Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6048–6052, August 1986
Genetics

Mutagenesis of the Ha-*ras* oncogene in mouse skin tumors induced by polycyclic aromatic hydrocarbons

(chemical carcinogenesis/carcinomas/DNA transfection/papillomas/restriction fragment length polymorphism)

DIANE BIZUB, ALEXANDER W. WOOD, AND ANNA MARIE SKALKA*

Department of Molecular Oncology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

Communicated by Herbert Weissbach, April 28, 1986

ABSTRACT **The importance of mutational activation of the Ha-*ras* protooncogene in polycyclic aromatic hydrocarbon-induced mouse skin tumors was investigated in a complete carcinogenesis model using repetitive applications of 7,12-dimethylbenz[*a*]anthracene (DMBA), or in an initiation–promotion model using a single application of dibenz[*c*,*h*]acridine (DB[*c*,*h*]ACR) or benzo[*a*]pyrene (B[*a*]P) followed by chronic treatment with phorbol 12-myristate 13-acetate. DNA isolated from carcinomas induced by DMBA or DB[*c*,*h*]ACR, but not by B[*a*]P, efficiently transformed NIH 3T3 cells, and a high percentage of the transformed foci had an amplified Ha-*ras* gene. Restriction enzyme Southern blot analysis and DNA sequencing revealed that the amplified Ha-*ras* genes of the transformants had an A→T transversion in the second position of the 61st codon. The same mutation was also detected in primary tumor DNA in a high percentage of the DMBA- or DB[*c*,*h*]ACR-induced carcinomas. Identification of the mutation in NIH 3T3 cells transformed with DNA from DB[*c*,*h*]ACR-induced benign skin papillomas suggests that it is an early event in skin carcinogenesis. Thus, mutation of the 61st codon of the Ha-*ras-1* gene appears to be a critical step in the formation of mouse skin tumors induced in both of the two models tested. Our analyses also delineate two other classes of hydrocarbon-induced carcinomas—namely, tumors whose DNAs efficiently transform 3T3 cells but do not contain mutated *ras* genes and tumors whose DNAs do not transform 3T3 cells.**

The identification of oncogenes has provided an important conceptual framework for the study of cancer. It has established a genetic basis for the disease, which allows the consolidation of results from studies in viral oncology, chemical carcinogenesis, and other disciplines as well. For chemical carcinogenesis, the oncogenes provide a focus for the evaluation of mutagenic interactions.

Polycyclic aromatic hydrocarbons, like a number of other cancer-causing compounds, have to be metabolized to chemically reactive electrophiles to initiate the carcinogenic process by covalent interaction(s) with DNA (1, 2). Over the last decade, metabolism, mutagenicity, DNA binding, and tumorigenicity studies have established that bay-region diol epoxides† are ultimate carcinogens of at least a dozen polycyclic aromatic hydrocarbons (3). While much is known about the chemical and electronic properties of these diol epoxides that determine their biologic effects, relatively little is known about the critical cellular targets that are involved in polycyclic hydrocarbon-initiated tumors.

To investigate this question, we used mouse skin tumor models (4, 5) in which carcinomas were induced either by repetitive application of a polycyclic hydrocarbon or by single treatment with a hydrocarbon followed by repetitive treatment with the noncarcinogenic phorbol ester tumor promoter phorbol 12-myristate 13-acetate. In both models, tumor development can be easily visualized, tissue is readily accessible, and development of carcinomas is preceded by the appearance of benign papillomas. In the latter model, the initiation and promotion steps are distinct stages in the carcinogenic process and the ratio of papillomas to carcinomas is much higher than in the complete carcinogenesis model. The papillomas and carcinomas produced in both models appear similar by standard pathological criteria.

The presence of a transforming Ha-*ras* oncogene in mouse skin papillomas and carcinomas induced by a single treatment with the polycyclic hydrocarbon DMBA and repetitive treatments with phorbol 12-myristate 13-acetate has been reported by Balmain and coworkers (6, 7). However, the mechanism of activation was not determined. The critical change may not involve overexpression of the cellular protooncogene since Toftgard *et al.* (8) found no increase in RNA levels of Ha-*ras* or seven other protooncogenes in polycyclic hydrocarbon-induced skin tumors. Our initial studies, which included both RNA analysis and protein analysis using monoclonal antibodies to detect the *ras* gene product (to be published elsewhere), are consistent with these findings. Thus, we looked for qualitative rather than quantitative changes in *ras* and used the NIH 3T3 transfection system to facilitate these analyses.

MATERIALS AND METHODS

Treatment Protocols. All skin tumors were induced in female CD-1 mice (Charles River Breeding Laboratories) by topical treatment of the shaved dorsal area with carcinogens dissolved in 200 μl of acetone. Tumors from DMBA treatment were induced by twice weekly treatment with 50 nmol of the carcinogen for 15 weeks. DB[*c*,*h*]ACR- and B[*a*]P-induced tumors were initiated by a single treatment of 200 and 250 nmol of compound, respectively, followed 10 days later by twice weekly treatment with 16 nmol of phorbol 12-myristate 13-acetate for 20–25 weeks. Tumors were excised at least several weeks after final treatment, frozen in liquid nitrogen, and high molecular weight DNA was prepared for analyses.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; DB[*c*,*h*]ACR, dibenz[*c*,*h*]acridine; B[*a*]P, benzo[*a*]pyrene; kb, kilobase(s).

*To whom reprint requests should be addressed.

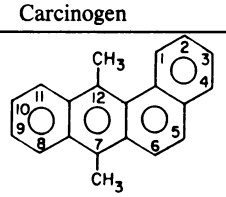
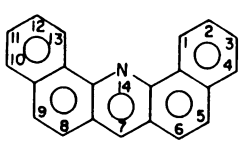
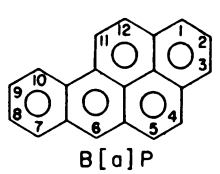
†A bay region is the sterically hindered area of a polycyclic hydrocarbon that results from the juxtaposition of an angular benzo-ring with a nonadjacent benzene ring. The bay regions in 7,12-dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (B[*a*]P) occur between carbon atoms 1 and 12, and 10 and 11, respectively. Dibenz[*c*,*h*]acridine (DB[*c*,*h*]ACR) is a symmetrical molecule and the regions between the nitrogen heteroatom at position 14 and carbon atoms 1 and 13 are equivalent bay regions (see Table 1 for structures and numbering system).

Molecular Cloning and Nucleotide Sequencing of the *Xba* I$^+$-Activated *ras* Gene. The mutant *Bam*HI Ha-*ras-1* fragment from DB[*c,h*]ACR focus 6b (cf. Fig. 4), which was slightly larger and could be distinguished from the endogenous 3.3-kilobase (kb) Ha-*ras* fragment, was used for cloning of the transforming gene. Procedures were as described (9). The vector was plasmid pSP65 DNA (Promega Biotec, Madison, WI) and competent *Escherichia coli* DH5 host cells (Bethesda Research Laboratories) were transformed according to the supplier's directions. The purified recombinant plasmid DNA was digested with *Acc* I, end-labeled with [γ-^{32}P]ATP, and then digested with *Bam*HI. The gel-purified 630-base-pair *Acc* I to *Bam*HI fragment was sequenced according to Maxam and Gilbert (10).

RESULTS AND DISCUSSION

Transfection with Mouse Skin Tumor DNAs. Table 1 shows representative results from transfection assays using DNA from carcinomas induced by each of the three hydrocarbons studied. Comparison with a control DNA isolated from the T24 human bladder carcinoma cell line, known to contain an activated H-*ras* oncogene, shows that both DMBA- and DB[*c,h*]ACR-induced tumors contain oncogenes that transform NIH 3T3 cells with similar efficiency. In contrast, DNA from three independent B[*a*]P-induced carcinomas was not transforming in the NIH 3T3 assay. A few foci were detected several weeks after detection of foci induced by the other DNAs, but their number and appearance was similar to the "spontaneous" foci observed on control plates that had not been exposed to tumor DNA.

Table 1. Transfection with mouse skin tumor DNAs

Carcinogen	Source of transfecting DNA	Foci per μg of DNA
 7,12-DMBA	Carcinoma 3	0.6
	Carcinoma 4	0.3
 DB [c,h] ACR	Carcinoma 6	0.6
	Carcinoma 7	0.1
 B[a]P	Carcinoma 9	0*
	Carcinoma 10	0*
	Carcinoma 11	0*
None	T24 human bladder carcinoma DNA	0.5

NIH 3T3 cells were seeded at 1×10^5 cells per 100-cm^2 plate and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% heat-inactivated calf serum. Cultures were transfected the next day using 20 μg of high molecular weight DNA per plate and the calcium chloride precipitation method (11). After 18 hr, the medium was changed and the cells were maintained on twice weekly changes of DMEM/5% heat-inactivated calf serum. Foci were scored 3 weeks after transfection. At this time, no foci were detected in control cultures not exposed to DNA.
*A few foci were seen 8 weeks after transfection.

Identification of a Diagnostic Restriction Fragment Length Polymorphism. All *ras*-activating mutations detected in DNAs from tumors or tumor cell lines have been mapped to regions in or near the 12th and 61st codons (12–17). To determine whether restriction analysis might be useful, we performed a computer study in which single base changes were substituted systematically at each position in these regions of the mouse Ha-*ras-1* exons. Data for the murine cellular Ha-*ras-1* sequence, which appears to differ from the murine v-Ha-*ras* sequences in only the 12th (AAA = GGA) and 43rd (AAA = GAA) codons (18), were provided by E. P. Reddy (personal communication). The computer study revealed several changes that might be identified by the generation of new restriction sites (Fig. 1*A*). Results of comparison of normal NIH 3T3 DNA with that from a focus transformed by DNA from a DB[*c,h*]ACR-induced carcinoma are shown in Fig. 1*B*. *Bam*HI was used in all cases because it was known that the v-Ha-*ras* probe detects a single 3.3-kb *Bam*HI fragment in mouse DNA (6). The presence of amplified amounts of this fragment in the transformed focus DNA (Fig. 1*B*, compare first two lanes) suggests that transformation resulted from activation of the Ha-*ras-1* gene (6, 23). Of the five additional enzymes used, only *Xba* I revealed a transformation-specific difference. We conclude from their relative intensity that the resultant fragments of $\approx$2.7 and $\approx$0.6 kb are derived from the amplified transforming gene. The normal 3.3-kb fragment, unaffected by *Xba* I, is also detected in this DNA sample. The appearance of the new *Xba* I cleavage site (*Xba* I$^+$) in the activated amplified gene suggests a change from CAA to CTA (Gln to Leu) in the 61st codon of the mouse Ha-*ras-1* gene. A computer scan of the cellular Ha-*ras-1* exons revealed only three other sites in which single base changes might produce new *Xba* I sites. Two of these result in termination codons and can be eliminated from consideration. The third is at codon 36 in exon 1. Restriction analysis (not shown) placed the *Xba* I$^+$ mutation near a known *Acc* I site in exon 2, as expected for codon 61. DNA sequencing of an activated *ras* gene fragment molecularly cloned from a focus obtained from DB[*c,h*]ACR-induced carcinoma DNA showed the predicted base change at the 61st codon (Fig. 2). An A$\rightarrow$T transversion has been detected in the 61st codon in an activated Ha-*ras* oncogene in a human lung carcinoma cell line and in a primary human urinary tract tumor by direct DNA sequencing (15, 24). However, because the sequence in this region is different in the mouse and human genes, the change in human DNA does not produce a new *Xba* I site.

The 61st Codon A$\rightarrow$T Transversion Can Be Detected in Primary Tumor Tissue. Restriction blot analysis of DNA from 11 independent primary carcinomas induced by all three hydrocarbons (Fig. 3) shows that this mutation was readily identified by the appearance of the characteristic 0.6-kb *Xba* I/*Bam*HI band in three of four DMBA and in three of four DB[*c,h*]ACR-induced tumors but in none of the three B[*a*]P-induced tumors analyzed. The latter observation is consistent with our inability to detect a dominant transforming gene in NIH 3T3 transfection assays of the B[*a*]P-induced tumor DNA.

Molecular Analysis of Transformants Induced by DNA from the B[*a*]P, DMBA, and DB[*c,h*]ACR-Induced Carcinomas. Analysis of DNA from individual NIH 3T3 foci obtained after transfection with DNA from several of the tumors represented in Fig. 3 is shown in Fig. 4. DNA from tumors that contained the *Xba* I$^+$ mutation gave rise to foci with amplified Ha-*ras-1* genes that contained the same mutation. For DB[*c,h*]ACR tumor 6, two independent foci were analyzed. Both contained an amplified Ha-*ras-1* gene, but in one of

A

Enzymes	Codon 11 12 13	Amino Acid Codon 12	Rel.Transf. Efficiency[a]
	GCT GGA GGC	Gly	-
PstI	C	Ala	++
XhoI	C	Arg	+++
TaqI	C	Arg	+++

Enzymes	Codon 60 61 62	Amino Acid Codon 61	Rel.Transf. Efficiency[b]
	GGT CAA GAA	Gln	-
TaqI	G	Arg	++
AvaII	C	Pro	-
XbaI	T	Leu	+++

B

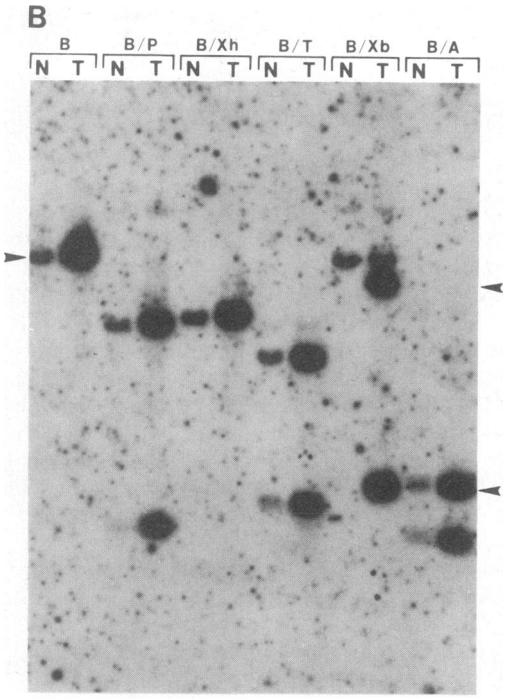


FIG. 1. Diagnostic restriction endonuclease digestions of DNA from NIH 3T3 cells transformed by DNA from a DB[*c,h*]]ACR-induced carcinoma. Fifteen micrograms of DNA from NIH 3T3 cells and NIH 3T3 cells transformed with DNA from a DB[*c,h*]ACR-induced carcinoma were digested with a 5-fold excess of the indicated restriction enzymes for 1 hr at 37°C. After electrophoresis in 1% agarose, the DNA was transferred to Biodyne A filters (Pall Products, Vauxhall, NJ) as described by Southern (19). The 500-base-pair v-Ha-*ras* insert of pBS9 (20) was nick-translated to a specific activity of 2×10^8 cpm/μg as described in the Amersham nick-translation kit. Hybridization was at 42°C for 48 hr with 1×10^7 cpm per ml of nick-translated probe under stringent conditions. Filters were covered with clear plastic wrap and exposed to Kodak XAR5 film with a Lightning Plus screen for 18 hr at 70°C. (*A*) New restriction sites predicted by the indicated changes in the regions of the 12th and 61st codons. Regions included in our computer scan extended from codons 10–15 and 56–64. Relative transforming efficiencies predicted for genes with the indicated changes are extrapolated from results of (a) Seeburg *et al.* (21) using rat-1 cells as recipients and (b) Der *et al.* (22) using NIH 3T3 recipients. (*B*) Digestion patterns for NIH 3T3 cells (N), and NIH 3T3 transformant (T). Arrow on left marks position of the 3.3-kb *Bam*HI fragment; arrows on right mark positions of the 2.7- and 0.6-kb *Bam*HI/*Xba* I fragments. B, *Bam*HI; B/P, *Bam*HI/*Pst* I; B/Xh, *Bam*HI/*Xho* I; B/T, *Bam*HI/*Taq* I; B/Xb, *Bam*HI/*Xba* I; B/A, *Bam*HI/*Ava* II.

these (6b) it was included in a larger *Bam*HI fragment. Two *ras* fragments are also produced after *Bam*HI/*Xba* I digestion of this DNA. Since one of them is the same size as in the parental DNA, we conclude that only the upstream *Bam*HI

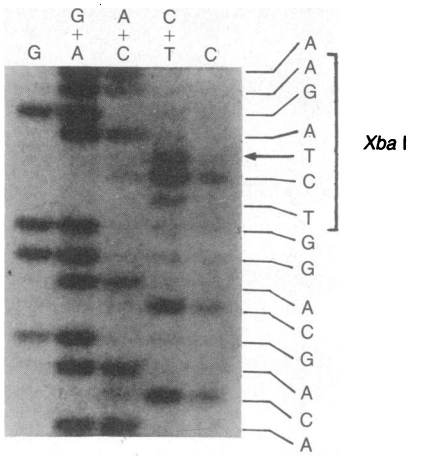


FIG. 2. Nucleotide sequence in the region of the *Xba* I$^+$ mutation of the activated *ras* gene in DNA from a DB[*c,h*]ACR-induced carcinoma. A section of the sequencing gel from codon 58 (bottom) to 62 (top) is shown. Arrow indicates position of the A→T transversion.

site was changed, presumably as a result of rearrangement during transfection. DNA from the DMBA tumor 4 and the DB[*c,h*]ACR tumor 7, both of which lacked the *Xba* I$^+$ mutation, gave rise to foci that also lacked this mutation. In addition, the foci were different in appearance from the *Xba* I$^+$ foci; they were less refractile and had a greater tendency to remain attached to the culture dish. Because there was also no evidence for an amplification of the Ha-*ras* gene, or of N- or K-*ras* genes in these cases (data not shown), it is possible that a different dominant transforming oncogene was activated in the primary tumor. This possibility is being inves-

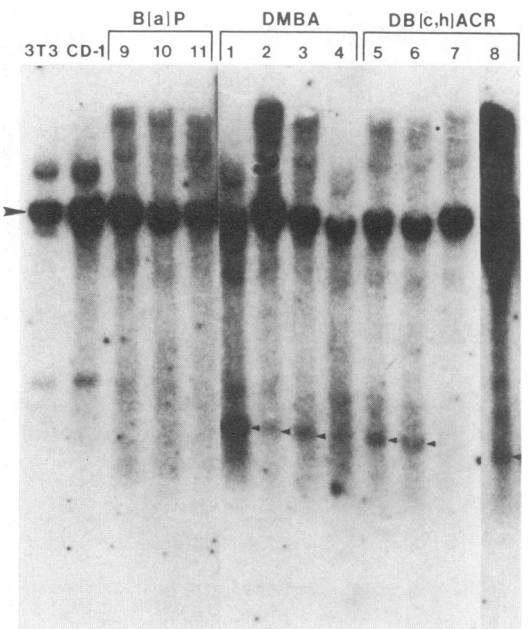


FIG. 3. Restriction endonuclease analysis of DNA from carcinomas induced by three polycyclic aromatic hydrocarbons. After isolation, DNA (20 μg) was digested with *Bam*HI and *Xba* I and analyzed as described in Fig. 1. The filter was exposed to Kodak XAR5 film for 4 weeks with a Lightning Plus screen. Lanes: 1, NIH 3T3 DNA; 2, normal CD-1 mouse skin DNA; 3–5, B[*a*]P-induced carcinomas (nos. 9–11); 6–9, DMBA-induced carcinomas (nos. 1–4); 10–13, DB[*c,h*]ACR-induced carcinomas (nos. 5–8). Arrow on left shows position of the 3.3-kb *Bam*HI fragment. Small arrows in the figure show position of the 0.6-kb *Xba/Bam* fragment.

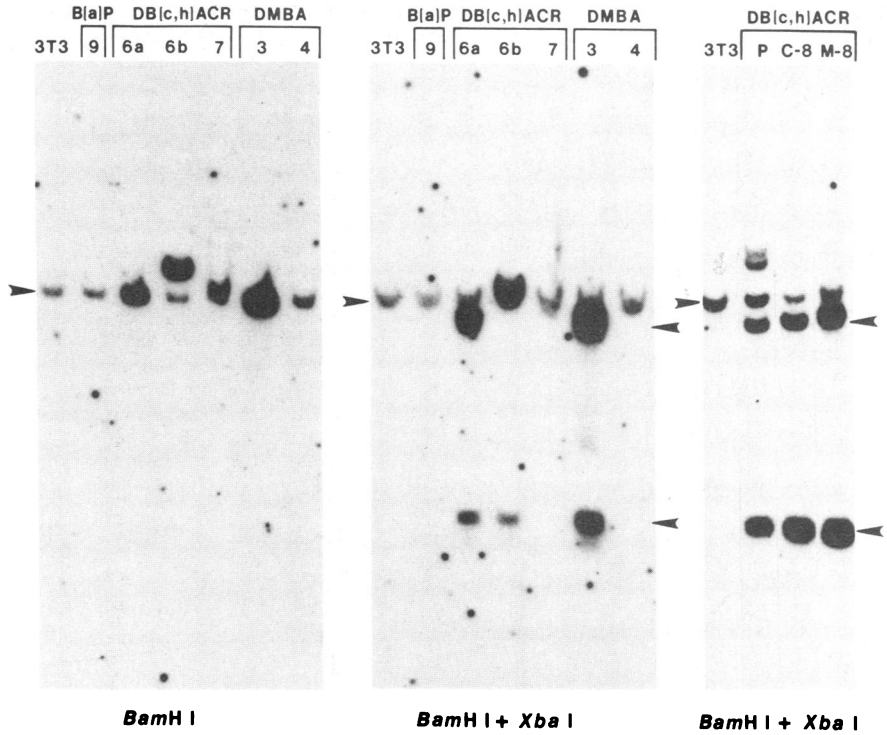


FIG. 4. Restriction endonuclease analysis of DNA from NIH 3T3 foci obtained by transfection with DNA from papillomas or carcinomas induced with three polycyclic hydrocarbons. Transformed foci were picked and expanded to mass culture and the extracted DNA was analyzed as described in Fig. 1. (*Left* and *Center*) Terminology is explained in Fig. 3 (6a and 6b were two separate foci). (*Right*) DNA for the NIH 3T3 cell transfections was obtained from a pool of skin papillomas (lane P), a skin carcinoma (lane C-8), and a mammary carcinoma (lane M-8) obtained from the same mouse bearing the skin carcinoma. Arrows on left of each panel mark the position of the 3.3-kb *Bam*HI fragment; arrows on right show the positions of the 2.7- and 0.6-kb *Bam*HI/*Xba* I fragment.

tigated further. From our inability to detect the *Xba* I^+ mutation or amplification of H- (or N- or K-) *ras* genes in "spontaneous" foci picked from the B[*a*]P DNA-treated cultures, we conclude that the B[*a*]P-induced tumors analyzed did not contain a dominant NIH 3T3 transforming gene. This result was somewhat unexpected because it has been reported recently (25, 26) that the human Ha-*ras*-1 cellular gene can be activated by mutation at the 12th and 61st codons after *in vitro* modification of the cloned DNA with the carcinogenic bay-region diol epoxide diastereomer of B[*a*]P (27, 28). We conclude that some aspect of the *in vivo* reaction modifies this activity, and that there is more than one pathway for oncogenesis in this model.

Restriction Analysis of Other Tumor DNAs. Fig. 4 (*Right*) shows a comparison of results from restriction analysis of DNA from foci transformed with DNA from a pool of three DB[*c,h*]ACR-induced papillomas, a DB[*c,h*]ACR-induced skin carcinoma, and a mammary carcinoma that arose in the same mouse as the skin carcinoma. The appearance of the *Xba* I^+ mutation at the papilloma stage is consistent with previous results with DMBA-induced papillomas (7), which suggested that *ras*-activation is an early event in oncogenesis induced by these agents. Since mammary tumors are rarely seen in the skin tumor model system, it seemed possible that the carcinoma obtained in this instance was a spontaneous random event. Our demonstration of the characteristic *Xba* I^+ mutation suggests that this tumor was either induced directly by DB[*c,h*]ACR or arose as a result of metastasis from one of the skin tumors. Unfortunately, histological analyses were not performed on this tumor so that this question could not be investigated further. It seems apparent, nonetheless, that such molecular analyses should provide a useful tool for diagnostics. Table 2 summarizes results obtained with all of the tumors included in this study.

Comparison of Results with Other Systems. Recent results with a rat mammary carcinoma model have demonstrated that the carcinogen nitroso-*N*-methylurea can activate the Ha-*ras-1* gene via a G→A transition in the 12th codon (29). This mutation is also presumed to be an early event since nitroso-*N*-methylurea has a very short half-life in biological systems and malignant neoplasia do not appear until 6–12 months after the single administration of the carcinogen. The relevance of *ras* activation to oncogenesis in this rat system was recently verified by the finding that mammary carcinomas induced by DMBA also contained a *ras* mutation. The DMBA mutation apparently involved the 61st codon (30) but its exact nature was not determined. It seems probable that the mutagenic selectivity of DMBA is the same in these two systems.

The Frequency of the 61st Codon Mutation Is Consistent with the Chemical Properties Known for Some of the Polycyclic Hydrocarbons. The covalent adducts formed between DNA and the ultimate carcinogenic metabolites of B[*a*]P and DMBA have been studied extensively (cf. refs. 3 and 31 and refs. therein) and significant differences have been observed that are relevant to the results of the present study. In mouse embryo cell cultures and in mouse skin, binding of DMBA and B[*a*]P to DNA is primarily a property of bay-region diol epoxide metabolites. In the case of B[*a*]P, adducts to the 2-amino group of guanine predominate, although minor amounts of deoxyadenosine adducts have been observed. In contrast, with DMBA deoxyadenosine adducts constitute a major covalent modification of DNA (32, 33). Thus an A→T transversion is consistent with the DNA binding selectivity of the relevant metabolites of DMBA but not of B[*a*]P. It is also noteworthy that a higher frequency of transversions involving guanine, as compared to adenine residues, has been observed in studies of nonsense mutations induced by the bay-region diol epoxide of B[*a*]P in the *lacI* gene of *E. coli* (34). Although the tumorigenic activity of DB[*c,h*]ACR has been known for some time (35), it has been found only recently (36, 37) that activation occurs through the formation

Table 2. Summary of analyses

Tumor	NIH 3T3 transfection	*Xba* RFLP Tumor	*Xba* RFLP Foci	"Activated" oncogene
DMBA				
C-1		+		Ha-*ras*-61
C-2		+		Ha-*ras*-61
C-3	+	+	+ (2)*	Ha-*ras*-61
C-4	+	−	− (5)*	Unidentified
DB[*c,h*]ACR				
C-5		+		Ha-*ras*-61
C-6	+	+	+ (2)*	Ha-*ras*-61
C-7	+†	−	− (5)*	Unidentified
C-8	+†	+	+	Ha-*ras*-61
M-8	+		+	Ha-*ras*-61
P(3)*	+	+	+	Ha-*ras*-61
B[*a*]P				
C-9	−	−	− (5)‡	None detected
C-10	−	−	− (5)‡	None detected
C-11	−	−	− (5)‡	None detected

Skin carcinomas, mammary carcinoma, and papillomas are represented by C, M, and P, respectively. Plus (+) in the NIH 3T3 transfection column signifies a value in the same order of magnitude as T24 carcinoma DNA as specified in Table 1. Minus (−) signifies a value indistinguishable from control plates not exposed to DNA. Identification of the activated oncogene is based on *Xba* restriction fragment length polymorphism (RFLP) data, transfection data, or both as described in the text. The 61 after Ha-*ras* indicates the mutated codon surmised or determined by indicated analyses and computer and sequence data described in the text and in Fig. 2.

*Numbers in parentheses indicate the number of tumors (pooled) or the number of individual transformed foci analyzed.

†Foci from I° transfection plates were picked and expanded to mass cultures. DNA from indicated foci were also positive in subsequent II° (CA-7 and -8) and III° (CA-7) transfections.

‡Number of spontaneous foci analyzed from B[*a*]P tumor DNA-treated NIH 3T3 cultures.

of a bay-region diol epoxide. The DNA adducts formed by the ultimate carcinogen have not been examined, but the results of the present study suggest that this nitrogen heterocycle, like the alkyl-substituted hydrocarbon DMBA, may bind preferentially to adenine residues in DNA.

Mutation at the 61st codon of the Ha-*ras-1* appears to be a critical step in formation of mouse skin tumors in both the initiation–promotion model with DB[*c,h*]ACR and the complete carcinogenesis model with DMBA. Because of the reproducibility and ease of detection of this mutation, these models should be of use in evaluating the mechanism and significance of oncogene activation and in the development of diagnostic and therapeutic regimens for neoplasia in which such activation may be critical.

We thank our colleagues, T. Curran, E. P. Reddy, and members of his group for advice and assistance and for communication of unpublished data. We also thank R. E. Lehr and D. M. Jerina and their colleagues for providing us with the sample of DB[*c,h*]ACR, which was synthesized as described (38).We also thank R. Ellis for providing pBSq for isolation of the v-Ha-*ras* probe.

1. Miller, E. C. & Miller, J. A. (1976) in *Chemical Carcinogens*, ed. Searle, C. E. (Am. Chem. Soc., Washington, DC), pp. 737–762.
2. Dipple, A., Moschel, R. C. & Bigger, C. A. H. (1984) in *Chemical Carcinogens*, ed. Searle, C. E. (Am. Chem. Soc., Washington, DC), 2nd ed., pp. 41–163.
3. Thakker, D. R., Yagi, H., Levin, W., Wood, A. W., Conney, A. H. & Jerina, D. M. (1985) in *Bioactivation of Foreign Compounds*, ed. Anders, M. W. (Academic, New York), pp. 177–242.
4. Verma, A. K., Conrad, E. A. & Boutwell, R. K. (1982) *Cancer Res.* **42**, 3519–3525.
5. Slaga, T. J. (1984) in *Mechanisms of Tumor Promotion*, ed. Slaga, T. J. (CRC Press, Boca Raton, FL), pp. 1–16.
6. Balmain, A. & Pragnell, I. B. (1983) *Nature (London)* **303**, 72–74.
7. Balmain, A., Ramsden, M., Bowden, G. T. & Smith, J. (1984) *Nature (London)* **307**, 658–660.
8. Toftgard, R., Roop, D. & Yuspa, S. H. (1985) *Carcinogenesis* **6**, 655–657.
9. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
10. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
11. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52**, 456–467.
12. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. & Chang, E. H. (1982) *Nature (London)* **300**, 143–149.
13. Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. (1982) *Nature (London)* **300**, 149–152.
14. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M. & Wigler, M. (1982) *Nature (London)* **300**, 762–765.
15. Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. & Aaronson, S. A. (1983) *Nature (London)* **303**, 775–779.
16. Bos, J. L., Verlaan-deVries, M., Jansen, A. M., Veeneman, G. H., van Boom, J. H. & van der Eb, A. J. (1984) *Nucleic Acids Res.* **12**, 9155–9163.
17. Fasano, O., Aldrich, T., Tamanoi, F., Taparowsky, E., Furth, M. & Wigler, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4008–4012.
18. Reddy, E. P., Lipman, D., Andersen, P. R., Tronick, S. R. & Aaronson, S. A. (1985) *J. Virol.* **53**, 984–987.
19. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
20. Ellis, R. W., DeFeo, D., Shih, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowy, D. R. & Scolnick, E. M. (1981) *Nature (London)* **292**, 506–511.
21. Seeburg, P. H., Colby, W. W., Capon, D. J., Goeddel, D. V. & Levinson, A. D. (1984) *Nature (London)* **312**, 71–75.
22. Der, C. J., Finkel, T. & Cooper, G. M. (1986) *Cell* **44**, 167–176.
23. Guerrero, I., Calzada, P., Mayer, A. & Pellicer, A. (1984) *Science* **225**, 1041–1043.
24. Fujita, J., Srivastava, S. K., Kraus, M. H., Rhim, J. S., Tronick, S. R. & Aaronson, S. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3849–3853.
25. Marshall, C. J., Vousden, K. H. & Phillips, D. H. (1984) *Nature (London)* **310**, 586–589.
26. Vousden, K. H., Bop, J. L., Marshall, C. J. & Phillips, D. H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1222–1226.
27. Kapitulnik, K., Levin, W., Conney, A. H., Yagi, H. & Jerina, D. M. (1977) *Nature (London)* **266**, 378–380.
28. Kapitulnik, J., Wislocki, P. G., Levin, W., Yagi, H., Jerina, D. M. & Conney, A. H. (1978) *Cancer Res.* **38**, 354–358.
29. Sukumar, S., Notario, V., Martin-Zanca, D. & Barbacid, M. (1983) *Nature (London)* **306**, 658–661.
30. Zarbel, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D. & Barbacid, M. (1985) *Nature (London)* **315**, 382–385.
31. Jeffrey, A. M. (1985) in *Polycyclic Hydrocarbons and Carcinogenesis*, ed. Harvey, R. G. (Am. Chem. Soc., Washington, DC), pp. 187–208.
32. Dipple, A., Pigott, M., Moschel, R. C. & Costantino, N. (1983) *Cancer Res.* **43**, 4132–4135.
33. Dipple, A. & Pigott, M. A. (1985) *Proc. Am. Assoc. Cancer Res.* **26**, 89 (abstr.).
34. Eisenstadt, E., Warren, A. J., Porter, J., Atkins, D. & Miller, J. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1945–1949.
35. Lacassagne, A., Buu Hoi, N. P., Daudel, R. & Zajdela, F. (1956) *Adv. Cancer Res.* **4**, 315–369.
36. Wood, A. W., Chang, R. L., Levin, W., Kumar, S., Shirai, N., Jerina, D. M., Lehr, R. E. & Conney, A. H. (1986) *Cancer Res.* **46**, 2760–2766.
37. Thakker, D. R., Shirai, N., Levin, W., Ryan, D. E., Thomas, P. E., Lehr, R. E., Conney, A. H. & Jerina, D. M. (1985) *Proc. Am. Assoc. Cancer Res.* **26**, 114 (abstr.).
38. Lehr, R. E., Kumar, S., Shirai, N. & Jerina, D. M. (1985) *J. Org. Chem.* **50**, 98–107.