

Genetically Altered Mouse Models for Identifying Carcinogens

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Introduction

The evolving approach to the detection of environmental carcinogens

The empirical approach to the prospective identification of environmental carcinogens has been evolving for more than four decades. During most of this period, the experimental basis has remained two-year to lifetime bioassays for cancer, usually conducted in rodents, which involve extensive post-mortem microscopic examination of tissues. While experiments of this type are widely recognized as a logical approach for collecting relevant information about the carcinogenic potential of diverse classes of chemicals, they have both economic and utilitarian liabilities. For example, while the part of the bioassay *in vivo* may be of a specified duration, extensive preparatory experimentation, detailed histopathological assessment and lengthy debates about interpretation during the post-mortem phase may increase the overall assessment period from five to seven years. The demand for human, physical and financial resources severely limits the numbers of chemicals that can be assessed per unit time (Yamamoto *et al.*, 1996). The US National Toxicology Program, which has a large but finite budget, is obliged to exercise great selectivity in choosing candidate chemicals for investigation. Approaches that allow conservation of human, economic and/or physical resources will always be met with enthusiasm and anticipation.

From a utilitarian viewpoint, the use of results of bioassays is limited, as they must be used in an *extrapolative* manner to merely *predict* whether a carcinogenic risk is likely to attend human exposure to the chemical. Chemicals found to cause cancer in multiple species (trans-species carcinogens) are generally anticipated to pose a greater cancer risk to humans; whereas, at the opposite end of the spectrum, agents that do not increase tumour inci-

dence may be regarded as posing no risk, unless information becomes available that rodent models are not applicable for a particular chemical or class of chemicals. The results of about one-half of all cancer bioassays fall between these two extremes (Ashby & Tennant, 1991), many being considered equivocal or uninterpretable. For instance, treatment-associated excesses of tumour incidence may occur in a single species or even in animals of one sex of that species; furthermore, tumours may appear at a high, spontaneous (background) rate in a particular strain, species or sex. The predictive validity of equivocal results is always arguable and may indicate that additional experiments are required, further delaying safety assessment, regulatory decisions and, ultimately, the protection of human health.

The search for methods to support long-term cancer bioassays

Longstanding recognition of the difficulties involved in applying the results of cancer bioassays to establish and support regulatory decisions fuelled a search for other experimental methods that could be used as adjuncts in prospective carcinogen identification. Thus, knowledge about structure-activity relationships (Ashby & Tennant, 1991) and genotoxicity and mutagenic potential *in vitro* (Zeiger *et al.*, 1990) can assist in the interpretation of the results of bioassays. The currently available adjunct procedures cannot be used alone to identify carcinogens accurately and consistently; in the absence of information from surrogate animal models, predictions based on chemical characteristics or toxicological potential do not correlate well with the results of cancer bioassays. There is a greater likelihood that a genotoxic chemical will cause increased incidences of tumours in rodents than would a chemical that cannot interact with DNA, but even

this association is not absolute (Ashby & Tennant, 1991). Furthermore, some agents with no demonstrable genotoxic potential have been found to increase tumour incidences in rodents, e.g. trichloroethylene (US National Toxicology Program, 1976, 1990a), pentachlorophenol (US National Toxicology Program, 1989a), Mirex (US National Toxicology Program, 1990b), and 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (US National Toxicology Program, 1982a,b).

Although efforts to devise valid short-term, predictive tests for prospective identification of environmental carcinogens have yet to achieve the ultimate goal, steady progress has enhanced our understanding of chemically induced neoplasia and increased our knowledge about the role played by genetics. In fact, it now seems highly likely that most of the uncertainties and interpretative controversies involved in extrapolating from the results of bioassays in rodents to human health hazards stem from specific genetic differences between species (Tennant *et al.*, 1996).

In most studies of chemical carcinogenesis, highly inbred rodent strains are used in order to reduce experimental variability and to enhance the interpretability of the results. Allelic enrichment, the intended outcome of generations of brother-to-sister matings, has, however, resulted in one of the thorniest problems in interpreting the results of cancer bioassays—treatment-associated increases in tumour incidence that are more species-specific than reflecting a broad biological response to exposure to the chemical. Inevitably, in-breeding-associated allelic enrichment reduces the polymorphisms and genetic variability that characterize outbred populations, such as humans and feral rodents. Many polymorphic genes, acting through the specific proteins they encode, quantitatively and qualitatively modulate the complex interactions between the organism and the chemical environment within which it functions. Reduction in polymorphism limits the variety of possible responses of the organism, which in turn reduces experimental variability; however, at the same time, increasing degrees of allelic enrichment make the organism less and less representative of the entire population.

In-breeding affects the interpretation of the results of carcinogenesis bioassays in two main ways.

First, inbred rodent strains often have characteristic patterns of *spontaneous* tumour incidence. Reference to the historical tumour rates in a rodent strain shows that the finite rates vary quantitatively, but the tumours remain qualitatively constant generation after generation (Haseman *et al.*, 1985). Patterns of background tumour incidence are to be expected if the tumours are determined by heritable factors (Tennant *et al.*, 1996). Thus, the propensity of these inbred strains to develop neoplastic disease is usually of familial origin. A second confounding consequence of allelic enrichment is strain-specific response to chemicals. Dichotomous pharmacological and toxicological responses have long been recognized and have been attributed to genetic differences between species, strains and sexes. In a series of 296 bioassays in B6C3F₁ mice and Fischer 344/N rats in which the chemical was adequately tested in animals of each sex, 26% (76) were carcinogenic in one species but not in the other, and 43% (33) of these species-specific responses occurred in a single sex. Thus, when tumour incidences in treated and untreated groups of rodents are compared, it must be remembered that the tumour incidence is often imposed over a background of strain-specific, 'normally' occurring, spontaneous neoplasms.

Thus, interpretation and use of the results of bioassays for carcinogenesis in rodents requires the incorporation of a variety of factors, not the least of which is the genetic make-up of the test species. As early as 1985, the US Office of Science and Technology Policy counselled that the spontaneous nature of a neoplasm should be considered in interpreting treatment-associated increases in tumour incidence. Thus, for example, less predictive weight might be given to increased incidences of hepatocellular carcinomas in B6C3F₁ mice or of monoclonal-cell leukaemia in Fischer 344 rats than to neoplasms that occur at lower background frequencies in these species.

Interpreting the results of bioassays

Allelic enrichment and its attendant strain-specific influences on pharmacotoxic responsiveness and spontaneous tumour incidence are major confounding factors in the interpretation and use of the results of carcinogenesis bioassays. Extrapolation

from observations of tumour excesses in an inbred strain to a rational prediction of what is likely to happen when humans are exposed to the same chemical is difficult and probably inaccurate. The guidance of the expert committee of the US Office of Science and Technology Policy (1985) notwithstanding, few researchers can be completely comfortable in summarily dismissing statistically significant, treatment-associated increases in the incidence of neoplasms, even if they are common lesions in the strain or species in question.

One approach to the extrapolative dilemma posed by these bioassays is to stratify the experimental results according to the strength of evidence for carcinogenesis. Evidence of a chemically induced increase in tumour incidence in multiple species (i.e. trans-species carcinogenesis) is generally considered to constitute stronger evidence of carcinogenic potential for humans than responses in a single species or sex (Tennant, 1993).

Trans-species carcinogenesis is considered to provide evidence that a chemical can interact with proto-oncogenes and/or tumour suppressor genes. These two classes of gene, which have been identified both in neoplasms induced experimentally in animals and in human neoplasms of unknown aetiology, are known to have key roles in the induction and progression of tumours (Bishop, 1991; Weinberg, 1994). These genes have been highly conserved throughout the evolutionary process and are thus likely to be similar in (outbred) humans and in highly inbred rodent species. If chemicals that are carcinogenic in several species are more likely to mediate their effects through interactions with highly conserved proto-oncogenes and/or tumour suppressor genes than through interaction with a strain-specific locus, the importance of trans-species carcinogenesis is logical. We speculate that proto-oncogenes and tumour suppressor genes present in rodents are likely to be present in humans; however, the strain-specific loci or combinations of strain-specific loci, including unique modifier genes, that mediate susceptibility to cancers may not exist in humans or be overrepresented or contain unique polymorphisms that increase susceptibility in highly inbred rodent strains.

Oncogenes and transgenic models

Given the confounding effects of strain-specific carcinogenic responses on meaningful interpretation of the results of bioassays in rodents, the emergence of techniques for transgenesis (Gordon *et al.*, 1980) represented a significant step in the effort to differentiate carcinogenic chemicals from noncarcinogens. It is now possible to produce animals with modified genes that direct the response to carcinogenic chemicals in predictable ways. By inserting an inducible oncogene into the germ line, a strain of rodents can be created which has multiple copies of the gene in all their cells, thereby increasing the number of tissue-specific target cells for chemical-genome interaction. Conversely, deletion of tumour suppressor genes which protect against tumour development results in rodents that are specifically sensitive to genotoxic chemicals. With advances in molecular biology, applications have become available to improve the precision with which environmental carcinogens can be identified. Scientists at the National Institute of Environmental Health Sciences (NIEHS) in the USA and at the Central Institute for Experimental Animals in Japan are exploring use of transgenic mouse models to improve the identification of carcinogenic chemicals. On the basis of our own findings and those from other laboratories, we proposed a strategy (Tennant *et al.*, 1995; Tennant, 1997). Below, we describe the models, present our operational testing protocols and summarize the results obtained to date. Further information on our work in this field can be found at: http://ntp-server.niehs.nih.gov/Main_Pages/trangen/TransgenicPage.html.

The proposed testing and screening procedures must be shown to be reproducible, both within and between laboratories. We welcome collaboration with other laboratories to evaluate these and other models by systematic study of the responses of transgenic mice to chemicals with well-defined carcinogenic potential, publication of their results, whether confirmatory or discordant, and the sharing of applicable refinements to the method.

Useful transgenic mouse models

The transgenic mouse lines for the research initiative of the NIEHS were chosen with the objective of developing short-term assays *in vivo* that could

be used to differentiate carcinogenic from non-carcinogenic chemicals and mutagenic from non-mutagenic carcinogens. Such assays should allow identification of the majority of (ideally, all) trans-species carcinogens but not substances that do not induce tumours in conventional cancer bioassays.

Mouse lines with well-defined genetic alterations that result in overexpression or inactivation of a gene intrinsic to carcinogenesis, but that are insufficient alone for neoplastic conversion, appear to offer susceptible targets for carcinogenic chemicals (Stewart *et al.*, 1984; Donehower *et al.*, 1992; Tennant *et al.*, 1993; Mirsalis *et al.*, 1994; Morrison & Ashby, 1994). Chemically mediated events likely to meet the necessary criteria include mutation-mediated activation of the c-Ha-ras proto-oncogene, which alters signal transduction and growth control (Boguski & McCormick, 1993), and inactivation of the *p53* tumour-suppressor gene, which is critical to control of the cell cycle and DNA repair (Hartwell, 1992; Kastan *et al.*, 1992; Zambetti & Levine, 1993). These tumour genes have often been reported to be mutated and/or amplified (c-Ha-ras) or mutated and/or lost (*p53*) in both human and rodent tumours (Vogelstein, 1990; Hollstein *et al.*, 1991; Caamano *et al.*, 1992; Harris, 1993).

Few of the many transgenic and knockout models that have been developed have been assessed for use in identifying potential carcinogens (Tennant *et al.*, 1993; Storer *et al.*, 1995; de Vries *et al.*, 1995; Yamamoto *et al.*, 1996; Mitsumori *et al.*, 1997; Urano *et al.*, 1998; Yamamoto *et al.*, 1998). Another model for carcinogen identification that has not yet been extensively investigated is the XPA^{-/-} deficient mouse. Mice with defects in nucleotide excision repair due to inactivation of the xeroderma pigmentosum complementation group A-correcting (XPA) gene develop normally, but are highly sensitive to induction of skin tumours by ultraviolet B radiation (Nakane *et al.*, 1995; de Vries *et al.*, 1995; Berg *et al.*, 1997) and 7,12-dimethylbenz[*a*]anthracene (DMBA; de Vries *et al.*, 1997) and to benzo[*a*]pyrene-induced lymphomas. Tumours appeared earlier and at a higher incidence in XPA nullizygous^{-/-} than in treated co-isogenic wild-type and XPA^{-/-} heterozygous mice, indicating that XPA-deficient mice preferentially respond to muta-

genic carcinogens by rapid induction of tumours. Further investigation will be required to determine the potential of this model. We consider that the three models for which minimal data are available which are of the greatest potential usefulness are the Tg.AC, heterozygous (+/-) *p53*-deficient and TgHras2 lines.

Tg.AC (ζ-globin:v-Ha-ras) mice: Most of our early studies on the responses of transgenic mouse models to chemical carcinogens were conducted with the Tg.AC mouse line. These animals carry a v-Ha-ras oncogene fused to the promoter of the ζ-globin gene (Leder *et al.*, 1990). The v-Ha-ras transgene has point mutations in 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. The Tg.AC model is thus analogous to the classical, widely studied model of two-stage carcinogenicity in mouse skin (Boutwell, 1964).

Skin carcinogenesis is conventionally viewed as being a multistage process involving *initiation*, a mutagenic event, *promotion*, a proliferative process, and *progression to malignancy*, a process that most likely involves additional genetic and epigenetic modifications. Experimental initiation, usually accomplished by application of a single subcarcinogenic dose of a complete carcinogen, induces site-specific (or codon-specific) mutations that cause some percentage of cells to become latent neoplastic cells. In the presence of cell proliferative factors, the latent tumour cells may enter into the neoplastic process. This model has been used extensively to detect and differentiate between initiating agents, complete carcinogens and tumour promoting agents. The presence of the v-Ha-ras transgene in Tg.AC mice obviates the obligatory exposure to initiating agents that was necessary in the earlier models of skin carcinogenesis.

The transgene is not expressed constitutively in the skin of Tg.AC mice, so that untreated skin is indistinguishable from the skin of the wild-type FVB/N parent strain (Leder *et al.*, 1990; Hansen & Tennant, 1994). Repetitive dermal applications of recognized mouse skin tumour promoters to otherwise untreated skin of Tg.AC mice result in prompt, dose-related development of benign squamous-cell

papillomas which can progress to malignancy (Leder *et al.*, 1990; Spalding *et al.*, 1993). The uninitiated dorsal skin of FVB/N mice, however, remains unresponsive to repeated applications of tumour promoting agents (Spalding *et al.*, 1993). The Tg.AC line is useful for differentiating carcinogens from non-carcinogens but it cannot be used to distinguish genotoxic complete carcinogens from agents that have only tumour promoting capability (Tennant *et al.*, 1995, 1996). Thus, another model is required to further classify the carcinogenic potential of a test agent.

Heterozygous $p53^{+/-}$ deficient mice: Mice that are heterozygous for the $p53$ tumour suppressor gene are viable and have a low background incidence of tumours during the initial 12 months of life. Nullizygous (homozygous for the null allele) mice ($p53^{-/-}$), however, generally develop a higher incidence of spontaneous tumours within the first three to six months of life. The qualitative nature of the tumours seems to be determined by the genetic background of the strain (Harvey *et al.*, 1993a,b). Mice that are heterozygous for the suppressor gene are at increased risk for tumour development, since a mutational event may result in loss of the wild-type $p53$ allele, thereby removing proliferative restraint. Mice with a single $p53$ allele have been considered to be analogous to humans at risk for heritable forms of cancer such as the Li-Fraumeni syndrome (Malkin *et al.*, 1990). Initial investigations with heterozygous $p53$ -deficient mice focused on potent carcinogens. *N*-Nitrosodimethylamine administered in the drinking-water rapidly induced haemangiosarcomas of the liver in 14 of 17 mice (Harvey *et al.*, 1993a). Skin initiation with 25 μ g DMBA and promotion with 10^{-4} mol/litre 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced both squamous-cell carcinomas and spindle-cell carcinomas in 28/28 mice (Kemp *et al.*, 1993). The number of tumours produced was not significantly different from that in the co-isogenic wild-type control, but the latency for tumour induction was decreased and the degree of malignant progression was increased. Loss of heterozygosity at the wild-type allele locus had occurred in 14/22 tumours examined. Radiation (^{60}Co ; 4 Gy \times 1) was also a potent inducer of malignant lymphomas, sarcomas

and osteosarcomas in heterozygous $p53$ -deficient mice, and the incidence of loss of heterozygosity in the tumours was high (Kemp *et al.*, 1994). In contrast, DMBA (1 mg/week \times 6 weeks) induced mammary gland tumours, malignant lymphomas and ovarian and gastrointestinal adenocarcinomas at similar rates in homozygous $p53$ wild-type and heterozygous $p53$ -deficient mice with no loss of heterozygosity (Jerry *et al.*, 1994). Overall, these studies demonstrate that potent carcinogens can decrease the latency of cancer induction and the induction of loss of heterozygosity in heterozygous $p53$ -deficient mice.

TgHras2 mice: Transgenic mice that carry the human protooncogene *c-Ha-ras*, designated CB6F1-TgHras2, have the endogenous promoter enhancer for the *c-Ha-ras* gene. This line, produced by pronuclear injection, has five or six tandem copies of the gene. The transgene is constitutively overexpressed in normal tissues of the mouse and in spontaneous tumours that develop at sites such as the skin (papillomas), spleen (haemangiosarcomas), lymphatic system (lymphomas), lung (adenocarcinomas) and Harderian gland (adenocarcinomas) as the animals age. Thus far, mutations of the transgene have been detected only in tumour cells, although all carcinogen-induced tumours do not contain mutations; however, neither tumours nor preneoplastic lesions are observed in animals up to six months of age. The animals used in studies of carcinogenicity are the F_1 progeny of transgenic male C57BL/6J and female BALB/c ByJ \times C57BL/6J mice (Saitoh *et al.*, 1990; Yamamoto *et al.*, 1996; Mitsumori *et al.*, 1997; Yamamoto *et al.*, 1997; Mitsumori *et al.*, 1998; Urano *et al.*, 1998; Yamamoto *et al.*, 1998). This line has been most extensively evaluated for the effect of carcinogens by scientists at the Central Institute for Experimental Animals in Kawasaki, Japan (Urano *et al.*, 1998; Yamamoto *et al.*, 1998).

Experimental findings

Experience with dermal applications to Tg.AC mice

The untreated or intact skin of Tg.AC mice appears normal in all respects when compared with the skin

of the wild-type FVB/N parent strain. Spontaneous papillomas in the Tg.AC strain are rare; however, skin lesions, such as full-skin-thickness surgical wounding (Cannon *et al.*, 1997) and bite-inflicted wounds (Leder *et al.*, 1990), are associated with the development of squamous papillomas. Dermal application of classical tumour promoting agents, such as TPA, benzoyl peroxide and 2-butanol peroxide, causes prompt epithelial proliferation and papilloma formation in Tg.AC but not in FVB/N parent mice (Spalding *et al.*, 1993). Table 1 shows the relative responses of Tg.AC and non-carrier FVB/N mice to repeated dermal applications of TPA, the prototypical tumour promoter. Mice homozygous for the *v-Ha-ras* transgene had a robust tumorigenic response to twice weekly applications of 5 µg TPA for five weeks (total dose, 50 µg), but the FVB/N mice remained papilloma-free for up to

20 weeks, during which time they received a total dose of 310 µg TPA.

Large tumour responses like those observed in Tg.AC mice treated with 5 µg TPA are difficult to quantify, as, when the tumour burden exceeds 30-40 per mouse, the individual tumours frequently coalesce and continue to grow as a single mass. Furthermore, advanced keratinization of lesions results in sloughing or removal by biting or scratching. These factors, rather than tumour regression, may account for the apparent decrease in papilloma incidence that occurred at 16 and 20 weeks in some groups. We circumvent this problem by establishing a maximal limit of 30 tumours per animal, whether or not the actual papilloma count exceeds that number.

Another approach to avoiding large tumour responses is to use more moderate doses of TPA

Table 1. Skin tumour responses of groups of male and female homozygous Tg.AC and male and female non-carrier FVB/N mice to repeated doses of 12-O-tetradecanoylphorbol 13-acetate (TPA)

Dose of TPA (µg) ^a	No. of mice	Sex	Length of treatment (weeks)	Total dose (µg)	Time to first tumour (weeks)	Mean no. of papillomas per mouse at week no.				
						4	8	11	16	20
Tg.AC										
5.0	4	F	5	50	4	32	48	53	14	16
5.0	4	M	5	50	3	16	31	40	32	26
2.5	4	F	1.5	7.5	5	1	1	1	1	1
2.5	4	F	5	25	5	< 1	6	15	13	13
2.5	3	F	20	100	3	30	–	55 ^b	55 ^c	55
2.5	3	M	20	100	3	34	–	67 ^b	65 ^c	60
FVB/N										
10 ^d	4	F	20	310	–	0	0	0	0	0
10 ^d	4	M	20	310	–	0	0	0	0	0

From Spalding *et al.* (1993)

F, female; M, male

^a Dissolved in acetone and administered in a volume of 200 µl twice a week

^b Tumour incidence at week 10

^c Tumour incidence at week 15

^d Administered at 10 µg for 11 weeks then 5 µg for 9 weeks

for the positive controls. Table 1 also shows a dose-response relationship between the dose of TPA administered and the tumour incidence in Tg.AC mice. A total dose of 7.5 µg administered during the initial 1.5 weeks of the experiment failed to elicit a response, while 25 µg administered over the first five weeks produced unequivocal tumorigenesis that peaked at about week 11 and remained relatively constant throughout the remainder of the experiment. When 100 µg TPA were administered at a rate of 2.5 µg twice weekly for 20 weeks, the response was so large that counting tumours was again problematical. We used a dose of 1.25 µg TPA administered twice weekly for 20 weeks as the positive control dose in our early studies. Table 2 summarizes the results of four experiments in groups of 15–20 female homozygous Tg.AC mice at this dose, each of which came to the laboratory in separate shipments from the animal supplier (Taconic Laboratory Animal and Services, Germantown, NY). As shown in Table 2, twice weekly application of the lower dose of TPA (1.25 µg) resulted in a tumorigenic response that was unequivocal, but which was somewhat variable between experiments. We therefore propose use of a 2.5-µg dose as the positive control in order to obtain a reproducible response (see Box 1).

We selected acetone and ethanol as the solvents,

as application of 200 µl of these solvents in the regimens involving two to five doses per week did not elicit tumorigenic responses in Tg.AC mice (Table 3). Few Tg.AC mice exposed to either ethanol or acetone develop papillomas and, even when tumours do appear, positive and negative control groups are readily differentiated. Papillomas in negative control animals usually appear late in the study (after 15 weeks). Another differentiating factor is tumour multiplicity, since multiple tumours are rare in negative control animals.

Individual housing of Tg.AC mice is recommended because fighting with cage mates may influence animal responses to both positive and negative control treatments. The results obtained in an experiment influenced by fighting among cage mates are shown in Table 4. Papillomas developed in 57% of the Tg.AC mice treated with acetone and housed in cages where there was fighting but in only 15% of the historical controls. In addition, the times to both initial and maximal tumour yield were markedly reduced relative to historical data, and there was unequivocal tumour multiplicity, calculated on the basis of either tumour-bearing mice or animals at risk. The responsiveness to TPA was similarly affected by fighting. The time to appearance of the first TPA-associated tumour was reduced by nearly 40%, and the extent of tumour multiplicity was doubled. Confounding factors

Table 2. Skin tumour responses of groups of female homozygous Tg.AC mice to repeated doses of 12-O-tetradecanoylphorbol 13-acetate (TPA)

No. of animals per group	Total dose (µg) ^a	Time to first tumour (weeks)	Mean no. of papillomas per mouse at week no. (± SD)				
			5	9	11	16	20
20	50	8	0 ± 0.0	0.2 ± 0.5	0.4 ± 0.6	3.0 ± 3.8	9.8 ± 7.4
15	50	6	0 ± 0.0	0.3 ± 0.8	0.5 ± 0.9	5.1 ± 5.1	11.4 ± 8.6
20	50	5	0.1 ± 0.2	7.7 ± 5.7	9.1 ± 5.6	21.7 ± 11.9	27.4 ± 6.7
15	50	6	0 ± 0.0	0 ± 0.0	0.1 ± 0.4	3.1 ± 3.5	8.3 ± 7.6
		Mean	0.025 ± 0.05	2.1 ± 3.8	2.5 ± 4.4	3.9 ± 3.7	14.2 ± 8.9

From Spalding *et al.* (1998)

^a 1.25 µg TPA was dissolved in acetone and administered in a volume of 200 µl twice a week for 20 weeks.

Table 3. Incidences of skin tumours in female homozygous Tg.AC mice treated with 200 μ l acetone or ethanol 2–5 times per week for 20 weeks

Replicate	Animals with tumours	Time to first tumour (days) (mean \pm SD)	No. of tumours per affected animal (mean \pm SD)	No. of tumours per animal at risk ^a (mean \pm SD)	Time to maximal tumour yield (days) (mean \pm SD)
Ethanol					
1	1/19 (5.3%)	133 \pm 0.0	1.0 \pm 0.0	0.05 \pm 0.2	133 \pm 0.0
2	2/15 (13%)	91 \pm 59.4	1.0 \pm 0.0	0.1 \pm 0.4	91 \pm 59.4
Mean	9.2% \pm 5.4	112 \pm 29.7	1.0 \pm 0.0	0.08 \pm 0.04	112 \pm 29.7
Acetone					
1	4/14 (29%)	92.8 \pm 14.4	1.0 \pm 0.0	0.3 \pm 0.5	92.8 \pm 14.4
2	0/19	> 140	0	0	> 140
Mean	15% \pm 20	> 116.4 \pm 33.4	1.0 \pm 0.0	0.15 \pm 0.2	> 116.4 \pm 33.4

From Spalding *et al.* (submitted for publication)

^a Animals were considered to be at risk after 10 weeks of dosing.

Table 4. Effect of animal fighting on incidences of skin tumours in female homozygous Tg.AC mice treated with acetone or 12-O-tetradecanoylphorbol 13-acetate (TPA) for 20 weeks

Treatment	Animals with tumours	Time to first tumour (days) (mean \pm SD)	No. of tumours per affected animal (mean \pm SD)	No. of tumours per animal at risk ^a (mean \pm SD)	Time to maximal tumour yield (days) (mean \pm SD)
Acetone					
Fighting	8/14 (57%)	39.4 \pm 23	5.6 \pm 4.8	2.8 \pm 4.4	75.3 \pm 41.6
Historical data ^b	15% \pm 20	> 116.4 \pm 33.4	1.0 \pm 0.0	0.15 \pm 0.2	> 116.4 \pm 33.4
TPA					
Fighting	15/15 (100%)	44.8 \pm 17.7	30.6 \pm 5.4	30.6 \pm 5.4	74.2 \pm 19
Historical data ^b	95.2% \pm 4.1	75.9 \pm 20.9	14.5 \pm 8.1	13.6 \pm 8.5	116.7 \pm 24.7

^a Animals were considered to be at risk after 10 weeks of dosing.

^b Based upon two replicate experiments conducted on 38 acetone-treated animals and four replicate experiments on 70 TPA-treated animals.

such as these can affect the interpretation of experimental data.

An attractive feature of the dermal application protocol, in which the induction of papillomas at the site of application serves as a reporter phenotype for chemical activity, is rapid generation of dose-response curves. The data summarized in Table 5 and Figure 1 show the results of an experiment in which the known human leukaemogen, benzene, was applied to the shaven backs of groups of female homozygous Tg.AC mice. The dosing protocol was designed to elicit a range of responses, from no effect to a maximal response. Benzene was unequivocally active and induced a clear dose-related response at doses of 300–750 μl per week (Table 5). No effect was seen at doses of 75 and 150 μl per week, while the maximal response was observed at 600 μl . There were also clear dose-related decreases in tumour latency, the average time to the first tumour and time to maximum tumour yield; benzene caused a dose-related increase in tumour multiplicity, in every animal in each dose group

(Figure 1). At the two lowest doses, three animals in each group developed a single papilloma; at the two highest doses, 67% of the mice developed 30 or more papillomas each. The responses of individual mice at the two intermediate doses were more variable and approached the range of responses seen among the TPA-positive control animals. Such variability of response among individual animals is a characteristic most often associated with doses of chemicals that fall on the linear part of the dose-response curve. Although this experiment was continued to its scheduled 20-week termination, the three highest doses had produced unequivocal responses (an average of 8–17 tumours per animal) by the tenth week. Had the objective of this assay simply been to identify carcinogenic potential, it could have been terminated at week 10.

Experience with other routes of administration in Tg.AC mice

Other routes of administration of chemicals to Tg.AC mice are being investigated in order to

Table 5. Skin tumour incidences in female homozygous Tg.AC mice treated for up to 20 weeks with benzene

Treatment	No. of mice	Dose ($\mu\text{l}/\text{week}$)	Incidence of animals with tumours (%)	Mean time to first tumour (weeks)	Mean no. of tumours per animal at risk ^a	Mean time to maximal tumour response (weeks)
Acetone	19		0.0	–	–	–
Benzene ^b	13	75	23	18	0.23	17.7
	14	150	21	18	0.21	18.3
	15	300	80	12	2.7	17.8
	14	450	100	8	20.8	17.6
	15	600	100	7	31.5	13.6
	14	750	100	7	28.1	14.9
TPA ^c	15		93	12	9.2	20.0

From French *et al.* (1998a)

^a Animals were considered at risk after 10 weeks of treatment.

^b Benzene was applied three times per week diluted with acetone, so that the total volume per dose delivered was 200 μl .

^c 12-O-Tetradecanoylphorbol 13-acetate was applied at 1.25 μg twice per week for 20 weeks.

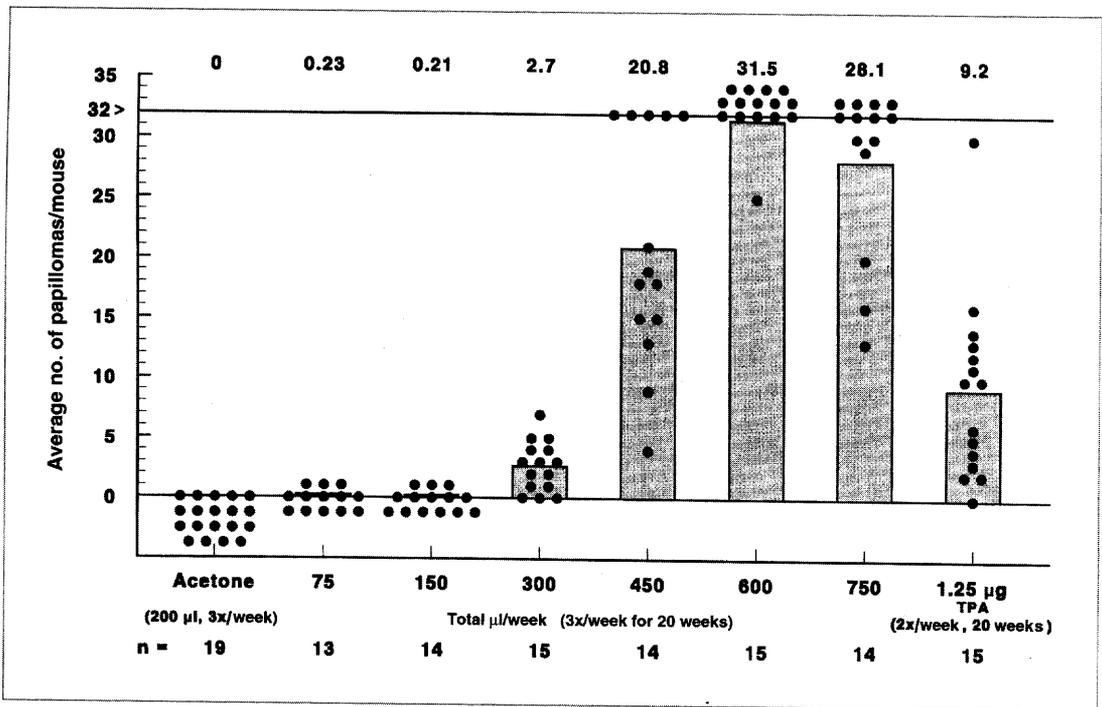


Figure 1. Incidences of papillomas induced by benzene per mouse. TPA, 12-O-tetradecanoylphorbol 13-acetate

expand use of the model. Studies were initiated in which benzene and dimethylvinyl chloride (CAS 513-37-1) were given by gavage at the same doses as those used in two-year bioassays in B6C3F₁ mice. Benzene was selected because of the wide range of primary neoplasms it induces in Fischer 344/N rats and B6C3F₁ mice (US National Toxicology Program, 1986a), and dimethylvinyl chloride was chosen because it induced a high incidence of forestomach squamous-cell carcinomas in animals of each sex of both species (US National Toxicology Program, 1986b). Both compounds were administered in corn oil by oral gavage at doses of 50, 100 or 200 mg/kg bw on five days per week to 14-week-old homozygous female Tg.AC mice. Dimethylvinyl chloride was given for 20 weeks, and the mice were held an additional 10 weeks for observation; benzene was administered for 26 weeks and the mice held for an additional four weeks. At terminal sacrifice, the incidence and multiplicity of forestomach tumours were tabulated.

At the end of the observation period, all mice were killed, and the numbers of squamous-cell papillomas of the forestomach were tabulated. No other treatment-related gross lesions were seen at necropsy. The responses to all three doses of benzene were not significantly different from the incidence observed in controls given corn oil (Table 6). Since forestomach papillomas are rarely observed in untreated Tg.AC mice, the low incidence in the controls is believed to be due to chronic irritation caused by gavage rather than to the corn oil. There was a significant, dose-related response to dimethylvinyl chloride, with an average maximum tumour multiplicity at 100 mg/kg bw. Because of the difficulty in counting the large numbers of forestomach papillomas accurately, the maximum number of tumours tabulated was set at 15. Of the mice given the two higher doses of dimethylvinyl chloride, 30/35 had 15 or more tumours.

The results of these initial studies are encouraging and provide evidence that different routes of admi-

Table 6. Incidences of forestomach papillomas in groups of 20 female homozygous Tg.AC mice induced by benzene or dimethylvinyl chloride (DMVC) administered by gavage

Treatment	Dose (mg/kg bw)	Average weight change (% control)	Incidence of forestomach papillomas ^a	Average tumour multiplicity ^b
Corn oil	5 (125 µl/dose)	26.8 g 26 weeks 27.1 g 20 weeks	15/20	2.4
Benzene (5x/week, 26 weeks)	50	100.0	10/13	3.5
	100	97.4	19/19	4.0
	200	98.9	14/18	2.7
DMVC (5x/week, 20 weeks)	50	98.9	11/14	7.6
	100	99.6	16/17	14.1
	200	95.9	16/18	12.6

^a Number of mice with tumours/number of mice in the dose group surviving 10 weeks

^b Mean number of papillomas/forestomach; the maximum number tabulated was 15 tumours per forestomach.

nistration should be explored in the Tg.AC model in order to broaden its usefulness.

Experience with p53-deficient mice

The heterozygous *p53*-deficient mouse used in rapid (26 weeks' exposure) studies for the identification of mutagenic carcinogens has one functional wild-type allele and one inactivated null allele and remains generally free of sporadic neoplastic disease during short-term studies. Inactivation of the remaining wild-type *p53* allele by mutation or loss due to exposure to a mutagenic carcinogen would be expected to give a selective growth advantage to clonally derived neoplasia and to result in shortened tumour latency.

The carcinogens benzene, *para*-cresidine, phenolphthalein and 4-vinyl-1-cyclohexene diepoxide each rapidly induced site-specific tumours (histiocytic sarcoma, urinary bladder carcinoma, thymic lymphoma and squamous-cell carcinoma of the skin, respectively) in heterozygous *p53*^{+/-} deficient C57BL/6 mice (het *p53* def) (see Table 7). Tumours at the same sites were also identified in two-year cancer bioassays in wild-type B6C3F₁ mice. The findings are consistent with the hypothesis that inac-

tivating mutations or loss of heterozygosity involving the *Trp53* locus are critical in both rodent and human cancers. Under the conditions of this experiment, chemicals that were carcinogenic only in B6C3F₁ mice in two-year studies (methylphenidate, *N*-methyl-*ortho*-acrylamide and reserpine) did not cause a response. In addition, *para*-anisidine, which is mutagenic in the gene mutation assay in *Salmonella typhimurium* but is not carcinogenic, also induced no response in these studies. Overall, these initial studies demonstrate that the heterozygous *p53*-deficient mouse model can be used to identify mutagenic carcinogens.

Loss of heterozygosity or p53 allelic loss in p53^{+/-}-deficient mice

The tumours induced in the het *p53* def mice by each carcinogen had different levels of wild-type *p53* allelic loss (phenolphthalein > benzene > *para*-cresidine) (French *et al.*, 1998b). When either a high frequency of loss (benzene) or complete loss (phenolphthalein) of the wild-type allele (Southern analysis) occurred, no inactivating mutations in the *p53* gene could be found in the wild-type allele, and mutations in the null allele were inconsequential. In

Table 7. Comparison of results of two-year bioassays in B6C3F₁ mice and 26-week studies in heterozygous p53^{+/-}-deficient C57BL/6 mice^a

Chemical	Reference	Sal ^b	B6C3F ₁		p53 ^{+/-} -deficient	
			Route	Target organ	Route	Result
Benzene	US National Toxicology Program (1986a)	-; + ^c	Gavage	Multiple site	Gavage	+ ^d
<i>para</i> -Cresidine	US National Toxicology Program (1978a)	+	Feed	Bladder, liver	Feed	+ ^d
4-Vinyl-1-cyclohexene diepoxide	US National Toxicology Program (1989a)	+	Skin painting	Skin, ovary	Skin painting	+ ^d
Phenolphthalein	US National Toxicology Program (1996)	-; + ^c	Feed	Liver, thymus, ovary	Feed	+ ^d
Methylphenidate	US National Toxicology Program (1995)	-	Feed	Liver	Feed	-
<i>N</i> -Methylolacrylamide ^e	US National Toxicology Program (1989c)	-	Gavage	Kidney, liver	Gavage	-
Reserpine	US National Toxicology Program (1980)	-	Feed	Mammary gland	Feed	-
<i>para</i> -Anisidine	US National Toxicology Program (1978b)	+	Feed	None	Feed	-

^a Two-year bioassays conducted for the US National Cancer Institute/US National Toxicology Program, which were replicated in the 26-week studies by using the same route of administration and doses

^b *Salmonella*/microsome assay for mutagenicity *in vitro*; -, negative result; +, positive result

^c Positive result in assay for micronucleus formation *in vivo*

^d Tumour incidence in treated animals significantly greater than that in concurrent controls at $p \leq 0.05$ (Fisher's exact test)

^e Synonym: *N*-(hydroxymethyl)acrylamide

final-stage transitional-cell carcinomas of the urinary bladder induced by aromatic amines, no inactivating mutations were observed in exons 5–9 of the *p53* alleles by either *p53* immunohistochemistry, cold single-stranded conformational polymorphism analysis or limited sequencing of polymerase chain reaction-amplified exons 5–8 of the *p53* alleles. Using mice of the same strain carrying the lambda *liz_x*-shuttle vector with the *lacI* neutral reporter gene, however, we could show that mutations had occurred in the bladder (Jakubczak *et al.*, 1996). There are, therefore, large differences in carcinogen- and tissue-specific *p53* allele loss, and we have established selected tissue-specific carcinogen-induced models to investigate the relationship between the *p53* gene, inactivating mutations and the potential mechanism of chromosomal instability (French *et al.*, 1998b).

A possible strategy for analysing the molecular changes in the wild-type *p53* allele of carcinogen-induced cancers in the heterozygous *p53*-deficient mouse is as follows (see French *et al.*, 1998b): First, consideration should be given to Southern analysis of genomic DNA. This technique, with the appropriate probes, is the most precise and efficient method for determining intragenic or intra-chromosomal alterations and loss over extended genetic sequences, because it involves precise requirements for hybridization of complementary probe and wild-type gene sequences. Pairwise comparison of representative genomic DNA samples from non-tumour tissues (ear or tail) and from the tumour may be used concurrently to demonstrate the expected genotype and loss of the wild-type allele. Tumours approximately 4–5 mm in diameter should be selected for dissection, to provide adequate tissue for both molecular analysis and histopathology. If loss of heterozygosity involving the wild-type allele is frequent in tumour DNA, no further analysis is required because *p53* mRNA or *p53* protein may not be observed. If infrequent or no loss of heterozygosity involving the wild-type allele can be demonstrated, additional analysis will be required to determine if the wild-type *p53* allele has been inactivated. Mutations in the *p53* allele may be analysed in a number of ways, including single-stranded conformational polymorphism analysis followed by sequencing of the polymorphisms to

identify mutations. In the absence of polymorphisms or mutations identified by sequencing, other strategies and methods with RNA and/or protein will be required to determine the mode or mechanism of *p53* inactivation.

Use of transgenic models in carcinogen assessment

The use of transgenic models holds promise for improving both the accuracy and efficacy of experimental assessments of carcinogenic potential. It is unlikely that a single model or protocol will ever suffice to differentiate carcinogens adequately from noncarcinogens, but the results obtained in two or more transgenic lines may predict the effects of chemicals in conventional long-term bioassays for carcinogens.

Earlier, we proposed that transgenic models could be used, in conjunction with tests for genotoxicity *in vitro* and assessments of systemic toxicological effects, in the identification of carcinogens and to further separate agents that act through genetic and epigenetic mechanisms (Tennant *et al.*, 1995; Tennant, 1997). Judicial incorporation of transgenic models into the carcinogen identification process could improve the precision with which carcinogens are differentiated from noncarcinogens and the predictive validity of the overall assessment by providing new information on mechanisms. Finally, the use of transgenic models could help to conserve some of the human, physical and financial resources that are currently expended in efforts to identify environmental carcinogens. A generalized scheme for integration of transgenic models into the overall assessment of carcinogenic potential is shown in Figure 2.

We envision that carcinogen identification could be conducted initially in two broad phases. Phase 1 would consist of examining and weighting all existing data, including information on chemical structure and biotransformation; on mutagenic potential *in vivo* and *in vitro*; on subchronic toxicity, with identification of target organs and determination of maximum tolerated doses; and the results of bioassays for carcinogenicity in at least two rodent species. Interpretation of these data could lead to an acceptable decision about the carcinogenic potential of the chemical, and further efforts

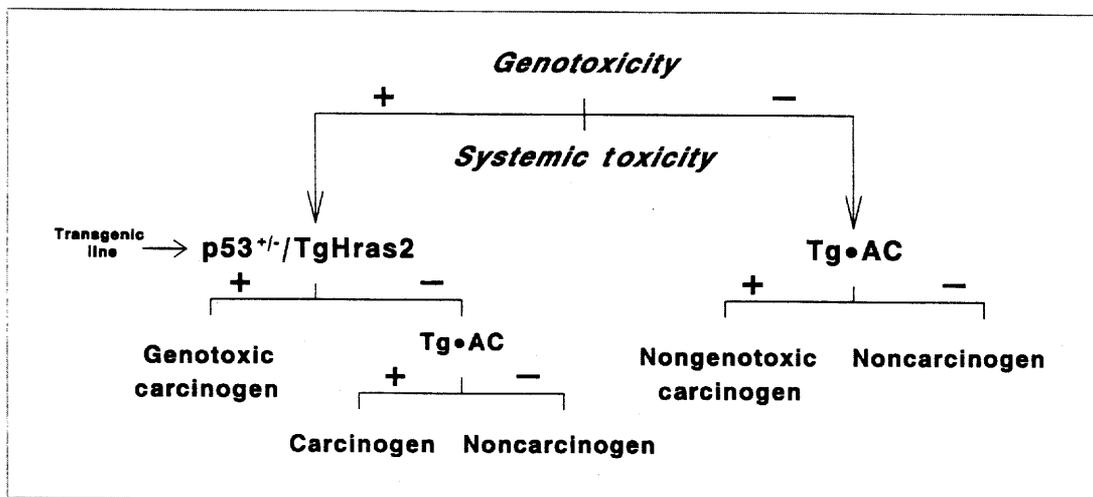


Figure 2. Decision-tree strategy for use of transgenic mouse lines to complement conventional rodent bioassays. Adapted from Tennant *et al.* (1995)

to identify and define carcinogenic effects would be unnecessary. A number of factors commonly confound the interpretation of classical data sets, however, so that decision-making is difficult. These include the appearance of tumours in a single sex or species, marginally increased incidences of common species-specific tumours and the apparent absence of genotoxic potential. When a scientifically logical decision cannot be reached by interpreting the data available in phase 1, the identification programme would proceed to phase 2.

Phase 2 involves evaluation of the effects of the chemical in transgenic rodent models. We currently favour use of mice of the Tg.AC, $p53^{+/-}$ and TgHras2 lines, which are given maximum tolerated doses and at least two lower, incremental doses daily for six months or until an unequivocally positive result justifies early termination. These experiments could be conducted simultaneously in most laboratories. A positive result in both lines is indicative of a genotoxic carcinogen with promotional activity and the ability to initiate neoplastic change. A positive result in only the $p53^{+/-}$ or the TgHras2 line is indicative of a mutagenic carcinogen. Positive results in only the Tg.AC line indicate a nonmutagenic carcinogen: a carcinogenic substance that acts through an epigenetic or tumour-promoting mechanism. Negative results in the transgenic mo-

dels indicate lack of carcinogenicity.

Each transgenic line is carried on a different genetic background ($p53^{+/-}$ on C57BL/6, TgHras2 on CB6F1, and Tg.AC on FVB/N), and this undoubtedly influences the penetrance of the transgene. These backgrounds are different from that of the B6C3F1, used in most conventional bioassays in mice, which undoubtedly influences responses to carcinogens, particularly in long-term bioassays. We believe that such background differences should be kept in mind but that their effect on the responses of the transgenic lines will be minimal in comparison with the conventional bioassay, as the 'genetic initiation' of the transgenic lines minimizes the strain-specific genetic effects often seen in conventional bioassays.

Interlaboratory development and evaluation of testing protocols

Before any test or group of tests can be used confidently for assessing issues associated with human health, the proposed procedures must withstand rigorous, critical evaluation. Methods should be evaluated in experiments designed to identify both the precision with which the results predict relative safety and the nature of the procedural and interpretative weaknesses. Unfortunately, flawless predictive validity, the ultimate goal of any testing

programme, can never be proven unequivocally, even if it is attainable. Consequently, circumstances must be identified that could lead to the most dangerous of all predictive errors — the false-negative result. At one level then, such experiments must ensure that the assay can result in unambiguous identification of chemicals known to be human carcinogens (IARC Group 1). Similarly, most if not all agents in Group 2A should be detected as carcinogens, whereas a lower percentage of those in Group 2B are likely to be identified as carcinogens (IARC, 1987).

We at the Laboratory of Environmental Carcinogenesis and Mutagenesis have begun the evaluation process in collaboration with other laboratories to establish the validity of the protocol and to determine any sources of interlaboratory variation. Our approach is to use Tg.AC mice treated dermally to test the effects of a variety of chemicals previously investigated in the National Toxicology Program's studies of toxicity and carcinogenesis (Table 8A). The results of experiments in which chemicals were tested in the *p53^{+/+}* and TgHras2 lines are presented in Tables 8B and 8C, respectively. Studies of chemicals that have not been tested in long-term bioassays by the National Toxicology Program (e.g. Spalding *et al.*, 1993; Albert *et al.*, 1996; Trempus *et al.*, 1998) are not included. Tables 8A, 8B and 8C show that although relatively few comparative studies have been completed the degree of correlation between results of the two-year bioassays and the experiments in transgenic mice are highly encouraging.

The scientific community is best served by laboratories that are willing to take part in an evaluation process that involves as much uniformity as possible in the design, conduct and interpretation of studies. While the activities of relatively few chemicals have been studied in the transgenic models, the experiments have afforded valuable insights, which have been used to develop operational protocols for assessing the effects of chemicals in two transgenic models (French *et al.*, 1998b; Spalding *et al.*, 1998). Examples of such protocols are shown in the boxes. Additional issues are addressed in Appendix 1. Although the suggested protocols will probably undergo developmental modifications as they mature, they are a starting point in the process.

Interlaboratory evaluations of the Tg.AC (Holden *et al.*, 1998) and the TgHras2 lines are being conducted. In addition, the International Life Sciences Institute is coordinating a large-scale evaluation of alternative models, including the three transgenic lines, which involves over 20 collaborating organizations (Robinson, 1998).

Summary and conclusions

For over 20 years, scientists have attempted to find reliable, specific, sensitive short-term methods for prospective identification of carcinogens. During that time, many, perhaps hundreds, of systems have been developed and evaluated; some, such as the *Salmonella* mutagenesis assay, are used extensively; however, while studies of chemical structure, genotoxicity and systemic toxicity provide predictive data, most chemicals and environmental agents cannot be judged prospectively for the actual risk they pose to human populations. While these factors may be determinants of carcinogenic potential, other information acquired over the same period show that cancers are genetic diseases, in that specific genes are involved in the neoplastic process and these confer individual and familial susceptibility to particular carcinogens. It has also been recognized that the rodent models used in conventional long-term bioassays for carcinogenicity are not always appropriate surrogates for humans. The strains of rodents used are highly inbred, which results in 'allelic enrichment', that is, many alleles of polymorphic genes are lost, and the rodents have high incidences of specific 'spontaneous' tumours and strain-specific responses to many agents. Humans have similar polymorphic gene families, but the alleles occur at variable frequencies in individuals because of random mating. It is therefore usually a challenge to judge which effects observed in rodents can be extrapolated meaningfully to humans.

We have proposed that the most important pattern of responses observed in bioassays is the induction of tumours in two rodent species, since such responses are intrinsically less likely to be strain- or species-specific and the agents are more likely to pose a significant risk to humans. In developing the transgenic bioassays, we therefore took as our goals the identification of most trans-species carci-

Table 8A. Chemicals tested in Tg.AC transgenic mice

Chemical	US National Toxicology Program bioassay		Transgenic mice	
	Result	Reference	Result	Reference
Benzene ^a	+	1986a	+	Tennant <i>et al.</i> (1995, 1996)
Benzethonium chloride ^b	-	1993	-	Tennant <i>et al.</i> (1995, 1996)
<i>o</i> -Benzyl- <i>p</i> -chloropheno ^b	+	1994	+	Tennant <i>et al.</i> (1995, 1996)
2-Chloroethanol ^b	-	1985	-	Tennant <i>et al.</i> (1995, 1996)
1-Chloro-2-methylpropene ^b	+	1986b	+	Tennant <i>et al.</i> (1996)
1-Chloro-2-propanol ^c	-	1997a	-	(unpublished)
Coconut oil diethanolamine ^c	+	1997b	-	(unpublished)
<i>p</i> -Cresidine ^b	+	1978a	+	Tennant <i>et al.</i> (1995, 1996)
Furfuryl alcohol ^c	+	1997c	-	(unpublished)
Lauric acid diethanolamine ^a	+	1997d	+	Hansen <i>et al.</i> (1995); Tennant <i>et al.</i> (1996)
Oleic acid diethanolamine ^c	-	1997e	-	(unpublished)
Mirex ^b	+	1990b	+	Hansen <i>et al.</i> (1995); Tennant <i>et al.</i> (1996) ^d
Pentachlorophenol ^c	+	1989b/1997f	+	(unpublished)
Phenol ^c	-	1982c	-	Spalding <i>et al.</i> (1993); Tennant <i>et al.</i> (1996)
Pyridine ^c	+	1997g	-	(unpublished)

^a Tested in homozygous and heterozygous Tg.AC mice

^b Tested in homozygous Tg.AC mice

^c Tested in heterozygous Tg.AC mice

^d Personal communication from Dr Robert Smart, North Carolina State University

genus and establishing lack of response to most substances that do not induce tumours in conventional bioassays. Thus, if a long-term bioassay is conducted in rats, the additional use of one or more transgenic models should result in the identification of most chemicals that have carcinogenic potential in both species. Chemicals that induce responses in rats only may elicit a species-specific response. It is anticipated that few chemicals will be active only in the transgenic models; that is, few 'false-positive' responses have been seen, which leads us to believe that they are specifically sensitive rather than generally supersensitive to potential carcinogens. A positive response in rats in a long-term bioassay and no effect in the transgenic models could reflect

either insufficient exposure (i.e. six versus 24 months) or insensitivity. The latter concern is related to the probability that not all tissue-specific pathways to tumour development necessarily involve the *p53* or *ras* genes, and chemicals that affect other important oncogenes or tumour suppressor genes could be missed in the current models (Ashby, 1997). These are legitimate concerns, and additional results are needed to determine if they are well founded. Another concern with regard to the Tg.AC model is that, since the transgene can be induced by either chemicals or physical wounding, false-positive responses might be induced by toxicity unrelated to carcinogenic potential. An additional concern relates to the value of negative

Table 8B. Chemicals tested in $p53^{+/-}$ transgenic mice

Chemical	US National Toxicology Program bioassay		Transgenic mice	
	Result	Reference	Result	Reference
<i>p</i> -Anisidine	-	1978b	-	Tennant <i>et al.</i> (1995, 1996)
Benzene	+	1986a	+	Tennant <i>et al.</i> (1995, 1996)
1-Chloro-2-propanol	-	1997a	-	(unpublished)
<i>p</i> -Cresidine	+	1978a	+	Tennant <i>et al.</i> (1995, 1996)
Coconut oil diethanolamine	+	1997b	-	(unpublished)
Glycidol	+	1990c	-	(unpublished)
Lauric acid diethanolamine	+	1997d	-	(unpublished)
<i>N</i> -Methylolacrylamide	+	1989c	-	(unpublished)
Methylphenidate	+	1995	-	(unpublished)
Oleic acid diethanolamine	-	1997e	-	(unpublished)
Pentachlorophenol	+	1989b/1997f	-	(unpublished)
Phenolphthalein	+	1996	+	Dunnick <i>et al.</i> (1997)
Pyridine	+	1997g	-	(unpublished)
Reserpine	+	1980	-	Tennant <i>et al.</i> (1995, 1996)
4-Vinyl-1-cyclohexene diepoxide	+	1989a	+	Tennant <i>et al.</i> (1995, 1996)

responses in the transgenic models. We believe that this is a 'weight-of-evidence' issue and that negative effects in two or more models are potentially important, particularly if the results of a conventional bioassay in rats are also negative.

One of the most important principles underlying the use of transgenic models is that, in the context of the multi-gene/multistep concept of carcinogenesis, they provide genetically 'initiated' animals in which specific genes that are known to be critical to many neoplastic pathways can be used as targets for chemical-gene interaction. While the results obtained to date with the three transgenic models are limited, empirical observations support the preferential identification of trans-species carcinogens. The *p53*-deficient and TgHras2 models show preferential responses to mutagenic carcinogens. These responses occurred with the same ranges of doses given by the same routes used in the conventional bioassays but within a fraction of the time required for the two-year bioassays, i.e. four to six months. The transgenic bioassays involve

fewer animals and permit dosimetric studies. In some cases, specific molecular events associated with the loss of the *p53* gene have been identified (Dunnick *et al.*, 1997). An important aspect of the studies is that the tumours are induced in a time-frame during which complications introduced by variable frequencies of background tumours are minimal.

The mechanism involved in the Tg.AC model differs from that in the *p53*-deficient model, and the results obtained to date indicate that specific activation of the expression of the transgene is the critical event underlying responses in the Tg.AC model (Hansen *et al.*, 1995; Cannon *et al.*, 1997). Responses to both trans-species chemical carcinogens and known tumour promoters have been elicited, with few false-positive responses to noncarcinogens (Table 8A). Studies on the mechanism of transgene activation are currently under way.

The heterozygous *p53*-deficient mouse model responds preferentially to mutagenic carcinogens (Table 8B). All four mutagenic carcinogens were

Table 8C. Chemicals tested in
CB6F₁-TgHras2 transgenic mice

Chemical	Result in bioassay	Result in transgenic mice
<i>p</i> -Anisidine	–	–
Benzene	+	+
<i>p</i> -Cresidine	+	+
Cyclophosphamide	+	±
Cyclosporin	+	+
1,2-Dimethylhydrazine	+	+
1,4-Dioxane	+	+
Ethyl acrylate	+	+
Ethylene thiourea	+	+
4-Hydroxyaminoquinoline-1-oxide	+	+
8-Hydroxyquinoline	–	–
Melphalan	+	+
Methylazoxymethanol	+	+
<i>N</i> -Methyl- <i>N</i> -nitro- <i>N</i> -nitrosoguanidine	+	+
3-(<i>N</i> -Methyl- <i>N</i> -nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	+	+
<i>N</i> -Methyl- <i>N</i> -nitrosourea	+	+
4-Nitro- <i>o</i> -phenylenediamine	–	±
4-Nitroquinoline-1-oxide	+	+
<i>N</i> -Nitrosodiethylamine	+	+
Phenacetin	+	+
Procarbazine	+	+
Resorcinol	–	–
Rotenone	–	–
4,4'-Thioaniline	+	+
Thiotepa	+	+
1,1,2-Trichloroethane	+	–
Vinyl carbamate	+	+
4-Vinyl-1-cyclohexene diepoxide	+	+
Xylenes (mixed)	–	–

Adapted from Yamamoto *et al.* (1998)

active at the same sites observed in the two-year cancer bioassay, and all 10 nonmutagenic carcinogens (primarily those carcinogenic only in mice at common sites) and one mutagenic noncarcinogen gave negative results in the studies reported to date. Three of the four chemicals that elicited responses (benzene, phenolphthalein and 4-vinyl-1-cyclo-

hexene diepoxide) were associated with frequent observation of loss of the wild-type allele, and the fourth, *para*-cresidine, a potent urinary bladder and liver carcinogen, with infrequent loss of the wild-type allele. Studies are in progress to further define the mode or mechanism of action for induction of cancer in the *p53*-deficient mouse model.

Box 1. Basic protocol for experiments with homozygous Tg.AC transgenic mice

Animals:	7–9-week-old homozygous male or female mice
Husbandry:	Housed singly in shoe-boxes with hardwood chips or in wire-bottom cages. Standard rodent diet, e.g. Purina Pico Chow No. 5058 (preferred) and tap water <i>ad libitum</i> . Animal room temperature and humidity maintained at 22 ± 1 °C and 40–60% respectively; 12-h light:dark cycle; tattoo or microchip for identification.
Route:	Preferred route is topical application on shaved dorsal surface (4–6 cm ²) in 200 μ l solvent. Acetone is the preferred solvent, although 70–95% ethanol has been used. Other possible routes are feed and water, 7 days/week; oral gavage or inhalation, 5 days/week. All treatment regimens are performed for 26 weeks followed by terminal sacrifice.
Dose selection:	Appropriate dose-range-finding studies by the route of administration should be performed to determine the maximum tolerated dose (MTD) that would be selected for a two-year bioassay. Three doses, the MTD and 1/2 and 1/4 MTD are the usual selection. The inclusion of a higher dose, 1.5 or 2 times the MTD is desirable if it is not considered to be life-threatening over the 26-week dosing period, as it reduces the likelihood of a false-negative response. Dose-finding and target organ toxicity studies may be conducted in the non-transgenic wild-type FVB/N parent strain. It is recommended that each dose group and the appropriate control groups comprise at least 15 male and 15 female mice.
Study design/end-point:	Skin papillomas are the reporter phenotype for topically applied test agents. Other target organs may be affected depending on systemic effects.
Negative and positive control groups:	Topical application: The solvent vehicle (200 μ l) serves as the negative control. 2.5 μ g TPA applied twice a week serves as the positive control.
Body weights/clinical observations:	Body weights should be noted at the beginning of the study and weekly thereafter. Clinical observations should be made for mortality and morbidity twice daily. The dorsal treated area should be examined for emerging skin papillomas from week 1 after the first dose. The number of papillomas should be noted weekly. Up to 30 papillomas can be counted with reasonable accuracy.
Necropsy:	At terminal sacrifice, an accurate tabulation of papillomas in the application area and a full gross necropsy of all tissues and gross lesions should be conducted.
Histopathology:	A limited histopathological evaluation of 12 tissues, including gross lesions and masses. Skin papillomas may be excluded, but skin malignancies should be examined.

Box 2. Basic protocol for experiments with heterozygous p53^{+/-}-deficient mice

Animals:	6–8-week-old male and/or female heterozygous p53 ^{+/-} -deficient mice
Husbandry:	Males housed singly; females housed 4–5/cage in shoe-boxes with hardwood chips or in wire-bottom cages; 12-h light:dark cycle; tattoo or microchip for identification; diet depends on calories and composition required
Route:	Any
Duration of treatment:	26 weeks followed by up to 2 weeks of holding for staggered termination of study. Dosing regimen: 7 days/week for diet and drinking-water; 5 days/week for skin painting, oral gavage or inhalation.
Dose selection:	If no maximum tolerated dose (MTD) is available from short-term studies, appropriate dose-range-finding studies must be conducted to establish the MTD for a two-year cancer bioassay. Cancer bioassay MTD (2× MTD or MTD; for mouse) may be used as the high dose, if desired. Inclusion of the next highest dose above the MTD (e.g., 2× MTD) that is not expected to be life-threatening over a six-month exposure may be desirable to reduce the potential for a false-negative result in a 26-week exposure regimen. Dose-finding and target tissue identification may be conducted in non-transgenic siblings.
Study design/end-point:	Target tissue-specific induction of cancer. Groups of 15 males and 15 females: vehicle controls, low dose (1/2 or 1/4 MTD), intermediate dose (MTD or 1/2 MTD) and high dose (2× MTD or MTD).
Body weights/clinical observations:	Weekly
Necropsy:	Full gross necropsy of all tissues and gross lesions
Histopathology:	Non-genotoxic agent: full histopathological evaluation (~40 tissues plus gross lesions, including masses); genotoxic agent: limited evaluation (~12 tissues including target tissues and gross lesions, including masses)

The TgHras2 model has thus far been evaluated for its response to approximately 30 chemicals, has shown the capacity for short-term (six months or less) response to a number of recognized carcinogens and has produced no unequivocally false-positive results (Table 8C). Since transgene mutations seem to be related to the generation of only certain types of chemically induced tumours (at a relatively low percentage), studies on the molecular mechanisms of enhanced tumour induction are now under way (Yamamoto *et al.*, 1998).

These transgenic models are therefore scientifically valid models that can be used to characterize potential carcinogens. It is important to empha-

size that the data derived from these models are best used in conjunction with all other knowledge about the chemical, including its structure, its genotoxicity *in vitro* and *in vivo*, its systemic toxicity and any information on its pharmacology in humans and levels of human exposure. These models can be used to generate dosimetric data, to permit better estimates of carcinogenic potential and to provide direct inferences about the mode of action of the carcinogen. It is also proposed that their deployment in evaluating drugs and chemicals will result in the rapid development of an expanded database about appropriate extrapolation of the results of bioassays to human health risks.

Box 3. Criteria for grading skin tumour responses in skin-painted Tg.AC mice

In order to be counted as a positive response, skin tumours (papillomas) must reach 1 mm in size and persist for at least three weeks. In practice, since individual tumours are not mapped, this requirement applies only to the first few tumours that appear, because as the papilloma multiplicity increases on any one mouse some papillomas may regress and others appear. Transient papillomas that appear in the application area for one or two weeks should be deleted from the computations. Loss of papillomas due to sloughing or scratching does occur and more often in animals with high (15–30) papilloma loads. True regression also occurs over the course of a study, even while the animals are being treated. The latency of the first tumour should be noted for each mouse and the tumour burden tabulated weekly for 26 weeks.

In order to compare papilloma responses among different treated groups, the papilloma burden of each animal at the end of 26 weeks is the value used for the computation. Exceptions to the above guidelines may be made, depending on the trend of responses among the animals in any particular dose group:

- If a papilloma appears at week 25–26 among the negative solvent controls, or among animals treated with an

inactive chemical agent, it should be tabulated even though it was not present for three weeks.

- Animals that are removed from the study between 11 and 26 weeks are given the attributed tumour burden that occurred in the last 2 or 3 weeks or the maximum (see item 3) tumour burden if regression has occurred, and this value is included in the computation for that dose group. If, as in positive control groups, tumour multiplicity per animal is increasing every week, then the tumour burden of the last week should become the value tabulated.
- When tumour regression occurs in an animal that survives for 26 weeks, an average of the highest maximum tumour burden over several (2–3) weeks should become the value tabulated.

In studies with active chemicals and a positive control group, a range of responses is observed among different animals in any dose group. Animals with low and high responses are found most often at doses that occur on the linear part of the dose-response curve; however, at more optimal inducing doses, e.g. 1.25 µg TPA 3x/week, individual papilloma burdens cluster in the range of 20–30 or more.

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Appendix 1. Basic considerations in the protocol for the Tg.AC skin-painting model

Animals and husbandry: Male or female Tg.AC mice are usually received at 4–5 weeks of age and held for another 2–3 weeks for acclimatization to laboratory conditions. Topical dosing is best initiated when the animals are 7 weeks old, when the hair cycle is in the resting (telogen) stage.

We house Tg.AC animals individually in plastic shoe-box cages over hardwood bedding. Animals have free access to food (a standard rodent diet, e.g. NIH 07, or Purina Pico Chow, No. 5058, Ralston Purina, St Louis, MO) and tap water. Clean cages are provided weekly. The animal room temperature and humidity are maintained at 22.2 ± 2 °C and 40–60%, respectively and fluorescent lighting is on a 12 h on, 12 h off cycle. Each animal has a unique number tattooed on its tail.

Chemicals: The identity and purity of the test chemicals, solvents and positive control agents is established before initiation of experiments. For experiments in Tg.AC mice, test chemicals are dissolved in acetone or 70–95% ethanol and administered in a volume of 200 μ l. Alternative solvent systems may be necessary. Regardless of the solvent selected, we encourage researchers to use the solvent or vehicle system as a negative control in order to ensure proper interpretation of the results obtained with the chemical under investigation.

In our laboratories, test chemical solutions and vehicle blanks (negative control solutions) are prepared simultaneously. Usually, solutions are prepared on the day of use, but if they are to be stored between uses, stability under the conditions of storage should be demonstrated before initiation of the experiment.

Positive control: The suggested positive control agent for experiments in the Tg.AC model is 12-O-tetradecanoyl 13-acetate (TPA). A TPA-treated positive control group of at least 15 animals of each sex is always included in the studies of dermal carcinogenicity because it serves as an indicator of the response of animals undergoing treatment with the test article. TPA readily dissolves in acetone and can be administered in a volume of 200 μ l at a dose

of 1.25 μ g per animal three days a week (e.g. Monday, Wednesday and Friday). This dose regimen can induce as many as 30 papillomas in some mice.

Preparation of animals for administration of compound: One to three days before initiation of dermal applications to Tg.AC mice, the application site is shaved so that an area of 4–6 cm² between the dorsal interscapular region and the base of the tail is revealed. During the dosing period, shaving should be repeated as necessary until the presence of skin tumours makes it inadvisable.

Dermal administration of test compounds and negative and positive control agents: The volume of the doses in our laboratories does not exceed 200 μ l. Whenever the dose cannot be delivered in a single 200- μ l volume, we repeat the dose but allow sufficient time for absorption before the second application.

Animal observations: Mice should be weighed on the first day of the study and then at weekly intervals until the study is completed. We observe all animals for mortality and morbidity twice daily.

In our experiments with Tg.AC mice, each animal is examined for evidence of systemic and/or local toxicity and for the development of tumours of the jaw at the time of dosing. Any animal found to have either a jaw tumour or malocclusion is removed from the study. Examination of the application area for skin tumours should begin on experimental day 7 and be continued weekly thereafter. In our experiments, a skin tumour that is counted as a positive response must be at least 1 mm in diameter and persist for at least three consecutive observation periods. The number of tumours at the application site is recorded weekly. We suggest imposing an upper tabulation limit of 30 tumours per animal because it is difficult to maintain an accurate count of more tumours. Animals with 30 tumours should continue in the study unless morbidity dictates early sacrifice. The criteria for tabulating papilloma incidence and multiplicity are summarized in Box 3.

Appendix 1 (contd)

Experimental design: *Animal selection.* All animals used in an experiment, i.e. negative and positive control groups and test groups, should be received from the supplier in a single shipment. We rotate the cage positions in order to reduce the possibility of an effect of position on the study outcome.

Numbers of animals per group. Experimental dose groups should contain 15–20 animals of each sex. Each of our experiments in Tg.AC mice also includes at least one negative control group that receives dermal applications of the solvent in the same volume administered to the experimental group receiving the largest dose of the test compound. We advocate the use of at least three doses of each test article. The highest dose should produce at least some minimal evidence of toxicity, such as the reduced body-weight gain that often defines the maximum tolerated dose (MTD). Appropriate lower doses would be one-half and one-quarter of the MTD. The lowest dose should produce no observable sign of toxicity. We have used data from carcinogenicity bioassays to select our doses. We use a dose that is 1.5 times the MTD if we consider that the animals can tolerate it for 26 weeks; this reduces the likelihood of a false-negative response. If the MTD is not known, range-finding studies should be conducted in the usual manner to determine it. Range-finding studies can be conducted in the wild-type FVB/N parental strain, as there is no evidence that there is any difference in the disposition or pharmacokinetics of chemicals between Tg.AC and FVB/N mice.

Duration of studies. A study duration of 26 weeks is optimal in studies with Tg.AC mice.

Necropsy/histopathology. For studies of skin-painting in

Tg.AC mice, quantification of papillomas, the reporter phenotype, at the application site should suffice; however, at necropsy, gross lesions should be noted and prepared for histopathological analysis. We recommend that the following tissues be examined microscopically: skin (site of application), kidneys, liver, lung, thymus, spleen, mandibular lymph node, mandibular salivary gland, stomach, adrenal glands, pituitary gland, thyroid gland and mammary gland with overlying skin. A basic protocol for Tg.AC mice is summarized in Box 1.

Interpretation of study results: Evaluation criteria. Experimental groups should be compared with negative control groups with respect to the percent of animals with one or more skin tumours, the average latency to appearance of the first skin tumour, the average number of tumours per animals at risk (Animals that survive 10 or more weeks of dosing are considered to be at risk.), the average number of tumours per tumour-bearing animal and the average latency to development of the maximal number of tumours observed.

Negative control responses. Each laboratory should develop a database for negative controls in order to judge the acceptability of results for concurrent negative controls. Experiments in which the concurrent negative control group has a skin tumour incidence that significantly exceeds the mean in the historical database may be discarded, as those data may compromise the interpretation of data for experimental groups.

Positive control responses. Experiments in which the positive control group does not have an unequivocal skin tumour response should be discarded.