully

susceptible to Py tumors. These results confirm and extend earlier studies indicating the importance of these two determinants and demonstrating their ability to act on heterogeneous genetic backgrounds.

An unexpected finding arose in the CBA/J × 129/SvJ cross in that a majority of backcross (F_1 × CBA/J) mice failed to produce p53 −/− offspring. Effects of p53 loss on mouse development as well as tumor susceptibility have been noted on 129/SvJ, C57BL6, and other backgrounds (39, 40). Neural tube closure defects (exencephaly) were seen in roughly 20% of the p53 −/− female embryos at around 13.5 days of gestation (41, 42). The limited penetrance of this effect contrasts with the complete absence of −/− offspring (0 of 81 live births) and no viable −/− embryos at 11–13 days (0 of 21) in mice of mixed CBA/J-129/SvJ background. p53 expression has been observed in 8.5-day embryos by *in situ* hybridization (43) and by monitoring expression of a p53-responsive reporter transgene after irradiation of pregnant mice (44). Expression of p53 may occur as early as 5 days, based on the sparing effect of p53 loss on mdm-2 knockout mice that die at about the time of implantation (45).

Current data do not allow conclusions to be drawn on the genetic basis of the apparent difference between 129/SvJ and CBA/J with respect to development of p53 −/− embryos. Our results are consistent with the hypothesis that 129/SvJ mice carry suppressor gene(s) that allow full development of p53 −/− embryos and that these gene(s) are lacking in CBA/J mice. Alternatively, lethality may result from some unusual gene interaction involving the two parental strains. In line with the former hypothesis is a recent report showing that the 129/SvJ mouse has dominant gene(s) that protect against postnatal defects in eye development attributable to p53 loss (46). A subline of mixed CBA/J-129/SvJ lineage that produces viable p53 −/− offspring has been established. The further development of this subline, based on repeated backcrossing to CBA/J and selecting for production of p53 −/− offspring, should facilitate the identification of the putative suppressor gene(s) as well as efforts to understand the physiological basis of their action(s).

Materials and Methods

Virus Strains and Establishment of Tumor Profiles. The A2 (large plaque) strain of polyoma virus (27) was inoculated intraperitoneally at a dose of approximately 2–5 × 10^6 plaque-forming units into newborn mice <20 h old. Animals were followed for tumor development and necropsied for histological confirmation of gross and occult tumors as described (27).

Mouse Strains. p53 homozygous knockout 129/SvJ mice were kindly provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Boston, MA) or purchased from The Jackson Laboratory (Bar Harbor, ME.) These mice carry a deleted *p53* gene lacking ~40% of the coding sequences spanning exons 2–7 (23). p53 knockout mice were crossed with polyoma-susceptible mice of the CBA/J and AKR/J strains (The Jackson Laboratory). Production and selection of backcross and test cross mice are described in the text.

Genotyping. Genomic DNAs from tail clips were prepared as described (47) and used for genotyping mice by PCR. The following primer pairs were purchased from Research Genetics, Inc. (Huntsville, AL): for p53, D11 MIT15; for H2, D17 MIT28; and for Mtv-7, D1 MIT36 (CBA/J cross) or D1 MIT206 (AKR/J cross). PCR was conducted in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). PCR products were separated on 6% polyacrylamide gels and visualized by ethidium bromide staining.

Western Analyses. Primary embryo fibroblast cultures from p53 +/+, +/−, and −/− embryos were prepared for use as controls. Secondary cultures were grown to 80% confluence and treated for 24 h ± actinomycin D (20 nM). Cells were extracted in the cold with buffer containing 1% NP40, 20 mM Tris/HCl (pH 7.5), 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 10 mM NaF, 50 mM β-glycerophosphate, 0.1 mM Na_3V0_4, and protease inhibitors (complete mini cocktail; Boehringer Mannheim) containing phenylmethylsulfonyl fluoride, leupeptin, and aprotinin at optimal concentrations. Extracts were clarified by centrifugation at 13,000 rpm for 10 min at 4°C. Approximately 70 μg of protein were separated by 7.5 or 12.5% SDS-PAGE and transferred to nitrocellulose. p53 was detected by chemiluminescence (Renaissance; DuPont NEN) using polyclonal rabbit anti-mouse p53 antibody CM5 (Novocastra Laboratories, Ltd.), which detects both wild-type and mutant p53 (48). $p21^{CIP1/WAF1}$ was detected using polyclonal rabbit antisera C-19 (SC-397; Santa Cruz Biotechnology, Inc). Primary cultures of fresh tumors were prepared by mincing and treating for 1 h or overnight at 37°C with collagenase (ICN; 500 units/ml). Cells were plated in DMEM containing 10% fetal bovine serum.

Immunostaining for p53. Py tumors were formalin fixed and processed for immunostaining using the p53 monoclonal antibody Ab-1 (Oncogene Research Products) and the *Citra* antigen retrieval system as described (49).

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