Loss of oncogenic *ras* expression does not correlate with loss of tumorigenicity in human cells

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ABSTRACT *ras* oncogenes are mutated in a variety of human tumors, which suggests that they play an important role in human carcinogenesis. To determine whether continued oncogenic *ras* expression is necessary to maintain the malignant phenotype, we studied the human fibrosarcoma cell line, HT1080, which contains one mutated and one wild-type N-*ras* allele. We isolated a variant of this cell line that no longer contained the mutated copy of the N-*ras* gene. Loss of mutant N-*ras* resulted in cells that displayed a less transformed phenotype characterized by a flat morphology, decreased growth rate, organized actin stress fibers, and loss of anchorage-independent growth. The transformed phenotype was restored following reintroduction of mutant N-*ras*. Although loss of the oncogenic N-*ras* drastically affected *in vitro* growth parameters, the variant remained tumorigenic in nude mice indicating that mutated N-*ras* expression is not necessary for maintenance of the tumorigenic phenotype. We confirmed this latter observation in colon carcinoma cell lines that have lost activated K-*ras* expression via targeted knockout of the mutant K-*ras* gene.

Mammalian Ras genes encode a family of plasma membrane-bound proteins that function as intermediates in signal transduction pathways involved in cell growth and differentiation. *ras* oncogenes are frequently mutated in human cancer, which suggests that they play a pivotal role in neoplastic transformation. Although studies of *ras*-mediated transformation led to claims of "dominantly acting" oncogenes (1), it is now clear that oncogenic *ras* expression is not sufficient to create the malignant state in primary human cells (2–6) but may predispose cells to transformation that occurs subsequent to other independent events, such as *ras* amplification (6), other oncogene activation (7), or loss of function of tumor suppressor genes (5). Even though oncogenic *ras* is not sufficient for neoplastic transformation, its continued expression might be necessary to maintain the malignant phenotype. Indeed, it has been suggested that the "dominance" of oncogenes exists once cells have undergone other genetic changes, such as loss of a growth suppressor gene (8). It is obviously important to determine whether *ras* has a direct role in maintaining the neoplastic behavior of cancer cells. Tacit acceptance of the notion of dominantly acting oncogenes is inherent in recent research focusing on the development of anti-*ras* drugs for cancer therapy (9–17).

To address whether oncogenic *ras* function is necessary for maintenance of tumorigenicity in human tumor cells, we exploited a variant of HT1080 human fibrosarcoma cells that no longer expressed the endogenous mutated N-*ras* allele. HT1080 cells are ideal for these studies because they have a predominantly pseudodiploid karyotype and contain one normal and one mutated N-*ras* allele. This mutated allele results

from a point mutation at codon 61 that converts Gln^{61} to Lys^{61} (18). HT1080 cells are completely transformed in culture and form progressively growing tumors in athymic mice. Furthermore, previous studies suggest that an absolute level of expression of the activated N-*ras* gene product controls the tumor-forming capability of HT1080 cells in a dosage-dependent manner (19). In this study, we report the effect of loss of oncogenic N-*ras* on the transformed and tumorigenic states of HT1080.

In addition, we have examined the oncogenic potential of colon carcinoma cells that have lost mutant K-*ras* expression as a consequence of targeted knockout of the relevant allele.

MATERIALS AND METHODS

HT1080 Cell Lines. The HT1080 variant, microcell hybrid (MCH) 603c8, which lacks the activated N-*ras* allele, was serendipitously isolated from a MCH fusion in the following way. HT1080 6TG, a hypoxanthine phosphoribosyltransferase-deficient variant of the HT1080 fibrosarcoma cell line (20, 21), was used as a recipient in microcell-mediated chromosome transfer experiments as described (22, 23). A *Escherichia coli* guanine phosphoribosyl transferase-tagged chromosome 1, containing the normal N-*ras* allele located at 1p13 (24) derived from the parental HT1080 cell line (21), was transferred into mouse A9 cells. The chromosome 1 subsequently was transferred from the donor A9 MCH into HT1080 6TG cells, and MCHs containing the transferred chromosome were selected in HAT (hypoxanthine/amethopterin/thymidine) medium (25). Pseudodiploid hybrids that contained the extra transferred chromosome were studied in further detail.

Cytogenetic Analysis. Metaphase spread preparation and Giemsa banding were performed as previously described (26). Fluorescence *in situ* hybridization was performed as described using a biotin-dUTP nick-translated chromosome 22 flow-sorted library (27).

Restriction Fragment Length Polymorphism (RFLP) Analysis. Genomic DNA (16 μg) was digested with *Bam*HI, separated by 0.9% agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with radiolabeled pMHZ14 (chromose 1p probe; American Type Culture Collection) as described (28).

Sequence Analysis. To sequence the N-*ras* gene encompassing codon 61, PCR was performed using two primers specific for the second exon: 5'-primer, CAAGTGGTTATAGATG-GTGA and 3'-primer, AGGAAGCCTTCGCCTGTCCT. The 110-bp PCR product was acrylamide gel-purified, subjected to

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asymmetric PCR as described (29), and sequenced directly with Sequenase 2.0 kit (United States Biochemical).

Sequence determination of the three *ras* genes (N-, K-, and H-) was carried out by reverse transcription (Moloney murine leukemia virus; GIBCO/BRL) of extracted total RNA (30) using oligo(dT) primers (GIBCO/BRL) and PCR amplification of the region encompassing codons 12, 13, 59, and 61, followed by cloning of the purified PCR product (Invitrogen). Plasmid DNA from 100 pooled colonies was purified and directly sequenced. The following primers were used for PCR: N-*ras*, 5′-ATGACTGAGTACAAACTGGT and 3′-GAGG-AAGCCTTCGCCTGTCCT; K-*ras*, 5′-CCTGCTGAAAAT-GACTGAAT and 3′-AAATACACAAAGAAAGCCCT; and H-*ras*, 5′-ATGACGGAATATAAGCTGGTG and 3′-ACT-TGGTGTTGTTGATGGCAA. PCR amplification resulted in 250-, 255-, and 265-bp products, respectively.

N-*ras* cDNA Construction and *in Vitro* Mutagenesis. Wild-type N-*ras* cDNA (pZIP-rasNwt4) was cloned into the *Bam*HI site of pCMVneo (31). Codon 61 (CAA; Glu) was mutated to AAA (Lys) via plasmid site-directed mutagenesis (32) using the primer TACAGCTGGAAAAGAAGAGTA to create the plasmid pCMVneo-N-ras^{61K}.

DNA Transfection. Subconfluent (70%) T75 flasks of MCH 603c8 were transfected overnight with 10 μg of linearized (*Hin*dIII) pCMVneo-N-ras^{61K} DNA or vector control DNA (pCMVneo) using 40 μl of lipofectin (GIBCO/BRL) in Opti-MEM medium (GIBCO/BRL). Clones were selected in medium containing 800 μg of G418 (Sigma) per ml.

Western Blot Analysis. Cells from subconfluent six-well dishes were lysed with 200 μl of RIPA buffer [150 mM NaCl, 1% Nonidet P-40, 0.4% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8), and 1 mM phenylmethylsulfonyl fluoride]. One hundred and twenty micrograms of cellular lysate was electrophoresed through a 12% SDS/polyacrylamide gel, and transferred onto an Immobilon membrane (Millipore) as described (33). Western blot analysis proceeded as described (34), using N-*ras* primary antibody (F155; Santa Cruz Biotechnology), anti-mouse IgG (horseradish peroxidase-conjugated) secondary antibody (Santa Cruz Biotechnology), and enhanced chemiluminescence detection (Amersham).

Growth Kinetics. To assess growth kinetics under serum conditions, cells (5×10^4) were seeded into duplicate T25 flasks containing DMEM/10% fetal calf serum (FCS). Flasks were harvested on the indicated days and counted on a Coulter counter. The medium was changed on day 4 and thereafter as necessary. For serum starvation kinetic assays, cells (2×10^5) were plated in DMEM/10% FCS in duplicate T25 flasks. The next day the flasks were washed three times with DMEM with no additives, and then refed with serum-free medium (DMEM/F-12; 1:1) supplemented with sodium selenite (5 ng/ml), insulin (5 μg/ml), and transferrin (5 μg/ml), or sodium selenite alone.

F-Actin Staining. Phalloidin staining was performed as described (35, 36).

Soft Agar Growth. Cells (1×10^5 or 1×10^4) were seeded in 0.3% top agar (in DMEM/10% FCS) above a 0.6% bottom layer (in DMEM/10% FCS) in duplicate 60-mm plates as described (37). Plates were fed periodically with 1 ml of DMEM/10% FCS. Colonies were counted after 3 weeks.

Colon Carcinoma Cell Lines. The DLD-1 colon carcinoma cell line, which is heterozygous for an activated K-*ras* allele and variants which have lost the mutant allele or the wild-type allele as a consequence of targeted knockout, were generated by Shirasawa and colleagues (38).

Tumorigenicity Assays. Tumorigenicity was assessed by subcutaneous injection of 1×10^7 cells, resuspended in 0.2 ml of phosphate-buffered saline, into the flanks of 4- to 6-week-old nude mice (three mice; two sites apiece for each cell line). Tumors were measured in three dimensions with linear calipers on the indicated days.

RESULTS

Oncogenic N-*ras* Influences Cell Morphology and Actin Filament Organization. In previous studies, we showed that the transfer of single copies of normal chromosome 1 via microcell fusion had no effect on the transformed or tumorigenic phenotypes of parental HT1080 cells (39). In this study, we transferred a copy of chromosome 1 derived from HT1080 cells, containing the wild type N-*ras* allele, into a hypoxanthine phosphoribosyltransferase-deficient clone of HT1080 (HT1080 6TG) (data not shown). With one exception, all of the HT1080 6TG/chromosome 1 MCH clones retained the transformed and tumorigenic phenotypes of the parental HT1080 6TG cells. These each were found to contain an intact copy of the transferred chromosome 1 (data not shown).

However, one clone, MCH 603c8, had a dramatically different phenotype in culture. This hybrid had a flat morphology and reduced growth rate compared to the parental cell line. Karyotypic analysis showed that the cell line contained two intact copies of chromosome 1 and an unbalanced translocation involving the long arm of chromosome 1 (Fig. 1*A*). The abnormal chromosome was identified as t(1q;22q) by fluorescent *in situ* hybridization using a 22-specific chromosome library (data not shown). The translocation resulted in loss of the short arm of the translocated chromosome 1. All other chromosomal abnormalities detected were also present in the parental HT1080 6TG cell line (Fig. 1*A*). Therefore, this cell line had a net increase in normal chromosome 1q material, and no net loss of chromosome 1p material. To determine the source of the chromosome 1 involved in the translocation, we performed RFLP analysis using a probe specific for the distal short arm of chromosome 1 (Fig. 1*B*). The transferred chromosome, containing the wild-type N-*ras* allele (represented by the 14-kb band), was present in the donor A9/chromosome 1 MCH, the parental HT1080 6TG cell line, a MCH containing an intact transferred chromosome 1 (MCH 603c1), and MCH 603c8. However, the copy of chromosome 1 containing the mutated N-*ras* allele (represented by the 9-kb band) was present in HT1080 6TG and MCH 603c1 but was absent from MCH 603c8 as well as the donor MCH. Sequence analysis of the region surrounding codon 61, showed that MCH 603c8 contained only the wild-type codon 61 sequence (CAA) and had lost the mutated copy (AAA; Fig. 1*C*). This confirmed that the copy of the endogenous chromosome 1 containing the mutant N-*ras* allele was involved in the translocation, resulting in loss of the endogenous chromosome 1p containing the mutant allele.

To determine whether the altered morphology and reduced *in vitro* growth rate of MCH 603c8 were due to loss of the mutated N-*ras* allele, we reintroduced a mutant N-ras^{61K} cDNA, under the control of the cytomegalovirus promoter, into MCH 603c8 by liposome-mediated transfection. Western blot analysis showed high expression of N-*ras* protein in expressing clones (due to the transfected mutant form; Fig. 2, lanes 3 and 4) as compared with the relatively low expression in HT1080 6TG (mutant and wild-type) and MCH 603c8 (wild-type only; Fig. 2, lanes 1 and 2, respectively). Repeated analyses showed that the total amount of N-*ras* protein was similar in HT1080 and MCH 603c8 cells (data not shown). Vector control clones were obtained by transfection of MCH 603c8 with the vector (pCMVneo) lacking an N-*ras* gene.

The parental HT1080 6TG cell line had a rounded, more refractile appearance upon light microscopic examination (Fig. 3*A*), whereas MCH 603c8 had an elongated, flat morphology (Fig. 3*B*). All vector control clones (V2–2 and V3–22) retained the flat appearance (Fig. 3*C*), whereas expressing mutant transfectants (M5–8 and M8–17; Fig. 3*D*) displayed a more rounded morphology reminiscent of the parental cell line.
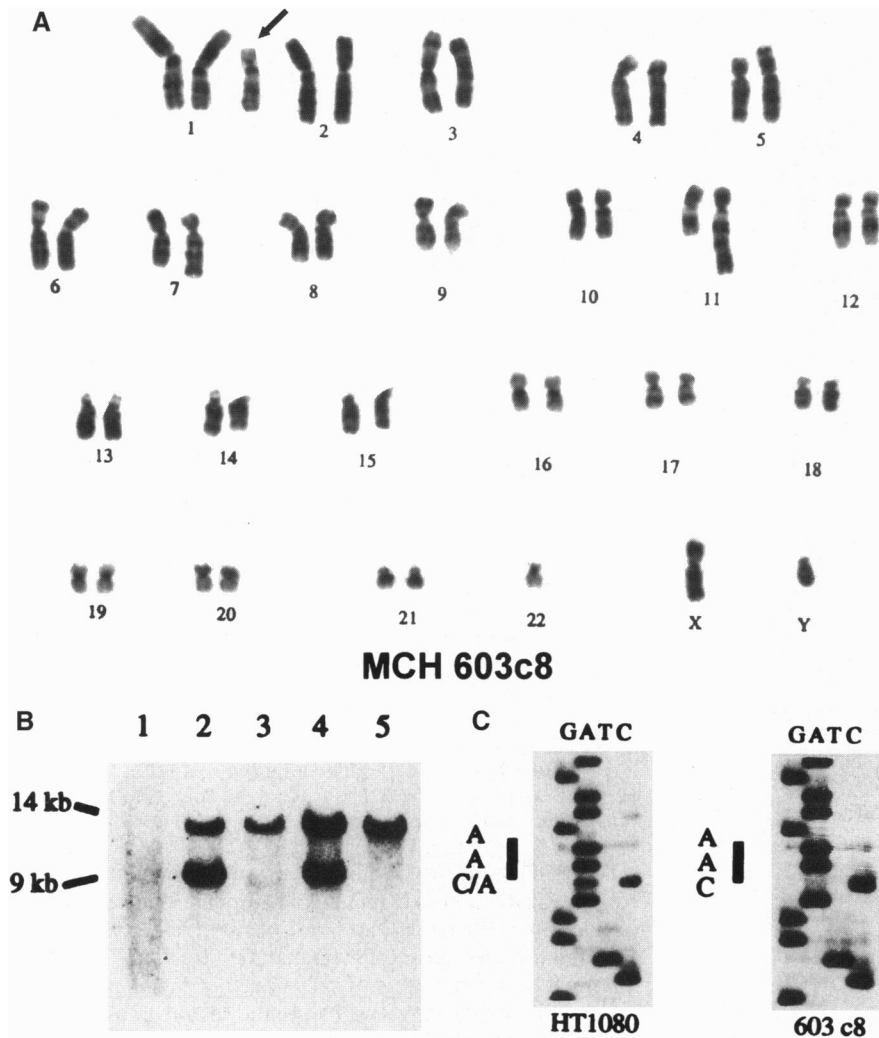
FIG. 1. Loss of the activated N-*ras* allele in MCH 603c8. (*A*) Representative GTG-banded karyotype. MCH 603c8 is pseudodiploid and contains three abnormal chromosomes [5p+, 11q+, and inv(7)] characteristic of the parental HT1080 6TG cell line. The additional, unique t(1q;22q) is indicated by an arrow. (*B*) RFLP analysis of chromosome 1 in MCH 603c8 with a probe specific for the short arm of chromosome 1 (pMHZ14). Lane 1, A9; lane 2, HT1080 6TG; lane 3, MCH 6 m2b2 (donor A9/chromosome 1 hybrid); lane 4, MCH 603c1 (contains an intact transferred chromosome 1); and lane 5, MCH 603c8. (*C*) Direct sequence analysis of the N-*ras* gene surrounding codon 61.

In fibroblasts, actin stress fibers form a major part of the cytoskeleton and are fused to the plasma membrane via focal adhesions. Actin reorganization plays a role in cell motility and cell shape, as well as in other cellular functions (40, 41). After transformation, flat, well-spread, fibroblast-derived cells become more round and display decreased cell–cell contact, which is accompanied by a loss of actin stress fibers in the cytoplasm and accumulation at the cell surface (ruffling; refs. 36 and 40). Membrane ruffling and stress fiber formation have been linked to the *ras*-related proteins, *rac* and *rho*, respectively (35, 36, 42). To determine the effect of loss of mutated N-*ras* expression on actin stress fiber organization in HT1080 cells, we stained with fluorescein isothiocyanate-conjugated phalloidin, which binds to filamentous F-actin (Fig. 3 *E–H*). The parental HT1080 6TG cell line had few detectable actin stress fibers under serum conditions (Fig. 3*E*), whereas MCH 603c8 had prominent stress fibers (Fig. 3*F*) reminiscent of primary fibroblasts. Transfection of mutant N-*ras* into MCH 603c8 resulted in a return to the parental HT1080 6TG

FIG. 2. Expression of N-*ras* protein in HT1080 6TG-derived cell lines. Western blot analysis using N-*ras* antibody that detects mutant and wild-type protein. Lane 1, HT1080 6TG; lane 2, MCH 603c8; lane 3, M5–8; lane 4, M8–17; and lane 5, M5–8TR (tumor reconstitute of M5–8).

phenotype (Fig. 3*H*), whereas vector controls retained the stress fibers observed in MCH 603c8 (Fig. 3*G*).

Oncogenic N-*ras* Affects *in Vitro* Growth. MCH 603c8 displayed altered *in vitro* growth as compared with the parental cell line. This was reflected in an increased doubling time and decreased saturation density (Fig. 4*A* and Table 1). Mutant transfectants grew more quickly than MCH 603c8 and had doubling times and saturation densities comparable with HT1080 6TG. Vector controls also grew slowly, with doubling times approximating that observed for MCH 603c8 (Fig. 4*A* and Table 1). Although MCH 603c8 and vector control clones had lower saturation densities, they did not exhibit complete contact inhibition.

Rodent cells transformed with *ras* oncogenes are able to grow in serum-free medium and have a decreased dependence on external growth factors, presumably because they secrete autocrine growth factors (43, 44). To determine whether loss of the activated N-*ras* results in HT1080 cells that are more serum-dependent, we cultured HT1080 6TG and MCH 603c8 under serum-free conditions, as well as supplemented with insulin and transferrin. Certain transformed cells that secrete autocrine growth factors are only able to grow in serum-free medium supplemented with adjuvant factors (insulin and transferrin; refs. 44 and 45). HT1080 6TG was able to grow under serum-free conditions, and the addition of adjuvant factors led to increased growth (data not shown). MCH 603c8 was able to divide in serum-free medium, but it grew at a slower rate and achieved a much lower maximum cell density. Adjuvant factors did not enhance this limited proliferation
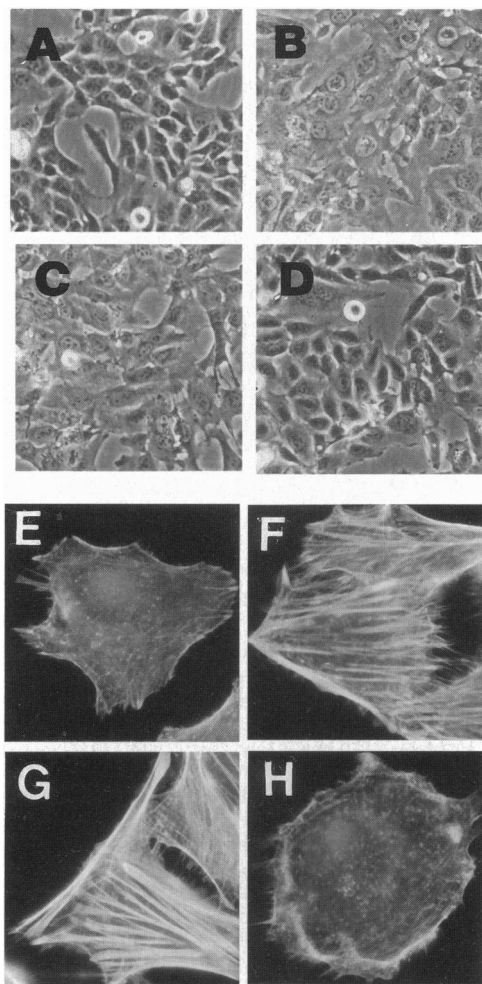
FIG. 3. *In vitro* characteristics. Comparative morphology (*A–D*). (*A*) HT1080 6TG, (*B*) MCH 603c8, (*C*) V2-2, and (*D*) M8–17. Actin stress fiber organization illustrated by fluorescein isothiocyanate-conjugated phalloidin staining of F-actin (*E–H*). (*E*) HT1080 6TG, (*F*) MCH 603c8, (*G*) V2-2, and (*H*) M8–17. Magnification: phase-contrast, ×50; and fluorescein isothiocyanate fluorescence, ×200.
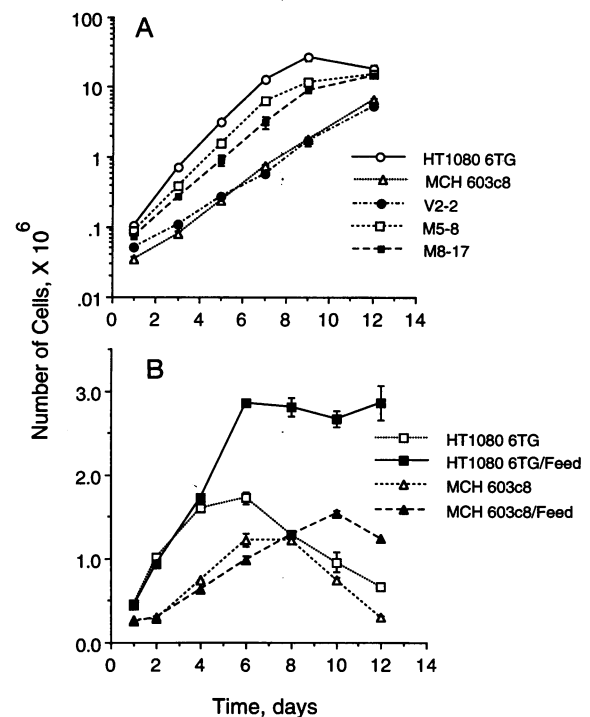

FIG. 4. *In vitro* growth kinetics. (*A*) Growth kinetics under serum conditions. (*B*) Serum deprivation growth kinetics. Flasks were fed on days 4, 7, and 10 (filled symbols), or were not fed at all during the course of the study (open symbols).

(data not shown). Both cell lines were able to grow to a higher maximum density when the medium was changed during the course of the assay (Fig. 4*B*). However, MCH 603c8 reached a maximum cell number and then began to die, whereas HT1080 6TG did not achieve serum-free cell death when fed regularly.

Mutant N-*ras* Controls The Capability For Anchorage-Independent Growth. The HT1080-derived cell lines were examined for their ability to display anchorage-independent growth. HT1080 6TG formed large colonies in soft agar (Fig. 5*A* and Table 1), whereas MCH 603c8 did not form colonies under the same conditions. Occasional colonies were observed when the cells were seeded at high density (10^5 cells per 60-mm dish; Fig. 5*B*). Vector control clones were all unable to grow in soft agar (Fig. 5*C* and Table 1). Mutant transfectants did not grow in soft agar when seeded at low density (Table 1), but formed numerous large colonies at high density (Fig. 5*D*).

Oncogenic N-*ras* Does Not Control Tumor-Forming Ability. *In vivo* tumorigenic potential was assessed by subcutaneous injection of the cell lines into the flanks of athymic nude (*nu/nu*) mice. HT1080 6TG formed aggressive tumors with no latency period (Fig. 6*A*). MCH 603c8 also formed tumors with no latency period, as did the vector control. However, these tumors grew at a slower rate than HT1080 6TG (Fig. 6*A*) but eventually reached the size of the tumors observed from the parental cell line. A smaller inoculum (1×10^6 cells) of

HT10806T6 and MCH 603c8 were also injected subcutaneously. Again, tumors formed in all animals. The lag period was longer for both cell lines, and again the MCH603c8 cells formed tumors more slowly than HT1080 6TG (data not shown). Lower numbers of cells were not inoculated, because in previous experiments we had found that at a dose of 5×10^5 cells, HT1080 6TG formed tumors in only a fraction of animals and the incidence of tumor formation was variable (E.J.S., unpublished observations). Histologic examination of excised MCH 603c8 tumors showed fibrosarcomas containing some necrotic tissue. Codons 12, 13, 59, and 61 of N-*ras*, K-*ras*, and H-*ras* genes were sequenced in tumor reconstitutes from MCH 603c8 and were found to remain wild-type, indicating that its ability to form tumors could not be explained by a *de novo ras* mutation (data not shown). Furthermore, tumor reconstitutes from MCH 603c8 had no additional cytogenetic abnormalities, and reinoculation of these tumor reconstitutes gave similar kinetics of tumor formation (data not shown). N-*ras* mutant transfectant (M5–8 and M8–17) tumors grew as aggressively as the parental HT1080 6TG cell line (Fig. 6*A*), retained a rounded morphology, and continued to express the mutant N-*ras* protein at a high level (Fig. 2, lane 5).

Colon Carcinoma-Mutant K-*ras* Does Not Control Tumor Formation. Shirasawa *et al.* (38) have reported that disruption of the mutant K-*ras* allele from human colon carcinoma cell lines results in loss of tumor-forming ability. Because of the differences in our results we obtained representative cell lines and examined their tumorigenic properties using the same inoculum size (1×10^7 cells per site) reported by the authors. In contrast to the published studies, we found that the two cell lines with a disrupted mutant K-*ras* allele (DKO3 and DKs8) were tumorigenic (Fig. 6*B*). As with MCH 603c8, they formed tumors more slowly than the parental DLD-1 colon carcinoma cells but eventually reached the same volume. Sequencing of the tumor reconstitutes showed no mutations in any of the N-, K-, or H-ras alleles (data not shown).

Table 1. Growth properties of HT1080 6TG-derived cell lines

Cell line	Morphology	Actin stress fibers	Doubling time,* hr	Saturation density,† ×10⁶	Growth in soft agar,‡ %	Tumorigenicity§
HT1080 6TG	Round	−	22.8	24	23	18/18
MCH 603c8	Flat	+	33.6	9	0.08 .	16/18
V2-2 (vector)	Flat	+	36.0	5.3	0.025.	6/6
V3-22 (vector)	Flat	+	38.4	5.3	0.55	6/6
M5-8 (mutant)	Round	−	24.0	17	0.1	6/6
M8-17 (mutant)	Round	−	26.4	18	0.045	6/6

*The number of hours required to produce one population doubling as determined from the linear portion of each curve averaged from two separate experiments.
†The highest number reached after cells became confluent. The number represents the average from two growth curves.
‡Each value represents the average of the percent of 1×10^4 cells that form colonies >0.1 mm, in duplicate 60-mm plates from two separate experiments.
§The fraction of mice with tumors after 2 months.

DISCUSSION

Loss of the mutant N-*ras* allele from HT1080 cells led to dramatic phenotypic changes, such as a flat morphology, appearance of organized actin stress fibers, and loss of anchorage-independent growth. These growth changes were only observed in the variant cell line that had lost the activated N-*ras* allele and were not observed in MCH clones containing an extra intact chromosome 1. These data show that the growth changes observed in our variant were not due to the net increase in normal chromosome 1q material in the cell line. In addition, reintroduction of the mutant N-*ras* into the variant led to reversion to the parental cell line morphology, actin filament organization, and *in vitro* and *in vivo* growth rates, suggesting that the oncogenic N-*ras* was responsible for the growth changes observed in the variant cell line. In the case of anchorage-independent growth, reintroduction of the mutant N-*ras* resulted in colony formation only at high cell density, and the mutant was not able to completely revert the phenotype. The reason for this observation is not known but may reflect differences due to the significantly higher level of expression of mutant N-*ras* in the transfectants compared to endogenous levels in the parental HT1080 6TG.

Our data show that like H-*ras*, oncogenic N-*ras* also plays an important role in controlling neoplastic cell shape and the cytoskeleton possibly by a *rac*- and/or *rho*-dependent pathway (35, 36, 42). Loss of oncogenic N-*ras* also resulted in cells that proliferated at a slower rate and had a diminished capacity to grow under serum-free conditions. *ras* is known to be involved in multiple signal transduction pathways, such as that involving the Raf/mitogen-activated protein kinase kinase/mitogen-activating protein kinase cascade (for review see ref. 46).
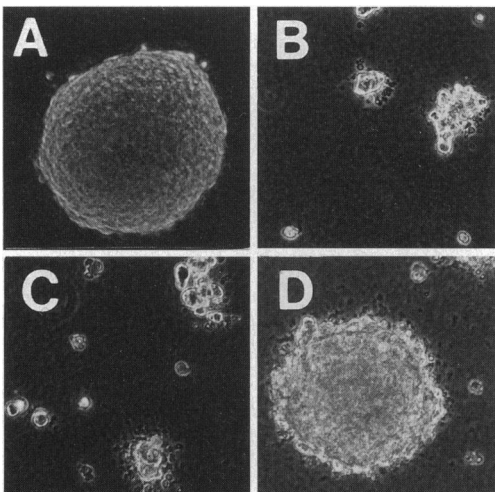
Constitutive *ras* expression, which occurs when cells contain an oncogenic *ras* allele, as in the parental HT1080 6TG cells, may lead to constitutive activation of kinases in the pathways, resulting in cell proliferation in the absence of external mitogenic stimulation. Loss of oncogenic *ras* expression could cause reversion to a regulated growth pathway, which may explain the slower *in vitro* and *in vivo* growth and decreased saturation density observed in MCH 603c8. However, MCH 603c8, despite loss of the mutant *ras* allele, still had a limited capability for serum-free growth. This may be because HT1080 and MCH 603c8 produce a growth factor (platelet-derived growth factor) that allows them to survive under serum-free conditions independent of *ras* status (47). The presence of a mutated N-*ras* allele may enhance the ability for serum-free growth, possibly due to the secretion of additional factors. We currently are in the process of investigating *ras* and platelet-derived growth factor signal transduction in these cells.

Although loss of the oncogenic *ras* allele resulted in altered morphology and growth kinetic changes possibly mediated by various signal transduction pathways, MCH 603c8 remained tumorigenic in nude mice. We are well aware that MCH 603c8 represents a single example of mutant N-*ras* deletion in this
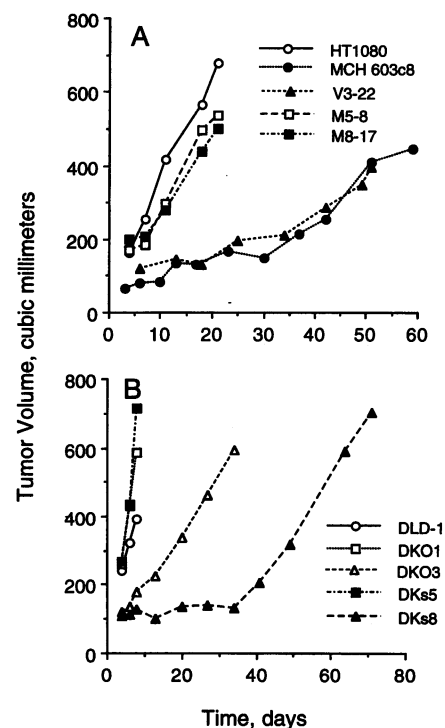

FIG. 5. Anchorage-independent growth. (*A*) HT1080 6TG, (*B*) MCH 603c8, (*C*) V2-2, and (*D*) M8-17. Phase-contrast magnification, ×50.

FIG. 6. *In vivo* growth kinetics. (*A*) HT1080 6TG-derived cell lines. (*B*) DLD-1-derived cell lines.

experimental model. However, several criteria suggest that this is a bona fide observation. Multiple MCH clones of HT1080 6TG containing an extra intact chromosome 1 have no effect on *in vitro* or *in vivo* growth (39). Thus, the extra 1q material in MCH 603c8 is not responsible for its phenotype. It might be argued that the translocation t(1q;22q) chromosome is critical. However, transfection of mutant N-*ras* cDNA into these cells restores the original HT1080 phenotype in multiple clones. It is possible, but highly unlikely, that *de novo* events (not involving *ras* mutations) are responsible for the tumorigenic phenotype of MCH 603c8 and the multiple subclones generated by vector transfection. To accommodate such a phenomenon, one would have to infer significant genomic instability in MCH 603c8, allowing for *in vivo* selection of tumorigenic variants. Classically, this is seen as a long latent period followed by rapid growth *in vivo*. Subsequent tumor reconstitutes then grow rapidly when reinjected in the host animal. We did not observe this phenomenon. MCH 603c8 and its vector transfectants form tumors more slowly than the parental HT1080 6TG and retain these kinetics of tumor formation after reinjection.

Similar results were obtained with the colon carcinoma DLD-1-derived cell lines. It is unclear, at this time, why there is a discrepancy between our tumorigenicity data and those reported by Shirasawa *et al.* (38). Sequence analysis shows that the cell lines and tumor reconstitutes do not contain any mutated *ras* genes, thereby ruling out any *in vitro ras* mutations. The tumorigenicity assays were performed in the same manner using the same amount of inoculum.

Thus the summation of these studies argues strongly that loss of mutant *ras* function has significant effects on *in vitro* and *in vivo* growth and morphologic parameters but does not abrogate tumor growth.

The results of our research are important in light of recent studies in the field of anti-*ras* cancer therapy, such as the use of anti-*ras* antibodies (9), anti-*ras* ribozymes (13), anti-sense *ras* strategies (11, 12), and inhibitors of *ras* function (farnesyl transferase inhibitors; refs. 14–17). If *ras* is not necessary to maintain tumorigenicity in human cells, as our study suggests, anti-*ras* therapies might not be effective treatments for cancer. Preliminary results regarding anti-*ras* therapies initially appeared promising. However, a close examination of the research shows that most studies used rodent rather than human cells (9, 12–14) and, with one exception (17), those that used human cells did not test the treatment in tumorigenicity assays (15, 16). Nagusa *et al.* (17), in their study of a farnesyl transferase inhibitor, treated nude mice injected with human cell lines. However, the inhibitor appeared to have little or no effect on the tumor growth rates in two out of three cell lines tested, including the HT1080 cell line (17). Further experimentation with human cells is clearly warranted to determine the efficacy of anti-*ras* therapies.

1. Bishop, J. M. (1987) *Science* **235**, 305–311.
2. Sager, R. (1984) *Cancer Cells* **2**, 487–493.
3. Wilson, D. M., Fry, D. G., Maher, V. M. & McCormick, J. J. (1989) *Carcinogenesis* **10**, 635–640.
4. Boukamp, P., Stanbridge, E. J., Foo, D. Y., Cerutti, P. A. & Fusenig, N. E. (1990) *Cancer Res.* **50**, 2840–2847.
5. Boukamp, P., Peter, W., Pascheberg, V., Altmeier, S., Fasching, C., Stanbridge, E. J. & Fusenig, N. E. (1995) *Oncogene* **11**, 961–969. Harris, C. C. & Fusenig, N. E. (1996) *Oncogene*, in press.
6. Finney, R. E. & Bishop, J. M. (1993) *Science* **260**, 1524–1527.
7. Parada, L. F., Land, H., Weinberg, R. A., Wolf, D. & Rotter, W. (1984) *Nature (London)* **312**, 649–651.
8. Hunter, T. (1991) *Cell* **64**, 249–270.
9. Montano, X. & Jimenez, A. (1995) *Cell Growth Differ.* **6**, 597–605.
10. Feramisco, R., Clark, R., Wong, G., Arnheim, N., Milley, R. & McCormick, F. (1985) *Nature (London)* **314**, 639–642.
11. Georges, R. N., Mukhopadhyay, T., Zhang, Y., Yen, N. & Roth, J. A. (1993) *Cancer Res.* **53**, 1743–1746.
12. Gray, G. D., Hernandez, O. M., Hebel, D., Root, M., Pow-Sang, J. M. & Wickstrom, E. (1993) *Cancer Res.* **53**, 577–580.
13. Kashani-Sabet, M., Funato, J., Florenes, V. A., Fodstad, O. & Scanlon, K. J. (1994) *Cancer Res.* **54**, 900–902.
14. Kohl, N. E., Mosser, S. D., deSolms, S. J., Giuliani, E. A., Pompliano, D. L., Graham, S. L., Smith, R. L., Scolnick, E. M., Oliff, A. & Gibbs J. B. (1993) *Science* **260**, 1934–1937.
15. Kohl, N. E., Wilson, F. R., Mosser, S. D., Giuliani, E., deSolms, S. J., Conner, M. W., Anthony, N. J., Holtz, W. J., Gomez, R. P., Lee, T. J., Smith, R. L., Graham, S. L., Hartman, G. D., Gibbs, J. B. & Oliff, A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9141–9145.
16. Sepp-Lorenzino, L. S., Ma, Z., Rands, E., Kohl, N. E., Gibbs, J. B., Oliff, A. & Rosen, N. (1995) *Cancer Res.* **55**, 5302–5309.
17. Nagasu, T., Yoshimatsu, K., Rowell, C., Lewis, M. D. & Garcia, A. M. (1995) *Cancer Res.* **55**, 5310–5314.
18. Brown, R., Marshall, C. J., Penne, S. G. & Hall, A. (1984) *EMBO J.* **3**, 1321–1326.
19. Paterson, H., Reeves, B., Brown, R., Hall, A., Furth, M., Bos, J., Jones, P. & Marshall, C. (1987) *Cell* **51**, 803–812.
20. Benedict, W. F., Weissman, B. E., Mark, C. & Stanbridge, E. J. (1984) *Cancer Res.* **44**, 3471–3479.
21. Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. & Gardner, M. B. (1974) *Cancer* **33**, 1027–1033.
22. Fournier, R. E. K. & Ruddle, F. H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 319–323.
23. Saxon, P. J., Srivatsan, E. S., Leipzig, G. V., Sameshima, J. H. & Stanbridge, E. J. (1985) *Mol. Cell. Biol.* **5**, 140–146.
24. Davis, M., Malcolm, S., Hall, A. & Marshall, C. J. (1983) *EMBO J.* **2**, 2281–2283.
25. Scott, A. F., Philips, J. A. & Migeon, B. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4563–4565.
26. Seabright, M. (1971) *Lancet* **II**, 971–972.
27. Pinkel, D., Landegant, J., Collins, C., Fuscoe, J., Segraves, R., Lucas, J. & Gray J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9138–42.
28. Sasaki, M., Okamoto, M., Sato, C., Sugio, K., Soejima, J., Iwama, T., Ikeuchi, T., Tonomura, A., Miyaki, M. & Sasazuki, T. (1989) *Cancer Res.* **49**, 4402–4406.
29. Gyllensten, U. B. & Erlich, H. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7652–7656.
30. Gough, N. M. (1988) *Anal. Biochem.* **173**, 93–95.
31. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. & Vogelstein, B. (1990) *Science* **249**, 912–915.
32. Inouye, S. & Inouye, M. (1987) in *Synthesis and Applications of DNA and RNA,* ed. Narang S. A. (Academic, Orlando FL), pp. 181–206.
33. Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203.
34. Towbin, H., Staehelin, T. & Gordin, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
35. Ridley, A. J. & Hall, A. (1992) *Cell* **70**, 389–399.
36. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. & Hall, A. (1992) *Cell* **70**, 401–410.
37. MacPherson, I. & Montagnier, L. (1965) *Virology* **23**, 291–294.
38. Shirasawa, S., Furuse, M., Yokoyama, N. & Sasazuki, T. (1993) *Science* **260**, 85–88.
39. Anderson, M. J., Casey, G., Fasching, C. L. & Stanbridge, E. J. (1994) *Genes Chromosomes Cancer* **9**, 266–281.
40. Bar-Sagi, D. & Feramisco, J. R. (1986) *Science* **233**, 1061–1068.
41. Bar-Sagi, D., Fernandez, A. & Feramisco, J. R. (1987) *Biosci. Rep.* **7**, 427–434.
42. Qiu, R., Chen, J., Kirn, D., McCormick, F. & Symons, M. (1995) *Nature (London)* **374**, 457–459.
43. Shirahata, S., Rawson, C., Loo, D., Change, Y. J. & Barnes, D. (1990) *J. Cell. Physiol.* **144**, 69–76.
44. Pironin, M., Clement, G., Benzakour, O., Barritault, D., Lawrence, D. & Vigier, P. (1992) *Int. J. Cancer* **51**, 980–988.
45. Kaplan, P. L., Anderson, M. & Ozanne, B. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 485–489.
46. Khosravi-Far, R. & Der, C. J. (1994) *Cancer Metastasis Rev.* **13**, 67–89.
47. Pantazis, P., Pelicci, P. G., Dalla-Favera, R. & Antoniades, H. N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2404–2408.