ANTICANCER RESEARCH 22: 2733-2740 (2002)

Oral Mucosal Carcinogenesis in SENCAR Mice

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Abstract. *Animal models of oral carcinogenesis have been developed but most use the hamster buccal pouch or rat oral mucosa. With completion of human and murine genome sequencing, the development of a mouse model of oral carcinogenesis may prove useful for future genomic studies of oral carcinogenesis. To achieve this objective, 30 SENCAR mice were initiated by brush application of palatal, buccal and tongue mucosa with 200 nmol 7,12-dimethylbenz[a]anthracene (DMBA) using 3 treatment regimens, and promoted by brush application with 5 nmol 12-O-tetradecanoylphorbol- 13 -acetate (TPA) for a total of 28 weeks. Alternatively, 5 mice were treated with 0.5% 4-nitroquinoline-1 -oxide (4NQO) alone by brush application for 28 weeks. There were another 6 control mice treated with vehicle alone. The tumor samples were analyzed for the presence of H-ras codon 61 gene mutations using a mutant-allele-specific amplification-polymerase chain reaction (MASA-PCR) technique. The results showed that among the group of 24 mice initiated with DMBA for 2 or 6 weeks, a range of papilliferous lesions were seen on the buccal mucosa comprising papillomas, papillomas with dysplasia and 7 squamous cell carcinomas (SCC). In those 6 mice initiated with 1 week of DMBA, only papillomas developed. In the 5 mice treated with 4NQO, one developed papillomas with dysplasia and two had SCCs in the tongue mucosa but not the buccal mucosa. Both carcinogens induced codon 61 mutation of the H-ras gene at a high frequency. The results indicated that DMBA/TPA and 4NQO in SENCAR mice reliably produced preneoplastic and malignant oral cavity lesions, which resemble the multistages for human oral carcinogenesis, and targeted to site-specific zones of the oral mucosa, namely the buccal mucosa and tongue, respectively. These results show that SENCAR mice can be used as a unique model of oral carcinogenesis with the potential for detailed molecular studies of neoplastic progression to SCC.*

Supported by: NIH Grant DE 11436, DE01193 and P01 DE 13904.

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Key Words: 12-Dimethylbenz[a] anthracene, carcinogens, H-*ras*, oncogene, mutant-allele-specific amplification, oral squamous

Oral cancer is the sixth most common cancer worldwide and is the most frequent form of head and neck cancer. It remains a serious public health problem, with more than 300,000 new cases annually worldwide (1). Over 90% of these cancers are squamous cell carcinomas (SCC). However, the molecular mechanisms leading to the development of oral SCC are poorly understood, and more studies are needed to define which specific genes are altered during oral tumor progression.

It is widely accepted that epithelial tumorigenesis involves a multistep process of genetic alterations. Based on their studies on colon tumors, Vogelstein and others have advanced the idea that certain tumors form as a consequence of sequential genetic changes (2, 3). There is now evidence that the development of SCC also occurs as a multistep process. A number of molecular alterations have been identified in the development of oral cancer (4). Many of these occur in known oncogenes and tumor suppressor genes resulting in dysregulated cell proliferation and apoptosis, and tissue invasion (4).

In both human and murine squamous carcinogenesis, *ras* gene is a frequent target of mutation (5,6). Indeed, in human head and neck cancers, H-*ras* mutations are detected in approximately 10% of carcinomas in Western countries (7). Moreover, H-*ras* mutations at codons 12 and 61 occur in 35% of oropharyngeal SCC in India (8-10). In animal models, DMBA-induced SCC of the skin and oral cavity are frequently associated with H-*ras* mutations (11, 12); 4NQO-induced oral cavity lesions in mice also frequently contain H-*ras* mutations (13). Activation of the *ras* oncogene occurs during the early stages of skin carcinogenesis induced by the carcinogen DMBA and the promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (14,15). Continuous exposure of squamous cells to DMBA and TPA induce H-ras mutations on chromosome 7 in more than 90% of mice (11).

Past studies have shown that carcinogenesis plays a critical

role in the malignant transformation of oral epithelial cells (16-18). To facilitate the study of oral SCC carcinogenesis, several animal models have been developed during the last two decades. Among the best studied are the 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced hamster cheek pouch model (14,19-21) and the 4-nitroquinolone-N-1-oxide (4NQO) rat oral model (22-24).

However, it has not been previously reported that DMBA could induce oral tumors in mice. Moreover, the availability of murine models with differing genetic or transgenic backgrounds using DMBA would permit detailed analysis of factors related to sensitivity of tumor progression using this carcinogen. In addition, a mouse model of oral carcinogenesis is desirable because the mouse genome has been sequenced offering the potential to detect and analyze sequence genetic alterations during cancer development.

SENCAR (SENsitive to CARcinogenesis) mice are widely used in skin carcinogenesis studies because of their enhanced susceptibility when initiated with DMBA and promoted with the phorbol ester TPA (25). Recent studies also showed that mammary cancer and lymphoma can be induced by intragastric use of DMBA in SENCAR mice (26). However, it has not previously been used to study oral carcinogenesis. Therefore, the purpose of the current study was to establish an alternative sensitive and reproducible animal model for oral carcinogenesis using SENCAR mice for the elucidation of molecular events leading to oral cancer. Our results show that DMBA/TPA and 4NQO are effective oral carcinogens and induce SCC in buccal mucosa and tongue, respectively, in the SENCAR mouse.

Materials and Methods

Animals. Seven-week-old female SENCAR mice were obtained from the National Cancer Institute (Bethesda, MD, USA) and acclimatized for at least one week in the UCSF Animal Care Facility before use. The mice were fed Lab. diet #5008 (PMI Nutrition International, Inc., St. Louis, MO, USA) and water *ad libitum*. The mice were kept on corn cob bedding and placed on a 12-hour light/dark cycle.

Chemicals. 4NQO, DMBA and RNase A were purchased from Sigma (St. Louis, MO, USA). TPA was purchased from Alexis Biochemicals, (San Diego, CA, USA). AmpliTaq DNA polymerase was purchased from Perkin-Elmer, Applied Biosystems, Foster City, CA, USA.

Dosing. Mice were randomly placed into one of the five groups (Table I). Group 1 was initiated with 200 nmol DMBA in 50 µl acetone three times weekly for 1 week and promoted with 5 nmol TPA in 50 µl acetone three times weekly for 27 weeks (n=6). Group 2 was initiated with 200 nmol DMBA in 50 µl acetone three times weekly for 2 weeks and promoted with 5 nmol TPA in 50 µl acetone three times weekly for 26 weeks (n=12). Group 3 was initiated with 200 nmol DMBA in 50 µl acetone three times weekly for 6 weeks and promoted with 5 nmol TPA in 50 µl acetone three times weekly for 22 weeks (n=12). Group 4 was treated with 50 µl acetone of 4NQO (0.5% w/v) three times weekly for 28 weeks (n=5). Group 5 was given applications of only the acetone vehicle three times per week for one week followed by TPA as above for 27 weeks (n=6) to serve as a control. DMBA, TPA and 4NQO were dissolved in acetone and applied to the palatal mucosa, buccal mucosa and tongue of the mice using a No. 3 sable paint brush. Previously, this method had been shown to deliver relatively constant amounts of carcinogen to the mucosa (27,28). The total dose of each carcinogen was based on the delivery of 50 µl of solution. The oral painting protocol used in the present studies is similar to previous models where DMBA was used to initiate carcinomas in the hamster cheek pouch model (28), or where 4NQO was used to induce carcinomas in rat oral mucosa (28). Furthermore, a similar protocol was used in the lacZ mouse where mutagenesis was shown to be induced by treatment with DMBA or 4NQO in oral cavity tissues (29). However, the precise amount of carcinogen applied in our study can only be estimated because small amounts may have remained on the brush and some may have been subsequently swallowed. However, a significant dose of drug appears to have been delivered since there was a consistently high incidence of carcinoma in these mice.

Histology. After 28 weeks of treatment, the mice were killed by cervical dislocation 18 hours after the last dose. Specimens taken from the tongue, buccal mucosa and hard palate were fixed in 10% neutral buffered- formalin for 18-24 hours. Normal adjacent tissues were used for histological comparison. Specimens were then processed and embedded in paraffin. Five-µm sections were cut and stained with hematoxylin and eosin. Step sections were evaluated for all lesions by an experienced pathologist (J.R). Criteria required for the diagnosis of dysplasia included disordered epithelial maturation, nuclear enlargement and hyperchromasia, and increased nucleus to cytoplasm ratio. The diagnosis of carcinoma was based upon loss of basement membrane and clear evidence of atypical epithelium within submucosa.

DNA isolation. DNA isolation from paraffin-embedded tissue sections was performed as previously described (30), with modification. Unstained sections were deparaffinized twice with xylene, rinsed twice with 95% ethanol, briefly stained with eosin, and air-dried. Specific histological fields of interest (*e.g.*, region of dysplasia, papilloma or SCC) were selected after examination by an oral pathologist (J.R.) and microdissected under the light microscope, using an 18G11/2 needle. Procured tissues were immediately resuspended in 10 µl buffer (Tris-HCl, pH 8.0, 0.1 mol/L EDTA, pH 8.0, 1% Tween 20, and 0.1 mg/ml proteinase K) and incubated overnight. DNA was then extracted from tissues with phenol/chloroform and precipitated with ethanol.

MASA assay. The mutant-allele-specific amplification (MASA) assay for detection of H-*ras* mutations was carried out (Figure 3) using the method described by Takeda *et al.* (31) and Shiraishi *et al.* (32) Different sets of polymerase chain reaction (PCR) primers were used to amplify the Exon 2 of the *ras* gene. Cycling conditions of MASA consisted of an initial denaturation at 99°C for 10 minutes, 40 cycles of 94°C for 20 seconds, 57°C for 15 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. AmpliTaq DNA polymerase (Perkin-Elmer) was used to effect a 'hot start' PCR to prevent non-specific annealing of primers during set-up. After MASA-PCR was completed, samples with positive results for the H-*ras* codon 61 mutation were sequenced to confirm the A→T transition. All specimens were analyzed at least twice to confirm the results.

Results

Four different treatment protocols were evaluated for induction of oral carcinomas, using DMBA/TPA or 4NQO as the carcinogen (Table I). After a total of 28 weeks of treatment, papillomas, papillomas with dysplasia, and SCCs were found in the different groups of mice (Table II). The

Table I. *Experimental design used in the study.*

Group	Initiation	Promotion	No. of mice
Group 1	DMBA → 1 week	TPA → 27 weeks	6
Group 2	DMBA → 2 weeks	TPA → 26 weeks	12
Group 3	DMBA → 6 weeks	TPA → 22 weeks	12
Group 4	4NQO ———	→ 28 weeks	5
Group 5	Acetone → 1 week	TPA → 27 weeks	6

Table II. *Histological findings in DMBA/TPA- and 4NQO-induced carcinomas.*

Histology	Group 1 DMBA 1 week	Group 2 DMBA 2 weeks	Group 3 DMBA 6 weeks	Group 4 4NQO	Group 5 Control	Total
No change	3	1	2	2	6	14
Papilloma	3	5	1	–	–	9
Papilloma w/ dysplasia	–	2	6	1	–	9
SCC	–	4	3	2	–	9
Total	6	12	12	5		35

results for DMBA/TPA are similar to those of previous studies of skin carcinogenesis in SENCAR mice. The incidence of papillomas was 18 out of 35 animals (50%), while the incidence of SCCs was 9 out of 35 animals (26%). Interestingly, more than one distinct tumor could frequently be observed in the oral cavity of DMBA-TPA-treated mice, suggesting that multiple simultaneous tumor induction was occurring.

Microscopic examination of specimens from different groups identified abnormal changes ranging from papillomas to invasive SCC in both buccal mucosa and tongue (Figures 1 and 2). In group 1, only papillomas were found on the buccal mucosa in 50% of the mice. However, in groups 2 and 3, papillomas with dysplasia and SCCs were evident in buccal mucosa. This may indicate that 1 week of initiation with DMBA is not sufficient to convert a papilloma to SCC. Multiple tumors in the same oral cavity were a frequent event and usually these were of different sizes. In group 4, both papillomas and SCCs were detected in the tongue, but not in the hard palate or buccal mucosa (Table II). Chronic inflammatory cell infiltration varied from slight in dysplasia to intense in carcinomas. Morphologically, the changes that developed in response to these carcinogens suggested a progression from dysplasia to SCC, similar to neoplastic transformation seen in many human oral cancers. Typically, the entire spectrum of histological changes consistent with transformation could be observed in the region of invasive SCC (Figure 1). Of the SCC lesions in the DMBA/TPA-treated mice, two showed progression to spindle cell carcinoma. Mice in control group 5, treated with vehicle alone, had histologically normal-appearing buccal, palate and tongue mucosa at 28 weeks.

We next studied the incidence of the codon 61 mutation of the H-*ras* gene in oral carcinogenesis induced by DMBA or 4NQO, since this alteration is common in DMBA-induced hamster cheek pouch cancers (33) and mouse skin lesions (11). We isolated genomic DNA from each tumor specimen and amplified codon 61 mutations in the c-Ha-*ras* genes by MASA-PCR, using primers specific for exon 2 (Figure 3). The amplification products were then examined using MASA and confirmed by sequencing. Using this sensitive technique, we determined that overall there were 18 out of 27 lesions (67%) with the codon 61 mutation (Table III). For oral SCCs, a majority of the lesions contained the codon 61 point mutations of the H-*ras* gene. A striking observation was that when multiple tumors arose in the same animal treated with DMBA/TPA, there was heterogeneity in the detection of the $A^{162} \rightarrow T$ mutation in these lesions. For example, in Figure 3 animal 118 had 3 buccal tumors but only one (118-2) had clear evidence of the H-*ras* mutation. This suggests that tumor progression is occurring asynchronously leading to tumors of different stages of development.

Discussion

We compared the potential for induction of SCC in the oral cavity by DMBA/TPA and 4NQO carcinogens. DMBA/TPA-induced papillomas eventually developed into malignancy on the buccal mucosa but not the tongue. By contrast, 4NQO treatment readily produced dysplastic lesions and SCCs, but consistently failed to generate similar lesions on the buccal mucosa. The reasons for this differential susceptibility to each carcinogen may be complex. However, recent work by von Pressentin *et al.* (29) suggests that the mutagenic rate may differ in different regions of the oral cavity. Thus, it has been reported in *lac* Z mice that 4NQO causes high rates of mutagenesis in the tongue but is less effective in other oral tissues. The reason for this may be due to the fact that 4NQO could be activated by reductases present in higher amounts in the tongue (34, 35). This probably contributes to the lack of carcinoma formation in the buccal site. These results therefore do not explain why carcinomas were produced by DMBA only at buccal mucosal sites.

There is extensive literature showing that DMBA/TPA

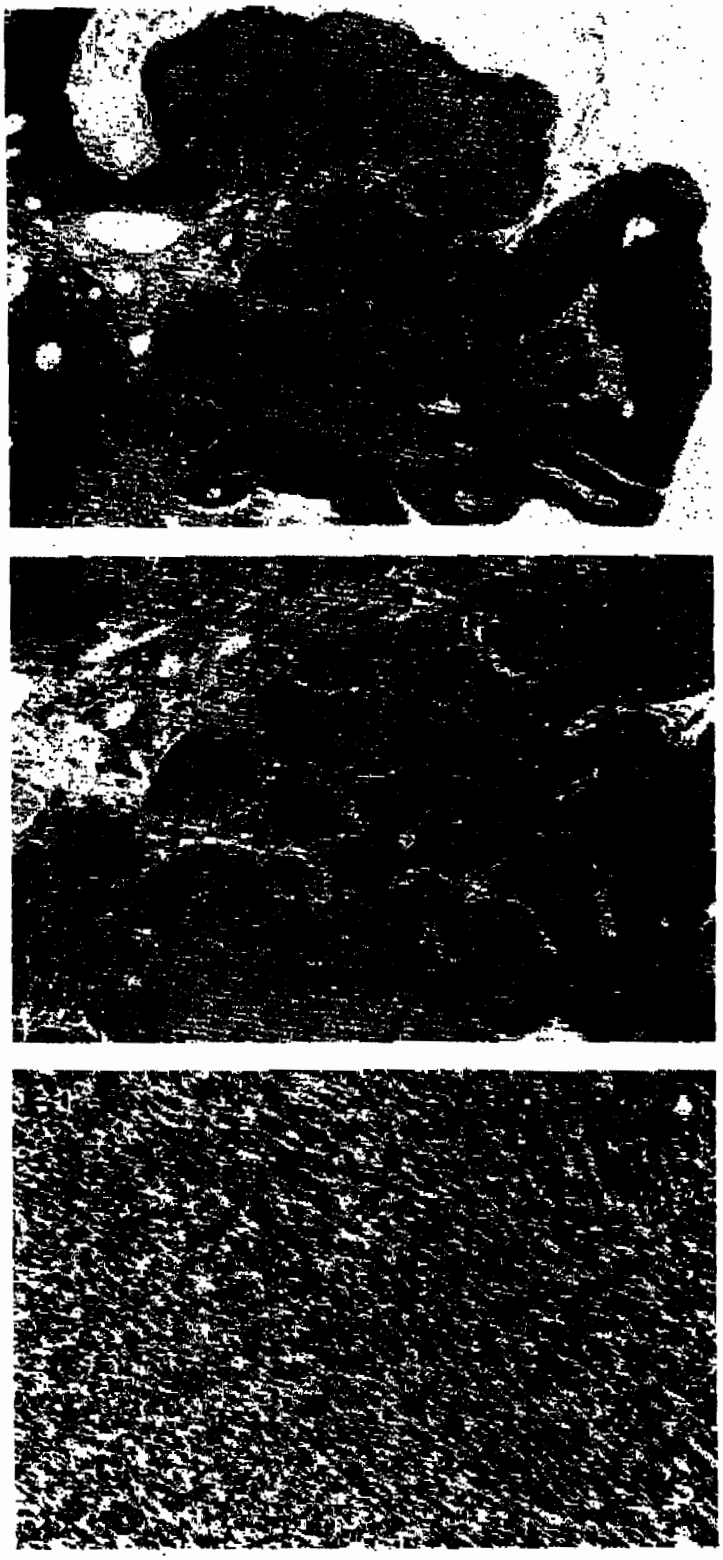

Figure 1. *DMBA-induced SCC of buccal mucosa in SENCAR Mice. A, invasive well-differentiated SCC, H & E staining, x 100; B, the same specimen as in figure A, x 250. C, spindle carcinoma of buccal mucosa; H & E staining, x 200.*

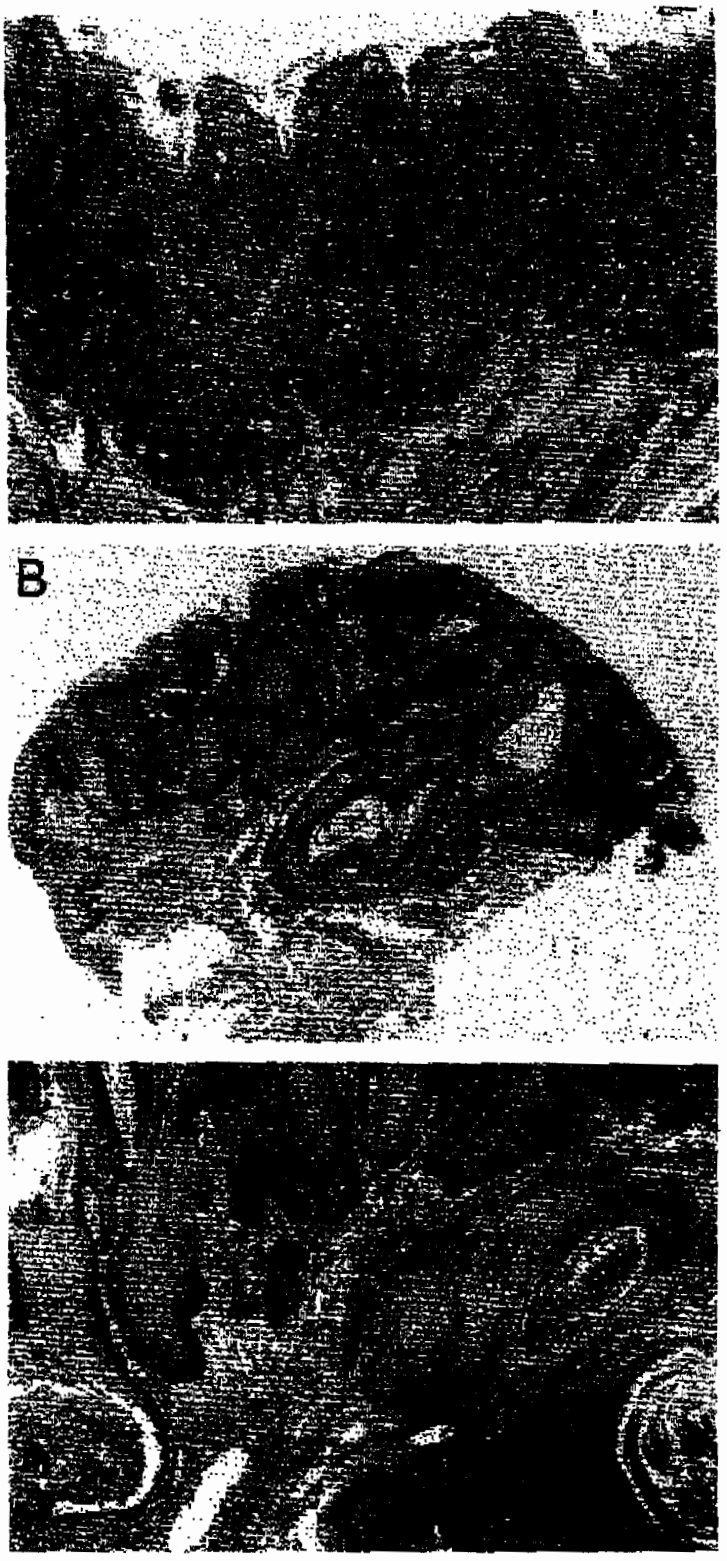

Figure 2. 4NQO-induced SCC of the tongue. A, verruciform hyperkeratosis with dysplasia. Note basal and parabasal cell hyperchromatin and crowding; H & E staining, x 250. B, papillary-verruciform carcinoma, showing invasion into submucosa and skeletal muscle; H & E staining, x 100. C, the same specimen as in Figure 2B, x 250.

Table III. *Incidence of codon 61 mutation of c-Ha-ras gene in oral lesions[1] from DMBA/TPA- or 4NQO-treated mice.*

Group[2]	Lesion incidence No. of mice (%)	Mutation (CAA→ CTA) frequency in tumors No. of lesions
Group 1	3/6 (50)	2/3 (67)
Group 2	11/12(92)	6/11(55)
Group 3	10/12(83)	8/10(80)
Group 4	3/5 (60)	2/3 (67)
Group 5	0/6 (0)	0/0(0)

[1] All lesions that developed (including papilloma, papilloma with dysplasia, and SCC).
[2] See Table I for a description of each experimental group.

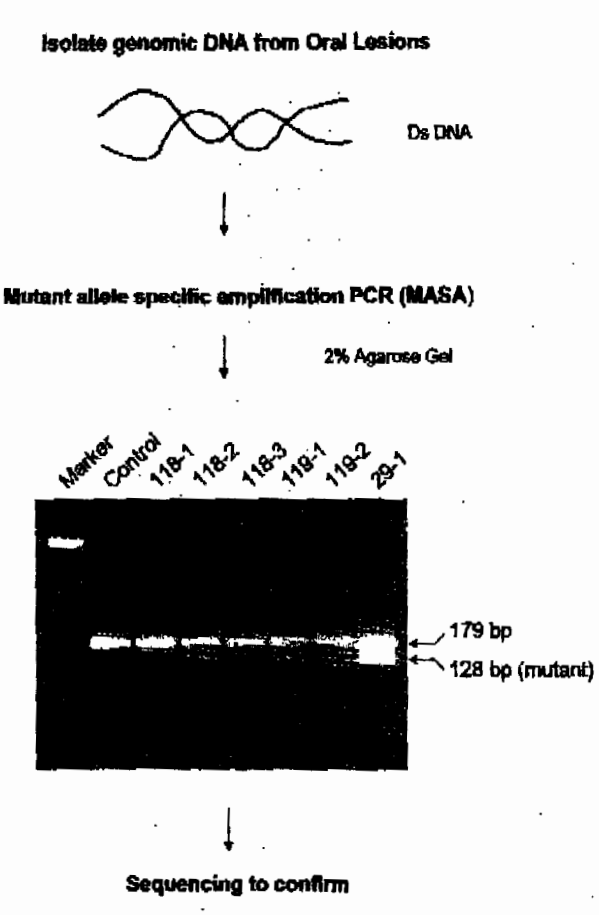

Figure 3. *Method and representative results of MASA analysis for the detection of c-Ha-ras A ^{182} → T mutation. Tumors were excised from the oral cavities of SENCAR mice treated with DMBA-TPA (Table I, Group 3) and isolated genomic DNA was processed for MASA as detailed in the Materials and Methods section. Ladder: 50 base pair molecular weight DNA ladder; Control: DNA isolated from normal mucosa of SENCAR mouse; Animals 118 and 119 had multiple buccal tumors that were individually analyzed whereas animal 29 had a single lesion. The presence of the A→ T mutation in codon 61 of the H-ras gene is evident by the detection of the 128 base pair mutant amplification fragment (128 bp) that migrates just below the wild-type, the 179 base pair amplification (179 bp) of H-ras. In animal 118, one tumor was positive and two were negative for the mutation whereas in animal 119 both lesions were positive for the mutation.*

efficiently induces skin squamous cell carcinoma (36) and other studies have documented the use of DMBA to initiate SCC on the tongue and buccal mucosa in the hamster (25, 37). The related carcinogen, polycyclic aromatic hydrocarbon benzo[a]pyrene, induces carcinoma of the tongue in mice when delivered in food (38). In a recent study, von Pressentin reported on DMBA applied by oral swabbing of mouse mucosa (29). While this study did not examine the formation of oral carcinoma, it was found that DMBA produced a significant incidence of mutation frequencies in the tongue. However, the frequency was approximately 18-fold less than 4NQO. The efficiency of carcinoma induction in the hamster pouch model by DMBA is related to the "pocket" in which the carcinogen is applied (14). These observations suggest that it is possible to target specific areas of the mouse oral cavity using different carcinogens.

A search of the literature indicates that DMBA has not previously been used successfully in mouse oral carcinogenesis except in salivary glands (39). The reason for this may be related to species and strain differences, since DMBA is oil-soluble and the saliva is thought to be protective. It may relate to resistance of the oral cavity to initiation by DMBA. Thus mouse strain selection will be important to establish a model of murine oral carcinogenesis using DMBA/TPA. The SENCAR mice we used in this study were developed by selective breeding for high susceptibility to skin carcinogenesis by initiation with DMBA and promotion with TPA, and they have been used widely and successfully in skin carcinogenesis studies (35, 37). Thus, from the results of our study, it seems SENCAR mice are suitable animals for studying oral carcinogenesis.

Several carcinogens produce mutations at multiple codons of the H-*ras* gene. DMBA has been shown to produce an A→ T mutation in codon 61 of the H-*ras* gene in mouse skin and hamster buccal mucosa (11, 34, 40). It has been observed that codon 61 H-*ras* mutations occurred in approximately 90% of murine skin papillomas and carcinomas (11, 41), while others have shown that approximately 60% of the hamster cheek pouch carcinomas have a mutation in codon 61 of the H-*ras* gene (34). The frequency of DMBA/TPA-induced mutations in our study was similar. A 4NQO-induced *ras* mutations in mouse oral cavity lesions has been reported by only one other group (13). They found about 50% of the carcinomas had G→ A mutations at codon 12 of the H-*ras* gene, but no codon 61

mutations. In our study, we found A→T mutations at codon 61 in 67% of the carcinomas instead. The reason for this codon difference may lie in the different mouse strains being used in these studies: CBA mice *vs.* SENCAR mice.

In summary, we report that in the SENCAR mouse, both DMBA/TPA and 4NQO treatment induces a high incidence of premalignant and malignant lesions in the oral mucosa. Importantly, each carcinogen is site-specific for induction of these carcinomas, with DMBA/TPA generating buccal SCC whereas 4NQO targets tongue mucosa. The DMBA protocol in mice with specific genetic or transgenic backgrounds offers a powerful tool for contributing factors involved in tumor initiation and progression of oral cancer.

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Received March 5, 2002
Accepted May 2, 2002