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Detection of mutant Ha-*ras* genes in chemically initiated mouse skin epidermis before the development of benign tumors

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ABSTRACT **An activated Ha-*ras* oncogene has been consistently found in chemically initiated benign and malignant mouse skin tumors, and an activated *ras* oncogene has been shown to initiate the process of mouse skin carcinogenesis. However, the exact timing of mutational activation of the Ha-*ras* gene relative to application of the chemical carcinogen is not known. A sensitive mutation-specific PCR technique was used to experimentally address the timing of Ha-*ras* gene mutational activation. This technique can detect mutant Ha-*ras* alleles in the presence of a very large excess of normal *ras* alleles. Activated Ha-*ras* genes with 61st codon A → T mutations were found in the epidermis of mice 1 week after topical initiation with 7,12-dimethylbenz[*a*]anthracene or urethane by using this assay. These results were confirmed by *Xba* I restriction fragment length polymorphism analysis and direct DNA sequencing. One week after initiation is 1–2 months before the appearance of benign papillomas that harbor activated Ha-*ras* oncogenes when the initiated mice are promoted with the tumor promoter phorbol 12-myristate 13-acetate. Our data support the hypothesis that initiated epidermal cells containing an activated Ha-*ras* gene can remain dormant in the skin until a tumor promoter induces regenerative hyperplasia that allows for outgrowth of these cells with an activated *ras* oncogene to give rise to a benign papilloma.**

Human and animal tumors are thought to develop as a consequence of changes in the structure or control of specific genes. Typically several sequential events are required. The carcinogenic nature of many chemicals has long been associated with their ability to bind DNA and cause somatic cell mutations (1). These lesions appear to occur at certain critical regions in the genome and are thought to affect key functions in initiating neoplastic development. Increasing evidence suggests that conversion of protooncogenes to their oncogenic forms by mutation plays an important role in the development of neoplasia and, thus, points to these genes as critical targets for chemical carcinogens (2, 3).

Point mutations in the ras family of protooncogenes are implicated in the pathogenesis of human cancers, including both hematologic tumors and solid tissue tumors (3, 4). The first evidence that mutational activation of *ras* oncogenes may be important in the early stages of carcinogenesis was provided by the study of premalignant mouse skin papillomas initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by phorbol 12-myristate 13-acetate (PMA) (5). It has since been shown that, with regard to chemically induced skin tumors, the type of carcinogenic agent used for tumor induction strongly correlates with the mutated locus of the *ras* gene (6). *N*-methy-*N*′-nitro-*N*-nitrosoguanidine resulted primarily in mutations involving codon 12 of the Ha-*ras* gene (7), whereas the polycyclic hydrocarbon DMBA (8) and urethane (9) predominantly resulted in mutations of the second position of Ha-*ras* codon 61. There is also some correlation between the nature of the carcinogen DNA adduct formed and the type of activating mutation seen in the Ha-*ras* oncogene (6, 7). These mutations apparently are tumor promoter independent because variation in promoting agent had no effect upon the mutations observed (6). These observations suggest a direct interaction between the initiating carcinogen and the critical *ras* DNA sequence and imply that mutational activation of *ras* oncogenes could be concomitant with initiation of benign and malignant skin tumors.

Because tumors do not develop immediately after carcinogen exposure, mutant *ras* oncogenes appear to remain latent within the target epidermal cells and require additional events to progress toward neoplasia. Support for this proposed mechanism has been provided by Kumar *et al.* (10) by using the rat mammary gland model of tumor formation. The use of high-resolution restriction fragment length polymorphism (RFLP) analysis and the PCR revealed both Ha-*ras* and Ki-*ras* gene mutations in mammary glands 2 weeks after carcinogen treatment and 2 months before the onset of neoplasia. In this rat mammary carcinogenesis model the *ras* oncogene appeared to remain latent within the mammary gland until exposure to estrogens. In the present study, we have addressed the question of whether mutant Ha-*ras* genes can be detected in the epidermis of mice initiated with chemical carcinogens before the appearance of benign papillomas.

MATERIALS AND METHODS

Chemical Initiation of Mouse Skin and Production of Benign and Malignant Mouse Skin Tumors. Female CD-1 mice were initiated with a topical application of DMBA (20 nmol) or urethane (0.7 mmol) dissolved in acetone according to described methods (8, 11). Epidermal DNA was isolated from the skin of animals at 1 day, 3 days, 7 days, and 30 days after initiation. A second group of mice was pretreated with acetone or PMA (8 nmol) 24 hr before chemical initiation. Next, the animals were initiated with DMBA or urethane at the doses described above. Epidermal tissue DNA was isolated 1 week after initiation.

Benign papillomas and malignant squamous cell carcinomas were generated by initiation with either DMBA (20 nmol) or urethane (0.7 mmol) and promotion with twice weekly applications of PMA (8 nmol) as described (8, 11).

Isolation of DNA. Genomic DNA was isolated from mouse skin epidermis, papillomas, and squamous cell carcinomas as described (12).

Oligonucleotide Synthesis and Design. Oligonucleotides, for use as "amplimers" (see Fig. 1), were synthesized on an Applied Biosystems 391 DNA synthesizer. After deprotec-

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Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MSPA, mutation-specific PCR assay; RFLP, restriction fragment length polymorphism; PMA, phorbol 12-myristate 13-acetate.

tion the oligonucleotides were purified by using Nensorb purification columns (13).

The amplimers used for specific amplification of the mouse Ha-*ras* containing a 61st codon A → T mutation were as follows: upstream primer, 5'-CTA AGC CTG TTG TTT TGC AGG AC-3'; downstream primer, 5'-CAT GGC ACT ATA CTC TTC TA-3'. The oligonucleotide amplimers were based on the sequence of the mouse c-Ha-*ras* gene and specified a 110-base-pair (bp) amplification unit (where upstream signifies the 5' amplimer, and downstream signifies the 3' amplimer).

DNA Amplification by Mutation-Specific PCR Assay (MSPA). A modification of the mutation-specific PCR technique described by Ehlen and Dubeau (14) was used in this study. The following modifications to the published technique were used: (*i*) the PCR reaction buffer contained 1.5 mM $MgCl_2$, (*ii*) the PCR reactions contained 100 ng of each amplimer, (*iii*) in some instances the PCR reactions included a ^{32}P-end-labeled upstream MSPA amplimer, (*iv*) *Taq* polymerase was obtained from Boehringer Mannheim. Before amplification, DNA (1 μg per reaction) samples were denatured for 5 min at 95°C. Each amplification cycle consisted of denaturation for 1 min at 95°C, hybridization for 1 min at 55°C, and extension for 1 min at 72°C. After 30 cycles of amplification, there was a final extension for 5 min at 72°C. In each experiment, DNA contamination of PCR reagents was monitored by performing a PCR in absence of added DNA template ("mock reaction"). After completion of the PCR reaction, 10-μl samples of the PCR products were analyzed by 3% agarose gel electrophoresis [2% NuSieve/1% agarose/90 mM Tris/6.4.6 mM borate/2.5 mM EDTA, pH 8.3 (TBE)] and stained with ethidium bromide or autoradiographed.

Direct DNA Sequencing. The 5'-end of upstream MSPA primer was end-labeled with [γ-^{32}P]ATP (specific activity 6000 Ci/mmol; 1 Ci = 37 GBq) with T_4 polynucleotide kinase and used as a primer in direct DNA sequencing of the MSPA product. Excess primer and unincorporated nucleotides were removed by selective isopropanol precipitation (15). The sequencing protocol was done as described by Neri *et al.* (16).

Digestion of Amplified DNA with *Xba* I. Samples (30 μl) of amplified DNA were mixed with 10× reaction buffer (New England Biolabs) and incubated with a 10-fold excess of *Xba* I at 37°C. Samples were analyzed as described above.

RESULTS

Initiation of mouse skin with either DMBA or urethane followed by tumor promotion leads to the development of benign and malignant skin tumors (8, 9). Molecular analysis of these tumors has revealed the presence of an A → T transversion mutation at the 61st codon position of the Ha-*ras* gene. This mutation creates an *Xba* I RFLP (8, 9). To address the temporal relationship between *ras* oncogene activation and the onset of neoplasia, we established a simple and highly sensitive approach for detection of point mutations within *ras* genes. This method, MSPA, is a modification of a procedure described by Ehlen and Dubeau (14). The MSPA is based on the rationale that a DNA primer with a 3' end complementary to a specific gene mutation will only allow chain elongation of mutant DNA sequences during PCR reactions (Fig. 1). We have tested the validity of the MSPA to detect specific 61st codon A → T mutations in the Ha-*ras* gene by using mouse skin tumor DNA samples from animals initiated with either DMBA or urethane. Oligonucleotide primers that allow for specific amplification of the mutant Ha-*ras* gene were used. The downstream primer was designed to be complementary to mutant Ha-*ras* DNA sequences. DNA from the mouse epidermal cell-line Car B is known to have an A → T mutation at the 61st codon of the Ha-*ras* gene (9) and was used as a positive control (Fig. 2). Normal epidermal DNA served as a negative control and did not amplify during the MSPA, nor did the mock reaction used to monitor for contamination of PCR reagents. The DNA from DMBA- and urethane-initiated skin tumors amplified the predicted 110-bp PCR product. The 110-bp MSPA product was seen in six different tumors from each treatment group. These data indicated that the MSPA selectively amplified the mutant sequences from the tumor DNA samples.

Because initiation is thought to be an infrequent event and to occur in a small fraction of cells in the epidermis, when Ha-*ras* activation is assumed to be the initiator, the MSPA must detect an activated Ha-*ras* gene among a vast excess of normal Ha-*ras* alleles. The experimental approach to test sensitivity of the MSPA involved mixing DNA from the Car B carcinoma cell line at various ratios with normal epidermal DNA. These ratios were achieved by serially diluting the Car B DNA and adding solutions containing normal epidermal DNA to achieve the proper weight ratios. In this sensitivity experiment, the MSPA product was radioactively labeled by using a ^{32}P-end-labeled upstream amplimer. The results of a MSPA sensitivity experiment are shown in Fig. 3. Neither the normal epidermal DNA sample nor the mock sample with no added DNA template produced a 110-bp radiolabeled PCR product. A signal was detected up to and including an apparent ratio of one mutant Ha-*ras* allele to 10^8 normal Ha-*ras* alleles. This is an apparent ratio because the mutated Ha-*ras* gene in the Car B cell line is amplified 5- to 10-fold. This cell line does not contain the normal Ha-*ras* allele (9). Thus, the sensitivity of this technique is such that specific point mutations can be detected in epidermal samples, even when only a few cells are affected by such mutations.

Next, we asked how soon after chemical carcinogen initiation of mouse skin could mutant Ha-*ras* genes be detected in the epidermis. Resting mouse skin or mouse skin stimu-

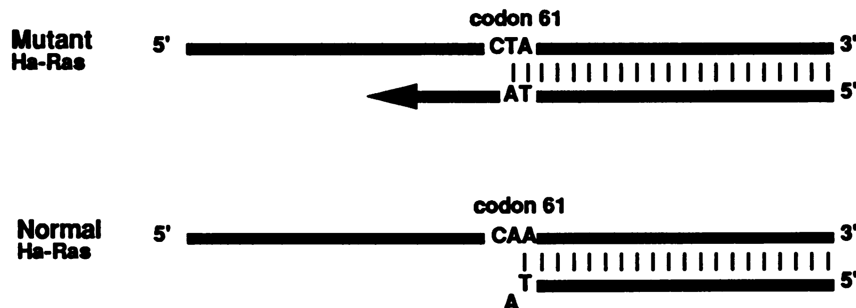

FIG. 1. Strategy for detection of specific point mutations by PCR using mutation-specific primers. Part of the second exon of the Ha-*ras* gene for either the mutant allele (*Top*) or the normal allele (*Bottom*) is represented. Amplification occurs from the mutant allele but not from the normal allele because the 3' end of the oligonucleotide is unpaired for the normal allele.

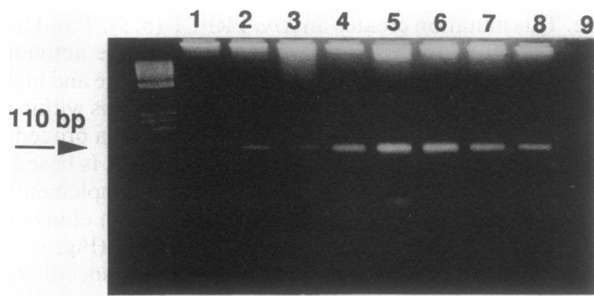

FIG. 2. Detection of mutant Ha-*ras* genes activated by an A → T transition mutation in mouse skin tumors initiated by DMBA or urethane. An ethidium bromide-stained agarose gel separating the MSPA products is shown. Note no amplification of epidermal normal DNA (lane 1), but the 110-bp MSPA products were amplified with DNAs obtained from tumors. The tumors tested were initiated with DMBA (lane 2, DMBA-initiated and PMA-promoted papilloma; lanes 3 and 4, DMBA-initiated and PMA-promoted carcinomas) or urethane (lane 5, urethane-initiated and PMA-promoted papilloma; lanes 6 and 7, urethane-initiated and PMA-promoted carcinomas). Lane 8 contains the positive control, amplified Car B cell-line DNA. Lane 9 is the mock reaction without any added DNA.

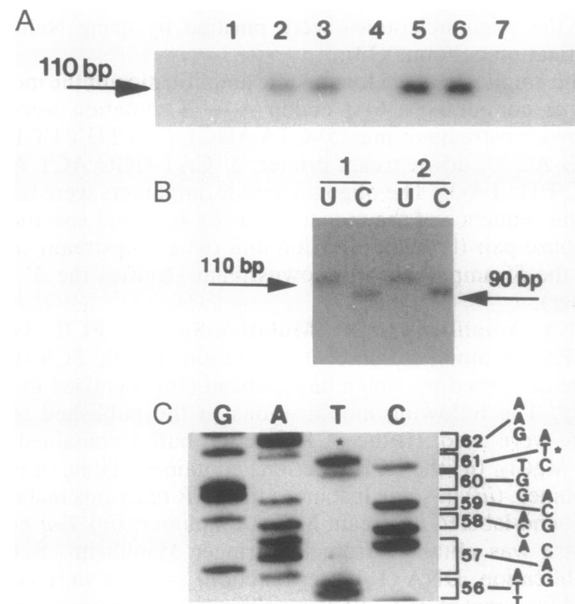

FIG. 4. (*A*) Identification of mutant Ha-*ras* genes in epidermal DNA isolated from the mouse skin treated with DMBA or urethane. An autoradiogram of an agarose gel-separated ^{32}P-labeled MSPA products is shown. The mutant Ha-*ras* gene was detected in epidermal DNA samples from mice initiated with DMBA alone (lane 2) and PMA-pretreated/DMBA-initiated mice (lane 5). Urethane initiation alone (lane 3), and PMA-pretreated/urethane-initiated mice (lane 6) are shown. Epidermal DNAs are from acetone-vehicle treated mice (lane 1) and PMA-pretreated mice (lane 4). Lane 7 is the mock control. (*B*) *Xba* I polymorphism in DMBA- and urethane-initiated epidermal DNA. Autoradiogram of an agarose gel separating *Xba* I restriction fragments of ^{32}P-labeled MSPA products. Lanes 1 are the DMBA PCR product, and lanes 2 are the urethane PCR product. U, uncut 110-bp amplified PCR product from DMBA and urethane treatments; C, 90-bp fragment resulting from digestion of 110-bp fragment with *Xba* I. (*C*) Representative direct DNA sequencing autoradiogram of Ha-*ras* gene in the region of codon 61 from MSPA-amplified epidermal DNA isolated from mouse epidermis 1 week after initiation with DMBA. *, A → T mutation.

lated to proliferate by pretreatment with PMA was initiated with either DMBA or urethane. We pretreated the mice with PMA to increase the probable frequency of initiation of the epidermal cells (11). In the first experiments the initiated mice were sacrificed 1 week after initiation, and epidermal DNA was isolated. The specific MSPA-radiolabeled 110-bp product was not detected with DNA from uninitiated mouse skin epidermis (Fig. 4*A*). However, the MSPA product was detected in DNA samples isolated from both DMBA- and urethane-initiated epidermis (Fig. 4*A*). Mutations were also detected in the epidermis of mice pretreated with PMA and initiated with either DMBA or urethane (Fig. 4*A*).

An A → T mutation at the 61st codon of the mouse Ha-*ras* gene creates an *Xba* I restriction enzyme site. If the 110-bp MSPA product represented the mutant Ha-*ras* gene, then digestion with *Xba* I would create a 90-bp and a 20-bp DNA fragment. The 90-bp fragment was observed (Fig. 4*B*) when the MSPA products from DNAs isolated from DMBA- and urethane-initiated mice were digested with *Xba* I. The specific amplification of mutant Ha-*ras* was further confirmed by direct DNA sequencing of an MSPA product obtained from DNA derived from epidermis 1 week after initiation with DMBA. The DNA sequence (Fig. 4*C*) read accurately from codon 56 to 62, except for a 61st codon A → T mutation. These data confirmed that the MSPA technique specifically amplified the mutant Ha-*ras* gene.

Next a time course for the appearance of mutant Ha-*ras* after initiation with DMBA or urethane was conducted. In these experiments PMA pretreatment was not used because we could detect the mutant Ha-*ras* alleles in animals given only DMBA or urethane. One, 3, 7, and 30 days after initiation were investigated (Table 1). No evidence for mutated Ha-*ras* was seen at 1 or 3 days after initiation with either DMBA or urethane. Again, the mutated Ha-*ras* gene was detected at 7 days for both DMBA and urethane initiation, whereas at 30 days mutated Ha-*ras* alleles were detected in DMBA-, but not urethane-initiated epidermis.

DISCUSSION

The mouse skin model system of carcinogenesis (17) has been useful in formulating the principles of initiation, promotion, and progression of tumors (for review, see ref. 18). In this model system, initiated mice (by operational definition) will not develop either benign or malignant skin tumors unless promoted with a tumor promoter such as the phorbol ester PMA for at least 4–5 weeks. Ninety percent of the papillomas initiated by either DMBA (8) or urethane (9) and promoted with PMA contain an activated Ha-*ras* gene with the same

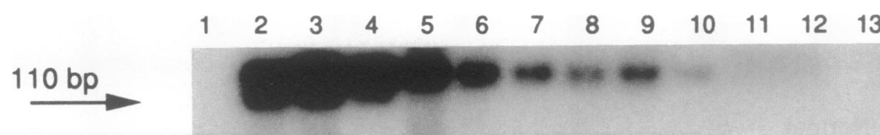

FIG. 3. Sensitivity of MSPA in detecting specific point mutations. An autoradiogram of an agarose gel separating ^{32}P-labeled MSPA products is shown. Mutant Car B DNA was mixed with normal epidermal DNA in the following weight ratios: Lanes: 1, mock; 2, 1/0; 3, $1/10^1$; 4, $1/10^2$; 5, $1/10^3$; 6, $1/10^4$; 7, $1/10^5$; 8, $1/10^6$; 9, $1/10^7$; 10, $1/10^8$; 11, $1/10^9$; 12, $1/10^{10}$; 13, 0/1. Lane 1 is the mock reaction. Lane 2 (1/0) contained only mutant Car B DNA, and lane 13 (0/1) contained only normal epidermal DNA. A total of 1 μg of DNA was added to each PCR reaction. The mutant Ha-*ras* sequence was detected in dilutions as low as $1/10^8$ (lanes 2–10). Note that normal epidermal DNA (lane 13) and the mock control (lane 1) did not produce a signal for the mutant Ha-*ras* gene.

Table 1. Time course for detection of mutant Ha-*ras* genes in mouse epidermal tissue after exposure to DMBA or urethane

Day	Treatment	Mutant Ha-*ras* positive*	RFLP analysis with *Xba* 1†
1	Acetone	0/4	NT
	Urethane	0/4	NT
	DMBA	0/4	NT
3	Acetone	0/4	NT
	Urethane	0/4	NT
	DMBA	0/4	NT
7	Acetone	0/4	NT
	Urethane	4/4	+
	DMBA	4/4	+
30	Acetone	0/4	NT
	Urethane	0/4	NT
	DMBA	4/4	+

Female CD-1 mice were given a topical application of acetone vehicle, DMBA (20 nmol), or urethane (0.7 mmol). At various time points epidermal DNA was isolated from the skins of mice and analyzed by MSPA. NT, not tested.

*Ratio of number of DNA samples testing positive for mutant Ha-*ras* genes to number of epidermal DNA samples tested from different mice.

†RFLP analysis was done on all samples testing positive in the MSPA assay. +, Each of the four samples demonstrated the *Xba* I RFLP.

mutation as seen in the epidermal cells 1 week after initiation with these agents. Thus, epidermal cells with an activated Ha-*ras* gene can exist in the epidermis before the appearance of benign tumors.

We did not observe mutant Ha-*ras* alleles at 1 day or 3 days after chemical initiation. To obtain initiated cells at least two rounds of cellular replication are necessary to "fix" mutations. The mean generation time of the epidermal cells of the skin is ≈3.5 days (19). Thus, it would require ≈6–7 days of cellular replication before "fixation" of the mutations caused by DMBA- or urethane-induced DNA adducts. Furthermore, the time for "fixation" could also be affected by exposure to the chemical initiators because DNA synthesis is known to be inhibited by chemical initiation (20). Therefore, it is not surprising that mutant Ha-*ras* alleles were not observed at 1 day and 3 days after DMBA or urethane topical application.

It is interesting to note that epidermal DNA obtained from animals 30 days after initiation with DMBA but not with urethane still had evidence of mutant Ha-*ras* alleles; this may be because DMBA is a more potent initiator than urethane. Chemical initiation with DMBA produces, on average, 10 papillomas per mouse (21), whereas urethane induces approximately 1 papilloma per mouse (11), using the initiating doses of the present study. Perhaps topical DMBA application leads to more initiated target cells than does urethane within the epidermis. Furthermore, 30 days after chemical initiation, the number of initiated cells may be less due to terminal differentiation of target basal cells within the epidermis. Therefore, detection of mutant Ha-*ras* alleles from animals initiated with DMBA is still possible at this time point because the amount of mutant Ha-*ras* remains within the detection limits of the MSPA assay. For urethane initiation, where the initial frequency of initiation was apparently lower, by 30 days the number of epidermal cells with mutant Ha-*ras* allele dropped below the sensitivity of the MSPA assay. The MSPA assay used in these studies, although very sensitive, is not quantitative in nature. A quantitative MSPA assay would help clarify some of the issues concerning relative numbers of epidermal cells with Ha-*ras* mutations.

Our finding that mutant Ha-*ras* genes can be detected in epidermal tissue 1 week and 30 days after carcinogen treatment is consistent with the findings of Kumar *et al.* (10). These investigators found both mutated Ha-*ras* and Ki-*ras* in rat mammary glands 2 weeks after carcinogen treatment and 2 months before the onset of neoplasia. In support of our findings, Balmain and his coworkers (22, 23) observed that transgenic mice expressing mutationally activated Ha-*ras* gene in their keratinocytes or mice that have been infected with a mutant *ras* retrovirus did not develop tumors unless wounding or tumor promotion occurred. Thus mutant *ras* oncogenes can remain dormant in the target tissue and require additional events, such as tumor promotion or wounding before tumors develop.

This report demonstrates detection of mutant Ha-*ras* alleles in the presence of a large excess of normal Ha-*ras* alleles. These results have important implications for both mechanistic studies and clinical evaluation of human cancer. Perhaps *ras* gene mutations in normal human tissues exposed to carcinogens can be detected; this could be useful in assessing the exposure level and risk of workers exposed to chemicals with known carcinogenic potential. The method could also be applied as a biomarker for chemoprevention. For cancers associated with mutated *ras* (such as colon and lung cancers), the MSPA technique could be used to detect premalignant changes within carcinogen-exposed normal tissues. If detection of *ras* mutations can be shown to predict the subsequent development of cancer, identification of *ras* mutations could be used as a marker to identify individuals at higher risk to be placed in cancer-prevention trials. As a tool in cancer treatment, MSPA could be used to detect minimal residual disease after cancer chemotherapy treatment.

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