

Increased Tumor Proliferation and Genomic Instability without Decreased Apoptosis in MMTV-*ras* Mice Deficient in p53

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Received 31 July 1996/Returned for modification 14 September 1996/Accepted 12 November 1996

We have used an in vivo tumor model to evaluate the consequences of p53 tumor suppressor protein deficiency in a tissue-specific context. By breeding MMTV-*ras* transgenic mice, which are highly susceptible to the development of mammary and salivary tumors, with p53^{-/-} mice, we generated three classes of animals which contained the MMTV-*ras* transgene but differed in their p53 functional status (*ras/p53*^{+/+}, *ras/p53*^{+/-}, or *ras/p53*^{-/-}). *ras/p53*^{-/-} mice developed tumors more rapidly than animals of the other two genotypes; however, the distribution of tumors was unexpectedly altered. Whereas the most frequently observed tumors in *ras/p53*^{+/+} and *ras/p53*^{+/-} mice were of mammary origin, *ras/p53*^{-/-} mice developed primarily salivary tumors. In addition, the mammary and salivary tumors from *ras/p53*^{-/-} mice consistently exhibited a number of unfavorable characteristics, including higher histologic grades, increased growth rates, and extensive genomic instability and heterogeneity, relative to tumors from *ras/p53*^{+/+} mice. Interestingly, the increased growth rates of *ras/p53*^{-/-} tumors appear to be due to impaired cell cycle regulation rather than decreased apoptosis, suggesting that p53-mediated tumor suppression can occur independent of its role in apoptosis.

Cancer development and progression are driven by the stochastic accumulation of genetic and epigenetic alterations which confer some selective advantage upon tumor cells. By far the most thoroughly documented changes have been those involving activation of cellular proto-oncogenes and inactivation of tumor suppressor genes. Of these genetic alterations, mutation of the p53 tumor suppressor gene is that most frequently observed in human cancer. Point mutations, deletions, rearrangements, and allelic loss of this gene have been documented in a variety of human tumor types (23). The significance of these mutations is underscored by their correlation with various markers of prognosis in human tumor specimens, including high S-phase fractions, aneuploidy, high histologic grade, and metastasis (11, 12, 15, 25, 47).

Recent progress in defining the biological functions of the p53 tumor suppressor protein has led to an increased understanding of why p53 gene mutations are observed so frequently in human cancer. Two critical cellular events can be regulated by the p53 protein: growth arrest at the G₁/S boundary and cell death by apoptosis (for a review, see reference 30). Both of these functions may contribute to p53's ability to act as a tumor suppressor, one by blocking cell cycle progression and the other by enhancing spontaneous cell loss. However, the factors regulating which of these p53-mediated events occurs in a given situation in response to a particular stimulus, and their relative importance in tumorigenesis and progression, remain poorly understood.

It is becoming increasingly apparent that tumor growth is determined by the relative levels of cell proliferation and cell death. Given the involvement of p53 in both of these processes, it is possible that the protein may contribute to tumor suppres-

sion by different mechanisms in different settings. The importance of p53-dependent apoptosis as a means of tumor suppression has been clearly demonstrated in a transgenic mouse brain tumor model, where it was shown that tumors arising in a null p53 background grew more aggressively than those arising in a wild-type p53 background as a result of decreased levels of tumor apoptosis (46). In other settings, however, high levels of tumor apoptosis can occur by p53-independent mechanisms. By using a transgenic mouse model of lymphomagenesis, it was shown that tumors had high levels of apoptosis regardless of p53 status (24). Thus, p53-dependent apoptosis can represent an important mechanism of tumor suppression in some settings but appears to be less involved in others.

Whether p53 mediates growth arrest or apoptosis may depend on the cellular context in which it is activated. When reintroduced into tumor-derived cell lines which lack it, p53 has been shown to induce growth arrest in some cell types (43, 54) or apoptosis in others (31, 36, 37), suggesting that the cellular environment may dictate the nature of the p53 response. Expression of genes like *myc*, *myb*, E1A, and E2F can stimulate the proliferation of cells but at the same time can enhance their susceptibility to apoptosis (14, 39, 41, 42), which in many cases is p53 dependent (8, 21, 35, 49, 52). Expression of other genes, such as *ras*, *raf*, and *src*, and the presence of certain growth factors, such as insulin-like growth factor I, platelet-derived growth factor, interleukin-3, and erythropoietin, can inhibit the cellular susceptibility to apoptosis (1, 4, 18). Interestingly, some of these factors have been shown to act by specifically inhibiting p53-dependent apoptosis while leaving intact the function of the p53-dependent G₁/S checkpoint (5, 33, 38).

We have used transgenic and knockout models of mouse tumorigenesis to investigate the nature of cooperativity between the *ras* oncogene and p53 deficiency in a tissue-specific context. MMTV-*ras* mice contain a transgene in which expression of an activated v-Ha-*ras* oncogene is under the control of the mouse mammary tumor virus (MMTV) transcriptional reg-

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ulatory elements. As a result, *ras* is expressed at highest levels in the mammary and salivary glands of mice which carry the transgene, predisposing them to tumors arising from these tissues (44). We have bred MMTV-*ras* mice with *p53*^{-/-} mice (10) in order to generate three classes of animals which differ only in their *p53* functional status (*ras/p53*^{+/+}, *ras/p53*^{+/-}, and *ras/p53*^{-/-}) and have compared various features of mammary and salivary tumors arising in these different genotypic groups. This study demonstrates that in the presence of a constitutively active *ras* oncogene, *p53* status does not influence tumorigenesis and progression at the level of tumor apoptosis. Nonetheless, *p53*-deficient tumors exhibit a more aggressive phenotype, as demonstrated by their accelerated onset, higher nuclear grade, increased growth rate, and extensive intratumor heterogeneity and multiclonality. These findings, taken together with recent reports in the literature, suggest that *p53*-mediated tumor suppression can occur by distinct mechanisms in different settings.

MATERIALS AND METHODS

Mice. MMTV-*ras* mice in an inbred FVB genetic background were obtained from Charles River, Inc. *p53*^{-/-} mice were obtained from Genpharm, Inc., and are maintained by our laboratory in a hybrid CB6F1 (C57BL6 × BALB/c) genetic background. Offspring of the various genotypes were generated as littermates from common matings so that all animals in the study were of a mixed genetic background derived from FVB, C57BL6, and BALB/c mice. Offspring from matings between MMTV-*ras* and *p53*^{-/-} (or *p53*^{+/-}) mice were screened by PCR for their *ras* and *p53* status. Briefly, a small piece of tail was cut from each animal at the time of weaning and then used to isolate genomic DNA by standard procedures (22) and for PCR analysis. Primers used for the detection of the MMTV-*ras* transgene were 5'-CCCAAGGCTTAAGTAAGTTTTGG-3' (5' sense primer) and 5'-GGGCATAAGCAGATAAAACACT-3' (3' antisense primer). For evaluating the *p53* status of offspring, PCRs using three primers allowed for detection of both the normal and mutant *p53* alleles in a single reaction. These primers consisted of a sense 5' primer specific for exon 4 (5'-GGGACAGCCAAGTCTGTTATGTC-3') and two antisense 3' primers specific for either exon 6 of *p53* (5'-CTGTCTCCAGATACTCGGGATAC-3') or the *neo* cassette (5'-TTTACGGAGCCTGGCGCTCGATGT-3'). Reactions were run for 35 cycles at an annealing temperature of 60°C.

Animals were checked twice weekly for the presence of tumors. Once a tumor was identified (either visually or by palpation) and had reached 200 to 400 mg in size, its growth was monitored for the next 10 to 14 days and the animal was sacrificed. Tumor weight was calculated according to the following formula: tumor weight (in milligrams) = $W^2 \times L/2$, where W is width and L is length. Female animals exclusively were used in this study; they were generally housed with male breeders continuously from the time they reached sexual maturity until the time of their sacrifice to maximize pregnancy and lactation.

Examining *p53* loss in *ras/p53*^{+/-} tumors. Tumor DNA was extracted, and Southern analysis was performed by standard methods. Fifteen micrograms of DNA was digested with *Bam*HI, electrophoresed in 1% agarose, and transferred onto a Nytran filter (Micon Separations Inc.). A 605-bp ³²P-labeled *Kpn*I fragment of plasmid LR10 (GenPharm) was used to identify the 6.5-kb mutant and 5.0-kb normal *p53* alleles. Semiquantitative analysis of band intensity ratios was performed with NIH Image analysis software.

Apoptosis analysis. Samples of tumors were fixed in 10% neutral buffered formalin and routinely processed for embedding in paraffin. In situ labeling of apoptotic cells was accomplished by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method (16). Positively stained cells were evaluated by light microscopy. Positive cells within a 10- by 10-mm grid in the eyepiece were counted in 10 450× fields. As expected, some staining occurred in clusters that were necrotic by light microscopy, and these regions were avoided. Apoptotic cells in nonnecrotic regions generally appeared to be uniformly distributed through viable areas of tumor. The total number of tumor cells in each field ranged from 500 to 800, depending on the histological pattern of the tumor.

Flow cytometry. At the time of sacrifice, a 25- to 50-mg piece of tumor tissue was flash frozen in liquid nitrogen and stored at -80°C until analyzed by flow cytometry. Tissue was processed by standard methods using a modified Krishan technique, involving propidium iodide staining of tumor cell DNA (28). All samples were analyzed with an EPICS ELITE flow cytometer (Coulter Cytometry, Miami, Fla.), using a 15-mW argon ion laser operated at 6 A of power at 488 nm. Histograms were analyzed for cell cycle compartments by using MultiCycle-PLUS version 3.0 (Phoenix Flow Systems, San Diego, Calif.). At least 50,000 events were collected in order to maximize statistical validity of the compartmental analysis.

Histological evaluation. Tissue was fixed in 10% formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin. Tumors were

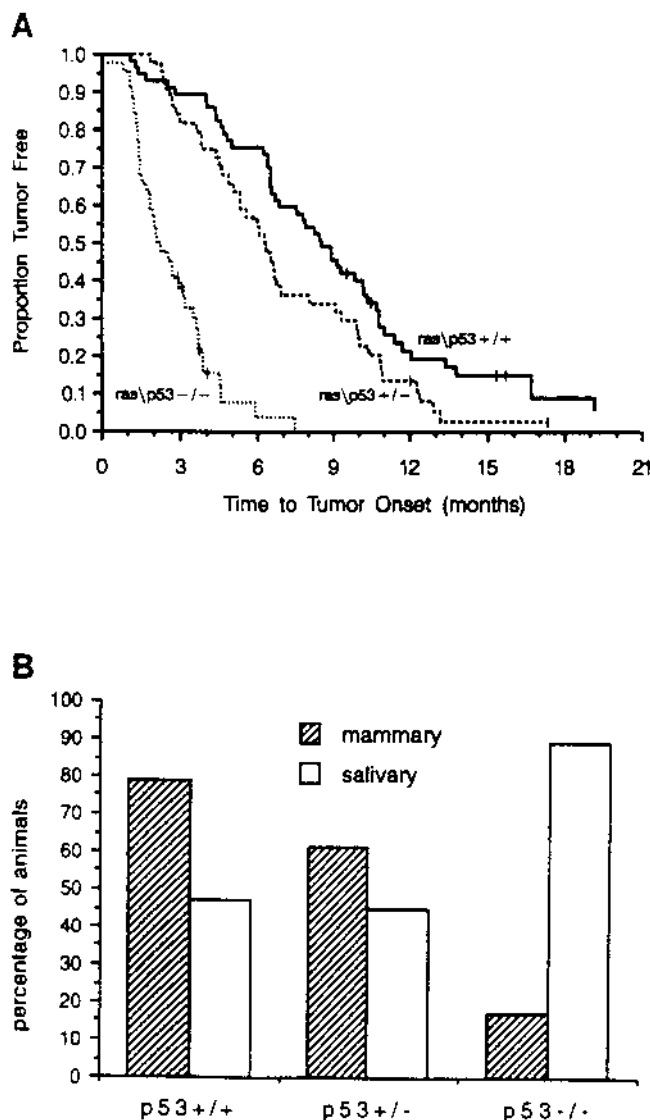


FIG. 1. Tumor onset and distribution in MMTV-*ras* mice of each *p53* genotype. (A) Survival curves showing the proportion of tumor-free female mice as a function of time for *ras/p53*^{+/+} ($n = 50$), *ras/p53*^{+/-} ($n = 39$), and *ras/p53*^{-/-} ($n = 42$) mice. Time to tumor onset represents age at which a palpable mammary or salivary tumor was first detected. (B) Proportion of animals of each genotype which developed mammary versus salivary tumors. Tumor type was assessed by a number of criteria, including location of tumor, tumor composition and morphology, and expression of α -amylase mRNA for salivary tumors or β -casein mRNA for mammary tumors (not shown).

graded according to a scoring system of 0 to 3, where 0 represented normal cells. Grades 1 and 2 were assigned to tumors showing increasing nuclear size, increased nuclear/cytoplasmic ratios, and pleomorphism. Grade 3 tumors showed, in addition, giant or multinucleated cells and a spindling cellular morphology. Tumors were also evaluated for the extent of necrosis present and were assigned a semiquantitative score of 0 to 3.

Cytogenetic analysis. At the time of sacrifice, a small piece of tumor was removed and placed in sterile medium. Cells were isolated by gently pushing the tissue through a 70- μ m-pore-size nylon cell filter. The cultures were established with serum-free medium, PC-1 (Hycor), supplemented with 1% L-glutamine and 0.1% gentamicin. Primary cultures were harvested for routine cytogenetics at approximately 70% confluency (between 7 and 21 days). Colcemid treatment for 1 to 2 h produced the highest mitotic index. Slides were prepared by standard methods and GTG banded (29).

Statistical analysis. Tumor onset data were analyzed for statistical significance by using survival analysis methods. Within each genotype, tumor-free survival curves were estimated by the Kaplan-Meier method (27) and compared by using

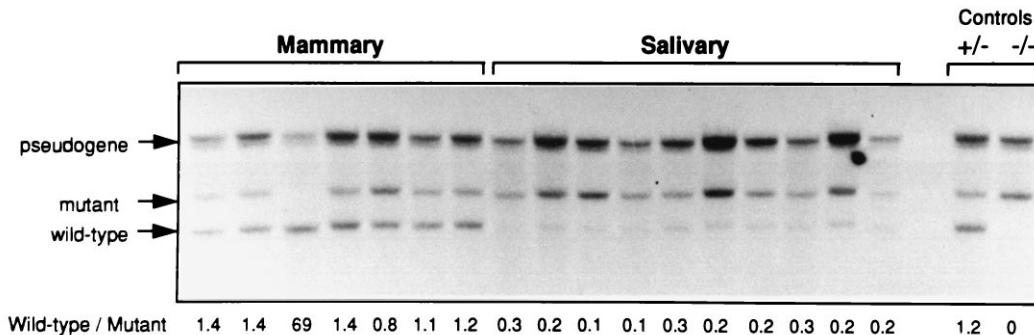


FIG. 2. Analysis of *ras/p53*^{+/-} mammary and salivary tumors for loss of the wild-type p53 allele. Tumor DNAs from 7 *ras/p53*^{+/-} mammary tumors and 10 *ras/p53*^{+/-} salivary tumors were analyzed by Southern analysis. The location of the p53 pseudogene, the mutant p53 allele, and the wild-type allele are indicated. The wild-type/mutant band intensity ratio is shown below each lane. Each lane contained 15 μ g of tumor DNA, while the controls contained 15 μ g of constitutional DNA from a *ras/p53*^{+/-} mouse and a *ras/p53*^{-/-} mouse.

the log-rank test (7). Tumor growth rates were analyzed in two stages for statistical comparisons. First, for each tumor, a tumor growth rate was estimated by modeling tumor growth as a function of time. Then a weighted one-way analysis of variance of the growth rates was used to compare the rates for each genotype and to estimate average growth rates (20). Levels of apoptosis for each tumor type and for each p53 genotype were compared by one-way analysis of variance. Flow cytometrically determined proportions of cells in the S and G₂/M phases of the cell cycle were analyzed for statistical significance first by multivariate one-way analysis of variance (50). After obtaining significant results, we used univariate analyses to determine the nature of the differences between genotypes.

RESULTS

Accelerated onset and altered distribution of tumors in MMTV-*ras* mice deficient in p53. By breeding MMTV-*ras* mice (44) with p53^{-/-} mice (10), we generated three genotypic groups of animals which carried the MMTV-*ras* transgene but differed in their p53 status (*ras/p53*^{+/+}, *ras/p53*^{+/-}, and *ras/p53*^{-/-}), and the mice were monitored over time for the development of tumors. Since the mammary and salivary tumors which arise in MMTV-*ras* mice can be detected while still very small, it was possible to accurately determine whether p53 status had any influence on the age of overt tumor onset. *ras/p53*^{-/-} mice developed tumors much more rapidly than mice from either of the other two genotypes (Fig. 1A). The median time to tumor onset for the *ras/p53*^{+/+} group, for example, was 8.5 months, whereas that for the *ras/p53*^{-/-} mice was 2.2 months ($P < 0.0001$). Tumors from *ras/p53*^{+/-} mice were intermediate with respect to tumorigenesis, with a median time to tumor onset of 6.3 months.

Although mammary tumors were the most frequent tumor type observed in *ras/p53*^{+/+} mice, salivary tumors were also common. For example, 76% of *ras/p53*^{+/+} mice developed mammary tumors, and 45% developed salivary tumors (27 *ras/p53*^{+/+} mice developed mammary tumors, 12 developed salivary tumors, and 10 developed both mammary and salivary tumors) (Fig. 1B). A similar distribution was observed in *ras/p53*^{+/-} mice. In contrast, *ras/p53*^{-/-} mice developed salivary tumors much more frequently than mammary tumors, reflecting a greater acceleration of tumorigenesis in the salivary gland than the mammary gland. Only 14% of the p53^{-/-} mice developed mammary tumors, whereas 90% developed salivary tumors (36 *ras/p53*^{-/-} mice developed salivary tumors, 4 developed mammary tumors, and 2 developed both salivary and mammary tumors) (Fig. 1B). These results demonstrate that p53 deficiency alters the normal distribution of tumor types arising in MMTV-*ras* transgenic mice, resulting in an increased frequency of salivary tumorigenesis compared to mammary

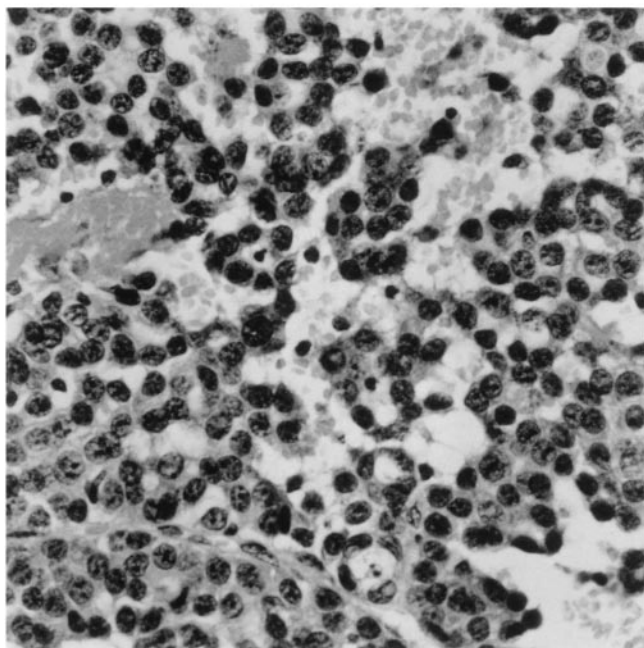
tumorigenesis. It should be noted, however, that the low incidence of mammary tumors observed in the *ras/p53*^{-/-} mice can be accounted for in part by the accelerated salivary tumorigenesis, which necessitated sacrifice of the mice prior to the time at which mammary tumors would likely have developed. Thus, it is difficult to determine the extent to which mammary tumorigenesis was accelerated by p53 deficiency.

A subset of *ras/p53*^{-/-} mice (approximately 10 to 15%) developed lymphomas before developing either mammary or salivary tumors (not shown). This was not unexpected since the most frequent tumor type found in p53^{-/-} mice were lymphomas (10), and there is some overlap between the age ranges of tumor onset for p53^{-/-} lymphomas and those for *ras/p53*^{-/-} mammary and salivary tumors. No lymphomas were observed in the *ras/p53*^{+/+} female animals used for the study.

Tissue-specific difference in the frequency of p53 loss in tumors from *ras/p53*^{+/-} mice. One possible explanation for the altered tumor type distribution observed is that the combination of *ras* and p53 deficiency may cooperate more effectively in the transformation of salivary tissue than mammary tissue, resulting in a more rapid acceleration of salivary tumorigenesis. We tested this indirectly by investigating *ras/p53*^{+/-} mammary and salivary tumors for loss of the wild-type p53 allele, scored as loss of heterozygosity (LOH) by Southern analysis. Of the seven *ras/p53*^{+/-} mammary tumors examined, none showed significant loss of the normal p53 allele. One tumor had actually lost the null allele, probably reflecting a random loss of this nonfunctional allele. In contrast, all of the 10 salivary tumors examined showed some degree of loss of the normal p53 allele (Fig. 2). Some tumors had lost the allele entirely, while several others had only a faint wild-type signal remaining. It is unclear whether this is a result of normal cell contamination (i.e., stromal or vascular cells present within the tumor) or whether a small subpopulation of tumor cells retained the wild-type allele. In either case, there was clearly a tissue-specific difference in the frequency of p53 loss in tumors from *ras/p53*^{+/-} mice. These results, together with the fact that salivary tumors are much more prevalent than mammary tumors in *ras/p53*^{-/-} mice, suggest that the combination of *ras* activation and p53 deficiency is more highly tumorigenic and transforming in the mouse salivary gland than in the mammary gland.

It might be expected, based on these results, that salivary tumors of the *ras/p53*^{+/+} genotype would have a higher frequency of de novo p53 mutations than mammary tumors of the same genotype. Surprisingly, however, no such mutations were

A



B

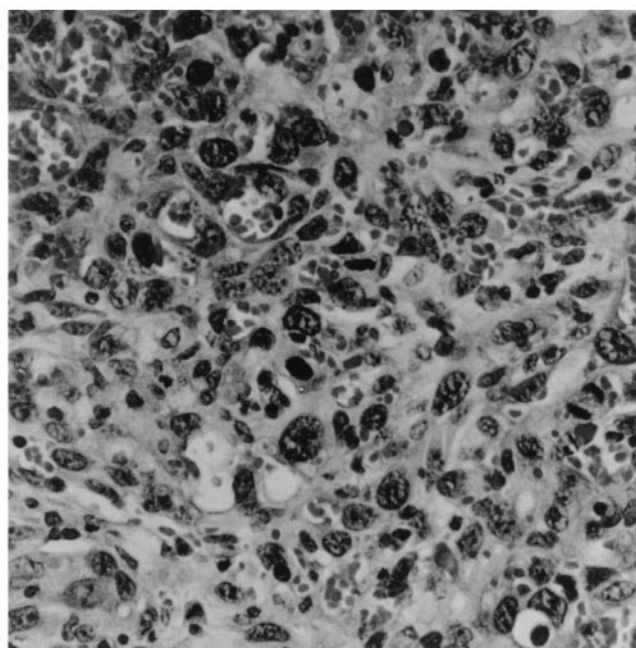


FIG. 3. Histological appearance of tumors arising in *ras/p53*^{+/+} and *ras/p53*^{-/-} mice. (A) Histological section from a *ras/p53*^{+/+} salivary tumor at a magnification of $\times 40$. (B) Histological section from a *ras/p53*^{-/-} salivary tumor at a magnification of $\times 40$. Tissue was fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin.

detected in any *ras/p53*^{+/+} or *ras/p53*^{+/-} mammary or *ras/p53*^{+/+} salivary tumors by single-strand conformation polymorphism analysis (data not shown). Salivary tumors from *ras/p53*^{+/-} mice were not subjected to single-strand conformation polymorphism analysis since they had lost their remaining wild-type p53 allele by Southern analysis.

Higher histologic grade in p53-deficient tumors. Histologic grading systems are used to describe the degree of malignancy of tumor specimens, and this grading has been demonstrated for a number of tumor types to have prognostic value (40). We therefore analyzed salivary tumors from *ras/p53*^{+/+} and *ras/p53*^{-/-} mice to determine whether p53 status had any influence on tumor histopathology. In general, tumors of either genotype were composed of nests and cords of epithelial cells which lacked distinguishing architectural features, such as acinar formation or squamous differentiation. Necrosis was sometimes found in small areas between broad, concentric bands of tumor cells. For grading, nuclear size, nuclear/cytoplasmic ratio, and nuclear pleomorphism were evaluated and incorporated into a final score of 0 to 4, with 4 representing the most pleomorphic and highest-grade cells. When tumors of each genotype were evaluated in this way, p53-deficient tumors generally had higher histologic grades. *ras/p53*^{+/+} tumors had a mean grade of 1.4 ($n = 9$; range = 1 to 2), whereas the mean grade for *ras/p53*^{-/-} tumors was 2.0 ($n = 14$; range = 1 to 3). Figures 3A and B are representative sections from a *ras/p53*^{+/+} tumor and a *ras/p53*^{-/-} tumor, respectively. The *ras/p53*^{+/+} cells shown in Fig. 3A are relatively uniform in size and shape (though still invasive and malignant), while the *ras/p53*^{-/-} tumor demonstrates more variability in cell and nuclear size and shape and less uniform distribution of chromatin within the nucleus. Similar features were observed in the small number of mammary tumors that arose in *ras/p53*^{-/-} mice.

Tumor growth characteristics: apoptosis versus proliferation. Given the involvement of p53 in both cell cycle regulation and apoptosis, and the fact that solid tumor growth reflects a balance between cell proliferation and cell death, we have investigated the dynamics of this balance in tumors from each genotypic class. The MMTV-*ras* mammary and salivary tumors are ideally suited for these types of analyses because both tumor types are palpable, allowing their size to be monitored over time by taking periodic caliper measurements. Additionally, upon sacrifice of the animal, tissue can be used to evaluate

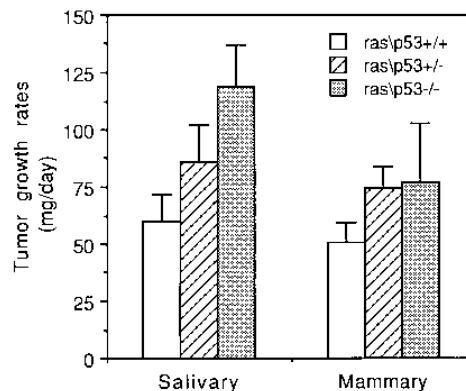


FIG. 4. Tumor growth rates. Growth was monitored from the time a tumor was first identified (generally when it had reached a weight of between 200 and 400 mg) for roughly 2 weeks, or until the tumor reached a size at which the general health of the animal appeared compromised. Tumor mass was calculated from caliper measurements as described in Materials and Methods.

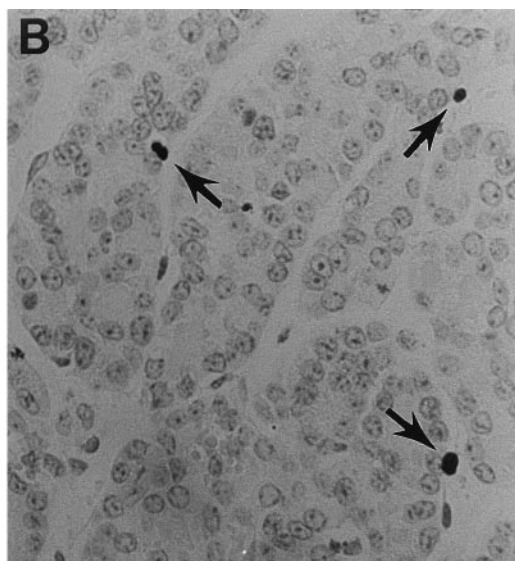
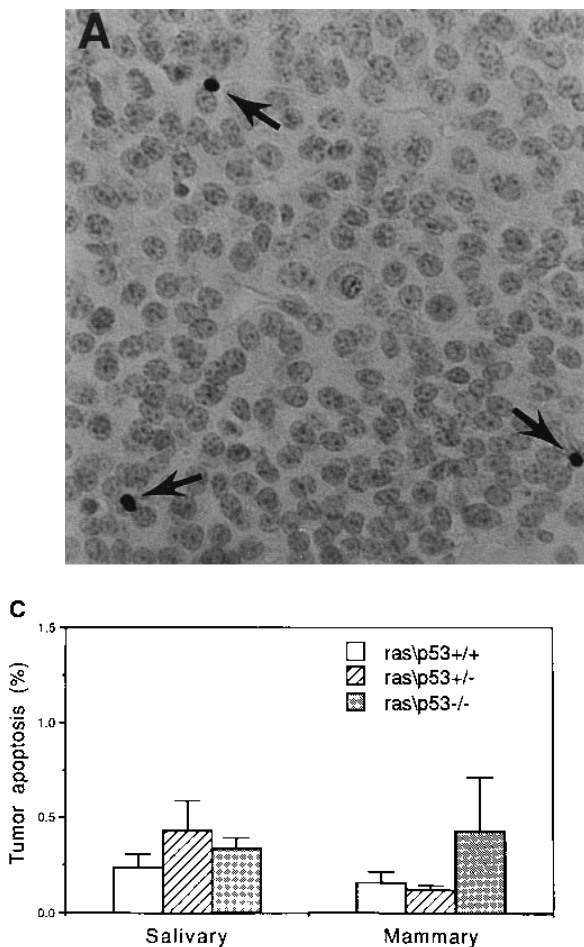


FIG. 5. Analysis of tumor apoptosis. Tumor cells undergoing apoptosis were detected on formalin-fixed and paraffin-embedded tissue sections by the TUNEL method (16). Shown are representative TUNEL-stained sections of a *ras/p53*^{+/+} salivary tumor (A) and a *ras/p53*^{-/-} salivary tumor (B). Arrows indicate TUNEL-positive cells. (C) Quantitation of apoptosis for salivary and mammary tumors of each p53 genotype. Positively stained cells were evaluated by light microscopy and quantitated as described in Materials and Methods. For the *ras/p53*^{+/+} group, *n* = 14 for salivary and *n* = 9 for mammary; for the *ras/p53*^{+/-} group, *n* = 6 for salivary and *n* = 14 for mammary; for the *ras/p53*^{-/-} group, *n* = 16 for salivary and *n* = 4 for mammary.

both the cell cycle and apoptotic characteristics of the same tumor for which growth data have already been obtained.

Animals were checked twice weekly for the presence of tumors. Once a tumor was detected in an animal, measurements were taken every other day for approximately 2 weeks, at which time the animal was sacrificed (tumor mass usually reached 750 to 2,000 mg by the time of sacrifice). Over this time period, mammary and salivary tumors of the *ras/p53*^{-/-} genotype grew more rapidly than those arising in *ras/p53*^{+/+} mice. For example, salivary tumors from *ras/p53*^{+/+} mice grew at a rate of 60 ± 11 mg/day (*n* = 18), whereas those from *ras/p53*^{-/-} mice grew at 119 ± 18 mg/day (*n* = 17; *P* = 0.02) (Fig. 4). Tumors from *ras/p53*^{+/-} mice grew at intermediate rates (85 ± 16 mg/day; *n* = 6). A similar trend was seen with mammary tumors, but the differences were not statistically significant, probably due in part to the small sample size and high variability for the few *ras/p53*^{-/-} mammary tumors that did arise. The growth rates for these tumors were 50 ± 8 mg/day for *ras/p53*^{+/+} tumors (*n* = 16), 74 ± 10 mg/day for *ras/p53*^{+/-} tumors (*n* = 19), and 77 ± 25 mg/day for *ras/p53*^{-/-} tumors (*n* = 5).

To determine whether the increased growth rate of p53-deficient tumors was a result of the loss of p53-dependent apoptosis, tumor tissue was taken at the time of sacrifice, and levels of apoptosis were quantitated by the TUNEL assay (16). Surprisingly, the *ras/p53*^{+/+} tumors did not have higher levels of apoptosis than *ras/p53*^{-/-} tumors. Regardless of p53 status, the levels in both mammary and salivary tumors were uni-

formly low as measured by the TUNEL assay (<0.5%), and no statistically significant differences were observed (Fig. 5). In addition, no DNA ladders were visualized when as much as 3 μ g of tumor DNA was electrophoresed on agarose gels (not shown). Thus, MMTV-*ras* tumors had consistently low levels of apoptosis, and p53 deficiency did not appear to contribute to tumorigenesis and progression in this model by attenuating apoptosis. We cannot, however, rule out the possibility that the rates with which the apoptotic process occurs vary in these different groups.

We next tested whether the increased growth rates of *ras/p53*^{-/-} tumors might be due to alterations in cell cycle characteristics, as measured by flow cytometry. Consistent with the involvement of p53 in a G₁/S checkpoint, tumors from *ras/p53*^{-/-} mice displayed significant differences in cell cycle distributions compared to *ras/p53*^{+/+} tumors. The former had increased cell numbers in S and G₂/M and a corresponding decrease in the proportion of cells in G₁ (*P* = 0.001 and *P* = 0.03 for salivary and mammary tumors, respectively [Fig. 6]). It should be noted that the cell cycle distributions for a substantial percentage of *ras/p53*^{-/-} tumors (~35%) were not analyzable due to the presence of multiple populations of tumors cells (see below). Thus, the cell cycle distributions shown reflect only those *ras/p53*^{-/-} tumors in which G₁, S, and G₂/M compartments were clearly identifiable. In contrast, all of the *ras/p53*^{+/+} tumors were analyzable in this regard.

p53-deficient tumors have an increased frequency of aneuploidy and extensive tumor heterogeneity. An indirect effect of p53 loss or mutation on tumor progression may result from the illegitimate propagation of DNA-damaged cells which occurs when the p53-dependent G₁/S checkpoint is impaired. Cells from p53-deficient mice and tumors expressing mutant p53 protein are often genetically unstable. Such cells display en-

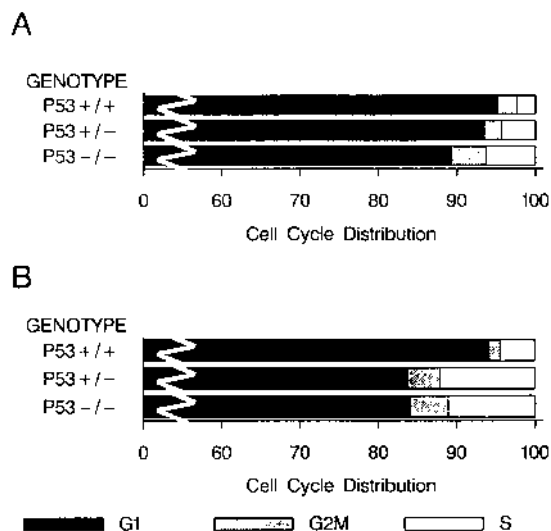


FIG. 6. Tumor cell cycle characteristics. G₁, G₂/M, and S-phase fractions based on flow cytometry analysis of propidium iodide-stained tumor cells are shown for mammary tumors (A) and salivary tumors (B). The G₁, S-phase, and G₂/M fractions for *ras/p53*^{-/-} mammary tumors were 87.2 ± 6.8, 6.9 ± 2.1, and 5.9 ± 4.6, compared to 95.3 ± 1.1, 2.2 ± 0.4, and 2.5 ± 1.2 for *ras/p53*^{+/+} tumors. The cell cycle distributions for analyzable salivary tumors from *ras/p53*^{-/-} mice were 84.0 ± 1.9, 10.6 ± 1.8, and 5.4 ± 0.9, compared to 94.9 ± 0.9, 3.7 ± 0.7, and 1.4 ± 0.3 for tumors from *ras/p53*^{+/+} mice. For the *ras/p53*^{+/+} group, *n* = 10 for salivary and *n* = 11 for mammary; for the *ras/p53*^{+/-} group, *n* = 6 for salivary and *n* = 13 for mammary; for the *ras/p53*^{-/-} group, *n* = 12 for salivary and *n* = 3 for mammary.

hanced karyotypic and molecular abnormalities and can readily amplify genes in response to selective pressure (3, 19, 34, 48, 53). Thus, the abrogation of p53 function may compromise the integrity of the genome, thereby fueling the multistep process of neoplastic progression.

To evaluate the effects of p53 status on genomic integrity in our model, mammary and salivary tumors were analyzed for DNA content by flow cytometry to assess the frequency with which aneuploid tumor populations were detected in the different groups and to determine the extent of intratumor heterogeneity or multiclonality. The majority (~70%) of *ras/p53*^{+/+} tumors were euploid by flow cytometry (Fig. 7A and B). The remaining tumors, which were aneuploid, generally consisted of a single, homogeneous population of tumor cells (i.e., were clonal with respect to DNA content). In contrast, most *ras/p53*^{-/-} tumors contained multiple aneuploid subpopulations of tumor cells which differed in DNA content, a few having as many as four distinct aneuploid peaks present in the small tumor sample analyzed (Fig. 7A and C). Thus, p53-deficient tumors had a much higher frequency of aneuploidy and were very heterogeneous or multiclonal in composition compared to those arising in a wild-type p53 background.

A smaller number of salivary tumors were also analyzed at the cytogenetic level by generating metaphase spreads from early-passage, cultured tumor cells. Chromosome counts were obtained on multiple metaphase spreads from different tumor-derived cultures. For the *ras/p53*^{+/+} tumor represented in Fig. 8A, most of the spreads contained either a diploid or a near-diploid chromosome number (mouse diploid number = 40). In contrast, two cultures derived from *ras/p53*^{-/-} tumors displayed much more heterogeneity with respect to chromosome number (Fig. 8B and C). For one of these tumor-derived cultures, more than 20 metaphase spreads were examined, and nearly all spreads had different chromosome numbers (Fig.

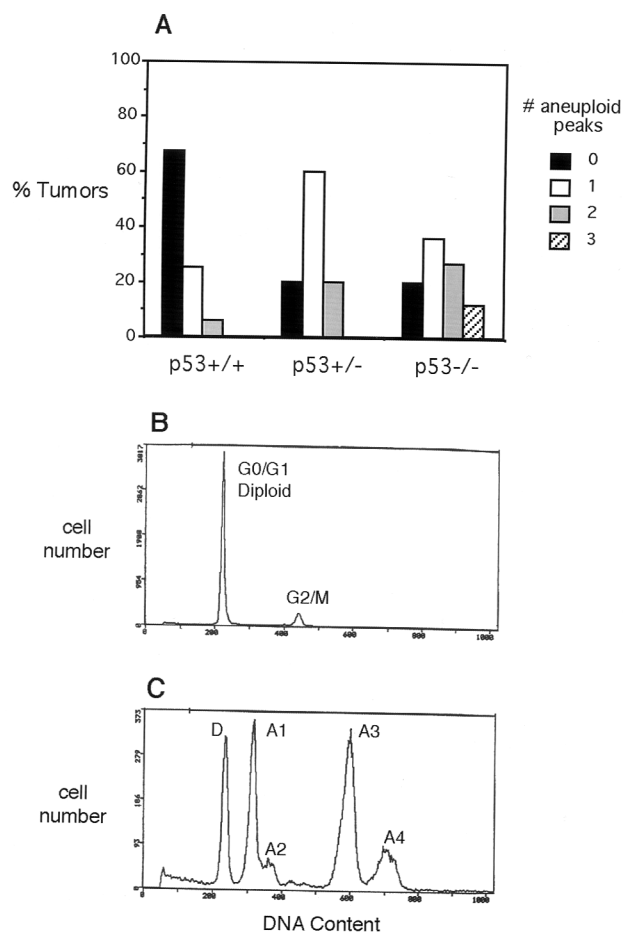


FIG. 7. Analysis of tumor aneuploidy and heterogeneity. (A) Graphical summary showing the percentage of tumors (including both mammary and salivary) of each p53 genotype which either contained no aneuploid peaks (i.e., were euploid) or contained one, two, or three or more distinct peaks with aneuploid DNA content. (B) Representative histogram from a *ras/p53*^{+/+} salivary tumor consisting of a homogeneous population of diploid tumor cells. The G₀/G₁ and G₂/M components are indicated. (C) Histogram from a *ras/p53*^{-/-} salivary tumor containing, in addition to a diploid peak (D), four distinct aneuploid G₁ populations (A1, A2, A3, and A4).

8C). Importantly, these are very early passage tumor cells, suggesting that these results reflect actual tumor characteristics rather than changes which have occurred as a result of in vitro cell culture.

DISCUSSION

Current technologies in mouse genetics provide powerful approaches to address specific questions regarding in vivo tumorigenesis and progression. We have used a cancer-susceptible MMTV-*ras* transgenic mouse strain in combination with *p53*^{-/-} knockout mice to develop a tissue-specific tumor model in which to evaluate the consequences of p53 loss. Using this approach, we have demonstrated that (i) the combination of *ras* activation and p53 deficiency may have differential transforming activities in the mammary and salivary glands, (ii) p53 deficiency appears to contribute to more aggressive tumor growth in this model through impaired cell cycle regulation rather than through decreased apoptosis, and (iii) p53-deficient tumors are more pleiomorphic, are more frequently ane-

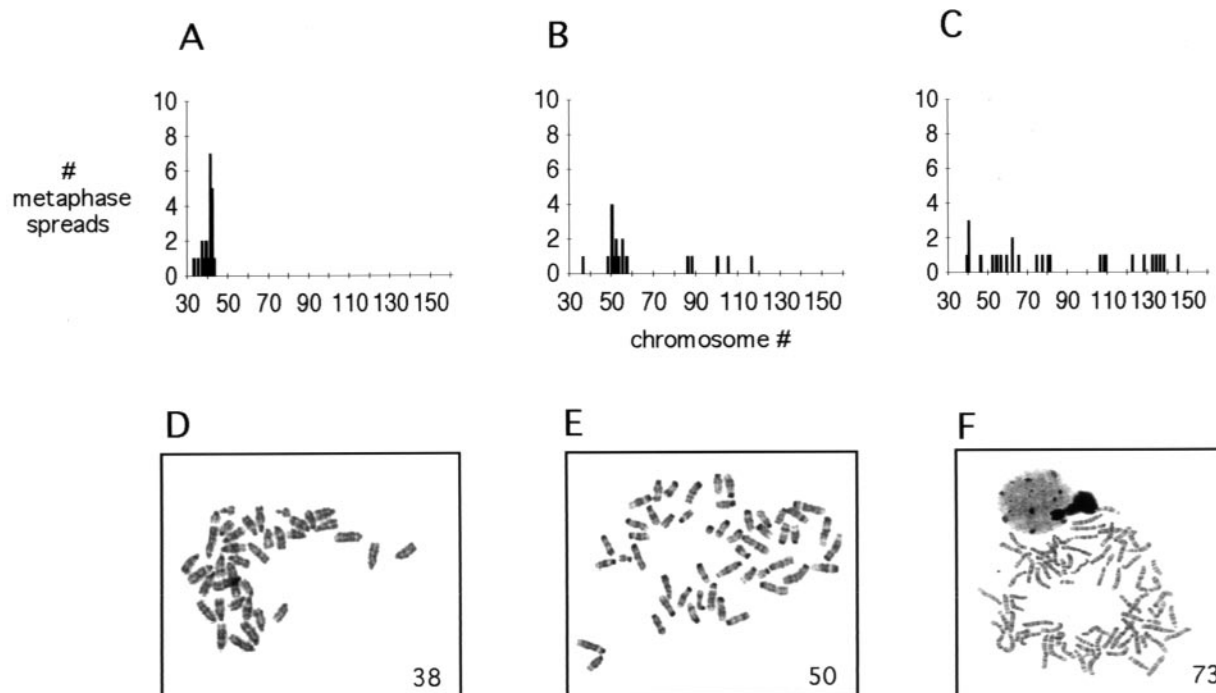


FIG. 8. Cytogenetic analysis of tumor heterogeneity. Chromosome numbers were determined on a minimum of 20 metaphase spreads from cultured tumor cells from one *ras/p53*^{+/+} (A) and two *ras/p53*^{-/-} (B and C) salivary tumors. Representative spreads from each of these tumors are shown in panels D, E, and F, respectively, and chromosome numbers of the spreads shown are indicated in the lower right corners.

uploid, and exhibit extensive genomic instability and heterogeneity.

The most frequent tumor type observed in *ras/p53*^{+/+} and *ras/p53*^{+/-} mice was mammary tumors, with a lower incidence of salivary tumors. However, in *ras/p53*^{-/-} mice, there was a significant shift in the distribution of tumors observed to primarily salivary tumors. This shift in distribution resulted from a dramatic acceleration of salivary tumorigenesis, which in many cases necessitated the sacrifice of mice prior to the development of mammary tumors. In view of the LOH results demonstrating that there was frequent LOH of the wild-type p53 allele in *ras/p53*^{+/-} salivary but not mammary tumors, it is possible that the advantage provided by p53 loss is greater, and therefore more strongly selected for, in the salivary microenvironment than in the mammary microenvironment.

Recently, Elson et al. (13) used an approach similar to that described here, in which MMTV-*myc* mice were bred with *p53*^{-/-} mice to generate offspring which contained the *myc* transgene but differed in their p53 status. Similar to our findings, mice which were positive for the MMTV-*myc* transgene and also null for p53 showed a profile of tumor types different from that of the parental MMTV-*myc* strain. These mice developed accelerated lymphomas with a very high frequency, whereas few mammary tumors were observed (13). Furthermore, Min mice, which contain a germ line mutation in the adenomatous polyposis coli gene, normally develop large- and small-bowel adenomas, which sometimes progress to malignant carcinomas. When these mice were bred into a p53-deficient background, they too showed a shift in phenotype relative to Min mice in a normal p53 background (6).

Since p53 deficiency does not appear to cooperate strongly with the *ras* or *myc* oncogene in the mammary tissue, it is possible that its loss or mutation is simply not an important event in mouse mammary tumorigenesis and progression, as it

is in human breast cancer. However, there are reports of p53 mutations in some mouse mammary tumor models (26). Furthermore, when MMTV-*Wnt-1* transgenic mice were bred into a nullizygous p53 background, they developed mammary tumors with accelerated onset and increased genomic instability compared to mammary tumors from MMTV-*Wnt-1* mice which were wild-type for p53, indicating that the combination of *Wnt-1* and p53 deficiency can successfully cooperate in mouse mammary tumorigenesis and progression. In addition, MMTV-*Wnt-1* mice which were heterozygous for the mutant p53 allele lost their wild-type allele in roughly half of the tumors analyzed (9), whereas we observed no selection for loss of the wild-type allele in *ras/p53*^{+/-} mammary tumors. Taken together, these findings indicate that the extent to which p53 loss can cooperate with a given oncogene or mutated tumor suppressor gene varies according to the cell type in which that cooperation occurs.

The relevance of p53-dependent apoptosis in different settings is not entirely clear. Symonds et al. (46) have demonstrated that p53-dependent apoptosis can represent a direct mechanism of tumor suppression in vivo. Mice expressing a mutant simian virus 40 T-antigen transgene (which binds Rb and related proteins but leaves p53 function intact) developed slowly growing brain tumors which were associated with very high levels of apoptosis (7 to 8%). When the mice were bred to nullizygosity for p53, tumors arose earlier, grew more rapidly, and had reduced levels of apoptosis (<1%) (46). Using the $\epsilon\mu$ -*myc* transgenic model of lymphomagenesis, however, Hsu et al. found that levels of apoptosis were high (7 to 10%) and not significantly different in tumors from $\epsilon\mu$ -*myc* mice which were wild type compared to those which were null for p53 function (24). Similarly, the MMTV-*ras* tumors examined here, regardless of p53 status, did not differ significantly in the levels of apoptosis detected. These and other results suggest that the

importance of tumor apoptosis in general, and p53-dependent apoptosis in particular, may be under oncogene- and/or tissue-specific influences.

The *ras* oncogene has been demonstrated to inhibit the cellular susceptibility to apoptosis in several experimental systems (1, 2, 4, 32, 51), in addition to its role in stimulating proliferation. Our results are consistent with a model in which activated v-Ha-*ras* can function to inhibit susceptibility to tumor apoptosis in vivo, as it has been shown to do in some in vitro settings. If the low levels of apoptosis observed in *ras/p53*^{+/+} tumors are a result of an oncogene-specific phenomenon (i.e., *ras* activation), then one might expect to observe variable levels of tumor apoptosis in other MMTV-oncogene models, depending on the oncogene being expressed. Indeed, preliminary results indicate that MMTV-*myc* (45) mammary tumors have levels of apoptosis significantly higher than those observed in MMTV-*ras* mammary and salivary tumors (data not shown). Thus, distinct oncogenes may differentially regulate the susceptibility to spontaneous tumor apoptosis in vivo.

Evidence has recently been reported regarding how p53-dependent processes can be modulated by the cellular environment. The presence of specific growth factors and/or the expression of genes like *src*, *raf*, *ras*, or the Epstein-Barr virus LMP1 gene appear to favor a p53-dependent G₁ arrest rather than apoptosis (5, 17, 33, 38). In this regard, the p53-mediated response to a particular stimulus may vary according to the context in which it is induced. It is possible that *ras* inhibits p53-dependent apoptosis in our model while the G₁/S checkpoint remains functional. The facts that there are statistically significant differences in cell cycle parameters between *ras/p53*^{+/+} and *ras/p53*^{-/-} tumors (the former have higher G₁ and lower S-phase fraction values) and that the G₁/S checkpoint is functional and can be activated by adriamycin in *ras/p53*^{+/+} but not *ras/p53*^{-/-} tumors (not shown) argue in favor of this possibility.

p53-dependent apoptosis did not appear to represent a mechanism of tumor suppression in *ras/p53*^{+/+} mammary and salivary adenocarcinomas, since the levels observed were uniformly very low (<0.5%), and no significant differences were observed between any of the groups. Nonetheless, p53 status was clearly related to tumor phenotype. Tumors from *ras/p53*^{-/-} mice had a number of features that are associated with poor prognosis in human cancer. These include higher histologic grade, high S-phase fractions, high frequency of aneuploidy, and extensive tumor heterogeneity and multiclonality. These findings strongly suggest that p53 can function as a tumor suppressor protein independent of its role in apoptosis.

Considering that the p53-mediated response (growth arrest versus apoptosis) can vary depending on the presence of certain growth factors or the activation of particular cellular signaling pathways, it is likely that the effects of p53 alterations on tumorigenesis and progression similarly depend on the cellular context in which it is mutated. In situations where p53-dependent apoptosis is actively suppressing tumor growth, loss of p53 function can result in reduced apoptosis, leading to enhanced tumor growth. In other settings where the p53-dependent G₁/S checkpoint is active, loss of p53 can result in more rapid tumor growth via enhanced proliferation and loss of genomic stability. The fact that p53 alterations may contribute significantly to tumor progression by different mechanisms in different settings may further explain why alterations in this gene are observed with such high frequency in diverse types of human cancer.

ACKNOWLEDGMENTS

We thank Barbara Koop and Angelica Lozano for technical assistance. We also thank John McGill and Vicki Mattern for helpful comments on the cytogenetic analyses and C. Kent Osborne and Craig Allred for many helpful discussions. We acknowledge the contributions of the San Antonio Cancer Institute Pathology, Cytogenetics, and Flow Cytometry cores to this research.

This work was supported by a developmental project subgrant (to J.J.W.) from National Institutes of Health (NIH) grant P50 CA58183, American Cancer Society grant DHP-150 (to J.J.W.), and San Antonio Cancer Institute Cancer Center Support grant P30 CA54174 from the NIH.

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