Identifying Chemical Carcinogens and Assessing Potential Risk in Short-term Bioassays Using Transgenic Mouse Models

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There has been a continuing, worldwide effort to control the public health and social burden of cancer. One aspect of this effort is the identification of agents that cause cancer in humans and the reduction of exposure to acceptable levels of risk by regulatory action. Human carcinogens have been identified with greatest certainty by epidemiological studies. However, the rodent bioassay has come into prominence because using epidemiology to identify causal agents is feasible only to a limited extent. Identification of human carcinogens by rodent bioassay, however, has a number of important limitations: the most perplexing problem is the uncertainty of whether all the agents that cause tumors in rodents do so in humans. A corollary to this problem is the uncertainty of whether the potency and dose–response relationships are the same in rodents and humans. There is also the cost and duration of the National Toxicology Program (NTP) bioassays, the gold standard for animal bioassays; the expense is so high that only a few chemicals per year can be evaluated. There are many chemicals in commercial use or in the environment that have not been tested, and thousands of new chemicals are synthesized each year. There is a clear need to improve the process of carcinogen identification not just so that more chemicals can be evaluated, but also to achieve a better understanding of human risk. Short-term testing of chemicals in bacteria and cultured cells contributes valuable information, but such assays are regarded as ancillary to epidemiology and animal testing because of their limited biological complexity. For the foreseeable future, improvements in identifying potential human carcinogens will come from improvements in the rodent bioassay.

A useful new tool which has emerged from developments in molecular biology is the transgenic rodent. Such animals have modified genes that cause them to respond to carcinogens in advantageous ways. This paper describes the response characteristics of two of the most promising of these transgensics, p53+/− and TG.AC, and discusses ways in which they may be used to improve carcinogen bioassay.

The p53+/− line responds rapidly to genotoxic carcinogens, and the TG.AC line responds rapidly to nongenotoxic carcinogens. Positive results in either could lead to an NTP bioassay modified to focus on low-level dose response; verification of carcinogenicity could be substantially accomplished by the transgenic assay, and the transgenic assay could provide guidance for dose selection. Thus, the use of these two transgenic models could accomplish several important objectives: 1) accelerated testing of environmental chemicals; 2) prioritization of chemicals for NTP bioassay; and 3) focusing the NTP bioassay on one primary objective, low-level dose response, instead of the dual objective of determining whether an agent is carcinogenic, which requires administration of high doses, and at the same time trying to determine dose–response characteristics at low doses. Conversely, as experience is accrued in the use of these transgensics, it may become evident that with agents that are clearly positive with structural alert evaluations, in vitro mutagenicity, and transgenic bioassays, the full NTP bioassay is not needed.

Transgenic Mouse Models

Appropriate transgenic mouse lines offer the opportunity to develop relatively short-term in vivo models to identify potential carcinogens and other toxic agents. Such models include transgenic mice carrying reporter genes that may serve as targets for mutagenic events (1,2) or mice carrying specific oncogenes or inactivated tumor-suppressor genes that are important factors contributing to the multistage process of carcinogenesis (3–5). Mouse lines with defined genetic alterations that result in overexpression or inactivation of a gene intrinsic to carcinogenesis, but that are insufficient alone for neoplastic conversion, are promising models for chemical carcinogen identification and evaluation. Likely gene targets for alteration that might result in this phenotype are 1) the activation (by mutation) of the c-Ha-ras protooncogene, which alters signal transduction and growth control (6, and 2) inactivation of the p53 tumor-suppressor gene, which is critical to cell cycle control and DNA repair (7–9). These tumor genes are often mutated and/or amplified (c-Ha-ras) or mutated and/or lost (p53) in human and rodent tumors (10–13).

p53 +/- mice. Mice with only a single wild-type p53 allele provide a distinct target for mutagens and are analogous to humans at risk due to heritable forms of...
cancer, such as the Li-Fraumeni syndrome (14). The reduction in p53 gene dosage by this "germline first hit" increases the probability that a second mutagenic event will cause either loss of p53 tumor-suppressor function or gain of transforming activity by requiring (at minimum) only a single mutation (10–13). Mice with inactive p53 genes are viable and, in the hemizygous state, have a low background tumor incidence for up to 12 months of age, whereas nullizygous mice have a higher rate of spontaneous tumors at sites apparently determined by the strain's genetic background (15,16).

We conducted concurrent 6-month carcinogenesis studies using male and female C57BL/6 mice hemizygous for the wild-type p53 gene together with their homozygous wild-type p53 siblings. We used two carcinogens that exhibited trans-species carcinogenicity (17) (i.e., induced tumors in both mice and rats), in long-term rodent bioassays and that were positive in the Salmonella mutagenesis assay (18–20): p-cresidine (21) and 4-vinyl-1-cyclohexene diepoxide (VCD) (22). For comparison, we used two nonmutagenic carcinogens that were carcinogenic in only one of two test species: N-methylolacrylamide (NMOA) (23) and reserpine (24). As a negative control, we used p-ansidine (25), an analogue of p-cresidine, which was positive in the Salmonella assay and negative in rats and mice in two-year carcinogenesis studies. These chemicals represent the opposite ends of the spectrum of biological activity observed in rodent bioassays (18–20). To facilitate this comparison, we used the same exposure conditions and chemical doses as used in the 2-year bioassays.

**TG.AC mice.** TG.AC mice carry a v-Ha-ras oncogene fused to the promoter of the β-globin gene (26). The v-Ha-ras transgene has point mutations at codons 12 and 59, and the site of integration of the transgene confers on these mice the characteristic of genetically initiated skin as a target for tumorigenesis in the context of the intensively studied mouse-skin tumorigenesis model (27). An important consideration of the TG.AC mouse model is that the transgene is not constitutively expressed in the skin, and the untreated skin appears normal when compared to the skin of the wild-type FVB/N parent strain (26,28). In addition, the spontaneous incidence of skin papillomas in the dorsal skin of untreated mice is very low to zero. Heterozygous or homozygous TG.AC mice receiving repetitive topical treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) or other well-described mouse-skin promoters (benzoyl peroxide, mezerein, or methyl ethyl ketone peroxide) readily develop, as early as 4–6 weeks after treatment, benign squamous papillomas which may progress to malignancy (26,29).

Our approach to evaluating and using the TG.AC mice is different from that for the p53+/- mice. Many of the chemicals tested initially in TG.AC were used to characterize the phenotype of the line rather than to directly assess its use as identifying carcinogens. As the studies progressed, the potential use of this line to identify putative nongenotoxic carcinogens and to permit extended dose–response analyses became apparent.

The activities of 20 chemicals have been evaluated in homozygous female TG.AC mice using a skin-painting protocol. These chemicals represent well-known mouse skin initiators and promoters (26,29), specific intermediate metabolites known to occur in the signal transduction pathways induced by TPA binding to its receptor, protein kinase C, and carcinogens and noncarcinogens identified in the NTP 2-year bioassay. These chemicals include genotoxic and nongenotoxic carcinogens and noncarcinogens that represent a spectrum of chemicals that have exhibited a wide range of biological activities in the 2-year NTP bioassays.

We have used the TG.AC mouse model in both retrospective and prospective studies to examine its potential to identify the activity of possible carcinogens and to complement the standard 13-week prechronic toxicity studies used by the National Toxicology Program (NTP) to identify target organs and dose–response relationships leading to a 2-year rodent bioassay. While the TG.AC mouse might seem most appropriate for evaluating the activity of those chemicals scheduled for a 2-year skin-paint study protocol, our retrospective studies with carcinogens identified in the 2-year NTP bioassays indicated that chemicals administered in the feed or via gavage can also exhibit activity in the skin of topically treated transgenic mice when using a dose range representative of that used in the 2-year bioassay protocol.

**Methods**

Mice were treated and maintained in accordance with the NIH Guidelines for Humane Care and Use and under an institutional, peer-reviewed animal study protocol.

**p53 +/- mice.** Male and female mice hemizygous for wild-type p53 (TG-p53) and homozygous wild-type siblings were obtained from GenPharm International (Mountain View, California). Treatments began in a staggered fashion at 15–18 weeks of age after a 3-week acclimation period. Male mice were housed singly, and female mice were housed in groups; both were fed a pelleted or powdered defined diet (#D10010, Research Diets, New Brunswick, New Jersey) and water ad libitum. Wild-type sibling and hemizygous p53 mouse control groups contained 10 mice each and hemizygous p53 treatment groups contained 15 (low-dose) or 20 (high-dose) mice. Assignment of uneven numbers was determined by the number of mice available and unknown effects of toxicants in these mice. Test chemicals were administered in the diet daily, by topical administration (2 times/week; 100 μl acetone vehicle), or by gavage (5 times/week; corn oil vehicle) for 24 weeks (Table 1). The mice were held an additional 4–6 weeks to allow tumor development after interim sacrifices indicated chemical-specific differences in the rate of appearance of gross lesions. Clinical observations and body weights were recorded weekly. All mice received a gross necropsy and microscopic examination of gross lesions and target tissues. Mice were held 4–6 weeks after cessation of treatment to allow for a staggered termination of the experiments, following determination of tumor progress in those mice with gross lesions.

**TG.AC mice.** Groups of 10–15 female homozygous TG.AC mice, housed 5 per cage, were first treated when 10–12 weeks old. Mice were fed Purina Pico Chow, no. 5058, and water ad libitum. The dorsal skin at the application site was clipped 1–2 days before administration of the first dose and as needed throughout the dosing period. Chemicals were administered topically to the clipped area with 200 μl of acetone or 70–95% ethanol 2 to 5 times per week for 20 weeks. Mice treated concurrently with the vehicle solvent or 1.25 μg TPA served as the negative and positive controls, respectively. Mice were examined weekly for the development of skin papillomas at the application site.

**Results**

**p53 +/- mice.** Topically administered VCD induced squamous cell and basal cell carcinomas in male and female F344 rats and male and female B6C3F1 mice in 2-year bioassays (22,30) that first appeared after 54 weeks of treatment (Table 1). In the studies described here, both hemizygous p53 and wild-type male and female mice showed an apparent dose-related decrease in body weight gain (not less than 10% of the concurrent control) throughout the VCD skin paint study that was reversed after the treatment period, especially in the females (data not shown). Microscopic analysis of gross lesions revealed that there
Table 1. Target organ tumor incidence in the 24-week study in C57BL/6 p53-deficient mice and the NTP 103-week study in B6C3F1 hybrid mice

| Chemical (route: target tissue) | Dose | Time (weeks) | Mouse strain (C57BL/6) | Mouse strain (B6C3F1) |
|--------------------------------|------|--------------|------------------------|-----------------------|
|                                |      | M            | F                      | M                      |
| 4-Vinyl-1-cyclohexene diepoxideb (topical: skin) | 0 mg | 24/4 | 2/7 | 0/5 | 0/5 | 0 mg | 0/50 | 0/50 |
|                                | 12.5 mg | 24/4 | 2/7 | 0/5 | 0/5 | 0 mg | 0/50 | 0/50 |
|                                | 25.0 mg | 24/4 | 2/7 | 0/5 | 0/5 | 0 mg | 0/50 | 0/50 |
| p-Cresidine (diet: urinary bladder) | 0% | 24/2 | 0% | 0% | 0% | 0.25 mg | 0.25% | 0.25% |
|                                | 0.25% | 24/2 | 0% | 0% | 0% | 0% | 0% | 0% |
| p-Anisidine (diet: none) | 0% | 24/6 | 0% | 0% | 0% | 0.25% | 0.25% | 0.25% |
|                                | 0.25% | 24/6 | 0% | 0% | 0% | 0% | 0% | 0% |
| N-Methylolacrylamide (gavage: liver) | 0 mg/kg | 24/6 | 0% | 0% | 0% | 0 mg/kg | 0% | 0% |
|                                | 25 mg/kg | 24/6 | 0% | 0% | 0% | 0 mg/kg | 0% | 0% |
|                                | 50 mg/kg | 24/6 | 0% | 0% | 0% | 0 mg/kg | 0% | 0% |
| Reserpinec (diet: M, seminal vesicle; F, mammary gland) | 0% | 24/6 | 0% | 0% | 0% | 0% | 0% | 0% |
|                                | 0.0005% | 24/6 | 0% | 0% | 0% | 0% | 0% | 0% |
|                                | 0.001% | 24/6 | 0% | 0% | 0% | 0% | 0% | 0% |

*Length of exposure plus holding (weeks) before euthanasia and necropsy.

NMOA was nonmutagenic in the Salmonella assay, although it contains a chemical structural alert indicating electrophilic potential and was carcinogenic to mice in 2-year bioassays when administered by oral intubation (18,23) (Table 1). In the 2-year bioassays, NMOA-treated B6C3F1 male and female mice exhibited an increase in body weight gain relative to controls and increased incidences of tumors in the Harderian gland, liver, lung, and ovary. In the studies with transgenic mice, NMOA depressed body weight gain in male mice (greatest in high-dose, wild-type siblings) and slightly increased body weight gain in female mice (data not shown). Mortality was slightly increased in high-dose male and female mice hemizygous for p53, but no induced tumors were observed (Table 2).

Reserpine was not mutagenic in the Salmonella assay, but in the 2-year dosed feed studies, it induced mammary gland tumors in B6C3F1 female mice (18,24) (Table 1). In these transgenic mouse studies, reserpine administered at 0.001% of the diet to p53 +/- mice resulted in lethargy, tremors, piloerection, and severe weight loss and death (Table 2), requiring immediate reduction of the high dose after 4 weeks of treatment to the lower dose level for the duration of the study in the hemizygous p53 mice to prevent further deaths. No tumors or gross toxic lesions were affected. No gross lesions or microscopic lesions in the urinary bladder were observed after 24 weeks of dietary administration and a 6-week holding period.

was an apparent dose-related increase in skin tumors (squamous cell or basal cell carcinoma or fibrosarcoma) in VCD skin-painted mice hemizygous for the p53 gene, but not in the respective p53 +/- vehicle controls or in the control or high-dose, homozygous wild-type sibling male or female mice (Table 1). The incidence of nodular epidermal hyperplasia appeared to be a continuum with the development of squamous cell carcinomas, which may be significant since carcinomas appeared virtually in the absence of papillomas. Repetitive exposure of the skin to carcinogens has been shown to result in complete loss of p53 and malignant skin tumors without progression through a benign tumor phase (31,32).

p-Cresidine was mutagenic in the Salmonella assay and caused urinary bladder tumors in rats and mice when administered in the diet (18,21). In the 2-year studies, the first bladder tumors were observed in B6C3F1 mice at 40 and 44 weeks of treatment for high-dose male and female mice, respectively (21) (Table 1). In the current dose-feed studies, p53 +/- and wild type mice treated with p-cresidine experienced a similar short-term transient and dose-related decrease in body weight gain, which was greater in males than in females of either mouse line and was similar to that observed in the 2-year studies. It is important that both p53 +/- and wild-type mice exhibited similar systemic toxicity, but the latter did not develop tumors. After 6 months of exposure and a 2-week holding period, microscopic analysis of gross lesions revealed an apparent dose-related increase in urinary bladder transitional cell carcinomas in male and female p53 hemizygous mice, but not in the p53 +/- controls or in the high-dose, homozygous wild-type siblings (Table 1). Tumors were grossly observed as thickened bladder walls or large masses, which frequently were associated with hydronephrosis due to bladder occlusion. These characteristics were similar to the tumors induced in the B6C3F1 mice used in the 2-year bioassay. Microscopically, the carcinomas were invasive masses composed of markedly anaplastic cells and/or cells exhibiting squamous differentiation. In one high-dose p53 +/- female, a squamous papilloma of the bladder mucosa was observed. Deaths related to tumor induction that occurred during the study (Table 2) were especially frequent in male mice hemizygous for wild-type p53.

p-Anisidine, an analogue of p-cresidine, was mutagenic in the Salmonella assay and was not carcinogenic to male or female F344 rats or B6C3F1 mice in 2-year dosed-feed studies (29). In those studies, the dose levels used induced body weight depression without an increase in mortality in the treated groups relative to the controls. In the present dose-feed study, p-anisidine (administered equimolar to p-cresidine) did not induce any significant clinical observations or depressed body weight gain relative to the control groups (Tables 1 and 2). However, 5 of 15 low-dose p53 +/- mice died of unexplained causes during the study. None of the high-dose p53 +/- mice were affected. No gross lesions or microscopic lesions in the urinary bladder were observed after 24 weeks of dietary administration and a 6-week holding period.

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observed in high-dose mice converted to the low dose, or in low-dose mice that survived to the end of the study.

**TG.AC mice.** In the retrospective studies, seven of eight chemicals were correctly identified as carcinogens or noncarcinogens when administered via the skin paint protocol in TG.AC female mice (Table 3). Benzene (18,33–35), a proven human carcinogen and a multisite, trans-species rodent carcinogen, readily induced papillomas in 70% of the transgenic mice by 10 weeks of exposure. Mirex (36), a nongenotoxic liver carcinogen when administered in the feed to rats, induced papillomas, which were observed as early as 7 weeks. In the NTP studies, o-benzyl-p-chlorophenol (37), a nonmutagenic carcinogen, caused kidney tumors in male B6C3F1 mice when administered by oral intubation (gavage) (Table 3). When administered topically as a promoter (3 mg, 3 times/week) for 50 weeks following an initiating dose of dimethylbenz[a]anthracene, o-benzyl-p-chlorophenol induced a low incidence of papillomas (2 papillomas/mouse) in 36% of the treated female mice (38). Topically applied o-benzyl-p-chlorophenol (3 mg, 3 times/week for 20 weeks) induced papillomas in 80% of the treated TG.AC mice, with an overall

### Table 2. Incidence of mortality during the in-life portion of the bioassay before termination

| Chemical | Dose | Timea (weeks) | Mouse strain | Hemizygous p53 | Wild type |
|----------|------|---------------|--------------|----------------|-----------|
| 4-Vinyl-1-cyclohexene dioxpide b | 0 mg | 24 + | 0/7 | 0/8 | 0/5 | 0/5 |
| | 12.5 mg | 24 + | 0/7 | 2/8 | — | — |
| | 25.0 mg | 24 + | 2/10 | 0/10 | 0/5 | 0/5 |
| p-Cresidine | 0% | 24 + | 0/5 | 1/5 | 0/5 | 0/5 |
| | 0.25% | 24 + | 2/8 | 0/7 | — | — |
| | 0.5% | 24 + | 7/10 | 2/10 | 0/5 | 0.5 |
| p-Anisidine | 0% | 24 + | 0/5 | 0/5 | 0/5 | 0.5 |
| | 0.225% | 24 + | 2/7 | 3/8 | — | — |
| | 0.45% | 24 + | 0/10 | 0/10 | 1/5 | 0/5 |
| N-methylolacrylamide | 0 mg/kg | 24 + | 0/7 | 0/7 | 0/5 | 0/5 |
| | 25 mg/kg | 24 + | 1/7 | 1/7 | — | — |
| | 50 mg/kg | 24 + | 2/10 | 2/10 | 0/5 | 1/5 |
| Reserpine c | 0% | 24 + | 0/7 | 0/8 | 0/5 | 0/5 |
| | 0.0005% | 24 + | 1/8 | 1/7 | — | — |
| | 0.001% | 24 + | 7/10 | 6/10 | 1/5 | 1/5 |

aLength of exposure plus holding (weeks) before euthanasia and necropsy.

bVCD dosage was administered (mg/mouse) in 200 ml acetone 2 x/week or 5 x/week in the 24 week and 105 week studies, respectively, resulting in an equivalent cumulative weekly dose.

cDue to excessive weight loss and mortality in the high dose group, survivors were converted to the low dose feed concentration after 4 weeks of treatment and maintained in the same cages for the remainder of the study without further loss. Data were also maintained separately because of sustained body weight depression and clinical observations.

### Table 3. Retrospective evaluation of NTP chemicals for activity in TG.AC transgenic mice

| Chemical | SAL a | Route b | Maximum dose | Rat c | Mouse d | LED d | TG.AC skin paint |
|----------|-------|---------|---------------|-------|---------|-------|-----------------|
| NTP bioassay | (weeks) | (weeks) | (weeks) | (weeks) | (weeks) | (weeks) | (weeks) |
| Benzene | — | G | 200 | 100 | + | + | + | 200 μL, 2 x/week | 5 | 7.4 | 77 | + |
| Benzetinium chloride | — | SP | 1.5 | 1.5 | — | — | — | 60 μg, 5 x/week | 8 | 0.55 | 22 | — |
| o-Benzyl-p-chlorophenol | — | SP | 240 | 480 | E (K) | + (K) | — | 3 mg, 3 x/week | 7 | 3.0 | 80 | + |
| 2-Chloroethanol | + | SP | 100 | 15 | — | — | — | 20 mg, 5 x/week | 10 | 0.10 | 11 | — |
| p-Cresidine | + | F | 1.0 | 0.46 | (UB) | (UB) | (UB) | (UB) | 80 μg, 2 x/week | 6 | 5.0 | 58 | + |
| Ethyl acrylate | — | G | 200 | 200 | + (S) | + (S) | + (S) | 30 mg, 3 x/week | 15 | 0.6 | 50 | — |
| Mirex | — | F | 0.005 | ND | + (L, AG)+ (L, HS) | ND | ND | 54.5 pg, 3 x/week | 7 | 12 | 70 | + |
| Phenol | — | W | 0.5 | 0.5 | — | — | — | 3.0 mg, 2 x/week | 7 | 0.20 | 0.16 | — |

ND, not done.

aSAL: Salmonella mutagenicity results provided by E. Zeiger, National Toxicology Program.

bRoute of administration of test chemical. G, oral gavage, dose in mg/kg, given 5 x/week; SP, skin paint, dose is in mg/kg except where otherwise indicated; F, feed, dose in %; W, drinking water, dose in %.

c334 rats and B6C3F1 mice except as noted. Tumor sites in parentheses: K, kidney; UB, urinary bladder; S, stomach; L, liver; AG, adrenal gland; HS, hematopoietic system. E, equivocal results.

dLED, lowest effective dose per mouse that induced papillomas at the site of application or highest "no effect" dose administered.

*TTFP, time (weeks) to first observation of a skin papilloma in any mouse of that dose group.

+At 20 weeks.

+CD-1 mice.

+Data from a collaborative study with R. Smart, North Carolina State University.
incidence of 3 papillomas/mouse (Table 3). 
P-Cresidine, the genotoxic urinary bladder carcinogen (18,21) described above in p53 +/− mice studies, not only induced papillomas in TG.AC mice, but showed systemic effects by causing the same preneoplastic lesions in the bladder epithelium that were observed in B6C3F1 mice. Benzenethionium chloride (39) and phenol (18,40), both nongenotoxic noncarcinogens, and 2-chloroethanol (18,41), a genotoxic noncarcinogen, were inactive in the TG.AC mice. Ethyl acrylate, a nongenotoxic carcinogen (19,42) induced only forestomach tumors in the 2-year bioassay, a consequence of gavage exposure (43−45). Ethyl acrylate was inactive in TG.AC mice, and no gross systemic effects were observed at the end of the 20-week skin-paint study that had induced chronic epidermal hyperplasia at the application site (Table 3). It is noteworthy that another trans-species forestomach carcinogen, diglycidyl rescinol ether (19,46), was also inactive in a long-term skin paint study in female Swiss mice (47).

Four chemicals undergoing skin-painting studies in NTP bioassays were evaluated prospectively for activity in TG.AC mice using dose ranges representative of those used in the 2-year studies (Table 4). Lauric acid diethanolamine (NTP studies in progress) and methyl ethyl ketone peroxide (48) readily induced papillomas as early as 4 and 5 weeks, respectively (Table 4). No papillomas were induced by 20 weeks of treatment with diethanolamine (NTP studies in progress) or triethanolamine (49). The accuracy of these predictive studies await the outcome of the NTP bioassays.

Discussion
Hemizygous p53 mice, but not their homozygous wild-type sibling controls, developed tumors after treatment with two model mutagenic carcinogens, but not with two model nongenotoxic carcinogens or with a mutagenic noncarcinogen. Tumors appeared much sooner, but at the same organ sites, in the hemizygous p53 mice as they did in B6C3F1 mice in the 2-year bioassays with the mutagenic carcinogens. These limited data suggest that the hemizygous p53 mice may exhibit a specificity of response for mutagenic carcinogens as well as increased sensitivity when compared to their normal, wild-type littermates. However, neither of the nonmutagenic carcinogens induced tumors in their expected target tissues in either hemizygous p53 or homozygous wild-type sibling mice when administered for 6 months by the same route used in 2-year bioassays. These short-term bioassay results are consistent with the 2-year NTP bioassays for the mutagenic carcinogens, indicating that the C57BL/6 strain responded similarly to the B6C3F1 hybrid. Although the number of mice used in these studies was small compared to long-term toxicology studies, these data indicate that these mice may have increased susceptibility to mutagenic carcinogens (15,16,32) and that mutagenic chemicals are required to initiate the carcinogenic responses observed.

The skin-paint studies in the TG.AC transgenic mouse model indicate that the model can complement the standard NTP 13-week subchronic bioassays that are used to identify potential target organ sites for toxic effects or neoplasia in the 2-year bioassay. Furthermore, the model is sensitive to chemicals that do not necessarily target the skin when administered by other routes of exposure. Some major advantages of this model are that the skin-tumor response can be visibly observed and induced within a 20-week dosing regimen; furthermore, the dosing protocol (e.g., dose frequency per week) can be varied according to the toxicity of the chemical. The target organ site, the dorsal skin, is normal until stimulated by a series of chemically induced events that lead to the induction of skin papillomas (26,29). The latency period is short, with the first tumors appearing as early as 5 weeks and usually by 10 weeks when using a 20-week dosing protocol. Further, in every instance where the treated mice were observed an additional 10−20 weeks after cessation of dosing, a subpopulation of the papillomas progressed to malignancy. Other important considerations are that the life span of homozygous TG.AC mice is well over a year, and the sporadic papilloma incidence at the target site, dorsal skin, is very low to zero in untreated or vehicle-control mice. Untreated TG.AC mouse skin has normal morphological and physiological characteristics that are maintained throughout the mouse’s life span. The transgene is not constitutively expressed at the target sites, but is expressed in association with the proliferative cell component observed in early papillomas (28). From these limited data, the TG.AC mouse model appears to identify genotoxic and nongenotoxic carcinogens and to discriminate a high proportion of carcinogens and noncarcinogens.

Dose and Dose-Rate Studies
Another potential advantage of the transgenic models is their capability of providing dosimetric data. A major controversy in toxicology is the nature of the dose response derived from rodent bioassays. Due to the variable potency and toxicity of chemicals and the variable and often high rate of site-specific sporadic tumors (50), few bioassays produce data that can be appropriately evaluated at relatively low doses. For example, benzene induced a broad spectrum of tumors in a relatively high proportion of mice in the rodent bioassay (33,35). When all significant sites of tumorigenesis were combined, even the low dose (20 mg/kg body weight) induced tumors in approximately 60% of male and female B6C3F1 mice. As shown in Figure 1, these results suggest that the response at all doses could represent the upper portion of a dose−response curve, and the pattern was similar for papillomas induced by benzene in TG.AC mice. However, the prospects that lower doses in B6C3F1 mice could produce data sufficient to allow the true shape of the dose response to be deter-

Table 4. Prospective evaluation of NTP chemicals for activity in TG.AC transgenic mice

| Chemical                  | NTP skin paint bioassay (maximum dose)#   | TG.AC skin paint |
|---------------------------|------------------------------------------|-----------------|
|                           | SALb                                      | B6C3F, mouse    |
| Diethanolamine            | −                                        | 160             |
| Lauric acid diethanolamine| 100                                       | 200             |
| Triethanolamine           | −                                        | 2000            |
| Methyl ethyl ketone peroxide| 3.6 mg/rat                              | 3.6 mg/mouse    |

#Results pending completion of 2-year bioassay study and peer review. Doses in mg/kg, except for methyl ethyl ketone peroxide, which was administered five times/week.

bSAL, Salmonella mutagenicity results provided by E. Zeiger, National Toxicology Program.

cLED, lowest effective dose that induced papillomas at the site of application or highest "no effect" dose administered.

dTTP, time (weeks) to first observation of a skin papilloma in any mouse of that dose group.

+At 20 weeks.
mined, even by extrapolation, is quite unlikely because of the high proportion of control mice that developed tumors at many of the same sites as the benzene-exposed mice by the end of the 104 week study. Because the frequency of spontaneous tumors increases with time, the distinction between induced and spontaneous tumors becomes more difficult to discern.

The principal attribute of the two transgenic mouse lines described here is that induced tumors develop within a time frame in which the spontaneous tumor incidence is effectively zero, thus reducing the confounding effects observed in an aging population.

The benzene study was repeated in TG.AC mice using doses as low as 75 µl/week/mouse. The high dose portion of the dose–response pattern, similar to that for the bioassay, was observed again, but it is noteworthy that the response curve also extended into the low-dose range (Fig. 2). These results suggest that it will be possible to compare the dose response of various chemicals, including known human carcinogens, and to study dose-rate effects of chemicals, including time-to-tumor analysis, which are critical (57). Further, in the case of the p53 +/- mouse line, it is possible to design molecular dosimetry studies because mutational spectra of the single functional wild-type p53 alleles may be determined (12).

Identification of Presumptive Carcinogens

Although large numbers of new chemicals are continually being synthesized for potential use, they need not all be subjected to a complete rodent bioassay. Over the past three decades, information has been developed in the characterization of the biological effects of most major structural classes of chemicals. It is possible to use available data to predict the relative toxicity or carcinogenicity of many untested chemicals.

The most predictive mechanistic determinant for chemical carcinogenicity is mutagenic potential (52). It has been recognized for many years that chemicals that demonstrate mutagenicity in vitro are also carcinogenic to rodents (34,53). An extensive evaluation of the association between mutagenicity and carcinogenicity of 114 chemicals that were subjected to rodent carcinogenicity bioassays showed that approximately 70% of the chemicals that were mutagenic in the Ames Salmonella mutagenicity assay were rodent carcinogens (53,54). Based on this relationship, mutagenicity, as well as other information relating to chemical structure and toxicity, were used to predict carcinogenic potential in two rodent species (55). Forty-four chemicals that were undergoing bioassays, for which the results were not yet available, were the focus of an effort to demonstrate that chemical structure, mutagenicity, and data from subchronic toxicity studies were sufficient to prospectively identify many potential carcinogens with a high degree of certainty. The principal results of the effort showed that a chemical of unknown carcinogenicity could be predicted to be in one of three possible categories: probably carcinogenic, probably noncarcinogenic, or of uncertain activity (56). All of the chemicals that induced trans-species carcinogenic effects were correctly predicted. Trans-rodent carcinogens demonstrate similar properties (mutagenicity and toxicity) of proven human carcinogens and represent a more proximate risk to human health than species-restricted carcinogens (17). The overall specific predictions were 75% concordant with the results of the rodent bioassays; the least success occurred with the chemicals that produced uncertain effects in the bioassay (i.e., equivocal evidence of carcinogenicity or single-site, sex-specific, and species-specific effects).

The overall results of this study demonstrated that the rat and human assays were superior to the mouse bioassay in their ability to predict carcinogenic potential but did not achieve the same precision as the rodent bioassays. It was not possible to predict the carcinogenic potential of chemicals that were not mutagenic or carcinogenic in the mouse bioassay. However, the overall results of this study demonstrated that the rat and human assays were superior to the mouse bioassay in their ability to predict carcinogenic potential but did not achieve the same precision as the rodent bioassays. It was not possible to predict the carcinogenic potential of chemicals that were not mutagenic or carcinogenic in the mouse bioassay.

Figure 1. Comparison of 2-year bioassay and 20-week dose–response studies for cumulative tumor burden in benzene-exposed mice. The graph shows tumors at all sites identified as resulting from chemical exposure in B6CF1 mice in the 2-year bioassay and papilloma incidence in TG.AC mice exposed to benzene. The doses represent gavage exposure (mg/kg) in B6CF1, mice or skin paint exposure (µl) in TG.AC mice for 20 weeks.

Figure 2. Benzene dose response. Groups of 10 TG.AC female mice exposed for 20 weeks to benzene via skin paint. Papillomas were counted weekly and the maximum countable limit was 30 papillomas/mouse.
able to reduce the uncertainty about the carcinogenic potential of nonmutagenic chemicals and reduce the number of false positives, i.e., in vitro mutagens that are incorrectly predicted to be carcinogens. The major impediments to successful predictions are the relatively weak chemicals that induce single-site, species-specific, or equivocal carcinogenic effects, which are generally the consequence of the use of inbred rodent strains (17).

**Strategy for the Use of Transgenic Mice**

Based on the responses of the p53 +/- and TG.AC mouse lines to specific chemical treatment regimens discussed here, we propose that these lines can be used selectively in conjunction with the 2-year bioassay to confirm predictions based on the aggregate of structure–activity relationships, in vitro genotoxicity, and systemic toxicity, ultimately reducing the number of chemicals (and mice) that must be subjected to 2-year rodent bioassays. The strategy is depicted in the decision-tree scheme outlined in Figure 3; a key element is the use of p53 +/- mice for the verification of predicted mutagenic carcinogens.

Few studies have specifically investigated the effects of environmental chemicals on the p53 tumor-suppressor gene in wild-type or genetically altered mice (16,29,61,62), and p53 mutations in experimentally induced rodent tumors have not been observed as frequently as in human tumors. This difference may be due to the methods of observation, tumor type, the degree of progression of the neoplasia, etc. Hemizygous p53 mice that lose the function of the remaining wild-type allele may allow a decreased latency through an increased rate of progression to malignancy without altering tumor multiplicity (32) or the tissue specificity of a chemical carcinogen. Trans-species carcinogens may involve chemical interaction with highly conserved genes involved in regulation of cell proliferation or differentiation (7,17,63,64). Interaction of mutagenic carcinogens with highly conserved genes in the genome may explain, at least partly, why hemizygous p53 mice exhibit a shortened latency while retaining tissue specificity. In addition, genomic stability may decrease with loss of both wild-type p53 alleles (7,63,64), possibly because of the loss of the ability to arrest the cell cycle to allow sufficient time for DNA repair and/or to induce apoptosis (8,65,66).

Although the number of chemicals evaluated in the p53 +/- line are limited, the results to date support further evaluation of the proposal of using this line to identify or verify mutagenic carcinogens. Because of the relatively rapid induction of tumors and the high proportion of the mice responding, we propose that chemicals with properties that predict mutagenic- and carcinogenicity be tested in this model before conducting a 2-year bioassay. Data relevant to potential target tissues and dose–response patterns can be obtained from a transgenic mouse study that can be conducted within 6-month exposure periods. The uniform sensitivity of the animals allows fewer mice to be used per dose group, and the absence of spontaneous tumors allows for a more categorical determination of carcinogenic potential at lower doses. If the toxicity of the chemical is not known from other studies, preliminary estimates can be made efficiently using wild-type nontransgenic siblings with the same genetic background. Application of the maximum tolerated dose (67) can be obviated because an extended dose–response characterization is possible. With a reduction in duration and overall cost, the transgenic mouse experiment may be repeated for verification or to determine additional doses required for dose–response characterization, which is rarely done with the 2-year bioassays.

The second component of the strategy is the use of the TG.AC mouse line. Although mutagenicity is the mechanistic determinant most clearly defined for a presumptive carcinogen, it is possible that some chemicals may not induce tumors through mutations but through epigenetic pathways. Therefore, chemicals that are not carcinogenic in the p53 +/- mouse line might still possess potential for nonmutagenic carcinogenicity. Since the TG.AC mouse line has shown sensitivity to nonmutagenic carcinogens and tumor promoters, a chemical acting via a nonmutagenic mechanism may be detected in these transgenic mice. Further, chemicals that were not tumorigenic in either line may be considered to be presumptive noncarcinogens or in the conventional category of "generally recognized as safe." A crucial question is whether 2-year or lifetime rodent bioassays will still be required to declare a chemical noncarcinogenic. Chemicals that are negative in both of these transgenic mouse lines may still require a complete rodent bioassay because the preliminary data indicate that neither transgenic model will detect all carcinogenic activity in rodents. In particular, nonmutagens that induce single-site carcinogenic effects would not be expected to be effective in the transgenic models. Such highly specific carcinogenic effects are unlikely to be predictable by any foreseeable strategy because the actions of such chemicals are the product of highly specific genetic effects or the interaction with specific gene products (17). It is plausible that the study of such chemicals in other transgenic models may provide insights into their modes of action, but the transgenic mouse lines proposed in this strategy will most likely fail to identify many site-specific carcinogens. The preliminary results with ethyl acrylate support this interpretation. Rodents are used as surrogates for humans, and it is essential to note that the aim is to identify carcinogens that may be predicted to be carcinogenic for humans. Several single-site responses in rodents now appear to be very unlikely to predict similar risk for humans (61,68).

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**Figure 3.** Decision tree of strategy for use of transgenic mouse lines to complement the rodent bioassay. Toxicity, in general, as indicated by the empirically determined maximum tolerated dose, increases for mutagenic carcinogens compared to nonmutagenic carcinogens (17).
Validation of this strategy can be accomplished by two approaches: one is retrospective, the other prospective. The retrospective approach, taken in the validation of in vitro or short-term test methods, has typically involved testing well-defined carcinogens. This approach led to overconfidence in the value of many short-term assays which, when they were more fully characterized for response to noncarcinogens, were found to lack sufficient specificity to be useful (52,53). While the selective testing of known carcinogens and noncarcinogens in the TG.AC and p53 +/- mouse lines is necessary, there is also the opportunity for a more direct and objective validation. This prospective approach involves testing chemicals that are currently undergoing long-term bioassays. By providing results, both qualitative and quantitative, before the outcome of the 2-year bioassay is known, even implicit investigator bias (such as in the selection of chemicals) is eliminated. We have implemented this approach with the TG.AC mouse line by prospectively testing four chemicals undergoing 2-year rodent bioassays (Table 4). It is important to emphasize that the hypothesis being tested by this type of validation is that these transgenic models will preferentially identify trans-species carcinogens and noncarcinogens. The TG.AC mouse line is responsive to a number of tumor promoters, but this is not a useful distinction in the context of the rodent bioassay. The principle of trans-species effects (17) should apply, since chemicals that can promote only in a single-species or strain are subject to the action of gene(s) or chemical specificity that limit their carcinogenic (or promotion) potential (67). Thus, while the TG.AC mouse line may identify chemicals with the capacity to promote tumor development in a mouse two-stage assay, its use is aimed principally at the detection of nonmutagenic carcinogens, for the verification of mutagenic carcinogens positive in the p53 +/- mouse line and for dose–response data.

This strategy can also be applied to problems associated with the development of new products for commercial or medicinal uses. Widespread application of the Salmonella mutagen assay has resulted in a marked decrease in the development of new products or chemicals that are positive in that assay. However, approximately 30% of such chemicals have not been carcinogenic when tested in 2-year bioassays. Because most mutagens are carcinogenic and because of the economic and regulatory risks (i.e., what information is needed to override a positive result in a Salmonella mutagenesis assay), the development of such products may be abandoned early. The use of the proposed predictive strategy outlined here can result in the identification of such mutagenic noncarcinogens and provide a scientific and economic justification for further development or for a more informed decision to conduct a costly bioassay to verify the lack of carcinogenic potential.

This strategy is not presented as a panacea to all of the public health issues associated with environmental carcinogens and risk assessment. It represents the use of new experimental tools to search for answers to such problems, and it can be objectively challenged and validated using ongoing and future bioassays. The strategy provides a mechanistic basis for judging the carcinogenicity of chemicals and for better defining the specific chemical–gene interactions that induce and promote the development of cancers.

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