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Genetics

Tumorigenic methylcholanthrene transformants of C3H/10T½ cells have a common nucleotide alteration in the c-Ki-*ras* gene

(malignant transformation/carcinogenesis/*ras* genes/cell culture)

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ABSTRACT **The polymerase chain reaction was used to amplify DNA surrounding the codon 12 region of the c-Ki-*ras* gene from C3H/10T½ cells and from a number of 3-methylcholanthrene (MCA)-transformed derivatives of these cells. Sequence analysis demonstrated that tumorigenic MCACl16/39 cells, known by DNA-mediated transfection to contain an activated c-Ki-*ras* oncogene, had a G → T transversion in the first position of codon 12 of this gene, resulting in a Gly^{12} → Cys mutation. A combination of polymerase chain-reaction amplification and oligonucleotide hybridization demonstrated that three additional tumorigenic MCA transformants of C3H/10T½ cells had an identical mutation in the c-Ki-*ras* gene. In contrast, this mutation was not present in an MCA-induced C3H/10T½ transformant that was not tumorigenic. The molecular specificity of this MCA-induced mutation resulting in C3H/10T½ tumorigenic transformants should provide an excellent system in which to study the roles of transcription, replication, repair, and exogenous factors in the establishment and expression of transformation and tumorigenicity.**

It has been argued that transformation in cell culture in response to chemical carcinogens should be a valuable model to elucidate mechanisms of carcinogenesis, because of the opportunity to closely control variables such as time of exposure to carcinogens, concentration of chemicals, cell cycle position, exposure to secondary effectors such as tumor promoters, etc. The murine C3H/10T½ cell transformation system, first described by Heidelberger and his colleagues (1), has been used to identify a wide variety of complete carcinogens, initiators, and promoters. DNA samples isolated from tumors derived from 3-methylcholanthrene (MCA)-transformed C3H/10T½ cell lines were among the first tumor DNA samples to be used to demonstrate the presence of dominant transforming oncogenes by DNA-mediated transfection (2). The activated oncogene in three tumors derived from MCA-transformed C3H/10T½ cells was identified as a c-Ki-*ras* gene (3).

Despite the early description of dominant oncogenes in MCA-transformed tumorigenic C3H/10T½ cells, the specific mutations associated with activation of oncogenes as a consequence of chemical carcinogenesis have been described in experimental animals, not in cultured cells. Exposure of female rats to the direct-acting carcinogen *N*-methyl-*N*-nitrosourea caused mammary tumors with an identical (G^{35} → A) transition in the second position of codon 12 of the c-Ha-*ras* gene in all 48 tumors analyzed (4). The extraordinary specificity of this mutation is surprising, since (*i*) studies with a shuttle vector (5) suggest that mutations in the 5′ G residue of GG pairs also occur in response to *N*-methyl-*N*-nitrosourea in mammalian cells and (*ii*) mutation at the first G residue of codon 12 of the c-Ha-*ras* gene is also transforming (6). In contrast, Reynolds *et al.* (7) have shown that a single carcinogen, furfural, can lead both to distinct mutations within the c-Ha-*ras* gene and to mutations in different *ras* genes, all of which are associated with hepatic tumors in the murine B6C3F1 carcinogen-testing system. The remarkable specificity of activating mutations in some instances of chemical carcinogenesis (4, 8) and the relative promiscuity of activating mutations in other systems (7, 9, 10) make it difficult to predict whether a common mutation or a variety of mutations will be responsible for the transformation of cells in culture in response to a single chemical carcinogen. We have examined this question by characterizing the c-Ki-*ras* genes from MCA-induced transformants of C3H/10T½ cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. All cell lines were passaged in Eagle's basal medium (GIBCO) containing 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. The MCATx1e cell line was isolated in our laboratory (11). MCACl16/39 (1) and MCA5 (12) cells were obtained from Robert Weinberg (Massachusetts Institute of Technology). MCACl15 (1) and MCASi-24 (13) cells were obtained from Joseph Landolph (University of Southern California). A separate sample of MCACl15 cells (CRL 1411) was purchased from the American Type Culture Collection.

DNA Isolation and Polymerase Chain Reaction (PCR) Amplification. DNA was isolated by resuspending cell pellets in 100 mM NaCl/10 mM Tris·HCl, pH 8.0/10 mM EDTA/0.5% SDS. The lysate was digested overnight with proteinase K (Boehringer Mannheim) at 200 μg/ml and purified by phenol/chloroform (1:1, vol/vol) extraction. DNA was digested for 1 hr with RNase A at 40 μg/ml, phenol/chloroform-extracted, and dialyzed overnight against 1 mM Tris·HCl, pH 7.6/0.1 mM EDTA. Oligonucleotides for amplification (20-mers) and analysis (17-mers) were synthesized by Dohn Glitz (University of California–Los Angeles) and are shown in Table 1. Ninety-base-pair regions surrounding codon 12 of the c-Ki-*ras* gene were amplified with *Thermus aquaticus (Taq)* DNA polymerase according to instructions from the manufacturer (Perkin–Elmer/Cetus), with the following modifications. In the first round of amplification, samples were incubated at 95°C for 7 min; 2.5 units of *Taq* polymerase was added and samples were incubated at 70°C for 2.5 min. For the remaining rounds of amplification, cycles consisted of 1 min of denaturation at 95°C, 1 min of annealing at 40°C, and 2 min of extension at 70°C. Thirty rounds of amplification

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Abbreviations: MCA, 3-methylcholanthrene; PCR, polymerase chain reaction.

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Table 1. Oligomers used for PCR (amplimers) and hybridization analysis

Oligomer	Sequence
Amplimers	
Codon 12 sense	5'-ATGACTGAGTATAAACTTGT-3'
Codon 12 antisense	5'-TCCACAAAGTGATTCTGAAT-3'
Probes	
Codon 12 wild type	5'-GGAGCTGGTGGCGTAGG-3'
Codon 12 mutant	5'-GGAGCTTGTGGCGTAGG-3'

were carried out. Amplified DNA was purified by electrophoresis in polyacrylamide gels and subsequent elution onto Whatman DE81 ion-exchange paper.

Cloning and Sequence Analysis. The amplified DNA was cloned into the *Sma* I site of phage M13mp19 DNA by standard techniques. To identify those phage with inserts, filter lifts (14) were probed with end-labeled (15) codon 12 sense amplimer (Table 1) used for PCR. Templates from a series of phage clones containing c-Ki-*ras* inserts were prepared and sequenced with [α-[^{35}S]thio]dATP by using the Sequenase kit according to instructions from the manufacturer (United States Biochemical).

To isolate additional mutant clones of the codon 12 region of the c-Ki-*ras* gene of MCACl16/39 for sequencing, filter lifts of M13 clones were hybridized with an end-labeled "wild-type" probe and a "mutant" probe (Table 1). Filters were baked and then incubated for 1 hr at 42°C in a prehybridization solution containing 5× SSC (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution (1× is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 50 mM sodium phosphate (pH 6.5), 1% glycine, and 250 μg of sonicated denatured salmon sperm DNA per ml (16). Filters were then washed and hybridization was carried out according to a modification of the procedure of Andersen *et al.* (17). Hybridization was for 3 hr at 42°C. Filters were washed in 6× SSC/0.1% SDS twice for 10 min at room temperature, followed by 2 min at 60°C for the wild-type oligomer probe. Additional mutant clones were picked and templates were prepared and sequenced.

Oligonucleotide Hybridization Analysis of Slot-Blotted, Amplified DNAs. DNA was amplified and electrophoretically purified as described above. Amplified DNA (4% of the eluted sample) was slot-blotted on nitrocellulose according to the manufacturer's instructions, using an apparatus purchased from Schleicher & Schuell. Nitrocellulose filters were hybridized with end-labeled (*i*) flanking probe used for amplification, (*ii*) wild-type GGT (Gly^{12}) probe, and (*iii*) mutant TGT (Cys^{12}) probe according to the conditions of Verlaan-de Vries *et al.* (18), with the exception that hybridization was performed at 42°C and filters were washed at 54°C after the initial wash at room temperature. Readers should note that, in each experiment, a C3H/10T½ sample was simultaneously subjected to amplification and oligonucleotide hybridization analysis, to assure that no contamination of the PCR product had occurred.

RESULTS

Identification of the c-Ki-*ras* Mutation in the Tumorigenic MCA Transformant MCACl16/39. Cultures of C3H/10T½ cells, MCACl16/39 cells, and the other cell lines used in this study are shown in Fig. 1. When compared to C3H/10T½ cells, all the MCA transformants grow to much higher cell densities and appear morphologically transformed. MCACl-16/39 is one of the tumorigenic MCA-transformed C3H/10T½ cell lines that Parada and Weinberg (3) identified, by DNA-mediated transfection, as containing an activated c-Ki-*ras* oncogene. We isolated genomic DNA from MCACl16/39 cells and used oligonucleotide primers to carry out a PCR amplification of a 90-base-pair region centered on codon 12 of the c-Ki-*ras* gene. We planned to identify the activating lesion, should it be in the amplified region of the c-Ki-*ras* gene, by direct sequencing of the amplified DNA. However, our sequence data were ambiguous. We thought that this might be due to the polyploidy of C3H/10T½ cells. If more than two copies of c-Ki-*ras* were present, a single mutated allele might be difficult to observe on a sequence "ladder." To circumvent this problem the PCR-amplified DNA was cloned into an M13 vector. Plaques from M13 phage containing inserts were identified by hybridization of filter lifts with the codon 12 sense amplimer used for PCR. When 12 clones were sequenced, the majority had the wild-type GGT (Gly^{12}) sequence at codon 12. One clone had a transversion at G^{34}, resulting in a TGT (Cys^{12}) mutation in the c-Ki-*ras* gene (Fig. 2).

We again amplified DNA from the 90-base-pair region around codon 12 of the c-Ki-*ras* gene of MCACl16/39 cells, cloned the amplified DNA into M13, and analyzed plaques. Triplicate lifts were made, and M13 phage containing inserts in the proper orientation were identified with a flanking

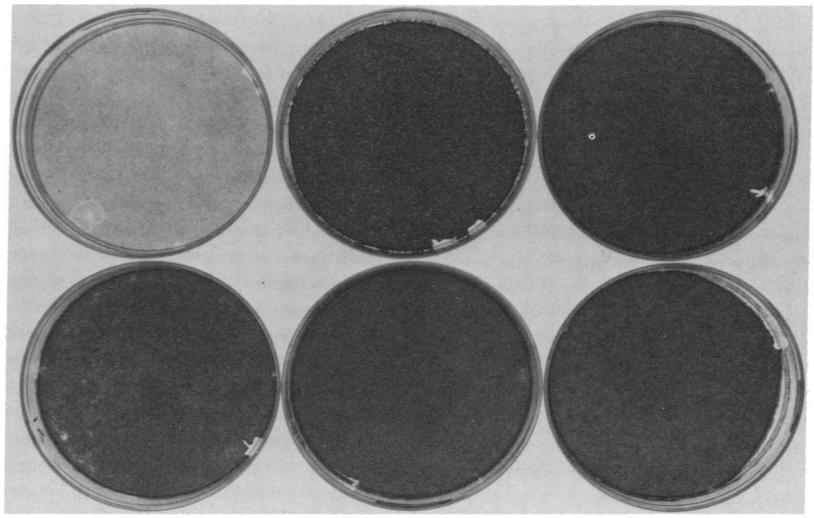

FIG. 1. Cultures of C3H/10T½ cells and the MCA-induced transformants of C3H/10T½ cells used in this study. Cells (10^5) were plated in 60-mm dishes, fed every third day, and stained with crystal violet 10 days after plating. Top row, left to right: C3H/10T½, MCACl16/39, MCA5. Bottom row, left to right: MCACl15; MCASi-24, MCATx1e.

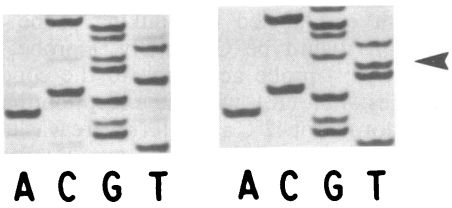

FIG. 2. Sequence of the wild-type and mutant codon 12 region of the c-Ki-*ras* gene from MCACl16/39 cells. A 90-base-pair region surrounding codon 12 of the c-Ki-*ras* gene of MCACl16/39 cells was amplified and cloned into M13. Phage plaques containing inserts were identified by hybridization of filter lifts with one of the flanking probes used for PCR. Templates were prepared and were sequenced by the dideoxy chain-termination method. (*Left*) Clone containing the wild-type GGT sequence. (*Right*) Clone containing the mutant TGT sequence. Arrowhead indicates the altered nucleotide.

oligonucleotide probe used for PCR. From this collection of clones, wild-type clones were identified with a 17-nucleotide probe containing the GGT sequence. Mutant clones, which did not hybridize with the GGT probe, were also positively identified with a 17-mer probe containing the TGT sequence. Approximately one in eight clones with inserts hybridized to the TGT probe. When sequenced, additional clones that hybridized with the mutant probe had the same $G^{34} \rightarrow T$ ($Gly^{12} \rightarrow Cys$) mutation.

Other Tumorigenic MCA Transformants of C3H/10T½ Cells Have the Same Mutation as MCACl16/39. We next wished to determine whether the mutation identified in MCACl16/39 occurred in other MCA-induced C3H/10T½ transformants. Parada and Weinberg (3) showed by DNA-mediated transfection that the tumorigenic MCA-induced C3H/10T½ transformant MCA5 also had an activated c-Ki-*ras* gene. MCACl15 and MCASi-24 are two additional high-dose MCA transformants known to give rise to tumors in experimental animals (1, 13). However, these two cell lines have never been analyzed for the presence or absence of dominant transforming oncogenes.

Genomic DNA was amplified around the codon 12 region of the c-Ki-*ras* gene from C3H/10T½ cells and from the four MCA-transformed, tumorigenic derivatives. Electrophoretically purified amplified DNA samples were slot-blotted onto nitrocellulose filters in triplicate and hybridized with end-labeled (*i*) flanking probe used for PCR, (*ii*) 17-nucleotide wild-type oligomer with the GGT (Gly^{12}) sequence, and (*iii*) 17-nucleotide "mutant" oligomer with the TGT (Cys^{12}) sequence. All four independently isolated tumorigenic MCA transformants have an activated c-Ki-*ras* oncogene with the same $G^{34} \rightarrow T$ ($Gly^{12} \rightarrow Cys$) transversion (Fig. 3). A second MCACl15 sample, obtained from an independent source, had the same mutation.

A Nontumorigenic MCA C3H/10T½ Transformant Does Not Have the $G^{34} \rightarrow T$ c-Ki-*ras* Mutation. A previous report (11) described a high-dose MCA C3H/10T½ transformant, MCATx1e, that is indistinguishable in culture from tumorigenic MCA transformants (Fig. 1). However, despite their loss of growth control in culture, MCATx1e cells cannot form colonies in soft agar or give rise to tumors in nude mice (19). The 90-base-pair region surrounding codon 12 of the c-Ki-*ras* gene of MCATx1e cells was amplified, slot-blotted, and analyzed with the three end-labeled oligonucleotide probes (Fig. 3). In contrast to the four tumorigenic MCA-induced C3H/10T½ transformants, MCATx1e cells did not have the $G^{34} \rightarrow T$ ($Gly^{12} \rightarrow Cys$) mutation in the c-Ki-*ras* gene.

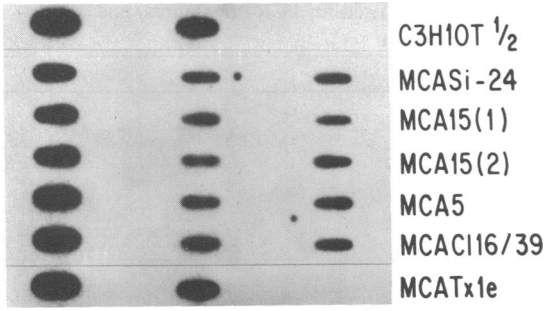

FIG. 3. Oligonucleotide hybridization analysis for the codon 12 region of the c-Ki-*ras* gene of MCA C3H/10T½ transformants. DNA surrounding the codon 12 region of the c-Ki-*ras* gene was amplified from each cell type and slot-blotted onto nitrocellulose. Slot blots were probed with flanking probe (left column), probe containing the GGT (Gly^{12}) sequence (center column), and probe containing the TGT (Cys^{12}) sequence (right column). MCACl15(1) was obtained from Joseph Landolph; MCACl15(2) was obtained from the American Type Culture Collection. In the experiment shown in this figure all DNA samples were amplified and analyzed concurrently. The autoradiograph was developed after 1 hr of exposure.

DISCUSSION

Our data suggest that the majority of, if not all, tumorigenic transformants of C3H/10T½ cells induced by MCA will share a common mutation, a $G^{34} \rightarrow T$ transversion in codon 12 of the c-Ki-*ras* oncogene. The four tumorigenic MCA transformants we have analyzed have, however, been extant for a number of years. They may have initially been chosen for study as a result of subjective criteria (growth, focus formation, etc.) that reflect, as a result of this mutation, a more prominent transformed phenotype. Alternatively, the $G^{34} \rightarrow T$ mutation may have occurred subsequent to their initial isolation, and been selected for during their extensive passage.

In the C3H/10T½ cell culture transformation system there are no developmental restrictions on replication; all cells are proliferating. There is no immune surveillance. In principle, any oncogene that is transcribed should be a potential target for transformation. We therefore find it surprising that all four tumorigenic MCA transformants share an identical lesion. Moreover, Smith and Grisham (20) have reported that an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced transformant of C3H/10T½ cells harbors an activated c-Ha-*ras* gene rather than an activated c-Ki-*ras* gene. Use of the PCR procedure followed by cloning and sequencing will make it possible to identify mutations in the various *ras* genes of C3H/10T½ cells, in response to a variety of chemical carcinogens, that lead to transformation and tumorigenicity. By coupled PCR and oligonucleotide hybridization, the mutational specificity of transformation in response to individual carcinogens can be rapidly evaluated in many independent transformants.

The roles of transcription and DNA replication in the transformation process have been the subject of considerable discussion. Both of these processes can be manipulated for the c-Ki-*ras* gene in the C3H/10T½ cell culture transformation system. Borek *et al.* (21) have shown that thyroid hormone is an important "cofactor" for chemical transformation of C3H/10T½ cells. Guernsey and Leuthauser (22) showed that the presence of triiodothyronine in the medium immediately prior to and during exposure to MCA increased the transformation frequency of C3H/10T½ cells by as much as 6-fold. Triiodothyronine also stimulated the transcription of the c-Ki-*ras* gene by an equivalent amount. These data suggest that thyroid hormone modulation of MCA-induced transformation of C3H/10T½ cells is a result of altered transcriptional activity of the c-Ki-*ras* gene at the time of MCA exposure. Several laboratories have demonstrated that C3H/10T½ cells are more susceptible to chemical transformation if cells are exposed to carcinogen in early S phase (23,

24). These data suggest that protooncogene replication influences the establishment of mutation(s) leading to tumorigenicity. A serum-free medium has been developed in which growth of C3H/10T½ cells can be made dependent on epidermal growth factor (25). The combination of PCR, M13 cloning, and screening of phage plaques with labeled oligonucleotides allows us to identify and quantitate the presence of the c-Ki-*ras* $G^{34} \rightarrow T$ mutation in only a few cells in a culture dish. We can now examine the appearance and propagation of this mutation in C3H/10T½ cultures in response to MCA exposure under a variety of culture conditions.

The presence of growth factors in the medium influences the emergence of the transformed phenotype for confluent C3H/10T½ cells. Mordan (26) reported that focus formation will not occur if MCA-treated C3H/10T½ cells are switched, immediately after attaining confluence, to platelet-poor plasma. Readdition of platelet-derived growth factor, a transforming growth factor β-like activity, and an epidermal growth factor-like activity restored focus formation, but only after a substantial lag period. By using the procedures described here it should be possible to determine both the presence in these cultures of cryptically transformed cells carrying the c-Ki-*ras* $G^{34} \rightarrow T$ lesion and the proliferation of these transformed cells in response to readdition of growth factors.

Exposure of C3H/10T½ cells to MCA results in a variety of transformed phenotypes. It will be of great interest to see whether additional nontumorigenic transformants, like MCATx1e, do not have the c-Ki-*ras* $G^{34} \rightarrow T$ mutation identified in the four MCA tumorigenic transformants. Mordan *et al.* (27) isolated a single-step MCA transformant of C3H/10T½ cells, INIT/10T½, that appears untransformed when grown in the presence of retinoids, in contrast to most MCA transformants. If retinoids are removed INIT/10T½ cells will express a transformed phenotype, but only after a substantial lag period. Bertram and his colleagues (27) consider these cells to be "initiated" and suggest that events that contribute to the establishment of the transformed phenotype must occur after retinoid withdrawal. INIT/10T½ cells are tumorigenic in nude mice, but only after a longer latent period than MCA transformants such as MCAC116/39. It will be of great interest to determine whether INIT/10T½ cells carry the c-Ki-*ras* $G^{34} \rightarrow T$ mutation shared by all the MCA tumorigenic C3H/10T½ transformants we have analyzed. The procedures described here will also make it possible to determine whether single-stage and two-stage (28) MCA transformants of C3H/10T½ cells, like single- and two-stage murine skin tumors isolated in response to dimethylbenzanthracene (29), have a common activating *ras* mutation.

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