*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 9047–9051, November 1990
Cell Biology

# Overexpression of normal and mutated forms of *HRAS* induces orthotopic bladder invasion in a human transitional cell carcinoma

DAN THEODORESCU*†‡, ISABELLE CORNIL†, BERNARD J. FERNANDEZ§, AND ROBERT S. KERBEL†

*Department of Surgery, Division of Urology, University of Toronto, Toronto, ON M5G 2C4, Canada; †Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Room 880, 600 University Avenue, Toronto, ON M5G 1X5, Canada and Department of Medical Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada; and §Department of Pathology, Mount Sinai Hospital, Toronto, ON M5G 1X5, Canada

*Communicated by Allan H. Conney, July 19, 1990 (received for review, April 11, 1990)*

ABSTRACT **Recent studies have shown that orthotopic (transurethral) transplantation of human bladder cancer cell lines into nude mice permits tumor growth that accurately reflects their clinical malignant status in the original host. Thus, such a system allows a unique opportunity to analyze the genetic events involved in the conversion of low-grade bladder cancer, the vast majority of which are curable, to the high-grade life-threatening form of the disease. Since 5–10% of transitional cell carcinomas (TCCs) have been shown to contain a mutated *HRAS* gene, and protein expression levels of all forms of *HRAS* have been correlated with TCC progression, we chose to study the contribution of the *HRAS* oncogene in bladder tumor progression. We evaluated the effects of transfection of normal or mutated *HRAS* genes into a human TCC, called RT-4, that behaves as a superficial noninvasive papillary tumor after transurethral orthotopic inoculation into athymic nude mice. We found that overexpression of either transfected normal or mutated *HRAS* genes converted RT-4 cells to express an invasive phenotype remarkably similar in nature to the clinical behavior of high-grade bladder carcinomas. These results suggest a role for overexpressed normal or mutated *RAS* genes in human bladder carcinoma progression, and highlight the importance of using orthotopic inoculation systems for evaluation of the contribution of oncogenes to malignant tumor progression.**

Dominantly acting cellular transforming genes belonging to the *RAS* family of oncogenes have been detected in a wide spectrum of animal and human cancers by DNA-mediated gene transfer experiments in which immortalized nonneoplastic cells are used as recipients (1–3). By employing such assays in combination with gene cloning and sequencing analysis, it has been estimated that 5–10% of human transitional cell carcinomas (TCCs) contain activated/mutated *RAS* oncogenes (3–5). Moreover, of the three known *RAS* family members, by far the most common found to be mutated in urothelial malignancies is *HRAS* (4–6). TCCs can be broadly classified in two forms: superficial and invasive. Moreover, although the majority of superficial bladder carcinomas are curable, 2–25% progress to the invasive life-threatening form of the disease (7). This raises the question of whether the presence of activated *RAS* oncogenes is causally associated with the degree of invasiveness of such tumors. The various studies cited above suggest that based on prevalence comparisons of TCCs with mutated *RAS* and invasive TCCs this is probably not the case (4–6). However, in view of the low frequency of occurrence of activated *RAS* genes combined with the relatively small number of bladder tumors analyzed, it is difficult to rule out the possibility that patients having tumors with an activated *RAS* oncogene constitute a distinct clinical subgroup of invasive tumors.

The possible relationship of *RAS* gene expression to bladder cancer development and progression has also been analyzed by immunohistochemical techniques. These studies have, in the main, concentrated on estimating the level of the *RAS* gene protein product, p21, in tumors of various stages (8). The results have shown that in general, there is a correlation between levels of p21 and the degree of tumor invasiveness similar to what has been observed in some other types of tumor (9–11). Detailed staining for p21 in normal bladder tissue has revealed that the basal (progenitor) cells of the multilayered transitional epithelium stain with the highest intensity whereas more superficial (differentiated) compartments stain to a much lesser degree (12). Thus the level of normal *RAS* protein diminishes considerably with differentiation and *HRAS* overexpression *per se* is not restricted to the malignant state in bladder tissue. It is thus conceivable that, in the context of malignant disease, a deregulation of *RAS* gene expression (13) or expression of a mutant protein (14) may occur as a "second hit," and, when combined with an earlier cellular lesion or lesions, results in the induction of invasive bladder cancer. Support for this hypothesis comes from results demonstrating that transfection of a mutant *HRAS* gene will transform simian virus 40-immortalized (nonneoplastic) human urothelial cells (15) into TCCs as assayed by subcutaneous growth in nude mice (16).

Thus there appears to be a role for altered *RAS* gene expression in the progression of human bladder cancer, but clearly more direct evidence is required to establish a mechanistic relationship. Such evidence is provided in this paper, and it derives from exploiting developments in orthotopic transplantation of human cancers in nude mice (17), in particular, bladder cancers (18). Such procedures often permit expression of invasive and/or metastatic spread that is otherwise not generally observed after conventional subcutaneous (ectopic) injection of human tumors in nude mice (17–19). Thus, Jones and colleagues (18) have reported that when cell lines established from invasive bladder cancers were injected transurethrally into nude mice, they retained an invasive phenotype and invaded through the mouse bladder wall in much the same manner as they do clinically. In contrast, a grade 1 papillary bladder cancer cell line called RT-4 (20, 21) remained incompetent for local invasiveness. These findings were in stark contrast with results after subcutaneous injection of these cell lines that showed no evidence of tissue invasion by any of the cell lines. This system, therefore, provides an opportunity to evaluate the role of various genes, including *RAS* gene expression, in

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGF, epidermal growth factor; FBS, fetal bovine serum; SFM, serum-free medium; TCC, transitional cell carcinoma of bladder.

‡To whom reprint requests should be addressed at the † address.

human bladder cancer progression by examining the following questions: (*i*) Would transfection and expression of either mutated or normal forms of *RAS* into noninvasive RT-4 bladder cancer cells convert them to invasive carcinomas *in vivo*? (*ii*) If so, is there a relationship between *RAS* mRNA levels and acquisition of invasiveness? The results we obtained provide direct evidence for a contribution by altered *RAS* gene expression to bladder carcinoma progression. They also help clarify some of the discrepancies in the literature on this subject, and highlight the importance of using orthotopic transplantation techniques when attempting to assess the contribution of oncogenes to tumorigenicity and malignant tumor progression *in vivo*.

## MATERIALS AND METHODS

**Animals and Tissue Culture.** Female athymic (*nu/nu*) Swiss nude mice were bred on site in the animal care facility of the Samuel Lunenfeld Research Institute of Mount Sinai Hospital (Toronto). They were first used for the experiments described at 6–8 weeks of age. The animals were maintained in pathogen-free isolated barrier facilities provided with sterile food, water, bedding, and cages.

RT-4 (20, 21) is a cell line derived from a 63-year-old patient who had a cystectomy because of recurrent multifocal grade 1 papillary TCC. A431 is a human squamous carcinoma cell line that overexpresses the epidermal growth factor (EGF) receptor. NIH 3T3 are immortalized murine fibroblasts. All of these lines were obtained from the American Type Culture Collection. C1 is a murine mammary adenocarcinoma that overexpresses a transfected mutated *HRAS* oncogene ([Val$^{12}$]HRAS) (22). Cell lines were maintained under sterile conditions without antibiotics in standard tissue culture incubators (37°C, 5% $CO_2$/95% air) and laminar flow hoods. The cells were maintained in Eagle's minimal essential (A431, NIH 3T3 C1) and McCoy's (RT-4) media (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS). Serum-free medium (SFM) was DMEM/F12, 1:1 (vol/vol), supplemented with transferrin (20 μg/ml), ethanolamine (0.5 μg/ml), and bovine serum albumin (0.2 μg/ml). The cells were harvested with 0.25% trypsin, pelleted, washed in medium, repelleted, and resuspended in isotonic phosphate-buffered saline (PBS) for implantation or passaging.

**DNA Constructs and Transfection.** Oncogene constructs were expressed in the Homer 6 vector and contained genomic *HRAS* oncogenes generously provided by N. M. Wilkie (23). Their detailed structure has been published elsewhere (23). In brief, these constructs contain either a 6.4-kilobase (kb) normal cellular *HRAS* (pHO6N1) gene or 6.6 kb from a mutated *HRAS* ([Val$^{12}$]HRAS; pHO6T1), both under the control of a Moloney long terminal repeat promoter/enhancer and simian virus 40 enhancer sequences and produce a 1.2-kb mRNA transcript with the same size as the endogenously produced transcript. The plasmid DNA used for transfection was prepared by the standard cesium chloride gradient method followed by transfection of the RT-4 cells by the Polybrene/dimethyl sulfoxide shock method (24). Control cell line transfectants were generated by transfection with the plasmid pSV2neo (22). Selection of colonies that had stably integrated the plasmid DNA was done by their continued growth for 2 weeks in medium containing G418 (500 μg/ml; GIBCO). The morphologic transformation ability of RT-4 total cellular DNA was assessed by the standard NIH 3T3 focus-forming assay (14). Positive controls were generated by transfection with the mutated *HRAS* plasmid construct described above.

**RNA Extraction and Northern Blot Analysis.** Total RNA was isolated from the RT-4 cell clones as described (24). For Northern blot analyses, equal amounts of RNA (10–15 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde and transferred to GeneScreen (DuPont/New England Nuclear). Blots were hybridized with $^{32}$P-labeled probes as described (24). Controls for loading were the intensity of ethidium bromide stain-mRNA on gels and probing with a 1.7-kb *Pst* I fragment of pA1 (25), a β-actin-specific probe. Analysis of *RAS* gene expression was performed on 10 RT-4 colonies transfected with either the normal or mutated forms of *HRAS* and probed with a *RAS* fragment from a *Sac* I-digested transfected construct.

***In Vitro* Growth.** To generate growth curves, cells were seeded in either McCoy's plus 10% FBS or SFM in 6-well plates (Falcon) at $1 \times 10^4$, $5 \times 10^4$, or $1 \times 10^5$ cells per well. Proliferation of cells was followed every other day for 7 days, by counting the number of cells using a hemocytometer. Trypan blue staining was used to assess viability before plating the cells and after each harvest. Assessment of the degree of anchorage-independent growth (colony formation in soft agar) was done as described (18) in either FBS-containing medium or SFM.

**Injection Procedures and Tissue Histology.** *Subcutaneous (ectopic) inoculation.* Mice were given injections in the anterior flank alternately with $4 \times 10^6$ or $1 \times 10^6$ tumor cells in 0.2 ml of SFM. The animals were sacrificed when the skin over the tumor became necrotic, which occurred ≈3 months after inoculation. If no tumor appeared, the mice were observed for at least 3 months. The primary tumor, lung, liver, and spleen were histologically examined by routine hematoxylin/eosin staining.

*Intravenous inoculation.* Cells were resuspended in 0.1 ml of PBS and $1 \times 10^6$ cells were injected into mouse tail veins with 25-gauge needles, as described by Fidler (17). Animals were sacrificed 6–8 weeks after inoculation or sooner if they developed signs of distress.

*Intravesical (orthotopic) inoculation.* The implantation technique was similar to that of Ahlering *et al.* (18) used without electrocautery. Mice were anesthetized with intraperitoneal pentobarbital (5 mg/100 g). Aseptically, a 22-gauge catheter (Angiocath) was placed transurethrally into the bladder and $5 \times 10^6$ cells were injected. Mice were examined daily and sacrificed 8–12 weeks after implantation or earlier if they developed signs of distress. Autopsies were performed immediately to establish the presence and extent of tumor. The lungs, liver, spleen, pancreas, bladder, and kidneys were processed for routine histological examination; the bladder tumors were examined carefully for extent of invasion into the bladder wall.

*Tissue histology.* The pathologist (B.J.F.) responsible for examining the sections of various organs and primary tumors was "blinded" with respect to the identity of the cell clone injected into the animal but was aware of the hypothesis, purpose, and methodology of the experiment.

## RESULTS

**Transforming Ability of RT-4 Total Cellular DNA.** The NIH 3T3 focus formation assay did not reveal any evidence for transforming activity of RT-4 total cellular DNA (data not shown). The positive controls, obtained by transfection of NIH 3T3 cells with the mutated [Val$^{12}$]HRAS plasmid construct yielded numerous NIH 3T3 refractile colonies, exhibiting the transformed phenotype. The assay was done twice in triplicate. Although the limitations of this assay in detecting transforming oncogenes are well known, it is suitable for detecting mutant *HRAS* genes (1, 2).

**Selection of Transfected Clones with Various Levels of *HRAS* Expression.** Ten G418-resistant colonies of cells transfected with either the mutated or normal forms of *HRAS* or the pSV2neo construct were screened for *RAS* mRNA expression. Seven or 8 of 10 colonies in each group had elevated

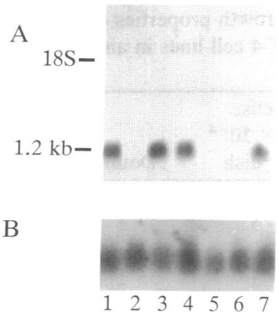


FIG. 1. Northern blot analysis for *RAS* and β-actin expression of various *HRAS*-transfected cell lines. Total RNA was isolated from the RT-4 cell clones. For Northern blot analyses, equal amounts of total RNA (10–15 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde and transferred to GeneScreen. Blots were hybridized using $^{32}$P-labeled probes of a *Sac* I-digested *HRAS* gene fragment (*A*) and the β-actin gene (*B*). Lanes: 1, C-1; 2, RT-4-neo; 3, RT-4-cr-1; 4, RT-4-cr-2; 5, RT-4-cr-3; 6, RT-4-mr-7; 7, RT-4-mr-10.

levels of *RAS* expression with the other 2 or 3 having no detectable expression. None of the 10 pSV2neo clones showed any detectable *RAS* expression in this assay. High and baseline (nonexpressing) colonies of either the normal or mutated forms were selected for further study (Fig. 1). A pool of >50 G418-resistant pSV2neo-transfected RT-4 clones was used as the control in all of the subsequent experiments. Henceforth, "parental line" or RT-4-neo refers to this pooled population of pSV2neo transfectants. The high *RAS* expressors were RT-4-mr-10, RT-4-cr-1, and RT-4-cr-2, and the low expressors were RT-4-mr-7 and RT-4-cr-3 ("mr" stands for *m*utated *r*as whereas "cr" designates *c*ontrol *r*as). These cell lines were selected for the next series of experiments. Control probing with β-actin shows equal amounts of mRNA in all lanes. RT-4-mr-10, RT-4-cr-1, and RT-4-cr-2 had a large increase in their *RAS* mRNA signal compared to the RT-4-neo clones or pooled population, which did not reveal any signal. RT-4-mr-7 and RT-4-cr-3 had undetectable *RAS* mRNA levels similar to the RT-4-neo population.

Despite not being able to discriminate between endogenous mRNA species and those resulting from expression of the construct, we feel that expression is due to the construct for the following reasons: (*i*) The plasmid had integrated into the cells since the constructs contain the *APH* (G418 resistance) gene and all clones were found to be G418 resistant. (*ii*) It would be extremely unlikely that the clones selected for further study were all overexpressing endogenous mRNA since 7 or 8 of 10 oncogene-transfected clones expressed the *RAS* gene whereas none of the 10 control pSV2neo clones or the pSV2neo pool containing >50 clones expressed it. Thus the frequency of RT-4 clones endogenously overexpressing *RAS* is likely to be at the most 10%.

Table 1. Tumorigenicity assay of parental and *RAS*-transfected cell lines in ectopic (subcutaneous) and orthotopic (intravesical) sites

| Cell line | No. animals with tumors/no. total animals inoculated: Subcutaneous, $1 \times 10^6$ cells per site | Subcutaneous, $4 \times 10^6$ cells per site | Intravesical ($5 \times 10^6$ cells) |
|---|---|---|---|
| RT-4-neo | 2/10 | 10/10 | 8/19 |
| RT-4-cr-1 | 7/10 | 10/10 | 10/20 |
| RT-4-cr-2 | 6/10 | 10/10 | ND |
| RT-4-cr-3 | 3/10 | 10/10 | 7/18 |
| RT-4-mr-7 | 2/10 | 10/10 | 6/19 |
| RT-4-mr-10 | 10/10 | 10/10 | 8/20 |

ND, not done. Subcutaneous inoculation of nude mice was done by injection into the anterior flank alternately with $4 \times 10^6$ or $1 \times 10^6$ tumor cells suspended in 0.2 ml of SFM. The intravesical implantation technique was essentially that of Ahlering *et al.* (18) used without electrocatery.

***In Vivo* Growth and Invasiveness of Transfectants.** Various amounts of RT-4-neo, RT-4-mr-7, RT-4-mr-10, RT-4-cr-1, RT-4-cr-2, and RT-4-cr-3 were injected subcutaneously into nude mice (Table 1). At $4 \times 10^6$ cells per site, all lines exhibited 100% tumorigenicity and measurements 20 and 40 days after injection revealed tumor sizes to be similar (data not shown). At $1 \times 10^6$ cells per site, RT-4-mr-10, RT-4-cr-1, and RT-4-cr-2 were significantly more tumorigenic. No evidence of metastatic spread was noted with any of the cell lines. This was assessed by routine hematoxylin/eosin staining of lung, liver, and splenic tissues. All tumors grew as pseudoencapsulated masses without any evidence of tissue invasion at the subcutaneous site. Intravenous inoculation of all cell lines resulted in pulmonary metastases. This has been reported for RT-4 (26). Lung nodules varied in size and in number in each animal and thus a meaningful average cannot be given. However, upon semiquantitative analysis, there seemed to be no significant difference among the lines.

Finally, and most significantly, the intravesical (orthotopic) inoculations revealed a marked histopathological difference between the cell lines. Sections of urinary bladder from the mice inoculated with RT-4-neo, RT-4-cr-3, and RT-4-mr-7 showed focal areas of superficial transitional cell carcinoma (Fig. 2*A*). This was characterized by disorderly proliferation of cells with loss of maturation at the surface and dysplastic features including course nuclear chromatin, large nucleoli, high nuclear–cytoplasmic ratios, and occasional mitoses. The

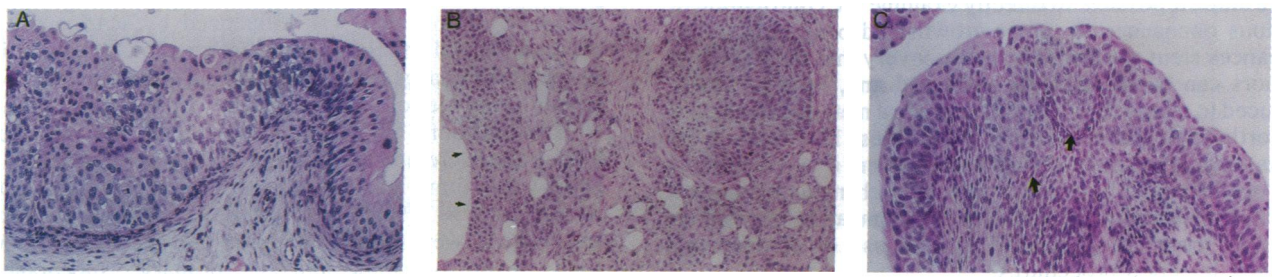


FIG. 2. (*A*) Section of bladder mucosa after intravesicular inoculation of RT-4-neo cells. TCC *in situ* is seen on the left side, with proliferation and dysplasia involving the full thickness of the epithelium. Normal epithelium is seen on the right side. Histologic appearance of RT-4-cr-3 and RT-4-mr-7 was identical. (Hematoxylin/eosin stain; ×50.) (*B*) Section of bladder mucosa after intravesicular inoculation of RT-4-mr-10 cells. On the surface (arrows) there is *in situ* carcinoma. In the underlying muscularis propria, there is extensive invasion by irregular nests and sheets of TCC. (Hematoxylin/eosin stain; ×30.) (*C*) Section of bladder mucosa after intravesicular inoculation of RT-4-cr-1 cells. Microinvasive carcinoma is present with irregular protrusions of tumor from the basal epithelium into the lamia propria (arrows). (Hematoxylin/eosin stain; ×50.)

epithelium consisted of multiple layers (more than six) of cells and the surface was flat but not ulcerated. The basement membrane was well delineated with no evidence of invasion. The underlying lamina propria showed edema with few inflammatory cells. The rest of the surface epithelium appeared normal with one to three layers of cells that were mature and flattened toward the surface. In marked contrast, sections of urinary bladder from the mice inoculated with RT-4-cr-1 and RT-4-mr-10 showed evidence of invasive TCC. In respect to RT-4-mr-10, there were broad areas of invasive TCC deep into the muscularis propria accompanied by inflammatory cells among which eosinophils were prominent. In other sections there was invasion of the entire muscularis propria with extension into the surrounding adipose tissue and tumor noted in vascular spaces (Fig. 2*B*). For RT-4-cr-1, we noted that in some areas the entire surface epithelium was involved but in other sections there was only localized involvement that was sharply demarcated from the adjacent normal epithelium. The surface was elevated with areas of ulceration and necrosis. The cells demonstrated markedly dysplastic features with frequent mitosis. In the earliest lesions, there was microinvasion of the lamina propria characterized by irregular jagged projections of tumor downward from the basal layer, but no invasion into the muscularis propria was noted (Fig. 2*C*). Finally, no contiguous or metastatic spread was found in other organs. In summary, acquisition of invasive competence similar to that of invasive bladder carcinoma in patients was observed in the transfectants expressing activated *RAS* (RT-4-mr-10) or high levels of normal *RAS* (RT-4-cr-1).

***In Vitro* Growth of Transfected Lines.** *In vitro* morphology of clones expressing low levels of *RAS* was identical to that of the parental cell line. High expressors were slightly different, having more definite cell contours and a decreased, although still significant, tendency to grow in intimate cell contact as islands. The *in vitro* growth properties of the six cell lines selected for study were examined in two ways. Doubling times were determined (Table 2). No significant differences were observed among the lines at the various cell concentrations or cultured in serum-containing medium or SFM. As expected, cells grew more slowly in SFM than in serum-containing medium and their viability based on trypan blue staining dropped from >95% to ≈70% on day 7 of the growth curve. There was, however, a major difference among the lines in their ability to form colonies in soft agar. Five of the six cell lines were similar; however, RT-4-mr-10 was ≈60% more proficient at forming colonies than other lines. No colonies were observed for any cell line when serum-free agar was used.

Table 2. *In vitro* growth properties of parental RT-4 and various *RAS*-transfected RT-4 cell lines in anchorage dependent and independent assays

| Cell line | Cells, no. × $10^{-4}$ per dish | Doubling times, hr | Number of colonies in agar |
|---|---|---|---|
| RT-4-neo | 1 | 35 ± 1.3 (59 ± 2.2) | 60 ± 4 |
| | 5 | 37 ± 1.5 (61 ± 2.3) | |
| | 10 | 34 ± 1.7 (58 ± 3.0) | |
| RT-4-cr-1 | 1 | 36 ± 2.0 (60 ± 3.0) | 60 ± 4 |
| | 5 | 38 ± 1.9 (60 ± 3.5) | |
| | 10 | 40 ± 1.3 (56 ± 2.0) | |
| RT-4-cr-2 | 1 | 37 ± 1.8 (58 ± 3.1) | 62 ± 3 |
| | 5 | 39 ± 1.7 (59 ± 2.8) | |
| | 10 | 34 ± 1.5 (58 ± 3.2) | |
| RT-4-cr-3 | 1 | 36 ± 1.3 (58 ± 3.1) | 71 ± 5 |
| | 5 | 37 ± 1.8 (59 ± 2.2) | |
| | 10 | 41 ± 1.1 (56 ± 2.1) | |
| RT-4-mr-7 | 1 | 33 ± 1.1 (61 ± 2.1) | 55 ± 5 |
| | 5 | 36 ± 1.7 (63 ± 3.0) | |
| | 10 | 38 ± 1.5 (65 ± 2.8) | |
| RT-4-mr-10 | 1 | 40 ± 1.4 (59 ± 2.0) | 108 ± 4 |
| | 5 | 36 ± 1.8 (60 ± 2.1) | |
| | 10 | 39 ± 1.5 (62 ± 1.8) | |

Cells were seeded in either McCoy plus 10% FBS or SFM in 6-well plates (Falcon) at 1 × $10^4$, 5 × $10^4$, and 10 × $10^4$ cells per well. Proliferation of cells was followed every other day for 7 days, by counting the number of cells by using a hemocytometer. Trypan blue staining was used to assess viability. Assessment of the degree of anchorage independence was done as described (24) in medium containing 5% FBS. Data are mean ± SEM. Data in parentheses are doubling times in SFM.

## DISCUSSION

Recent advances in the field of human tumor xenografting have made it possible to directly examine the contribution of various oncogenes to tumor growth and progression. The advances stem mainly from the discovery that the growth of tumors can be significantly enhanced and, in some cases, induced to mimic their respective clinical metastatic behavior by orthotopic transplantation procedures (17–19). Such results highlight the possibility that even if a transfected oncogene was to have major effects in changing the growth and/or malignant potential of a cell population, the effects may be completely masked by the use of conventional ectopic injection procedures.

We have used this reasoning to study the contribution of the *RAS* oncogene to human bladder carcinoma progression. Thus, Jones and colleagues (18) demonstrated that a low-grade human TCC (RT-4) was noninvasive after transurethral inoculation into nude mice whereas high-grade bladder carcinomas containing an endogenous mutated *HRAS* gene (e.g., EJ and T-24) were highly invasive. These observations prompted us to determine whether RT-4 cells could be converted to express a high-grade invasive form of the disease in an *in vitro* orthotopic system by manipulating the ability of the cells to express an activated *HRAS* gene or to overexpress the normal form of the gene. Since the expression level of *RAS* in bladder carcinoma appears to be the factor that is correlated with the invasive phenotype in previous studies, the transfected cell clones selected for further comparative in-depth analysis were those having either high or low levels of *RAS* expression. The control cells used consisted of a pooled population of pSV2neo RT-4 transfectants (27).

When the *in vitro* growth parameters of the various transfectants were examined, some differences were noted. Thus, monolayer growth curves of the selected clones under nonlimiting (10% FBS) or limiting (SFM) conditions did not reveal any differences between low and high expressors or those containing either normal or mutated forms of the *RAS* gene. In contrast to the monolayer culture results, colony formation in soft agar was influenced by *RAS* oncogene expression level and the presence of an activating mutation, although the changes were not striking. Likewise, when the growth properties of the transfectants were analyzed *in vivo* after conventional subcutaneous (ectopic) injection, some differences were noted. At 4 × $10^6$ cells per mouse, all the lines were tumorigenic and grew at comparable rates, whereas at 1 × $10^6$ cells per mouse, RT-4-mr-10, RT-4-cr-1, or RT-4-cr-2 were found to be more tumorigenic. None was found to be metastatic and all "primary" subcutaneous tumors grew as pseudoencapsulated masses without any evidence of tissue invasion at the subcutaneous site.

In contrast to their *in vitro* growth properties or *in vivo* growth characteristics after s.c. injection, the orthotopic injection studies of the transfectants revealed some remarkable differences. Histopathologic findings revealed that these lines behaved similarly to human TCC in patients. The

maintenance of the noninvasive phenotype of the control pSV2neo-transfected pooled parental line RT-4 and of the RT-4-cr-3 and RT-4-mr-7 plus the acquisition of an invasive phenotype by RT-4-cr-1 and RT-4-mr-10 was clear and striking. This modulation of the invasive phenotype by manipulation of the level of *HRAS* appears to be consistent with clinical data showing correlations of enhanced invasion with greater degrees of p21 expression (8). In addition, it is consistent with a model postulating possible mutations in the promoter (13) or in the coding regions (14) of the *HRAS* gene as "second hits," inducing tumor cell invasion.

Although we have documented that a high *HRAS* expression level correlated with the orthotopic invasive phenotype, it was interesting to note that the cells overexpressing the mutated form of *RAS* had an added advantage with respect to both their *in vitro* and *in vivo* clonogenic ability. Thus, although overexpression of either mutated or normal forms of *RAS* induced invasion through the basement membrane, it is conceivable that those cells overexpressing a mutated *RAS* may possess enhanced abilities of bladder muscle invasion and, ultimately, would be more capable of metastatic spread. We did not observe any muscle invasion with the RT-4-cr-1 cell line, whereas RT-4-mr-10 cells exhibited muscularis propria and vascular invasion, both signs of enhanced malignancy. However, these results must be considered in the light of the limited number of animals with tumors studied and the possibility of sampling error, although the latter is not very likely.

A possible explanation to account for the observed similarities in orthotopic invasive ability and subcutaneous tumorigenicity but differences in colony formation in agar between the RT-4-cr-1 and RT-4-mr-10 transfectants is that an exogenous factor from the microenvironment may be capable of selectively enhancing the growth and clonogenicity of the RT-4-cr-1 cells, both subcutaneously and in the bladder. Since urine (28) and subcutaneous tissues (29) have been shown to contain EGF in biologically significant amounts, it would be interesting to know whether differences existed between RT-4-cr-1 and RT-4-mr-10 with respect to their ability to proliferate after exposure to EGF, especially as there is evidence showing EGF receptor levels increase during human bladder carcinoma progression (30, 31). Thus we evaluated surface protein EGF receptor expression in our transfectants by fluorescence-activated cell sorting analysis and found its level to correlate with the invasive phenotype of the cell and thus its *HRAS* expressing level (D.T. and R.S.K., unpublished observations).

In summary, orthotopic transplantation of *RAS*-transfected human bladder carcinoma cells into nude mice has enabled us to directly implicate alterations in *RAS* gene expression in the conversion of superficial to invasive bladder carcinoma. Both overexpression of normal *RAS* and expression of mutated/activated *RAS* were competent to induce this conversion, a finding that helps clarify much of the confusion regarding the possible contribution of *RAS* genes to bladder carcinoma progression. The orthotopic transplantation model would appear to have considerable promise as a means of investigating other possible genetic events in bladder carcinoma progression such as inactivation or loss of suppressor genes [e.g., p53 (34)]. Our results emphasize the fact that previous *in vivo* functional studies of oncogene-transfected tumor cells have rarely employed orthotopic transplantation techniques (33) and, as a result, certain functional properties attributable to the transfected genes such as invasive and/or metastatic competence may have gone undetected.

We thank Shan Man and Capucine Sheehan for their excellent technical assistance and Frances Hogue and Lynda Woodcock for their excellent secretarial assistance. This work was supported by grants from the National Cancer Institute of Canada, Medical Research Council of Canada, and National Institutes of Health (CA-41233) to R.S.K. R.S.K. is a Terry Fox Associate Cancer Research Scientist of the National Cancer Institute of Canada.

1. Cooper, G. M. & Lane, M. A. (1984) *Biochim. Biophys. Acta* **738**, 9–20.
2. Weinberg, R. A. (1982) *Adv. Cancer Res.* **36**, 149–163.
3. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56**, 779–827.
4. Fujita, J., Yoshida, O., Yuasa, Y., Rhim, J. S., Hatanaka, M. & Aaronson, S. A. (1984) *Nature (London)* **309**, 464–466.
5. Fujita, J., Srivastava, S. K., Kraus, M. H., Rhim, J. S., Tronick, S. R. & Aaronson, S. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3849–3853.
6. Malone, P. R., Visvanathan, K. V., Ponder, A. J., Shearer, R. J. & Summerhayes, I. C. (1985) *Br. J. Urol.* **57**, 664–667.
7. Heney, N. M., Ahmed, S., Flanagan, M. J., Frable, W., Corder, M. P., Hafermann, M. D. & Hawkins, I.R. (1983) *J. Urol.* **130**, 1083–1086.
8. Viola, M. V., Fromowitz, F., Oravez, S., Deb, S. & Schlom, J. (1985) *J. Exp. Med.* **161**, 1213–1218.
9. Horan-Hand, P., Thor, A., Wunderlich, D., Murano, R., Caruso, A. & Schlom, J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5227–5231.
10. Thor, A., Horan-Hand, P., Wunderlich, D., Caruso, A., Murano, R. & Schlom, J. (1984) *Nature (London)* **311**, 562–565.
11. Viola, M. V., Fromowitz, F., Oravez, S., Deb, S., Finkel, G., Lundy, J., Hand, P., Thor, A. & Schlom, J. (1986) *N. Engl. J. Med.* **314**, 133–137.
12. Furth, M. E., Aldrich, T. H. & Cardon-Cardo, C. (1987) *Oncogene* **1**, 47–58.
13. Cohen, J. B. & Levinson, A. D. (1988) *Nature (London)* **334**, 119–124.
14. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. & Chang, E. H. (1982) *Nature (London)* **300**, 143–149.
15. Christian, B. J., Loretz, L. J., Oberley, T. D. & Reznikoff, C. A. (1987) *Cancer Res.* **47** (2), 6066–6073.
16. Christian, B. J., Kao, C. H., Wu, S. Q., Meisner, L. F. & Resnikoff, C. A. (1990) *Cancer Res.*, in press.
17. Fidler, I. J. (1986) *Cancer Metastasis Rev.* **5**, 29–49.
18. Ahlering, T. E., Dubeau, L. & Jones, P. A. (1987) *Cancer Res.* **47**, 6660–6665.
19. Cornil, I., Man, S. M., Fernandez, B. & Kerbel, R. S. (1989) *J. Natl. Cancer Inst.* **81**, 938–944.
20. Rigby, C. C. & Franks, L. M. (1970) *Br. J. Cancer* **24**, 746–754.
21. Masters, J. R. W., Hepburn, P. J., Waler, L., Highman, W. J., Trejdosiewicz, L. K., Povery, S., Parkar, M., Hill, B. T., Riddle, P. R. & Franks, L. M. (1986) *Cancer Res.* **46**, 3630–3636.
22. Waghorne, C., Breitman, M. L. & Kerbel, R. S. (1988) *Tumor Progression and Metastasis*, UCLA Symposia on Molecular and Cellular Biology, eds. Nicolson, G. L. & Fidler, I. J. (Liss, New York), Vol. 78, pp. 135–141.
23. Spandidos, D. A. & Wilkie, N. M. (1984) *Nature (London)* **310**, 469–475.
24. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1988) *Current Protocols in Molecular Biology* (Wiley, New York), Vol. 1.
25. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Ruther, W. J. & Kirschner, M. V. (1980) *Cell* **20**, 95–105.
26. Kovnat, A., Armitage, M. & Tannock, I. (1982) *Cancer Res.* **42**, 3696–3703.
27. Kerbel, R. S., Waghorne, C., Man, M. S., Elliott, B. & Breitman, M. L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1263–1267.
28. Starkey, R. H., Cohen, S. & Orth, D. N. (1975) *Science* **189**, 800–802.
29. Tsutsumi, O., Tsutsumi, A. & Oka, T. (1987) *Cancer Res.* **47**, 4651–4653.
30. Neal, D. E., Bennett, M. K., Hall, R. R., Marsh, C., Abel, P. D. & Harris, A. L. (1985) *Lancet* **i**, 366–368.
31. Smith, K., Fennelly, J. A., Neal, D. E., Hall, R. R. & Harris, A. L. (1989) *Cancer Res.* **49**, 5810–5815.
32. Tsai, Y., Nichols, P. W., Hill, A. L., Williams, Z., Skinner, D. G. & Jones, P. A. (1990) *Cancer Res.* **50**, 44–47.
33. Radinsky, R. & Fidler, I. J. (1989) *J. Natl. Cancer Inst.* **82**, 166–168.