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Malignant transformation of human fibroblasts caused by expression of a transfected T24 *HRAS* oncogene

(human cell transformation/infinite life-span human fibroblasts/pHO6T1/T24 *HRAS* expression/malignant fibrosarcoma)

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ABSTRACT **We showed previously that diploid human fibroblasts that express a transfected *HRAS* oncogene from the human bladder carcinoma cell line T24 exhibit several characteristics of transformed cells but do not acquire an infinite life-span and are not tumorigenic. To extend these studies of the T24 *HRAS* in human cells, we have utilized an infinite life-span, but otherwise phenotypically normal, human fibroblast cell strain, MSU-1.1, developed in this laboratory after transfection of diploid fibroblasts with a viral v-*myc* oncogene. Transfection of MSU-1.1 cells with the T24 *HRAS* flanked by two transcriptional enhancer elements (pHO6T1) yielded foci of morphologically transformed cells. No such transformation occurred if the plasmid containing T24 *HRAS* had only one enhancer or none at all or if the normal human *HRAS* gene was transfected in the pHO6 vector (pHO6N1). Cell strains derived from such foci expressed high levels of T24 *HRAS* product p21, formed colonies in soft agar at high frequency, proliferated rapidly in serum-free medium that does not support growth of the parental cell line, and formed progressively growing, invasive fibrosarcomas. These foci-derived T24 *HRAS*-transformed cell strains, as well as cells from the tumors derived from them, had the same near-diploid karyotype as that of the parental MSU-1.1 cells. Transfection of pHO6T1 into two other infinite life-span human fibroblast cell lines, cells that had not been transfected with v-*myc*, also resulted in malignant transformation, suggesting that the infinite life-span phenotype of MSU-1.1 cells, and not necessarily expression of the v-*myc* oncogene, was the factor that complemented T24 *HRAS* expression to cause malignant transformation.**

Oncogenes of the *ras* family have been detected in numerous and diverse human malignancies (1). A growing body of evidence from both *in vivo* and *in vitro* studies suggests that the p21 products of *ras* oncogenes are involved in the genesis of such malignancies. For example, transgenic mice harboring the *HRAS* oncogene of the human bladder carcinoma cell line T24 (2) or the v-Ha-*ras* oncogene of Harvey murine sarcoma virus (3), linked to a promoter specific for expression in mammary tissue, developed mammary tumors. However, they did so only after a long latency, and the tumors were clonal in origin, suggesting that additional changes at the cellular level were required to cause tumor formation. Clues to the nature of the steps or events that may complement mutational activation of *ras* genes in tumorigenesis have come primarily from *in vitro* experiments utilizing rodent fibroblasts in culture (for a review, see ref. 1). For example, when primary or early-passage rodent fibroblasts with a finite life-span in culture were transfected with an *HRAS* oncogene, no transformation was seen, but when cells that had acquired an infinite life-span were used, fully transformed cells were obtained (4). Similarly, when primary or early-passage rodent cells were transfected with an *HRAS* oncogene and any one of a number of genes considered to be involved in causing cellular immortalization, malignant transformation resulted (5–7). Not surprisingly, transfection of *HRAS* oncogenes into infinite life-span rodent fibroblast cell lines resulted in malignant transformation (5, 8) in the majority of the studies (9).

To test models proposed for *ras* transformation generated from experiments with rodent fibroblasts for their relevance to human cell transformation, we and our colleagues (10–12) and several other groups (13–21) have used human fibroblasts as target cells for transfection of *HRAS* or *NRAS* oncogenes or for infection with the Harvey or Kirsten murine sarcoma viruses. Two groups (14, 15, 17) reported that early-passage diploid human fibroblasts are resistant to transformation by transfected *HRAS* oncogenes. However, Hurlin *et al.* (10) showed that if such cells are transfected with the pHO6T1 plasmid, which contains the human T24 *HRAS* oncogene flanked by Moloney murine sarcoma virus long terminal repeat and simian virus 40 (SV40) transcriptional enhancer sequences in order to increase expression of this gene, they become morphologically transformed, develop distinct foci, and form colonies in soft agar. In a similar study, Tubo and Rheinwald (20) showed that diploid human fibroblasts transfected with the T24 *HRAS* oncogene in a plasmid that contains a SV40 transcriptional enhancer sequence become morphologically transformed and exhibit reduced growth-factor requirements. As discussed by Hurlin *et al.* (10), the negative results referred to above (14, 15 17) most probably reflect lack of expression of the transfected *HRAS* oncogene or expression at a level too low to cause measurable effects. In the positive studies (10, 20), where expression of the transfected T24 *HRAS* oncogene was higher than that of the normal *HRAS* gene, the transformants exhibited several characteristics of tumor-derived cells but did not acquire an infinite life-span or tumorigenicity.

The majority of the studies of the transforming ability of oncogenes carried out in rodent cells in culture have used fibroblasts that have acquired an infinite life-span in culture but are otherwise apparently normal. Until now, no comparable human cell lines have been available for use in testing the applicability of the results of such studies to human fibroblasts. The few infinite life-span, nontumorigenic human fibroblast cell lines that exist have been generated by using SV40 (22) or repeated exposure to carcinogen (23, 24) and consist of cells that are morphologically transformed and exhibit other transformed characteristics. Recently, a human fibroblast cell strain with an infinite life-span, designated MSU-1.1, was generated in this laboratory after transfection of foreskin-derived, normal diploid human fibroblasts with a plasmid carrying a v-*myc* oncogene. These cells, which have

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Abbreviation: SV40, simian virus 40.

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a normal morphology and have exhibited the same near-diploid karyotype for more than 200 population doublings postcrisis, do not form foci or produce colonies in soft agar and are nontumorigenic. (A full description of the generation of this cell strain and its properties will be published elsewhere.) Using these cells, we have determined that transfection of the T24 *HRAS* oncogene in the pHO6T1 plasmid into human cells with an infinite life-span causes them to exhibit not only the characteristics previously seen with finite life-span cells (10) but also the ability to form rapidly growing, malignant fibrosarcomas in athymic mice.

METHODS AND MATERIALS

Cells and Culture Conditions. Cell line LG1, from which the MSU-1.1 cell strain arose, was initiated from foreskin material of a clinically normal newborn male as described (25). The MSU-1.1 cell line, described above, is resistant to G418 (GIBCO) because the plasmid used to transfect the LG1 cells carried the aminoglycoside phosphotransferase gene. The infinite life-span human fibroblast cell lines KMST-6 and SUSM-1, generated after repeated carcinogen treatment (23, 24), were obtained from M. Namba. The SV40-transformed infinite-life-span cell line GM0637B was purchased from the National Institute of General Medical Sciences (Human Genetic Mutant Cell Repository, Camden, NJ). The human bladder carcinoma cell line T24 was purchased from the American Type Culture Collection, and the chicken hepatoma, MC29 virus-producer cell line LSCC-DU72, was provided by K. Nazerian. The cells were routinely cultured in Eagle's minimal essential medium as described (10). For studies of growth factor independence, McM medium [ref. 26; a modification of MCDB110 medium (27)], formulated for use with serum-replacements, was used.

Plasmids and DNA Transfection. Plasmid pHO6T1 containing the T24 *HRAS* oncogene, pHO6N1 containing the normal human *HRAS* gene, and pHO6, which has no additional sequences (28), were provided by N. Wilkie. Plasmid pT24 was purchased from the American Type Culture Collection, and pSV2-T24 was constructed in this laboratory by inserting the T24 *HRAS* oncogene into the *Bam*HI site of pSV2-neo (29). Target cells (2×10^5) were transfected with DNA at 1.3 μg per ml by using the Polybrene/dimethyl sulfoxide method (30) as described (10).

Assay for Growth Factor Independence. Cells (5×10^4) were plated in McM medium supplemented with 1% fetal bovine serum. After 24 hr, the number of cells was determined, and the medium was exchanged for McM medium containing 0.1 mM calcium and the serum replacements of Ryan *et al.* (26) but lacking epidermal growth factor. The medium was renewed 3 days later, and the number of cells was determined after another 3 days.

Assay for Anchorage Independence. Cells were assayed for the ability to form colonies in soft agar as described (10) with the following modifications. Cells (5000 per dish) were plated in top agar consisting of McM medium supplemented with 2% fetal bovine serum and 0.33% SeaPlaque agar (FMC, Rockland, ME). McM medium containing 2% fetal bovine serum was provided to cultures weekly. Colonies with diameters larger than 80 μm were counted and sized electronically after 3 wk.

Tumorigenicity Assays. Exponentially growing cells (1×10^7) were injected subcutaneously into 4- to 8-wk-old BALB/c athymic mice that had been irradiated with 300 rads (1 rad = 0.01 Gy) from a ^{60}Co source 24 hr previously.

DNA Analysis. DNA was isolated as described (31) and analyzed for transfected sequences by the method of Southern (32).

Analysis for T24 *HRAS*-Encoded p21 and v-*myc*-Encoded p110. Cells to be analyzed for all human *RAS*-encoded p21 were labeled with [^{35}S]methionine (New England Nuclear) at 250 μCi (1 μCi = 37 kBq) per ml for 18 hr in McM medium lacking methionine but supplemented with 1% fetal bovine serum. Cell lysis, immunoprecipitation with the *ras* (any species) p21-specific antibody Y13-259 (33), NaDodSO$_4$/PAGE, and fluorography were performed as described (10). For analysis of v-*myc*-encoded p110, cells were labeled as above, but for only 1.5 hr. Cells were washed in ice-cold phosphate-buffered saline, lysed in lysis buffer (10 mM Tris·HCl, pH 8.2/150 mM NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% NaDodSO$_4$/5 mM EDTA/2 mM phenylmethanosulfonyl fluoride), and clarified by centrifugation at 10,000 $\times$ *g* for 20 min. Supernatants containing 7.5×10^7 cpm were immunoprecipitated with a sheep antibody (Cambridge Research Biochemicals, Valley Stream, NY; antibody OA-11-801) that reacts broadly with the proteins of the *myc* family. Protein A-Sepharose (Pharmacia) coated with goat anti-sheep IgG (Cooper Biomedicals, Malvern, PA) was added, and the mixture was shaken for 30 min at 4°C. The Sepharose fraction was washed twice with lysis buffer, once with 10 mM Tris·HCl, pH 7.5/1 M $MgCl_2$, and again with lysis buffer. NaDodSO$_4$/PAGE sample buffer containing 8 M urea was added, and the samples were heated for 5 min at 95°C and centrifuged. Aliquots of the supernatant, along with prestained molecular weight markers (Bethesda Research Laboratories) were electrophoresed (34) in NaDodSO$_4$/10% polyacrylamide gels, and the gels were fixed, treated with enhancer, dried, and exposed to x-ray film as described.

Karyotype Analysis. For each cell line, at least 25 G-banded karyotypes (35) were examined for chromosomal rearrangements, and 100 conventionally stained metaphases were counted to determine the modal chromosome number.

RESULTS

Transformation to Focus Formation by the Human T24 *HRAS* Oncogene. After transfection, the MSU-1.1 cells were cultured for 3 wk in Eagle's medium containing 10% fetal bovine serum and screened for colonies of cells that overgrew the monolayer (foci). Cells that had been transfected with pHO6T1 formed distinct foci of morphologically altered cells at a frequency of 10 per 10^6 cells transfected. No foci or indications of transformation were observed after transfection with control plasmids either containing the normal human *HRAS* gene (pHO6N1) or lacking a *ras* gene (pHO6). Transfection of 5×10^6 MSU-1.1 cells with plasmids containing the T24 *HRAS* oncogene but no enhancer sequences (pT24) or with only one SV40 transcriptional enhancer (pSV2-T24) did not induce focus formation. Six populations of morphologically transformed cells (designated MSU-1.1-T24 strains 1–6) were isolated from foci in separate dishes and were expanded for subsequent characterization. Fig. 1 shows an example of the morphology of such cells, along with that of the parental MSU-1.1 cell strain and LG1 cells, from which it arose. The MSU-1.1-T24 cells were smaller, rounder, and more refractile than the other two cell types. These six cell strains have maintained their transformed

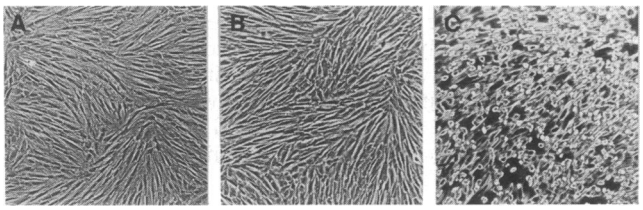

FIG. 1. Morphology of LG1 fibroblasts (*A*), MSU-1.1 fibroblasts (*B*), and MSU-1.1-T24 cells (*C*).

morphology for >50 population doublings to date. This stability contrasts with what Hurlin *et al.* (10) found with finite life-span human fibroblasts transformed following transfection of the T24 *HRAS* oncogene in pHO6T1.

Growth of MSU-1.1-T24 Fibroblasts in Serum-Free Medium. Schilz *et al.* (36) found that human fibrosarcoma-derived cell lines, but not normal human fibroblasts, were capable of proliferating in McM medium containing 0.1 mM calcium instead of the usual 1 mM, supplemented with the serum replacements of Ryan *et al.* (26), but lacking epidermal growth factor. To determine their growth factor independence, MSU-1.1-T24 cell strains, the parental MSU-1.1 cells, and LG1 cells were compared for ability to proliferate in this medium. The MSU-1.1-T24 cell strains proliferated 6- to 7-fold more than the MSU-1.1 cells did (Table 1). As expected, LG1 cells could not proliferate. The number of cells attained by the MSU-1.1-T24 cell strains was similar to that attained in medium containing 10% fetal bovine serum.

Anchorage Independence of MSU-1.1-T24 Cell Strains. When tested for ability to form colonies in agar, LG1 cells and MSU-1.1 cells did so with an efficiency of <0.007% (i.e., no colonies were detected out of a total of 15,000 cells plated), whereas MSU-1.1-T24 cell strains formed colonies with efficiencies from 3.5% to 23% (Table 1). There was variation in the size range of colonies detected. For example, although MSU-1.1-T24 strain 6 exhibited an agar cloning efficiency of only 3.5%, it gave rise to the highest proportion of very large colonies (i.e., having diameters of >300 μm).

Tumorigenicity of MSU-1.1-T24 Cell Strains. Four of the six MSU-1.1-T24 cell strains injected into athymic mice formed progressively growing tumors that reached 1 cm in diameter within 30–36 days (Table 1). These were diagnosed as poorly differentiated, invasive fibrosarcomas with a high mitotic index and a moderate degree of anaplasia. A low proportion of giant multinucleated cells were observed in several tumors. A histological section of a representative tumor is shown in Fig. 2. Several criteria indicated that the tumors were formed by the injected MSU-1.1-T24 cell strains (see below).

Presence of T24 *HRAS* Oncogenes. After digestion of genomic DNA with *Xba* I and *Hin*dIII, fragments of 4.5 and 2.5 kb that hybridize with the *Sac* I 2.9-kb fragment of the T24 *HRAS* oncogene are diagnostic for plasmid pHO6T1 sequences, which include the T24 *HRAS* oncogene and flanking 5′ and 3′ transcriptional enhancer sequences (28). Genomic DNA from each of the MSU-1.1-T24 cell strains contained *HRAS* hybridizing fragments close to these predicted sizes (Fig. 3). No such sequences were seen in parental MSU-1.1 cells or in LG1 cells.

Table 1. Growth properties and tumorigenicity of MSU-1.1-T24 cell strains

Cell strain	Cloning efficiency in agar, %	Cells × 10^{-5} attained in serum-free 0.1 mM Ca^{2+} medium*	Tumor incidence†	Days for tumor to reach 1-cm diameter
1	15	16	2/2	36
2	23	NT	6/6	30
3	10	NT	6/6	30
4	18	17	0/5	—
5	8.5	19	0/3	—
6	3.5	17	4/4	34
MSU-1.1	<0.01	2.7	0/9	—
LG1 cells	<0.01	0.4‡	0/9	—

*Duplicate cultures were counted 7 days after plating 5 × 10^4 cells per dish.
†Ratio of animals with tumors to animals injected subcutaneously. Animals were examined for tumor formation for at least 8 months after injection. NT, not tested.
‡This is the same no. of cells as was determined 1 day after plating.

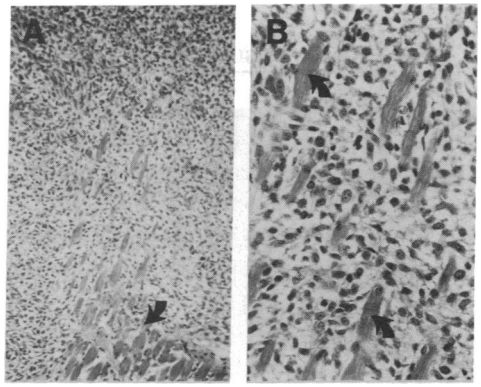

FIG. 2. Histology showing a poorly differentiated fibrosarcoma formed by a MSU-1.1-T24 cell strain. A 2.5-cm diameter tumor, removed 8 wk after subcutaneous injection of 10^7 cells, was sectioned and stained with hematoxylin/eosin. Mouse skeletal muscle is indicated by the arrows. [×50 (*A*) and ×250 (*B*).]

Expression of T24 *HRAS* Oncogenes. Radioimmunoprecipitation analysis of the MSU-1.1-T24 cell strains, using the *ras* p21-specific monoclonal antibody Y13-259, indicated that each of them overexpressed a 21-kDa protein (see arrow) that migrated to the same position as the overexpressed T24 *HRAS* oncogene-encoded p21 of the bladder carcinoma cell line (Fig. 4). To determine the relative levels of *RAS* p21 expression, the radiolabeled p21 bands were cut out of duplicate gels and quantitated by scintillation counting. The level of total *RAS* p21 expression in the MSU-1.1-T24 cell strains was 3–5 times that expressed in parental MSU-1.1 cells or in LG1 cells and was only slightly increased over that observed in bladder carcinoma-derived cells. Radioimmunoprecipitation analysis of *RAS* p21 was also carried out on fibroblasts taken from the malignant tumors derived from two of the MSU-1.1-T24 cell strains (37). The *RAS*-encoded p21 levels in these cells were comparable to those in their respective focus-derived cell strains, indicating that *in vivo* selection of cells expressing higher (or lower) levels of the transfected oncogene did not occur.

Presence and Expression of the v-*myc* Oncogene. Southern blot analysis with v-*myc*-specific DNA fragments from MC29

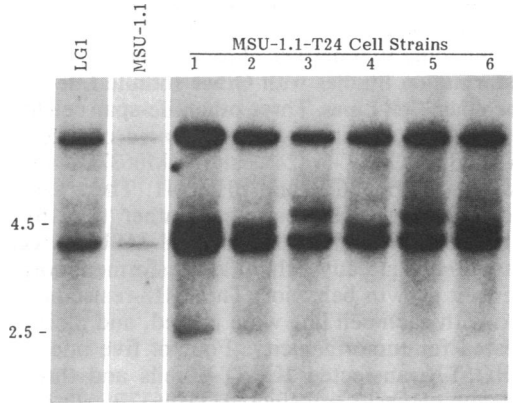

FIG. 3. Southern blot analysis of DNA from LG1 cells, MSU-1.1 cells, and the six MSU-1.1-T24 cell strains for the presence of T24. DNA was digested with *Hin*dIII and *Xba* I and hybridized with the 2.9-kb *Sac* I fragment of the T24 *HRAS* oncogene from pHO6T1. The locations of the expected 4.5-kb and 2.5-kb pHO6T1-specific fragments are indicated.

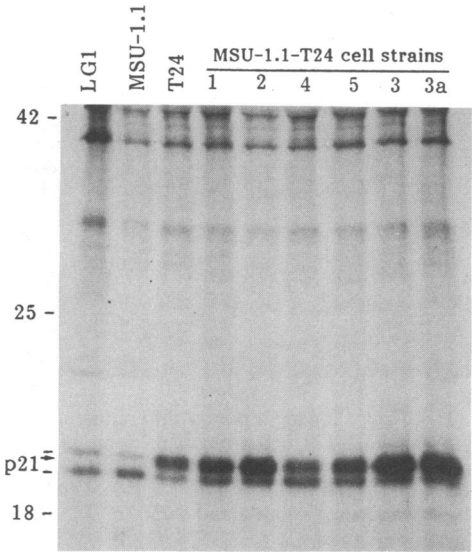

FIG. 4. Expression of T24 *HRAS*-encoded p21 in MSU-1.1-T24 cell strains. The *RAS*-encoded p21 proteins were immunoprecipitated with antibody Y13-259 (24) from [^{35}S]methionine-labeled cell lysates and were analyzed by electrophoresis as described. The location of the endogenous *RAS*-encoded p21 protein doublet is indicated by the bars, and the T24 *HRAS*-encoded p21 location is indicated by the arrow. T24 is the human bladder carcinoma cell line; 3a is a clonal derivative of MSU-1.1-T24 strain 3. The location of molecular mass markers in kDa on the gel is indicated.

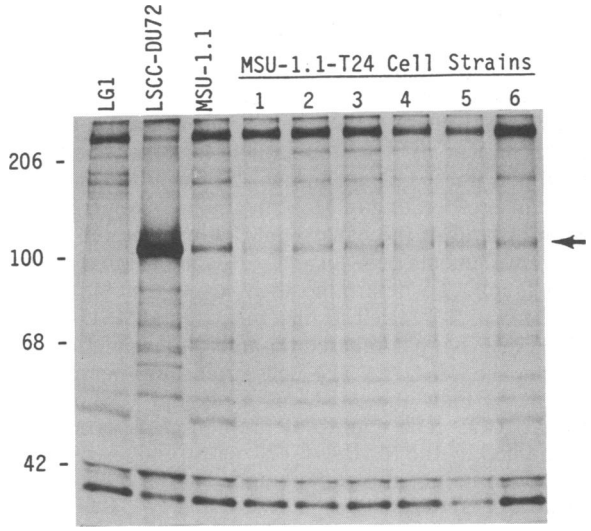

FIG. 5. Expression of v-*myc*-encoded p110 in MSU-1.1-T24 cell strains, v-*myc*-encoded p110 was immunoprecipitated from [^{35}S]methionine-labeled cell lysates with an antibody that reacts with proteins of the *myc* family and was analyzed by electrophoresis. The location of the v-*myc*-encoded p110 is indicated by the arrow. LSCC-DU72 is a chicken hepatoma; MC29 is a virus-producer cell line. The location of molecular mass markers in kDa on the gel is indicated.

virus indicated the presence of the v-*myc* gene in the six MSU-1.1-T24 cell strains (37). The v-*myc*-hybridizing DNA fragments were of identical size and intensity as those detected in the parental MSU-1.1 cells, indicating that no rearrangements or amplification of this oncogene had occurred subsequent to introduction of pHO6T1.

Radioimmunoprecipitation analysis indicated that in the MSU-1.1-T24 cell strains, v-*myc*-encoded p110 was expressed at levels comparable to that found in the parental MSU-1.1 cells (Fig. 5). As expected, no v-*myc* p110 was detected in the LG1 cells. A chicken hepatoma cell line expressing a v-*myc* p110 was used as a positive control in this analysis.

Karyotype Analysis of MSU-1.1-T24 Cell Strains. G-banded and conventionally stained chromosomes from MSU-1.1-T24 cell strains and cells from malignant tumors derived from them were analyzed and compared with that obtained with the parental MSU-1.1 cells. The G-banded karyotype of each set of cells was identical. Their modal chromosome number was 45, including two marker chromosomes.

Transformation Studies with Other Infinite Life-Span Human Fibroblast Cell Lines. Three other life-span cell lines that had not been transfected with the v-*myc* oncogene—i.e., SUSM-1 (23), KMST-6 (24), and GM0637B—also were transfected with pHO6T1 and pHO6N1. The transfectants were selected for G418 resistance rather than for focus formation because, unlike the parental MSU-1.1 cell line, these target cells already exhibit a transformed morphology and aberrant growth behavior. The G418-resistant colonies obtained with each cell line were pooled, and their progeny were tested for tumorigenicity. Four of five mice injected with pHO6T1-transfected KMST-6 cells and three of six injected with pHO6T1-transfected GM0637B cells developed progressively growing malignant fibrosarcomas. No tumors were formed in five mice injected with pHO6T1-transfected SUSM-1 cells generated in two separate experiments or with any of the pHO6N1-transfected G418-resistant controls. Immunoprecipitation studies with these pooled populations (37) indicated that the KMST-6 and GM0637B-T24 transfectants expressed the T24 *HRAS* oncogene, but the SUSM-1 transfectants did not. In contrast to the 30–36 days required for the tumors formed by the focus-derived MSU-1.1-T24 cell strains to reach a diameter of 1 cm, those formed by the progeny of these pooled G418-resistant transfectants required 4–5 months to reach this size. Therefore, the cells cultured from such tumors were reinjected in athymic mice to determine if *in vivo* selection for tumorigenic cells had occurred. This time two of two mice injected with tumor-derived cells formed with the KMST-6-T24 cell strains developed 1-cm-diameter tumors within 25 days, but no decrease in time for tumor development was observed with tumor-derived cells formed with the GM0637B-T24 cell strains.

DISCUSSION

From the results of this study with the infinite life-span MSU-1.1 cells and those of our earlier study (10) cells, we conclude that "immortalized" nontumorigenic human fibroblasts can be transformed into malignant cells by transfection of the T24 *HRAS* oncogene and its subsequent expression. The tumors formed by the MSU-1.1-T24 cell strains grew progressively and were diagnosed as invasive fibrosarcomas. The two cell strains that did not give rise to tumors, even though they expressed the T24 *HRAS* gene and exhibited the other transformed characteristics, were retested in a separate experiment and again proved to be nontumorigenic. The reasons for their lack of tumor formation are not yet understood.

Since the karyotype of the malignant cells derived from the tumors matched the karyotype of the MSU-1.1-T24 cells injected and both karyotypes were identical to that observed in the nontumorigenic parental MSU-1.1 cell line from the earliest passage tested postcrisis, we conclude that the tumorigenic phenotype in these T24 *HRAS*-transformed cell strains did not arise as the result of chromosome changes.

Although the MSU-1.1 cell strain arose after transfection of diploid human fibroblasts with a plasmid containing a v-*myc* oncogene and the cells express that oncogene, we cannot

conclude that this expression is responsible for acquisition of an infinite life-span because we have studied only one such cell strain. It has been proposed from studies with rat embryo fibroblasts (5–7) that the v-*myc* oncogene cooperates with a *ras* oncogene to cause cells to become malignantly transformed. The results of our studies do not disagree with this hypothesis, but the fact that transfection and subsequent expression of T24 *HRAS* oncogene also induced the malignant transformation of two non-v-*myc*-transfected infinite life-span cell lines suggests that the infinite life-span phenotype, rather than v-*myc* expression, is the characteristic of MSU-1.1 cells that complemented *HRAS* oncogene expression and allowed the cells to be malignant.

Our results with the latter two lines support the findings of others who investigated the transforming potential of *ras* oncogenes in infinite life-span human fibroblast cell lines—viz., O'Brien *et al.* (18), who utilized SV40-transformed cell line Va2, and Namba *et al.* (19, 21), who utilized KMST-6 cells. In their studies, introduction of *ras* oncogenes by either virus infection (18, 19) or by transfection (21) resulted in cells able to form tumors in athymic mice, although, in contrast to our results with the SV40-immortalized GM0637B cells, the tumors produced with the Va2 cells did not grow progressively (18)—an important criterion for malignant transformation, along with the ability to invade.

The significance of studies of the *in vitro* transformation of human fibroblasts by activated *RAS* genes ultimately depends on whether activated *RAS* genes play a causal role in the malignant transformation of human fibroblasts *in vivo*. Of particular relevance, therefore, are the findings that activated (mutated) *NRAS* genes have been found in human fibrosarcoma-derived cell lines HT1080 (38) and SHAC (39). Whether the mutation occurred in the cells that gave rise to the original tumor or arose during passage of the cells in culture cannot be ascertained from such studies. However, the observation that the tumorigenicity of the HT1080 cell line is dependent upon the cells' containing more copies of the mutant *NRAS* gene than of the normal gene (40) suggests that the activated *RAS* gene was required for tumorigenicity *in vivo*. Although it has often been reported that human fibroblasts are refractory to transformation, in fact, they readily express various transformed phenotypes (cf. ref. 41). The present study and those discussed above indicate that activated *HRAS* and *NRAS* genes can malignantly transform infinite life-span human fibroblasts. Finite life-span human fibroblasts transfected with activated *RAS* oncogenes express identical characteristics but do form malignant tumors (10–12, 20). The simplest explanation of why an infinite life-span is required for malignant transformation is that without it, the transformed cell has too few potential doublings left to form a tumor (42). Since infinite life-span human fibroblast cell lines have never been reported to arise spontaneously, and only very rarely arise after SV40 or carcinogen treatment, the apparent "refractoriness" of these cells to malignant transformation appears to result from the low frequency at which infinite life-span cell strains arise.

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1. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56**, 779–827.
2. Andres, A., Schonenberger, C., Groner, B., Hennighausen, L., LeMeur, M. & Gerlinger, P. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1299–1303.
3. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R. & Leder, P. (1987) *Cell* **49**, 465–475.
4. Newbold, R. F. & Overell, R. W. (1983) *Nature (London)* **304**, 648–651.
5. Land, H., Parada, L. & Weinberg, R. (1983) *Nature (London)* **304**, 596–602.
6. Ruley, H. E. (1983) *Nature (London)* **304**, 602–606.
7. Parada, L., Land, H., Weinberg, R., Wolf, D. & Rotter, V. (1984) *Nature (London)* **312**, 649–651.
8. Goldfarb, M., Shimizu, K. & Wigler, M. (1982) *Nature (London)* **296**, 404–409.
9. Franza, B. R., Maruyama, K., Garrels, J. I. & Ruley, H. E. (1986) *Cell* **44**, 409–418.
10. Hurlin, P. J., Fry, D. G., Maher, V. M. & McCormick, J. J. (1987) *Cancer Res.* **47**, 5752–5757.
11. Wilson, D. M., Maher, V. M. & McCormick, J. J. (1987) *Proc. Am. Assoc. Cancer Res.* **28**, 604 (abstr.).
12. Fry, D. G., Hurlin, P. J., Maher, V. M. & McCormick, J. J. (1988) *Mutat. Res.* **199**, 341–351.
13. Doniger, J., DiPaolo, J. A. & Popesco, N. C. (1983) *Science* **222**, 1144–1146.
14. Sager, R., Tanaka, K., Lau, C. C., Ebina, Y. & Anisowic, A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7601–7605.
15. Sager, R. (1984) in *Cancer Cells: Oncogenes and Viral Genes*, eds. Vande Woude, G. F., Levine, A. J., Topp, E. V. & Watson, J. D. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 487–493.
16. Sutherland, B., Bennett, P. V., Freeman, A. G., Moore, S. P. & Strickland, P. T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2399–2403.
17. Spandidos, D. A. (1985) *Anticancer Res.* **5**, 485–498.
18. O'Brien, W., Stenman, G. & Sager, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8659–8663.
19. Namba, M., Nishitani, K., Fukushima, F., Kimoto, T. & Nose, K. (1986) *Int. J. Cancer* **37**, 419–423.
20. Tubo, R. A. & Rheinwald, J. G. (1987) *Oncogene Res.* **1**, 407–421.
21. Namba, M., Nishitani, K., Fukushima, F., Kimoto, T. & Yuasa, Y. (1988) *Mutat. Res.* **199**, 415–424.
22. Sack, G. H. (1981) *In Vitro* **17**, 1–19.
23. Namba, M., Nishitani, K. & Kimoto, T. (1978) *Jpn. J. Exp. Med.* **48**, 303–311.
24. Namba, M., Nishitani, K., Hyodoh, F., Fukushima, F. & Kimoto, T. (1985) *Int. J. Cancer* **35**, 275–280.
25. McCormick, J. J. & Maher, V. M. (1981) in *DNA Repair: A Laboratory Manual of Research Procedures*, eds. Friedberg, E. C. & Hanawalt, P. A. (Dekker, New York), pp. 501–522.
26. Ryan, P. A., Maher, V. M. & McCormick, J. J. (1987) *Exp. Cell. Res.* **172**, 318–328.
27. Bettger, W. J., Boyce, S. T., Walthall, B. J. & Ham, R. G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5588–5592.
28. Spandidos, D. A. & Wilkie, N. M. (1984) *Nature (London)* **310**, 469–475.
29. Southern, P. J. & Berg, P. (1982) *J. Mol. Appl. Gen.* **1**, 327–341.
30. Morgan, T. L., Maher, V. M. & McCormick, J. J. (1986) *In Vitro Cell. Dev. Biol.* **22**, 317–319.
31. McCormick, J. J., Yang, D., Maher, V. M., Farber, R. A., Neuman, W., Peterson, W. D., Jr., & Pollack, M. S. (1988) *Carcinogenesis* **9**, 2073–2079.
32. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
33. Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. (1982) *J. Virol.* **43**, 294–304.
34. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
35. Yunis, J. J. & Chandler, M. E. (1978) *Prog. Clin. Pathol.* **7**, 267–288.
36. Schilz, R. J., Maher, V. M. & McCormick, J. J. (1988) *J. Cell. Biochem. Suppl.* **12a**, 156 (abstr.).
37. Hurlin, P. J. (1988) Dissertation (Michigan State Univ., East Lansing).
38. Brown, R., Marshall, C. J., Pennie, S. G. & Hall, A. (1984) *EMBO J.* **3**, 1321–1326.
39. Andeol, Y., Nardeux, P. C., Daya-Grosjean, L., Brison, O., Cebrian, J. & Suarez, H. (1988) *Int. J. Cancer* **41**, 732–737.
40. Paterson, H., Reeves, B., Brown, R., Hall, A., Furth, M., Bos, J., Jones, P. & Marshall, C. (1987) *Cell* **51**, 803–812.
41. McCormick, J. J. & Maher, V. M. (1988) *Mutat. Res.* **199** (2).
42. McCormick, J. J. & Maher, V. M. (1988) *Mutat. Res.* **199**, 273–292.