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Carcinogen-induced mutations in the mouse c-Ha-*ras* gene provide evidence of multiple pathways for tumor progression

(mouse skin/oncogene/papilloma/carcinoma/initiation)

KEN BROWN*, ALBRECHT BUCHMANN†, AND ALLAN BALMAIN

Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, Scotland

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ABSTRACT **A number of mouse skin tumors initiated by the carcinogens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methylnitrosourea (MNU), 3-methylcholanthrene (MCA), and 7,12-dimethylbenz[*a*]anthracene (DMBA) have been shown to contain activated Ha-*ras* genes. In each case, the point mutations responsible for activation have been characterized. Results presented demonstrate the carcinogen-specific nature of these *ras* mutations. For each initiating agent, a distinct spectrum of mutations is observed. Most importantly, the distribution of *ras* gene mutations is found to differ between benign papillomas and carcinomas, suggesting that molecular events occurring at the time of initiation influence the probability with which papillomas progress to malignancy. This study provides molecular evidence in support of the existence of subsets of papillomas with differing progression frequencies. Thus, the alkylating agents MNNG and MNU induced exclusively G → A transitions at codon 12, with this mutation being found predominantly in papillomas. MCA initiation produced both codon 13 G → T and codon 61 A → T transversions in papillomas; only the G → T mutation, however, was found in carcinomas. These findings provide strong evidence that the mutational activation of Ha-*ras* occurs as a result of the initiation process and that the nature of the initiating event can affect the probability of progression to malignancy.**

The carcinogenic nature of many chemicals has long been associated with their ability to bind to DNA and to cause somatic mutations (1). These lesions, if they occur at certain critical locations in the genome, are thought to effect key functions in initiating or furthering neoplastic development. Increasing evidence suggests that the activation of cellular protooncogenes to their oncogenic forms plays a central role in the development of neoplasia and thus identifies protooncogenes as candidate targets for chemical carcinogens (2–4).

The most frequently detected oncogenes, both in human tumors and in many animal tumor model systems, are members of the *ras* gene family—Ha-, Ki-, and N-*ras* (2–4). These oncogenes differ from their normal cellular counterparts by having acquired a single point mutation at codon 12, 13, 61, or 117 (2). Studies on several experimental model systems have identified, for a number of chemically induced tumors, the particular activating mutations involved (5–7). From these has developed a correlation between carcinogen and *ras* mutation, suggesting a causal relationship between carcinogen treatment, *ras* activation, and, in these tumor systems at least, the initiation of tumorigenesis.

One of the best studied animal tumor models is the mouse skin system (8) in which tumor formation occurs in discrete stages. Initial treatment with carcinogen is considered to cause an irreversible genetic event, resulting in the formation of a population of "initiated" cells within the epidermis. These initiated cells remain dormant until stimulated by a tumor promoter, such as a phorbol ester, to proliferate, clonally expand, and become premalignant lesions or papillomas. A small number of these papillomas then progress to fully malignant carcinomas.

One important issue that has not been fully resolved lies in the exact nature of the progression pathway. Previous studies have suggested that papillomas are heterogeneous in their ability to progress to carcinomas (9). Most papillomas are promoter dependent and regress upon cessation of promoter treatment; the remainder are promoter independent and it is from this group that carcinomas appear to develop. Promoter-independent papillomas may arise from preexisting dependent papillomas in a sequential manner (10). Alternatively, they may be formed directly from initiated cells without involving an intermediate promoter-dependent papilloma, suggesting that initiation produces different populations of initiated cells, with differing potentials to progress to malignancy (11, 12). Indeed, the existence of papillomas with a high probability of progression to carcinomas has been demonstrated (13).

The presence of discrete premalignant stages in the mouse skin system has allowed the sequential molecular events involved in tumor development to be studied. Earlier work from this laboratory has demonstrated a high incidence of Ha-*ras* gene activation in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mouse skin tumors (14, 15). More recently, it has been shown that in the majority of these tumors the activating mutation is an A → T transversion in the middle base of codon 61, resulting in the creation of an *Xba* I restriction fragment length polymorphism (RFLP) (7, 16). This *Xba* I mutation was not detected in the DNA from a series of tumors initiated with the carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Thus, the mutation appeared to be carcinogen specific, as had previously been found for Ha-*ras* mutations in rat mammary tumors (5).

To date, no complete analysis has been reported, using the same experimental tumor system, on the types of mutations induced by commonly used polycyclic aromatic hydrocarbons, such as DMBA and 3-methylcholanthrene (MCA), and alkylating agents such as MNNG and methylnitrosourea (MNU). For this reason, we carried out a comparison of mutations at codons 12, 13, and 61 of Ha-*ras* in tumors initiated with these carcinogens. The present study provides further evidence that Ha-*ras* gene activation involves carcinogen-specific mutations and so further strengthens the argument that such activation occurs at initiation. In contrast, however, to our previous results on DMBA-initiated

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MCA, 3-methylcholanthrene; MNU, methylnitrosourea; RFLP, restriction fragment length polymorphism; TPA, 12-*O*-tetradecanoyl-13-acetylphorbol.

*To whom reprint requests should be addressed.
†Present address: Institute of Biochemistry, German Cancer Research Centre, Im Neuenheimer Feld 280, 6900 Heidelberg, F.R.G.

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tumors (7), the distribution of Ha-*ras* gene mutations differs substantially between papillomas and carcinomas. This finding suggests that the initiating event may play some role in determining the probability of tumor progression.

MATERIALS AND METHODS

Chemically Induced Mouse Skin Tumors. Female NIH/Swiss mice 8–12 weeks old were used in DMBA, MCA, and MNNG experiments. These mice, originally obtained from Olac (Bichester, U.K.), had been bred in the Beatson Institute for 7 years. The dorsal skin was shaved 24 hr before carcinogen treatment. The mice were then given MCA (200 μg) or MNNG (600 μg) dissolved in acetone (200 μl). Promotion was carried out twice weekly by treatment with an acetone solution of 12-*O*-tetradecanoyl-13-acetylphorbol (TPA) (200 μl at 0.1 mM). Mice were sacrificed and tumors were collected upon the appearance of carcinomas, generally after 7–15 months of promotion treatment. MNU papillomas were induced in RFM mice by G. Harris (London Hospital Medical School) by a single MNU treatment followed by twice weekly promotion with TPA.

Isolation of Tumor DNA. Tumors, frozen in liquid nitrogen, were finely ground and lysed in the presence of 5 M guanidinium thiocyanate. DNA was isolated by centrifugation through a cesium chloride density gradient as described (14).

Southern Blot Analysis. After restriction digest, DNA samples were electrophoresed in 1% agarose gels using Tris acetate buffer and then transferred to Biotrace RP nylon membrane (Gelman) by capillary blotting with ammonium acetate solution (17). Filters were prehybridized, hybridized, and washed according to procedures described by Reed and Mann (18). Autoradiograms were obtained by exposing the blots to Kodak X-Omat or XAR film at −70°C in the presence of intensifying screens. DNA fragments, for use as probes, were isolated from plasmids by electrophoresis in low melting point Seaplaque agarose gels (ICN) and labeled by the random-primer method (19).

Oligonucleotide Synthesis. Oligonucleotides, for use as "amplimers" (see Table 1) or probes (see Table 2), were synthesized on an Applied Biosystems 381A synthesizer, using the manufacturer's protocols and reagents, and after deprotection were used without further purification. Oligomers, for use as hybridization probes, were 5′ end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase.

DNA Amplification by Polymerase Chain Reaction. Tumor DNA was amplified as described by Saiki *et al.* (20). Briefly, a 100-μl reaction mixture, containing 1 μg of genomic DNA in 67 mM Tris·HCl, pH 8.8/16.7 mM NH_4SO_4/6.7 mM $MgCl_2$/10 mM 2-mercaptoethanol/6.7 μM EDTA, 17 μg of bovine serum albumin, each amplimer at 1 μM, each dNTP at 1.5 μM, and overlaid by several drops of mineral oil (100 μl), was incubated at 91°C for 7 min. After allowing 3 min at 45°C to allow amplimer annealing, 2 units of thermostable DNA polymerase from *Thermus aquaticus* (Anglian Biotechnology, Colchester, U.K.) was added and the extension reaction was carried out at 72°C for 2 min. Reactions were subsequently subjected to 35 cycles under the following conditions: 91°C for 1 min 30 s, 45°C for 1 min 30 s, 72°C for 2 min. Thermal cycling was controlled by a programmable heating block (Perkin-Elmer/Cetus).

Oligonucleotide Dot-Blot Hybridizations. Amplified DNA (1 μl) was spotted onto Biotrace RP nylon membranes, and the filters were dried at 80°C for 4 hr. Filters were prehybridized in 5× SSPE (1× SSPE = 180 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.4) containing sonicated salmon sperm DNA (500 μg/ml) at 56°C for 16 hr. ^{32}P-labeled oligomer probe was then added (2 ng/ml; specific activity, 10^9 dpm/μg) and the filter was subsequently hybridized for 16 hr at 56°C. The filters were washed in 2× SSPE/0.1% SDS twice for 30 min at 20°C, and then in 5× SSPE/0.1% SDS twice for 30 min at selective discriminating temperatures (see Table 2). Finally, the filters were autoradiographed by exposure to Kodak XAR or X-Omat film at −70°C with intensifying screens.

Table 1. Sequences of oligonucleotide primers for polymerase chain reaction amplification

Amplimer	Sequence
Ex1A	CTT GGC TAA GTG TGC TTC TCA TT
Ex1B	CAC CTC TGG CAG GTA GGC AGA GC
Ex2A	CTA AGC CTG TTG TTT TGC AGG AC
Ex2B	GCT AGC CAT AGG TGG CTC ACC TG

The oligonucleotides were based on the sequence of the mouse c-Ha-*ras* gene (22) and correspond to intron sequences immediately outside exon 1 (Ex1A and Ex1B) and exon 2 (Ex2A and Ex2B) (where A signifies the 5′ amplimer and B signifies the 3′ amplimer).

RESULTS

Mouse Skin Tumors Initiated by the Methylating Agents MNNG and MNU Contain $G^{35} \rightarrow A$ Transitions in Codon 12 of c-Ha-*ras*. As a direct comparison with the spectrum of *ras* mutations in tumors induced by the polycyclic aromatic hydrocarbon DMBA, we undertook to examine mouse skin tumors initiated by the methylating agents MNNG and MNU. DNA samples from tumors were analyzed for possible Ha-*ras* mutations, using the techniques of DNA amplification, by the polymerase chain reaction (20), together with allele-specific oligonucleotide hybridization (21). Oligonucleotides, corresponding to intron sequences and designed to specifically amplify either exon 1 or exon 2 of the murine c-Ha-*ras* gene (22), are shown in Table 1. Dot blots containing the amplified DNA were probed with individual oligonucleotides, comprising either the wild-type sequence or sequences containing a single mutation at codon 12, 13, or 61, as shown in Table 2.

Except for the wild-type oligomer 12A, the only oligonucleotide to give a positive signal with MNNG or MNU tumor DNA samples was the 12B probe, indicating the occurrence of a $G^{35} \rightarrow A$ transition. This lesion was detected in DNA from 13 of 28 MNNG tumors (Fig. 1*A*) and from 5 of 12 MNU tumors (Fig. 1*B*). No other changes were detected at codons 12, 13, or 61 of Ha-*ras*. A similar $G^{35} \rightarrow A$ Ha-*ras* mutation

Table 2. Oligonucleotides used to detect point mutations at codons 12, 13, and 61 of the Ha-*ras* gene

Probe	Sequence	Discriminating temperature, °C
12A	TG GGC GCT GGA GGC GTG GG	67
12B	GAA	65
12C	GTA	65
12D	GCA	67
12E	CGA	67
12F	AGA	65
13A	TG GGC GCT GGA GNC GTG GG	63
13B	NGC	63
61A	ACA GCA GGT CAA GAA GAG TA	61
61B	AAA	59
61C	GAA	61
61D	CGA	62
61E	CCA	62
61F	CTA	61
61G	CAC	62
61H	CAT	61

Oligonucleotides containing thymidine at the first base of codon 12 or 61 were omitted since these would detect nonsense codons. Also omitted were probes for mutations in the third base of codon 12 or 13, as no change in amino acid would occur. Probes 13A and 13B were both mixtures of oligomers, with N representing A, C, or T.

has been found in the DNA from a high proportion of rat mammary carcinomas induced by MNU (5).

Surprisingly, in the case of the MNNG tumors, the distribution of $G^{35} \rightarrow A$ mutations was found to differ between papillomas and carcinomas, with the mutation occurring in 11 of 15 papillomas, but in only 2 of 13 carcinomas. Unfortunately, this analysis did not include carcinomas initiated by MNU and consequently, for this carcinogen, no conclusion could be deduced for the distribution of mutations.

Identification of Alternative Codon 61 Mutations in DMBA-Initiated Tumors. Although the majority of mouse skin tumors induced by DMBA and promoted with TPA show an *Xba* I RFLP at codon 61 of Ha-*ras* (7), there were a small number of DMBA papillomas (3 of 48) that were negative for this RFLP. DNA samples from these papillomas were amplified and analyzed with oligonucleotide probes for the presence of alternative Ha-*ras* mutations. All three of the tumor DNAs exhibited alternative codon 61 mutations. Two papilloma DNAs contained $A^{183} \rightarrow T$ transversions, while an $A^{182} \rightarrow G$ transition was found in the other DNA sample, as illustrated in Fig. 2. Thus, all of the papillomas studied arising from DMBA/TPA treatment contain mutations in Ha-*ras*, whereas the carcinomas show Ha-*ras* activation in 10 of 13 cases. Moreover, all of the DMBA-induced mutations appear to involve deoxyadenosine (or alternatively thymidine) adducts.

MCA-Induced Tumors Show a Different Codon 13 Mutation. We have also carried out a complete analysis of DNAs from five papillomas and seven carcinomas that were initiated with MCA. Two papilloma DNA samples were found to hybridize with probe 61F (Fig. 2*C*), indicating the occurrence of an $A^{182} \rightarrow T$ transversion, and this was confirmed by the presence of an *Xba* I RFLP (data not shown). The remainder of the tumor DNAs were negative for mutations at codons 12 or 61 of Ha-*ras*. DNA samples from four carcinomas, however, gave a positive signal with probe 13A, indicative of mutations at the middle position of codon 13 (Fig. 3). In each case, the mutation was subsequently identified as a $G^{38} \rightarrow T$ transversion. Such a lesion creates an additional *Hin*fI RFLP (Fig. 4*A*), which was confirmed by Southern analysis of the respective tumor DNA samples (Fig. 4*B*). In addition, one of the carcinomas had amplified the mutant *ras* allele (lane 3).

DNA samples from a further 15 MCA-initiated papillomas have been analyzed by Southern blotting for *Hin*fI or *Xba* I RFLPs. Of these papilloma DNAs, two were found to exhibit an *Xba* I site indicating an $A^{182} \rightarrow T$ alteration, whereas four samples contained a *Hin*fI polymorphism diagnostic of a $G^{38} \rightarrow T$ transversion (data not shown).

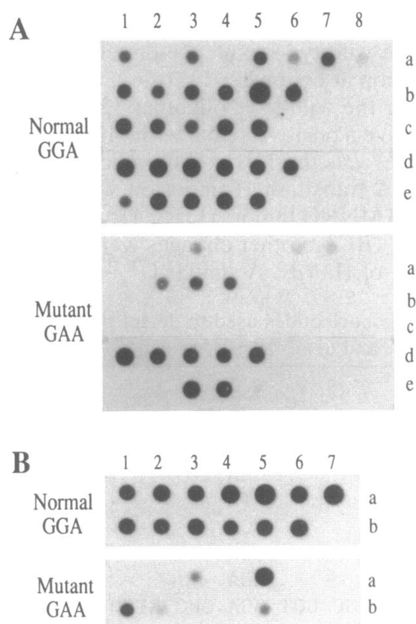

FIG. 1. Identification of Ha-*ras* codon 12 G → A transitions. DNAs from MNNG- or MNU-initiated tumors were amplified for exon 1 and spotted onto duplicate nylon filters. (*A*) MNNG-initiated tumors. Positions 2a–8a, 1b–3b, and 1d–4d contain papilloma DNAs (DNAs from tumors corresponding to 2a, 4a, and 8a, which failed to amplify originally, were reamplified and are shown in 2e–4e). Positions 1a, 4b, 5b, 1c–4c, 5d, 6d, 1e, and 5e contain carcinoma DNAs; 6b and 5c contain control DNA from normal mouse skin. The result of hybridizing with both the wild-type oligonucleotide 12A (GGA) and the mutant oligonucleotide 12B (GAA) is shown. (*B*) Analysis of MNU-initiated papillomas. Positions 2a–7a and 1b–5b correspond to tumor samples. Position 1a contains a negative control DNA. The result of probing with wild-type (GGA) and mutant (GAA) oligonucleotides is shown.

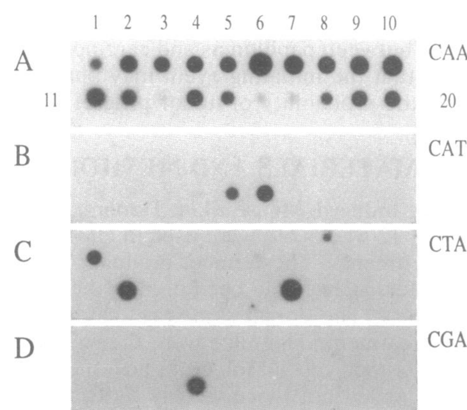

FIG. 2. Characterization of Ha-*ras* codon 61 mutations in DNAs from tumors initiated with DMBA and MCA. Tumor samples were amplified with exon 2 amplimers and probed with oligomers specific for codon 61 mutations. DNA samples were as follows: 1–4, and 12, MCA-initiated papillomas; 5–11, MCA carcinomas; 13–16, DMBA papillomas; 17, DMBA carcinoma that contains an *Xba* I RFLP; 18–20, normal mouse skin controls. The autoradiographs shown in *A–D* were obtained by hybridizing duplicate filters with probes 61A (wild type), 61H (CAT), 61F (CTA), and 61D (CGA), respectively.

DISCUSSION

In this study, we have characterized all Ha-*ras* codon 12, 13, and 61 mutations found in a series of mouse skin tumors initiated by several types of carcinogens, including polycyclic aromatic hydrocarbons and direct-acting methylating agents. The results are summarized in Table 3. For each carcinogen, a distinct pattern of mutations is observed, supporting the proposal that the *ras* mutations are a direct result of interaction between the carcinogen and DNA (5), and, conse-

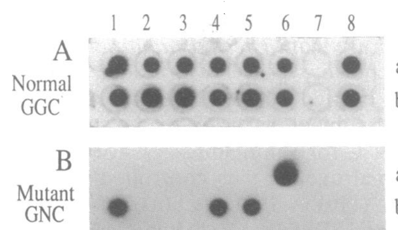

FIG. 3. Demonstration of Ha-*ras* codon 13 mutations in four carcinomas initiated by MCA. DNA samples were amplified *in vitro* and analyzed by selective oligonucleotide hybridization. Filters were hybridized with wild-type probe 12A (*A*) or probe 13A specific for a point mutation at the middle position of codon 13 (*B*). Papilloma DNA samples correspond to positions 1a–4a and 6b, and carcinoma DNA samples correspond to positions 5a, 6a, and 1b–5b. Positions 8a and 8b contain normal mouse skin DNA as controls.

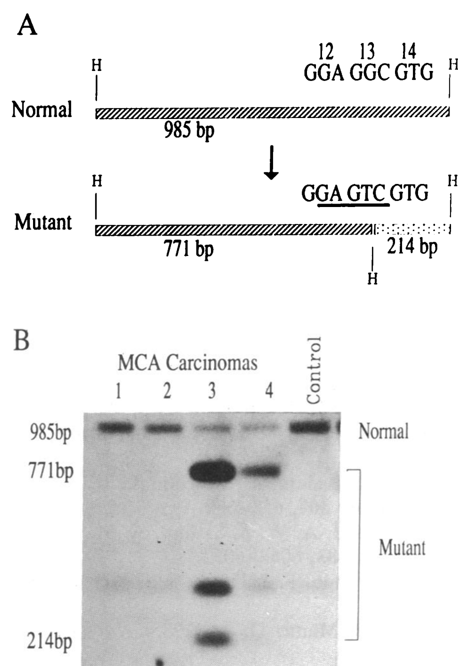

FIG. 4. *Hin*fI polymorphism in MCA carcinomas. (*A*) Induction of a *Hin*fI polymorphism by a specific mutation at codon 13 of c-Ha-*ras*. Digestion of normal mouse DNA with *Hin*fI gives rise to a 985-base-pair (bp) fragment containing exon 1, together with a 300-bp fragment containing exons 2 and 3. Mutation of the middle base of codon 13 from G to T would give rise to a new *Hin*fI site within the 985-bp fragment, generating mutation-specific fragments of 771 and 214 bp. (*B*) Southern analysis of representative samples of MCA carcinomas. DNAs were digested with *Hin*fI, transferred to nylon membrane, and hybridized with a mouse Ha-*ras* probe (22). Lanes 1–4 contain carcinoma DNAs corresponding to samples 2b, 5a, 6a, and 1b shown in Fig. 3. Also shown is DNA from normal mouse skin (Control).

quently, *ras* activation is an initiating event in two-stage skin carcinogenesis (7).

Both MNU and MNNG in mouse skin induce Ha-*ras* mutations at the middle base of codon 12, which are exclusively $G^{35} \rightarrow A$ transitions, as has been observed in MNU-induced rat mammary tumors (5, 23). This lesion is consistent with the production of an O^6-methylguanine adduct (24). Such adducts are known to result in G·C → A·T transitions by mispairing with thymidine during DNA replication (25).

One intriguing point is the selectivity of mutation for the middle base of codon 12. No mutations are seen at the first guanine or in either of the codon 13 guanine residues (GGC). Substitution of Gly^{12} by an amino acid other than proline results in the activation of *ras* (26), as determined by transforming activity. Not all Gly^{13} substitutions, however, appear to be equally effective in activating the gene (27, 28). Whereas

Table 3. Carcinogen induced Ha-*ras* mutations in mouse skin tumors

Tumor	Carcinogen	Mutation	Incidence
Papilloma	MNNG	$G^{35} \rightarrow A$	11/15
Carcinoma	MNNG	$G^{35} \rightarrow A$	2/13
Papilloma	MNU	$G^{35} \rightarrow A$	5/12
Papilloma	DMBA	$A^{182} \rightarrow T$	45/48
		$A^{182} \rightarrow G$	1/48
		$A^{183} \rightarrow T$	2/48
Carcinoma	DMBA	$A^{182} \rightarrow T$	10/13
Papilloma	MCA	$A^{182} \rightarrow T$	4/20
		$G^{38} \rightarrow T$	4/20
Carcinoma	MCA	$G^{38} \rightarrow T$	4/7

Val^{13} or Asp^{13} substitutions result in activation, although to a lesser degree than do Gly^{12} substitutions, replacement by Ser^{13} has little effect on transforming activity (28). Thus a $G^{37} \rightarrow A$ mutation, encoding Ser^{13}, may not result in a sufficiently abnormal *ras* protein to confer any selective advantage, in growth or differentiation *in vivo*, necessary for papilloma formation. The absence of $G^{34} \rightarrow A$ or $G^{38} \rightarrow A$ lesions, however, cannot be explained in this manner. Here, additional factors may play a role in determining the observed selectivity, such as the influence of neighboring bases, either on the carcinogen–DNA interaction (29) or on the mechanisms responsible for repair (30).

Tumors induced by polycyclic aromatic hydrocarbons show a more heterogeneous spectrum of Ha-*ras* mutations. From these and previous studies (7), the critical premutagenic lesions formed by DMBA metabolites would appear to involve almost exclusively deoxyadenosine adducts. Unlike many polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene and MCA, which form major adducts with guanine nucleotides (31), DMBA is known to react extensively with adenine moieties (32, 33).

Although two papillomas arising from MCA initiation contained an $A^{182} \rightarrow T$ mutant *ras*, the majority of carcinomas analyzed contained, surprisingly, a codon 13 $G^{38} \rightarrow T$ lesion. Thus, for MCA, deoxyguanosine adduct formation accounts for at least a large part of this compound's carcinogenicity. While MCA adduct formation has been far less extensively studied than that of either benzo[*a*]pyrene or DMBA, the pattern is reported to be similar to that of benzo[*a*]pyrene with the major species formed being the N-2 guanine adduct (34). In agreement with this interpretation, examination of a number of tumorigenic C3H/10T½ cell lines, which had been transformed by MCA treatment, has shown several of these lines to contain a Ki-*ras* gene activated by a $G^{34} \rightarrow T$ transversion (35).

The most surprising findings of our study were the differences in mutational patterns observed between papillomas and carcinomas initiated by the same carcinogen. Previous studies on DMBA-initiated tumors had shown the same $A^{182} \rightarrow T$ pattern in both papillomas and carcinomas (7). However, the results of the present investigation, using MCA or MNNG as initiating agents, clearly show marked differences between the mutation patterns for benign and malignant tumors. Thus, while most of the MNNG papillomas (11 of 15) have a $G^{35} \rightarrow A$ mutation in Ha-*ras*, this lesion is very infrequent in the carcinomas (2 of 13). Consequently, if skin carcinomas occur by progression from existing premalignant papillomas, there are at least two subsets of papillomas that arise by MNNG initiation; those with Ha-*ras* $G^{35} \rightarrow A$ transitions, which show a lower probability of progression and give rise to a minority of carcinomas; and those with no detectable Ha-*ras* mutation, which show a higher probability of progression, and so give rise to the majority of carcinomas. Alternatively, carcinomas may develop not from preexisting papillomas but directly from a separate initiated cell population. The majority of accumulated data, however, would discount the latter possibility (36).

The unequal distribution of mutations could also be explained by the possibility that the Ha-*ras* mutation is no longer needed to maintain the tumorigenic phenotype and is subsequently lost during the progression of papillomas to carcinomas. Recent results from this laboratory, however, contradict this theory. Tumors that contain Ha-*ras* mutations are also found to contain gross changes involving mouse chromosome 7 (R. Bremner and A. Balmain, unpublished results). Such changes are not found in carcinomas lacking Ha-*ras* mutations. Thus, if these carcinomas arise from progression of papillomas that contain mutant *ras*, this would require the loss of two independent genetic changes.

Several MNNG-initiated carcinoma DNAs were used in 3T3 transfections with negative results (data not shown). The initiating event for these tumors is, therefore, unlikely to involve Ki- or N-*ras* mutations, although the presence of a weakly transforming mutation cannot be formally excluded. Alternative possibilities for initiating events include activation of an oncogene that cannot transform 3T3 cells or inactivation of a tumor suppressor gene.

A difference in the distribution of mutations is also seen for MCA-initiated tumors, although, in this case, different Ha-*ras* mutations appear to show different probabilities of progression. Whereas papillomas were found to contain $G^{38} \rightarrow T$ (4 of 20) and $A^{182} \rightarrow T$ (4 of 20) mutations, only the $G^{38} \rightarrow T$ transversion was detected in the carcinomas examined. Thus, initiated cells that contain a $G^{38} \rightarrow T$ activated Ha-*ras* are apparently more likely to progress than those that contain an $A^{182} \rightarrow T$ mutation. No significant difference in the mean latency of carcinoma formation was observed between MCA and DMBA initiation in this study, and the mutational spectrum seen in carcinomas cannot simply be explained by a difference in progression rate between papillomas containing different *ras* mutations.

The absence of $A^{182} \rightarrow T$ mutations in MCA-initiated Ha-*ras* carcinomas may be a consequence of the relatively small number of tumors examined. Around 5–10% of DMBA-initiated papillomas, containing $A^{182} \rightarrow T$ transversions, progress to carcinomas. As only 20% (4/20) of MCA-induced papillomas contain this mutation, $\approx$100 papillomas would be required to produce one carcinoma containing such a mutation. To clarify this point, further experiments are necessary to obtain and analyze larger numbers of tumors.

The existence of subsets of papillomas with differing probabilities of progression (9) is supported by recent results from several groups. In experiments in which bromomethylbenzanthracene (BrMBA) initiation/BrMBA promotion versus DMBA initiation/BrMBA promotion were compared, a larger number of papillomas were produced by the latter protocol. No corresponding increase, however, in carcinoma yield was observed (11), suggesting that these additional papillomas do not progress to malignancy within the normal life-span of the animals. Hennings *et al.* (13) have identified papillomas with high probabilities of conversion to malignancy, including early developing papillomas promoted by TPA.

The identification of papillomas with a high probability of progression, as described in this report, should permit a more detailed analysis of the critical changes that lead to malignancy.

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