The Role of Oncogenes in Chemical Carcinogenesis

by S. Jill Stowers,* Robert R. Maronpot,† Steven H. Reynolds,* and Marshall W. Anderson*

Proto-oncogenes are cellular genes that are expressed during normal growth and developmental processes. Altered versions of normal proto-oncogenes have been implicated in the development of human neoplasia. In this report, we show the detection of activated proto-oncogenes in various spontaneous and chemically induced rodent tumors. The majority of activated proto-oncogenes found in these tumors are members of the ras gene family and have been activated by a point mutation. Characterization of the activating mutation may be useful in determining whether this proto-oncogene was activated by direct interaction of the chemical with the DNA. Comparison of activating lesions in spontaneous versus chemically induced tumors should be helpful in determining whether the chemical acts via a genotoxic or a nongenotoxic mechanism. All of this information may be helpful in the assessment of potential carcinogenic hazards of human exposure to chemicals.

Introduction
Recent evidence suggests that neoplastic development, at least in part, is the result of the abnormal activation of a small set of cellular genes. These genes, termed proto-oncogenes, were originally discovered as the transduced genes of acute transforming retroviruses (1–8). Subsequent studies have established that these proto-oncogenes can also be activated as oncogenes by mechanisms independent of retroviruses (4). Mechanisms for the conversion of proto-oncogenes to activated oncogenes include point mutations, gene amplification, chromosomal rearrangements, and promoter insertion (Fig. 1). The activation of proto-oncogenes by genetic alterations results in altered levels of expression of the normal protein product, or in normal or altered levels of expression of an abnormal protein.

Proto-oncogenes are expressed during regulated growth such as embryogenesis, regeneration of damaged liver, and stimulation of cell mitosis by growth factors. Proto-oncogenes are highly conserved. They are detected in species as divergent as yeast, Drosophila, and humans. Proto-oncogenes include genes that encode for growth factors (sis), growth factor receptors (neu, erbB, fms), regulatory proteins in signal transduction (ras family), nuclear regulatory proteins (myc, myb, fos), and tyrosine kinases (src, abl, ras). Thus, the encoded proteins appear to play a crucial role in normal cellular growth and/or differentiation.

The activation of proto-oncogenes in spontaneous and chemically induced tumors has been extensively studied during the past several years. Although oncogenes such as ras and myc can complement each other in the malignant transformation of a cell in vitro (2), the number of proto-oncogenes that must be activated in the multistep process of carcinogenesis is unclear at present. Also, new evidence from several laboratories suggests that in addition to the activation of positive factors (oncogenes), the loss of negative regulatory functions (tumor suppressor genes) may also be a necessary but distinct step in neoplastic development (5). This paper will discuss the detection of activated oncogenes in rodent tumors and the implication of oncogenes in risk analysis of carcinogen-induced rodent tumor data.

Detection of Activated Oncogenes in Tumors
Detection of activated oncogenes in neoplasia can be achieved by using several different techniques depending on how the particular oncogene might be activated. Abnormal expression of oncogenes in tumors due to amplification of the gene may be detected by dot blot or Southern blot analysis. Increased expression due to deregulation of the gene may be detected by dot blot as well as Northern blot analysis. Chromosomal translocations may be detected by cytogenetic analysis. Ex-
amples of abnormal expression of oncogenes detected in human tumors and tumor cell lines are shown in Table 1.

A number of oncogenes present in human tumors as well as animal tumors have been detected by the NIH/3T3 transfection assay. The NIH/3T3 transfection technique involves the ability of the NIH/3T3 mouse fibroblast to accept and express genes from donor tumor DNA, resulting in the formation of transformed cells. Only a few years ago, Shih et al. (6) were the first to show that DNA from carcinogen-transformed cell lines could cause transformation of NIH/3T3 cells after transfection. This transformation was characterized by a change in the morphology of the NIH/3T3 cells and by anchorage-independent growth. Other investigators using this technique were then able to show that dominant transforming genes or oncogenes were present in human tumors and in carcinogen-induced animal tumors. An extension of the NIH/3T3 transfection assay that affords greater sensitivity is the nude mouse tumorigenicity assay (7). This involves cotransfection of NIH/3T3 cells with tumor DNA and a selectable marker gene. The selected cells are then injected SC into the immunocompromised mice. The tumors that develop from the nude mice are then analyzed using the techniques described earlier to characterize the activated oncogenes.

The majority of activated genes in human tumors detected by the NIH/3T3 assay have been members of the ras gene family: the H-ras, the K-ras, and the N-ras. Early studies using the NIH/3T3 assay showed only ras gene activation in a low percentage of the human tumors (approximately 10%). Later studies have shown that other oncogenes can also be detected in the human tumors by this assay, including the lca (8), hst (9), and the trk (10) oncogenes. In addition, the percentage of certain tumor types that test positive for activated ras oncogenes are higher than 10%. For example, Verlaander-Vries et al. (11) detected activated ras genes in the 27% of acute myeloid leukemia examined. Anantheswamy et al. (12) detected Ha-ras genes in four of six human squamous cell carcinomas examined. The addition of the tumorigenicity parameter to the assay system appears to improve the efficiency in detection of activated oncogenes in human tumors.

A variety of animal tumor model systems have also been examined for activated genes using the NIH/3T3 assay. These include spontaneous tumors in rats and mice, tumors that arise after single or multiple doses of carcinogen, and tumors that arise after long-term exposure to a carcinogen. Examples of the activated genes in the different tumor model systems are shown in Table 2. Like the human tumors, the majority of activated oncogenes detected in the animal tumors are members of the ras gene family. Other oncogenes have also been detected in animal tumors using the NIH/3T3 assay (Table 3). One example is the activated neu oncogene found in nervous tissue tumors induced in rats by transplacental exposure to N-methyl-N-nitrosourea (MNU) or N-ethyl-N-nitrosourea (ENU). The c-raf on-
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Table 2. Activated oncogenes in rodent tumor models.

| Model                     | Tumor            | Number positive/Number tested | Oncogene                          | Reference |
|---------------------------|------------------|-------------------------------|-----------------------------------|-----------|
| Spontaneous               | Mouse liver      | 17/27                         | H-ras (15)*, raf (1), unknown (1) | (18,32)   |
| Single dose               | Rat              | 1/37                          | H-ras (1)                         | (18)      |
| NMU                       | Rat mammary      | 61/71                         | H-ras (61)                        | (14)      |
| DMBA                      | Rat mammary      | 6/29                          | H-ras (6)                         | (15)      |
| Single dose, neonatal    |                  |                               |                                   |           |
| HO-AAF                     | Mouse liver      | 10/10                         | H-ras (10)                        | (33)      |
| HO-DHE                    | Mouse liver      | 11/11                         | H-ras (10), K-ras (1)             | (33)      |
| VC                        | Mouse liver      | 10/10                         | H-ras (10)                        | (33)      |
| DEN                       | Mouse liver      | 14/33                         | H-ras (14)                        | c         |
| Multiple doses            |                  |                               |                                   |           |
| DMN-OMe                   | Rat renal        | 10/35                         | K-ras (2), unknown (9)            | (34)      |
| Aflatoxin B1              | Rat liver        | 10/11                         | K-ras (2), unknown (9)            | (35)      |
| Continuous dose           |                  |                               |                                   |           |
| TNM                       | Rat and mouse liver | 18/19, 10/10 | K-ras (18), K-ras (10)            | (19)      |
| Furans                    | Mouse liver      | 13/29                         | H-ras (10), raf (1), K-ras (2)    | (23)      |
| Furfural                  | Mouse liver      | 13/16                         | H-ras (9), K-ras (1), unknown (3) | (23)      |
| Benzidine-derived dyes    | Rat              | 34/58                         | H-ras (31), N-ras (3)             | e         |
| DEN                       | Rat liver        | 1/12                          | Unknown                           | c         |
| Initiation-promotion      |                  |                               |                                   |           |
| DEN-Farber protocol       | Rat liver        | 0/20                          | —                                 | (36)      |
| DEN + PB                  | Rat liver        | 0/10                          | —                                 | c         |
| DEN + EE2                 | Rat liver        | 2/19                          | non-ras (2)                       | f         |
| DMBA + TPA                | Mouse skin       | 33/37                         | H-ras                            | (37)      |
| Transplacental dose       |                  |                               |                                   |           |
| ENU                       | Rat neuroblastomas | 3/3                           | neu (3)                           | (38)      |
| MNU                       | Rat schwannomas  | 10/13                         | neu (10)                          | (16)      |

* Numbers in parentheses are the number of positive samples with that oncogene.

b Abbreviations: HO-AAF, N-hydroxy-2-acetylaminofluorene; HO-DHE, 1'-hydroxy-2,3'-dehydroestrageol; VC, vinyl carbamate; DEN, N-nitrosodiethylamine; DMN-OMe, methyl(3-methoxymethyl)nitrosamine; PB, phenobarbital; EE2, ethinyl estradiol. For other abbreviations, see text.

c Stowers et al., unpublished data.

d Benzidine-derived dye-induced rat tumors include preputial gland tumors, squamous cell carcinomas, basal cell tumors, clitoral gland tumors, and mammary tumors.

* Reynolds and Anderson, unpublished data.

f Goodrow et al., unpublished data.

cogene has also been detected in mouse liver tumors. The detection of unusual mutations in the ras genes, as well as the identification of new classes of oncogenes in animal tumors, should be enhanced by the addition of the nude mouse tumorigenicity assay.

Activation of Oncogenes by Carcinogens

Studies in animal tumor model systems suggest that the chemicals or radiation may play a role in the activation of oncogenes by point mutation. Point mutations resulting in the activation of ras proto-oncogenes in several chemically induced rodent tumors have been consistent with the known alkylation patterns of the carcinogens (13). For example, the mutation at the 12th codon of the H-ras gene detected in rat mammary tumors induced by methylnitrosourea (14) is consistent with the formation of the 06 methylguanine adduct. The activating mutation in the 61st codon of the H-ras gene found in 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors and skin tumors is consistent with DMBA binding to adenosine residues (15). The point mutation that activates the neu proto-oncogene in peripheral nervous system tumors in rats induced by ENU or MNU probably results from the binding of these potent genotoxic chemicals to DNA (16). “Hot spots” for activating mutations in these oncogenes have been observed (17). For example, the GGA → GAA mutation observed in the 12th codon of ras oncogenes detected in MNU-induced mammary carcinomas (14) is always at the second guanine of this codon, even though a similar mutation at the first guanine could also produce an activated ras oncogene. If the sequence of specificity for the binding of carcinogens to DNA corresponds to a known biological hot spot in an oncogene, then this chemical can be a very potent carcinogen.

Several studies have shown that oncogene activation may not be the direct result of chemical interaction with DNA. Activated oncogenes have been detected in a number of different types of tumors in the mouse. This
implies that activation of oncogenes in long-term carcinogenic studies in the mouse may or may not be the result of direct interaction of the chemical with DNA (U. Candrian, unpublished data; 18). It is possible in some instances that the chemical did activate the oncogene directly and is consistent with the chemical binding to the DNA. In other instances, the chemical may have increased the background tumor incidence by a mechanism such as cytotoxicity or receptor-mediated promotion. If the pattern of activated oncogenes in the chemically induced tumors is different from that in the spontaneously occurring tumors, then the chemical probably caused the mutations, at least in some of the tumors. One example in which the chemical’s role in oncogene activation is not known is the activated K-ras oncogene detected in tetranitromethane (TNM)-induced rat and mice lung tumors. TNM is a mutagen and an irritant. However, the interactions between TNM and DNA are not known. In a recent long-term carcinogenesis study conducted by the NTP, chronic exposure to TNM resulted in a high incidence of primary lung tumors in Fischer 344 rats and B6C3F1 mice (19). K-ras oncogenes with a GGT→GAT mutation in the 12th codon were observed in 18 of 19 rat lung tumors and 10 of 10 mouse lung tumors (Table 4). The activation of the K-ras oncogene in these TNM-induced lung tumors may be the result of one or more actions of the chemical: a direct consequence of TNM-induced DNA damage; the tumors may be spontaneously occurring; enhancement of spontaneously occurring K-ras by TNM-induced cell replication; or combination of direct TNM-induced DNA damage and enhancement of spontaneous occurrence.

It is a distinct possibility that these activated K-ras oncogenes with GC→AT transitions in the second base of the 12th codon are spontaneous, since an activated K-ras with the same mutation was observed in a spontaneously occurring pulmonary adenocarcinoma in the B6C3F1 mouse (U. Candrian, unpublished data). Even though spontaneous lung tumors in the Fischer 344 rat were not observed in this study, it is still possible that the irritant property of TNM could have promoted the cells, which activated the K-ras or enhanced the spontaneously occurring K-ras. The reproducible detection of the K-ras in lung tumors of mice and rats suggests that TNM could have directly induced the mutation. In support of this conclusion, mutagenicity studies have shown that TNM causes mutant bacterial strains to revert to the wild type by the same GC→AT transition. Studies on the possible interactions of TNM with DNA are required to precisely determine the origin of the activated K-ras oncogenes in these TNM-induced lung tumors.

Although gene amplification and chromosomal translocation have been observed in several types of human tumors, these activating mechanisms have not been extensively observed or studied in spontaneous or chemically induced rodent tumors. Sawey et al. (20) did observe c-myc gene amplification and restriction polymorphisms in addition to activated K-ras genes in rat skin tumors induced by ionizing radiation. Quintanilla et al. (21) suggested that amplification of the mutated H-ras gene may be involved in the progression of mouse skin papillomas to carcinomas. Further studies are required to determine the possible role of chemicals and radiation in the activation of proto-oncogenes by gene amplification, chromosomal translocation, and other mechanisms that can alter gene expression.

Carcinogen-induced rodent tumor models may be useful in determining the temporal activation of oncogenes in tumor development. Evidence in several animal studies suggests that activation of the ras proto-oncogene is an early event. The activated ras gene has been detected in many benign tumors, including mouse skin papillomas, mouse lung and liver adenomas, and basal cell and clitoral gland tumors of the rat. This implies that the activated ras was present in the cell that clonally expanded to these benign tumors. In addition, it was recently shown that mouse epidermal cells injected...
in vivo with the viral Ha-ras gene can be promoted with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to papillomas (22). Thus, activation of the ras proto-oncogene may be the initiation event in some model systems. Moreover, dormant initiated cells with the activated ras gene can survive surrounded by normal cells until stimulated to proliferate by some endogenous or exogenous agent.

Implications for Risk Analysis

The number of proto-oncogenes that must be activated in order to convert a normal cell into one that is tumorigenic is unknown at present. However, there is increasing evidence that the transformation of a normal cell into a tumorigenic cell involves the activation and concerted expression of several proto-oncogenes as well as, perhaps, the inactivation of suppressor genes. Continued research on mechanisms of oncogene activation in animal and in vitro models may provide new insights into several long-standing problems in chemical carcinogenesis and risk analysis of carcinogenesis data.

Oncogene analysis on tumors from long-term carcinogenesis studies are employed to help identify potential human carcinogens can be useful in several ways. The analysis can help identify chemicals that can activate proto-oncogenes in vivo to cancer-causing genes. Model systems very susceptible to chemically induced tumors, such as the B6C3F1 mouse liver tumors, appear to be suited for this purpose (23). The classification of chemicals as initiators, promoters, complete carcinogens, etc., may become clearer as we better understand the sequential requirements for activation of oncogenes in the various animal model and cell culture systems. In particular, comparison of patterns of oncogene activation in spontaneously occurring and chemical-induced tumors should assist in determining mode(s) of action of a carcinogen. Low-dose and species-to-species extrapolation of risk from carcinogenic data may become more reliable from examination of oncogene activation and expression in animal model systems for carcinogenesis. These and similar approaches to explore the mechanisms by which chemicals induce tumors in animal model systems may remove some of the uncertainty in risk analysis of rodent carcinogenic data.

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