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Chemical Induction of Oncogene Mutations and Growth Factor Activity in Mouse Skin Carcinogenesis

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The goal of understanding the molecular basis of human tumor development has been greatly facilitated by the use of animal model systems in which the etiology of tumor development can be carefully controlled. Environmental chemicals, either naturally occurring or artificially produced, are thought to make a major contribution to the human tumor burden. Many of the concepts of multistage carcinogenesis have been developed and refined using the mouse skin model system and the work described in this article has been carried out in an attempt to analyze the molecular changes that are associated with the initiation of tumor development, the selection of initiated cells to form papillomas, or the progression of premalignant tumors to carcinoma. We have analyzed a number of skin tumors induced in mice by a two-stage initiation and promotion protocol and have detected a high frequency of c-*ras* oncogene mutations in this system. The mutation found in each case correlates well with the known reactivity of the carcinogens used. It has also been shown that where *ras* activation occurs this represents an early event in the tumor model system. Transforming growth factor β is induced in mouse skin by tumor promoter treatment and may therefore play a role in the selection of initiated cells to form papillomas. Additional events, some of which involve the loss of normal *ras* alleles and possibly tumor suppressor genes, appear to take place at a later stage of carcinogenesis.

Introduction

A great deal of information has been accumulated on the genetic toxicology of chemical carcinogens present in the environment. Some of these chemicals give rise to specific DNA modifications and consequently induce mutations in cellular DNA. However, the nature of such events and the specific biological responses that are induced in exposed cells are still unclear.

Animal model systems have been very useful for studies of these questions. A wealth of information has been obtained on the processes of initiation, promotion, and progression using the mouse skin model system (*1*), which we have employed in studies on the molecular events associated with the different stages of carcinogenesis. Most of the work to be described will be related to mechanisms of initiation, but further events in promotion and progression will also be discussed.

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Initiation Protooncogenes as Targets for the Action of Chemical Carcinogens

The critical biological targets for mutagenic action of chemical carcinogens were unknown until the discovery of cellular protooncogenes. These have been shown to play an important role in the control of cell growth and differentiation (*2*) and may be capable of activation by environmental chemicals or radiation (*3*). Different genetic changes have been shown to activate protooncogenes to an oncogenic state, including single point mutations, gene amplification, translocations, or deletions (*4*).

ras Gene Activation

The individual members of the *ras* family of protooncogene known as Harvey-(Ha), Kirsten-(Ki), and N-*ras* are found to be activated in 10 to 40% of human tumors

(5). Our previous experiments on the role of oncogenes in mouse skin carcinogenesis showed that a member of this family (Ha-*ras*) was reproducibly activated by point mutations in both papillomas and carcinomas initiated with dimethylbenz[a]anthracene (DMBA) (6–8).

The Has-*ras* was also found to be mutated in chemically induced rat mammary gland (9), while either the N-*ras* or K-*ras* gene was activated in rat thymic lymphomas (10). Some tissue specificity of *ras* activation is also found in human tumors. The Ha-*ras* gene has been found most frequently activated in squamous carcinomas and melanomas, whereas N-*ras* is found predominantly in tumors of hematopoietic cells. The Ki-*ras* is activated in a wide spectrum of malignancies of different types but shows a very high prevalence for activation in human colon tumors (5). Almost all the activated forms observed were by point mutation in the coding sequence of *ras* genes, with hotspots located at codons 12, 13, and 61 (11).

The reasons for specific activation of members of the *ras* family in different tissues are unclear. One possibility is that specific mutagenesis takes place in one member of the *ras* family in a particular tissue. Alternatively, each *ras* gene may be equally susceptible to mutation, but tissue-specific selection mechanisms may operate to encourage the outgrowth of a cell containing a particular mutation. The activation pattern seen could also be due to varying transcript levels in different tissues (12). This seems unlikely to be the explanation, however, since epidermal cells express higher levels of Ki-*ras* transcripts than Ha-*ras* transcripts (unpublished results), but only the latter is found to be mutated in tumors.

Transcriptional Control of the Mouse Ha-*ras* Gene

The mouse Ha-*ras* gene has been demonstrated to have an important role in skin carcinogenesis. However, some tumors of either animal or human origin do not have point mutations in the structural gene but rather show increased expression of the normal protooncogene product (13). We therefore undertook a study of the transcriptional control elements of this gene, as these elements could be important alternative targets for chemical mutagenesis.

A genomic clone of the normal Ha-*ras* gene (λN1-*ras*) was obtained and restriction mapped. Full details of this sequence will be published elsewhere (14). Figure 1 shows the sequenced region of λ1-*ras* and indicates that the clone contains the first two coding exons of the Ha-*ras* gene, but lacks exons 3 and 4. The sequenced data of the clone has been compared to that of both rat (15) and human (16).

The presence in λN1-*ras* of the first exons together with 20 the kb upstream sequence enabled us to investigate the transcriptional control of this gene in some detail. A combination of nucleotide sequencing, primer extension analysis and S1 mapping was used to show that initiation of transcription starts at the three sites shown in Figure 1. The upstream region containing the transcription start sites (exon-1) is highly conserved between

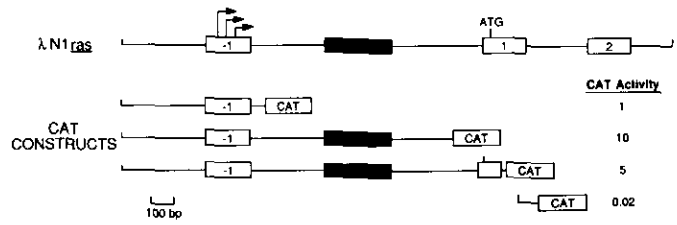

FIGURE 1. The structure and promoter activity of the mouse Ha-*ras* gene 5′ flanking sequence. Primer extension analysis and S1 mapping show three transcription start sites (arrows) in exon-1. The shaded box represents a sequence within the first intron that is highly conserved between mouse and human DNA. The open boxes represent either coding (1 and 2) or noncoding (−1) exons. The CAT enzyme activities obtained with three constructs have been compared. The first one showed significant enzyme activity compared to the promotorless gene. The inclusion of the first intron increased promoter activity by 5- to 10-fold.

mouse and human and contains several potential SP1 (promoter-specific factor) binding sites, similar to the promoter regions of a number of cellular housekeeping genes (17).

The promoter activity of this sequence was directly tested by fusion to a promoterless chloramphenicol-acetyl-transferase gene (CAT) followed by transfection into 208F fibroblast cells and an assay for CAT enzyme activity. The results are in agreement with the promoter activity found in the exon-1 region of the human gene (18,19). Interestingly, the inclusion of the first intron in the fusion construct increased substantially the promoter activity of the exon-1 region (Fig. 1), suggesting that a previously undetected enhancer element may be present within this intron (14). A possible candidate for this enhancer element could be a highly conserved sequence of about 300 bp found in the first intron (14).

To date, single point mutations within the coding exon seem to be the only differences detectable that confer dominant transforming activity. It remains possible that additional mutation may be found within the putative enhancer region or within the housekeeping promoter region, which could lead to elevated expression in some tumors.

Carcinogen-Specific Mutations in the Ha-*ras* Gene

A single treatment with a variety of carcinogens (Table 1) can accomplish the initiation of mouse skin carcinogenesis. The list of initiators includes both direct-acting alkylating agents and derivatives requiring metabolic activation such as polycyclic aromatic hydrocarbons and heterocyclic aromatic amines. Chemicals of these two classes are known to induce different types of point mutations by forming specific promutagenic lesions with the DNA. *N*-methyl-*N*-nitro-nitrosoguanine (MNNG) causes GC to AT transitions by miscoding during DNA replication (19). Bulky adducts formed by polycyclic hydrocarbons (20) and heterocyclic aromatic amines such as 4-nitroquinoline-1-oxide (4-NQO) (21,22) cause predominantly transversion and transition mutations. respectively. We have therefore investigated the types of on-

Table 1. Initiators and promoters of mouse skin carcinogenesis.

Initiators	Promoters
Benzo[a]pyrene	Those that bind, protein kinase C
Dimethylbenzanthracene	12-*O*-Tetradecanoylphorbol-13-acetate
Methylcholanthrene	Dihydroteleocidin
N,*N*'-Dimethylnitrosourea	Others
N-Methyl-*N*-nitro-nitrosoguanidine	Phenol
4-Nitroquinoline-1-oxide	Anthralin
β-Propiolactone	Chrysarobin
bis(Chloromethyl) ether	Skin wounding

cogene mutations that can be found in tumors initiated with these different mutagens. If the oncogene mutations detected have the correct carcinogen specificity, this would provide strong evidence that the mutation takes place during initiation by direct interaction between the carcinogen and the oncogenic DNA.

The main conclusion obtained from the results (Table 2) is that the type of Ha-*ras* mutation seen is dependent upon the initiating agent used. An AT→TA transversion mutation is seen in a very high proportion of the tumors initiated with DMBA. A substantial proportion of the MNNG-induced tumors exhibited the GC to AT transition predicted on the basis of the known mechanisms of action of this carcinogen (*19*). This mutation had not been seen in any tumors initiated with the polycyclic hydrocarbons, but has been found with 4-NQO (B. Bailleul and A. Buchman, unpublished data).

In contrast, no effect on the mutations has been observed by using different promoting agents. These results therefore support the conclusion that the *ras* mutations are induced at an early stage or carcinogenesis (*7*) and probably at the time of initiation (*8,9*).

Promotion: Molecular Events in Skin Tumor Promotion

Tumor promotions elicit a wide range of morphological and biochemical changes when applied to cells in culture or to mouse skin *in vivo* (*1*). These include the induction of the synthesis of RNA, DNA, and protein that accompanies the phases of cellular proliferation and differentiation. An important advance in our understanding of tumor promotion was the recognition of a cellular receptor for 12-*O*-tetradecanoylphorbol 13-acetate (TPA) which copurified with protein kinase C (*23*). Activation of this kinase by TPA is responsible for initiation of a complex chain of events, which in mouse skin culminates in the selection of initiated cells from the background of normal cells. Yuspa and co-workers have postulated that resistance to terminal differentiation induced by the tumor promoter TPA is an important factor in initiated cell selection (*24*).

The endogenous inducers of epidermal differentiation are unknown. We have investigated the early effects of TPA on the temporal and spatial expression patterns of the gene encoding TGF-β-1 (TGF-β), a negative growth regulator that has been shown to affect the growth and differentiation properties of epithelial cells *in vitro* (*25,26*). It has previously been postulated that TGF-β may play a role in tumor promotion (*27*), but the main source of TGF-β was thought to be platelets or macrophages released during the process of inflammation or wounding (*28*). Northern blot analysis using a cloned TGF-β probe (*29*) has now shown that this growth factor is highly induced in mouse epidermis by treatment with the tumor promoter TPA (*31*). Furthermore, *in situ* hybridization to tissue sections shows that the TGF-β expression takes place predominantly in the differentiating epidermal cells (*30*). TGF-β mRNA is also induced in the hair follicles by TPA treatment, again in cells that are undergoing terminal differentiation.

Table 2. Ha-*ras* gene activation in mouse skin tumors.

Tumor	Carcinogen[a]	Promoter	No. analyzed	Ha-*ras* activation	Mutation
Papilloma	DMBA	TPA	42	39	$A^{182}\rightarrow T$
				1	Codon 61
Papilloma	DMBA	Chrysarobin	5	5	$A^{182}\rightarrow T$
Carcinoma	DMBA	TPA	13	10	$A^{182}\rightarrow T$
				1	$G^{35}\rightarrow T$
Carcinoma	DMBA	DMBA	8	4	$A^{182}\rightarrow T$
Papilloma	MCA	TPA	5	2	$A^{182}\rightarrow T$
Carcinoma	MCA	TPA	7	1	Codon 12/13
Papilloma	BaP	TPA	2	1	$G^{35}\rightarrow T$
Carcinoma	BaP	TPA	3	1	$G^{35}\rightarrow T$
Papilloma	MNNG	TPA	14	11	$G^{35}\rightarrow A$
Carcinoma	MNNG	TPA	9	1	$G^{35}\rightarrow A$
Papilloma	4-NQO	TPA	4	2	$G^{35}\rightarrow A$

[a]Abbreviations: DMBA, dimethylbenzanthracene; MCA, methylcholanthrene; BaP, benzo[a]pyrene; MNNG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; 4-NQO, 4-nitroquinoline-1-oxide.

These results provide a possible explanation for the selection of initiated cells by TPA treatment. If initiated cells have a block in the growth inhibitory or differentiation responses to TGF-β, this could provide a selective advantage each time the TPA treatment is carried out. However, the process of promotion is undoubtedly complex and probably involves the action of both positive and negative growth regulators. A series of experiments is in progress to test these hypotheses and consequently elucidate the role played by growth factors in tumor promotion.

Chemicals in Tumor Progression

There is evidence for oncogene involvement in progression of the tumorigenic phenotype from studies on human tumors. In the late stages of development of human neuroblastomas, one of the *myc* gene family members is amplified, and the degree of amplification can be correlated with prognosis (*31*). In the mouse skin model system, changes in addition to single point mutation in *ras* genes seem necessary for tumor progression, since many papillomas with such mutations regress spontaneously.

Malignant conversion of mouse skin tumors is increased by treatment of the papillomas with chemicals such as MNNG or 4-NQO (*32*), but not by treatment with the tumor promoter TPA. These two compounds have been used to induce tumorigenic progression of human epidermal keratinocytes previously immortalized by adenovirus 12 and simian virus 40 (*33*).

Amplification of the Ha-*ras* gene locus may play a role in progression by increasing the expression level of the mutated *ras* allele (*8*). Other results have recently been obtained showing that the normal allele was absent from carcinomas that had amplified a mutated Ha-*ras* allele (unpublished results from this laboratory). A bias toward transcription of the mutant *ras* allele has been observed previously in human carcinoma cell lines (*16,34*).

Loss of anti-oncogenes or tumor suppressor genes may constitute a very important step in tumor progression, and it is possible that the karyotypic changes very often found in the late stage of carcinogenesis involve loss or inactivation of such sequences. Since the chemical agents that induce progression most efficiently are not the best initiators, it is tempting to speculate that qualitatively different events are involved. For example, DMBA is a very strong initiating agent, presumably since it efficiently induces point mutation in the coding sequence of Ha-*ras*, but is a relatively poor progressing agent. Other polycyclic aromatic compounds and 4-NQO are weak initiators but relatively good progressors, because they induce a higher conversion rate of papilloma to carcinomas (*35*; T. J. Slaga, personal communication).

One of the most effective progressing agents in mouse skin is 4-NQO, which has been studied in a forward mutagenesis in *E. coli* as previously used with aminoacetoxy fluorene (AAF) (*36*). This study showed that the main adduct formed by 4-NQO on the N-2 of the guanine (*22*) induced frameshift mutations in a hotspot sequence which is GC rich (M. H. Loucheux-lefebvre, personal communication). If similar frameshift mutations are seen in mammalian cells, these might be expected to lead to inactivation of genes rather than to activation of transforming genes by single point mutations.

In conclusion, the quantitative differences in the abilities of chemical mutagens to induce progression may reflect qualitative differences in their mechanisms of action. Certain agents may be more efficient progressors because the mutations they induce are more likely to delete or inactivate genes rather than cause point mutations that are normally associated with initiating capacity. The identification and cloning of genes involved in late stages of carcinogenesis will provide new approaches to analysis of the diverse mechanisms by which environmental carcinogens induce neoplastic changes.

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REFERENCES

1. Hecker, E., Fusenig, N. E., Kunz, W., Marks, F., and Thielman, H. W., Eds. Cocarcinogenesis and Biological Effects of Tumor Promoters, Vol. 7. Raven Press, New York, 1982.
2. Bishop, J. M. Cellular oncogenes and retroviruses. Annu. Rev. Biochem. 52: 301–354 (1983).
3. Klein, G., and Klein, E. Oncogene activation and tumor progression. Carcinogenesis 5: 429–435 (1984).
4. Weinberg, R. A. The action of oncogenes in the cytoplasm and nucleus. Science 230: 770–776 (1985).
5. Bos, J. L. The *ras* gene family and human carcinogenesis. Mutat. Res. 3: 255–271 (1988).
6. Balmain, A., and Pragnell, I. B. Mouse skin carcinomas induced in vivo by chemical carcinogens have a transforming Harvey-ras oncogene. Nature 303: 72–74 (1983).
7. Balmain, A., Ramsden, M., Bowden, G. T., and Smith, J. Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. Nature 307: 658–660 (1984).
8. Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature 322: 78–80 (1986).
9. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D., and Barbacid, M. Direct mutagenesis of Ha-ras1 oncogene by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315: 382–386 (1985).
10. Guerrero, I., and Pellicer, A. Mutational activation of oncogenes in animal model systems of carcinogenesis. Mutat. Res. 185: 293–308 (1987).
11. Barbacid, M. *ras* genes. Annu. Rev. Biochem. 56: 779–827 (1987).
12. Leon, J., Guerreo, I., and Pellicer, A. Differential expression of the *ras* gene family in mice. Mol. Cell. Biol. 7: 1535–1540 (1987).
13. Tanaka, T., Slamon, D. J., Battifora, H., and Cline, M. J. Expression of P21 *ras* oncoproteins in human cancers. Cancer Res. 46: 1465–1470 (1986).
14. Brown, K., Bailleul, B., Ramsden, M., Fee, F., Krumlauf, R., and Balmain, A. Isolation and characterisation of the 5′-flanking region of the mouse C-Harvey-ras gene. Molec. Carcinog., in press.
15. Damante, G., Filetti, S., and Rapoport, B. Nucleotide sequence and characterization of the 5′ flanking region of the rat Ha-ras protooncogene. Proc. Natl. Acad. Sci. (U.S.) 84: 774–778 (1987).
16. Capon, D. J., Chen, E. Y., Levinson, A. D, Seeburg, P. H., and Goeddel, D. V. Complete nucleotide sequence of the T24 human bladder carcinoma oncogene and its normal homologue. Nature 302: 33–37 (1983).

17. Dynan, W. S., and Tijan, R. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature 316: 774-778 (1985).
18. Ishii, S., Merlino, G. T., and Pastan, I. Promoter region of the human Harvey-ras proto-oncogene: Similarity to the EGF receptor proto-oncogene promotor. Science 230: 1378-1381 (1985).
19. Eadie, J. S., Conrad, M., Toorchen, D., and Topal, M. D. Mechanism of mutagenesis by O^6-methylguanine. Nature 308: 201-203 (1984).
20. Eisenstadt, E., Warren, A. J., Porter, J., Atkins, D., and Miller, J. H. Carcinogenic epoxides of benzo[a]pyrene and cyclopenta[cd]pyrene induce base substitutions via transversions. Proc. Natl. Acad. Sci. (U.S.) 79: 1945-1949 (1982).
21. Bailleul, B., Galiegue, S., and Loucheux-lefebvre, M. H. Adducts from the reaction of O,O'-diacetyl or O-acetyl derivatives of the carcinogen 4-hydroxyaminoquinoline 1-oxide with purine nucleosides. Cancer Red. 41: 4559-4565 (1981).
22. Galiegue-Zouitina, S., Bailleul, B., Ginot, T. M., Perly, B., Vigny, P., and Loucheux-lefebvre, M. H. N^2-Guanyl and N^6-adenyl arylation of chicken erythrocyte DNA by the ultimate carcinogen of 4-nitroquinoline 1-oxide. Cancer Res. 46: 1858-1863 (1986).
23. Castagna, M., Takai, T., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, T. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257: 7847-7851 (1982).
24. Yuspa, S. H., and Morgan, D. L. Mouse skin cells resistant to terminal differentiation associated with initiation of carcinogenesis. Nature 293: 72-74 (1981).
25. Jetten, A. N., Shirley, J. E., and Stoner, G. Regulation of proliferation and differentiation of respiratory tract epithelial cells by TGFβ. Exp. Cell Res. 167: 539-549 (1986).
26. Masui, T., Wakefield, L. M., Ledner, J. F., LaVeck, M. A., Sporn, M. B., and Harris, C. C. TGF-β is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. Proc. Natl. Acad. Sci. (U.S.) 83: 2438-2442 (1986).
27. Parkinson, K. Defective responses of transformed keratinocytes to terminal differentiation stimuli. Their role in epidermal tumor promotion by phorbol esters and by deep skin wounding. Br. J. Cancer 52: 479-493 (1985).
28. Sporn, M. B., Roberts, A. B., Wakefield, L. M., and Assoin, R. K. Transforming growth factor-β biological function and chemical structure. Science 233: 532-534 (1986).
29. Derynck, R., Jarrett, J. A., Chen, E. Y., and Goeddel, D. V. The murine transforming growth factor-precursor. J. Biol. Chem. 261: 4377-4379 (1986).
30. Akhurst, R. J., Fee, F., and Balmain, A. Localised production of TGF-β mRNA in tumour promotor-stimulated mouse epidermis. Nature 331: 363-365 (1988).
31. Alitalo, K., and Schwab, M. Oncogene amplification in tumor cells. Ad. Cancer Res. 47: 235-281 (1986).
32. Hennings, H., Shores, R., Wenk, M. L., Spangler, E. F. Tarone, R., and Yuspa, S. H. Malignant conversion of mouse skin tumour is increased by tumor initiators and unaffected by tumour promotors. Nature 304: 67-69 (1983).
33. Rhim, J. S., Fujita, J., Arnstein, P., and Aaronson, S. A. Neoplastic conversion of human keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. Science 232: 385-387 (1986).
34. Taparowsky, E., Suard, Y., Fasano, D., Shimizu, K., Goldfarb, M., and Wigler, M. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature 300: 762-765 (1982).
35. Scribner, J. D., Scribner, N. K., McKnight, B., and Motlet, N. K. Evidence for a new model of tumor progression from carcinogenesis and tumor promotion studies with 7-bromomethylbenz(a)anthracene. Cancer Res. 43: 2034-2041 (1983).
36. Fuchs, R. P. P., Schwartz, N., and Daune, M. P. Hot spots of frameshift mutations induced by the ultimate carcinogen N-acetoxy-N-2 acetylaminofluorene. Nature 294: 657-659 (1981).