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T24 human bladder carcinoma cells with activated Ha-*ras* protooncogene: Nontumorigenic cells susceptible to malignant transformation with carcinogen

(transformed phenotype/nude mice/rhodamine 123/secreted phosphoprotein)

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ABSTRACT **A comparative analysis of T24 human bladder carcinoma cells and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MeNNG)-transformed derivatives (MeNNG-T24 cells) revealed the following: (*i*) The presence of an activated c-Ha-*ras* gene (in the absence of the normal allele) is insufficient to confer upon T24 cells a tumor-associated phenotype. (*ii*) MeNNG-transformed T24 cells not only acquire tumor-associated (*in vitro*) traits (growth in soft agar and rhodamine retention) but, are highly tumorigenic in nude mice. (*iii*) It is possible to render T24 cells tumorigenic by chemical transformation; therefore, the reason that T24 cells lack tumorigenicity is not because of possible incompatibilities between these cells and nude mice but, in fact, because T24 cells are not malignant. (*iv*) The loss of expression of a transformation-related M_r 67,000 phosphoprotein by MeNNG-T24 cells after explantation of these cells from nude mouse tumors to *in vitro* culture indicates that culture conditions can be responsible for rapid phenotypic conversion of human tumor cell lines.**

At present, the only way to establish that a human tumor cell line contains malignant cells is to demonstrate that the cell line in question can produce tumors in nude mice or other immunodeficient hosts. Although many human tumor cell lines are tumorigenic in nude mice, some are not (1, 2). Even though it is logical to conclude that tumorigenic human cell lines consist (at least partially) of malignant cells and therefore bear at least some resemblance to the original tumor cells, the relationship between nontumorigenic cell lines and the tumor cells from which they were originally derived is uncertain. Nontumorigenic cell populations may be truly noncancerous, or, alternatively, they may include malignant cells that cannot establish tumors because of the artificiality of the site of implantation and/or incompatibility with the nude mouse host. However, if a nontumorigenic cell line is susceptible to chemical or viral transformation and its transformed derivatives are tumorigenic in the nude mouse assay, the nonmalignant status of the nontumorigenic cell line is more clearly established.

In addition to tumorigenicity in nude mice, there exist various other tumor-associated traits by which human tumor cell lines can be further evaluated. Best known among them is the ability of tumor cells to grow in soft agar or methylcellulose. This characteristic has been shown to correlate well with the tumorigenicity of cell lines with fibroblastic origins and for some, but not all, cells derived from epithelium (reviewed in refs. 3 and 4). Another tumor-associated trait is prolonged retention of rhodamine 123 by tumor cell mitochondria (5). Various tumor cell lines with epithelial origins have been shown to retain this dye far longer than normal epithelial cells (5–7). Yet another tumor-associated trait is the elevated secretion of a transformation-related phosphoprotein with a M_r of ≈62,000 (depending on the species of origin). A wide variety of rodent and human tumor cells with both fibroblastic and epithelial origins secrete this phosphoprotein at levels that are at least 10-fold greater than those for corresponding normal or nontumorigenic cells (8–11).

We have evaluated the T24 human bladder carcinoma cell line for tumorigenicity in nude mice as well as for the aforementioned tumor-associated characteristics. This cell line has received much attention in recent years; interest has been centered on the fact that T24 cells bear a point mutation within codon 12 of the c-Ha-*ras* gene (12, 13). EJ human bladder carcinoma cells, which appear to be related to T24 cells (14), have this same mutation (15). These mutated T24 and EJ proto-*onc* genes are capable of transforming mouse NIH 3T3 cells, whereas the homologous gene from normal human DNA does not have this potential; accordingly, the T24 and EJ c-Ha-*ras* genes have been termed "activated oncogenes" (12, 13, 15). EJ cells have been reported to be tumorigenic, whereas T24 cell lines (from different laboratories) with activated c-Ha-*ras* genes have been reported to be both tumorigenic and nontumorigenic (14, 16). In this report, we demonstrate that T24 cells with the activated c-Ha-*ras* gene are nontumorigenic as well as untransformed by other criteria and most importantly that they, like other untransformed cells, are susceptible to malignant transformation with carcinogen.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Nude Mice. All cell lines, with the exception of the chemically transformed T24 and J82 lines (see below), were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The T24 cell line was established from a human grade III urinary bladder carcinoma in 1970 (17). A culture of unknown passage number was provided to ATCC by C. O'Toole in 1971 (see ref. 14), and ATCC has subcultured these cells 35 times since the original deposit. The J82 cell line was derived from a poorly differentiated, invasive human transitional cell bladder carcinoma (stage T3) in 1972 (18). The J82 cells we obtained from ATCC had been serially subcultured 51 times since derivation from the tissue of origin.

All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. BALB/c *nu/nu* (athymic nude) mice (female, 4 to 5 weeks old) were obtained from the Animal Genetics and Production Branch,

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Abbreviations: MeNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ATCC, American Type Culture Collection.

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National Cancer Institute. C57/BALB/c *nu/nu* bg^J/bg^J (nude–beige; athymic and natural killer cell-deficient) mice (female, 5 weeks old) were obtained from Life Sciences (Saint Petersburg, FL).

Transformation of T24 and J82 Cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MeNNG). T24 cells and another nontumorigenic human bladder carcinoma cell line (J82) were incubated with the carcinogen MeNNG (Sigma) at a concentration of 0.01 μg/ml for 7 days according to the method of Rhim *et al.* (19). Eight weeks (14 passages) after exposure to carcinogen, both cell populations displayed uniform morphological changes, and 12 weeks after exposure to MeNNG, cells injected s.c. gave rise to progressively growing tumors. The transformed cell lines are designated MeNNG-T24 and MeNNG-J82.

Southern Blots. DNA was extracted from human lymphocytes; T24, MeNNG-T24, J82, and MeNNG-J82 cells; and MeNNG-T24 and MeNNG-J82 tumors as described (20). For analysis of restriction site polymorphism at codon 12, 20 μg of DNA from the various sources was digested with the restriction enzymes *Msp* I and *Hpa* II (Bethesda Research Laboratories) under the conditions suggested by the manufacturer, electrophoresed in horizontal agarose gels (1.8% wt/vol), and blotted to 0.05-μm nitrocellulose filters as described by Southern (21). The filters were baked in a vacuum oven for 3 hr at 80°C, prehybridized in 5× SSC (standard saline citrate; 1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) containing 50% (vol/vol) formamide, 10% dextran sulfate, 0.1% $NaDodSO_4$, and 4× Denhardt's solution (20) for 12 hr. They were then hybridized for 48 hr at 42°C in the same solution containing ^{32}P-labeled nick-translated (specific activity > 10^8 dpm/μg) pKY-1 probe subcloned from the T24 c-Ha-*ras* oncogene (22). The filters were then washed in 0.1× SSC/0.5% $NaDodSO_4$ at 65°C and were subjected to autoradiography.

Extraction of RNA and Blot-Hybridization Analyses. Total cellular RNA was isolated from T24 and MeNNG-T24 cell lines by using the guanidium thiocyanate method (23). Poly(A)-containing RNA was selected with oligo(dT)-cellulose (Collaborative Research, Waltham, MA) columns according to the manufacturer's recommendations. Poly(A)-selected RNA (2.5 μg) from T24 and MeNNG-T24 cells was electrophoresed on a 1.0% agarose gel containing formaldehyde, transferred to a nylon membrane (24), and hybridized with an insert probe of the c-Ha-ras-1 recombinant clone (25).

Clonal Growth of Cells in Agarose. Cells were plated in DMEM/10% fetal calf serum/0.48% agarose [Sea Plaque, FMC (Rockland, ME)] onto 60-mm dishes containing a basal layer of DMEM, 10% fetal calf serum, and 0.75% agarose. T24 cells were plated at a density of 1×10^4 cells per dish, MeNNG-T24 cells were plated at 1×10^3 cells per dish, and J82 and MeNNG-J82 cells were plated at 5×10^3 cells per dish. All cell types were also plated in DMEM/10% fetal calf serum on standard tissue culture plastic as a check for cell viability and inoculation density. Colonies were counted after 4 weeks. By this time, all viable colonies were large and visible to the naked eye.

Rhodamine 123 Retention. Cells were incubated with rhodamine 123 (Eastman Organic Chemicals, Rochester, NY) at 10 μg/ml for 10 min and then extensively washed and incubated for 24 hr in rhodamine-free medium (5). Cells were trypsinized and counted, and 1×10^6 cells were extracted with 2 ml of butanol. The fluorescence of the butanol-extracted rhodamine was determined with a Perkin–Elmer fluorescence spectrophotometer (excitation wavelength, 485 nm; emission wavelength, 532 nm).

Detection of a M_r 67,000 Secreted Phosphoprotein. Cells were labeled with [^{32}P]orthophosphate for 3.5 hr, and secreted proteins were subjected to immunoprecipitation with antisera specific for the transformation-related secreted phosphoprotein as previously described (11). Immunoprecipitated protein was electrophoresed on 7.5% (wt/vol) polyacrylamide slab gels (26), and the labeled proteins were visualized by autoradiography.

RESULTS

Tumorigenicity of T24, J82, and Other Human Tumor Lines in Nude Mice. T24 cells and a variety of other human tumor lines were tested for their ability to establish subcutaneous tumors in nude (BALB/c *nu/nu*) mice. Subcutaneous injection of T24 cells (1×10^7 cells) never resulted in the growth of a tumor (five experiments, 18 animals). In addition, we tested the tumorigenic potential of T24 cells in a nude–beige cross (C57/BALB/c; *nu/nu* bg^J/bg^J) that is both athymic and natural killer cell-deficient. No tumors arose in the 6 animals employed. In all cases, animals were followed for 3–4 months, and aliquots of tumor cell suspensions used in the inoculating procedure were retained and shown to be highly viable in culture. Similar analyses were performed with J82 cells, which were also consistently found to be nontumorigenic (a total of 14 animals). In contrast, lesser doses (1×10^6 cells) of seven other human tumor cell lines were highly tumorigenic in nude mice, as all consistently gave rise to progressively growing tumors within 14 days. These cell lines included MeNNG-HOS, COLO205, RD, HT1080, WiDr, PA-1, and HeLa.

Transformation of T24 and J82 Cells with Carcinogen. Within 8 weeks (14 passages) of exposure to MeNNG, carcinogen-treated T24 and J82 cell populations had undergone a morphological transformation, and within 12 weeks after treatment, transformed T24 and J82 cells were capable of giving rise to tumors in nude mice (5×10^6 cells injected s.c.). We explanted the resulting tumor cells to *in vitro* culture (sterile technique) and repassaged them in nude mice. In all, the tumorigenic MeNNG-T24 cells were passaged three successive times in nude mice as s.c. tumors; the resulting uncloned cell line as well as a cloned subline consistently (12/12) produced rapidly growing tumors (1.25 cm in diameter) within 16 days of s.c. implantation (5×10^6 cells). Tumorigenic MeNNG-J82 cells were passaged two successive times as subcutaneous tumors; these cells were also highly tumorigenic (11/11). Control cultures of T24 and J82 cells, which were passaged in parallel with the MeNNG-treated cultures, continued to remain nontumorigenic long after the MeNNG-treated cells began giving rise to tumors, thus demonstrating that malignant transformation was not merely due to extended *in vitro* culture. Isoenzyme analyses of T24, MeNNG-T24, cloned MeNNG-T24, J82, and MeNNG-J82 cellular extracts (performed by ATCC) indicated that, in all cases, zymogram migrations were identical to that expected for cells of human origin. Enzyme systems assayed included nucleoside phosphorylase, glucose-6-phosphate dehydrogenase (all five lines displayed the type B phenotype), malate dehydrogenase, mannose phosphate isomerase, peptidase B, glutamate oxaloacetate transaminase, and lactate dehydrogenase.

Comparison of T24 and J82 Cells with Their Tumorigenic Derivatives (MeNNG-T24 and MeNNG-J82). (*i*) *Morphology.* Both MeNNG-T24 and MeNNG-J82 cells displayed morphologies in culture that were distinctly different from the parental T24 and J82 cell lines (Fig. 1). MeNNG-T24 and MeNNG-J82 cells grew as tightly packed clusters even at low density, whereas T24 and J82 cells did not reveal much cell–cell contact until they reached nearly confluent densities.

(*ii*) *c-Ha-*ras *genotype and mRNA.* DNA from normal human lymphocytes, T24 cells, and MeNNG-T24 cells was digested with *Msp* I and *Hpa* II restriction enzymes, and the fragments derived were subjected to Southern blotting with the labeled pKY-1 insert probe derived from the activated

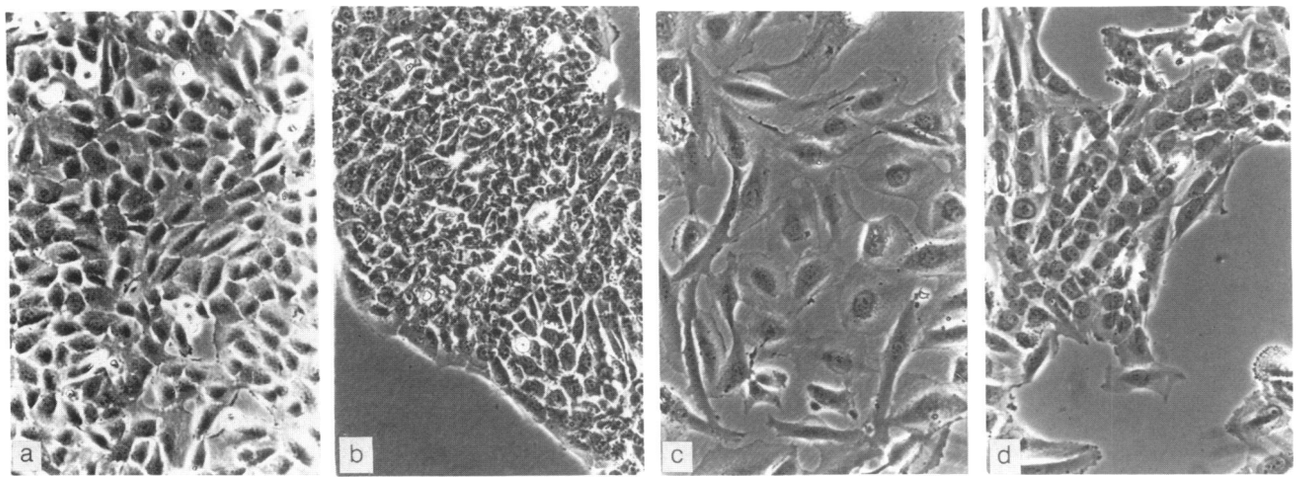

FIG. 1. Micrographs of T24 (*a*), MeNNG-T24 (*b*), J82 (*c*), and MeNNG-J82 (*d*) cells in culture. (×420.)

T24 c-Ha-*ras* oncogene (22). As shown in Fig. 2, the probe detected solely a 411-base-pair (bp) fragment in T24 cells, MeNNG-T24 cells, and MeNNG-T24 nude mouse tumor DNA digests, which is indicative of the c-Ha-*ras* codon 12 mutation (22). In contrast, DNA from both the J82 human cell line and normal human lymphocytes yielded 355-bp fragments that hybridized with the pKY-1 probe. This 355-bp fragment is indicative of the normal c-Ha-*ras* gene (22). As with normal human lymphocyte and J82 cell line DNAs, the pKY-1 probe detected exclusively 355-bp fragments in digests of both MeNNG-J82 cell line and MeNNG-J82 nude mouse tumor DNA (Fig. 2). Blot-hybridization analyses of RNAs extracted from T24 and MeNNG-T24 cells clearly demonstrated that the mRNA transcribed from the activated c-Ha-*ras* oncogene was equally present in both cell types (Fig. 3).

(*iii*) *Growth in agar.* The T24 cell line failed to produce colonies in soft agar (Table 1). In contrast, MeNNG-T24 cells consistently formed colonies in agar with high efficiency (33%). Thus, the relative colony-forming ability in agar of T24 (0%) and MeNNG-T24 (33%) cells is consistent with the tumorigenic potential of these two cell lines. Comparisons between J82 and MeNNG-J82 cell lines demonstrated similar differences (Table 1).

(*iv*) *Retention of rhodamine 123 by mitochondria.* T24 and MeNNG-T24 cells and also J82 and MeNNG-J82 cells were compared for retention of rhodamine 123 (5). In both cases, the tumorigenic derivatives (MeNNG-T24 and MeNNG-J82) demonstrated dramatic increases in 24-hr retention of this compound as compared with the parental cell lines (Table 2).

(*v*) *Secretion of a* M_r *67,000 transformation-related phosphoprotein.* T24 cells, MeNNG-T24 cells, and MeNNG-T24 tumors (which had just been explanted from nude mice) were analyzed for expression of a transformation-related secreted phosphoprotein (11). Parallel experiments were performed with J82 and MeNNG-J82 cells in culture and MeNNG-J82 tumors. Only the MeNNG-T24 and MeNNG-J82 cells freshly explanted from nude mouse tumors secreted detectable levels of the phosphoprotein (Fig. 4). Explanted MeNNG-T24 tumor cell populations (including those derived from a MeNNG-T24 clone) and MeNNG-J82 tumor cells ceased to express the ^{32}P-labeled protein within several weeks after explanation of cells from tumors to *in vitro* culture.

DISCUSSION

T24 human bladder carcinoma cells bearing the activated form of the c-Ha-*ras* oncogene are not tumorigenic in either athymic nude mice or nude mice that are both athymic and natural killer cell-deficient. Furthermore, these cells lack other phenotypic traits that are closely associated with neoplastic transformation (growth in soft agar and rhodamine 123 retention). Most significantly, these nontumorigenic T24

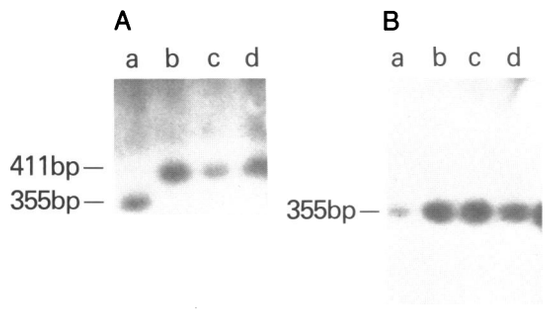

FIG. 2. Analysis of the point mutation at codon 12 in c-Ha-*ras* gene. DNAs were digested with *Msp* I and *Hpa* II, and Southern hybridization was performed with an insert probe of pKY-1 (22). (*A*) Lanes: a, normal human lymphocytes; b, T24 cell line; c, MeNNG-T24 cell line; d, MeNNG-T24 tumor. (*B*) Lanes: a, normal human lymphocytes; b, J82 cell line; c, MeNNG-J82 cell line; d, MeNNG-J82 tumor. Lane c in *A* and lane a in *B* contain less than 10 μg of DNA as compared with 20 μg of DNA in the other lanes.

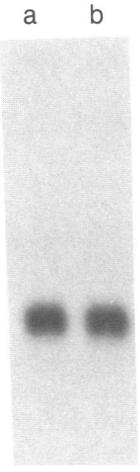

FIG. 3. c-Ha-ras mRNA from T24 and MeNNG-T24 cell lines. Poly(A)-selected RNA (2.5 μg) was analyzed by gel blotting; hybridization was carried out with a nick-translated insert probe of c-Ha-ras-1 recombinant clone (25). Lanes: a, T24; b, MeNNG-T24. The bands correspond to a size of 1.4 kilobases.

Table 1. Colony growth in soft agar

Cell type	% colony growth, mean ± SD
T24	0 ± 0
MeNNG-T24	33 ± 3
J82	3 ± 1
MeNNG-J82	13 ± 3

The results represent three experiments per cell type.

Table 2. Rhodamine 123 retention after 24 hr

Cell type	Fluorescence, mean ± SD
T24	7 ± 2
MeNNG-T24	99 ± 4
J82	8 ± 1
MeNNG-J82	79 ± 13

The results represent six comparisons per cell pair (T24/MeNNG-T24 and J82/MeNNG-J82).

cells can be chemically transformed with MeNNG to give rise to a population of cells that is not only highly tumorigenic in the subcutaneous space of nude mice but also produces colonies in agarose with high efficiency and demonstrates dramatic retention of rhodamine 123. MeNNG transformation of another nontumorigenic human bladder carcinoma cell line, J82, resulted in similar phenotypic changes. By demonstrating that nontumorigenic T24 and J82 cells can be transformed to yield highly tumorigenic derivatives, we have established that T24 and J82 cells lack tumorigenicity because they are not malignant. The alternative possibility—that the nude mouse assay unfairly evaluates the malignant potential of T24 and J82 cells—has been discounted by these findings.

MeNNG has been used previously to transform a nontumorigenic human osteosarcoma line (HOS) (19), which resulted in a cell population (designated MeNNG-HOS) that was tumorigenic and had tumor-associated phenotypic traits that were lacking or deficient in the parental HOS line (27). Thus, it appears that this carcinogen can be employed to transform both nontumorigenic human sarcoma and nontumorigenic human carcinoma cell lines with similar results. However, the effects of MeNNG on the genotype of HOS and T24 cell lines, at least in these particular cases, appear to be different. MeNNG-HOS cells have an activated *met* oncogene (absent in HOS cells), which arose by fusion of sequences from chromosomes 1 and 7 (28). MeNNG-HOS cells and NIH 3T3 cells transformed with MeNNG-HOS DNA express a 5-kilobase hybrid transcript encoded by the *met* oncogene (28). However, this messenger RNA was not detected in MeNNG-T24 cells or nude mouse tumors derived from these cells (M. Park and I.U.A., unpublished observations). Thus, it appears that tumorigenic transformation of T24 cells by MeNNG was not due to activation of the *met* oncogene. In addition, exposure to MeNNG did not result in any obvious differences at the c-Ha-*ras* locus (Fig. 2). Furthermore, both nontumorigenic T24 cells and highly tumorigenic MeNNG-T24 cells contained identical levels of c-Ha-ras transcripts (Fig. 3).

The original T24 cell line was reported to be tumorigenic in the hamster cheek pouch in 1973 (17), but since then it apparently has undergone a major reversion. We have shown here for MeNNG-T24 and MeNNG-J82 cells, and elsewhere (11) for some but not all tumorigenic human cell lines, that a partial phenotypic reversion involving the loss of one tumor-associated trait (secretion of a transformation-related phosphoprotein) occurs soon after explantation of cells from progressively growing tumors in nude mice to *in vitro* culture. This raises the important possibility that although a particular human tumor cell line may still be tumorigenic in nude mice, its phenotypic traits in culture may not be completely representative of those that it displayed *in vivo*. Our observations regarding rapid phenotypic alteration of tumorigenic human cells in culture parallel those of others with both normal and transformed rodent cells. For example, normal rat and hamster hepatocytes have been shown to alter their protein synthetic patterns within 1 week of transfer to culture on plastic (29), as have mouse mammary epithelial cells (30). In particular, clonal transformants of mouse BALB/3T3 cells reveal dramatic changes in their colony-forming efficiencies in agar when passaged from *in vitro* culture to tumors and again when explanted from tumors to *in vitro* culture (31, 32). Since cultivation of tumor cells on plastic substrata can lead to rapid phenotypic alterations, long-term *in vitro* culture of the T24 cell line may have been responsible for its total loss of malignancy. In another case, a human epidermoid carcinoma (HEP-3), which was established on the chicken chorioallantoic

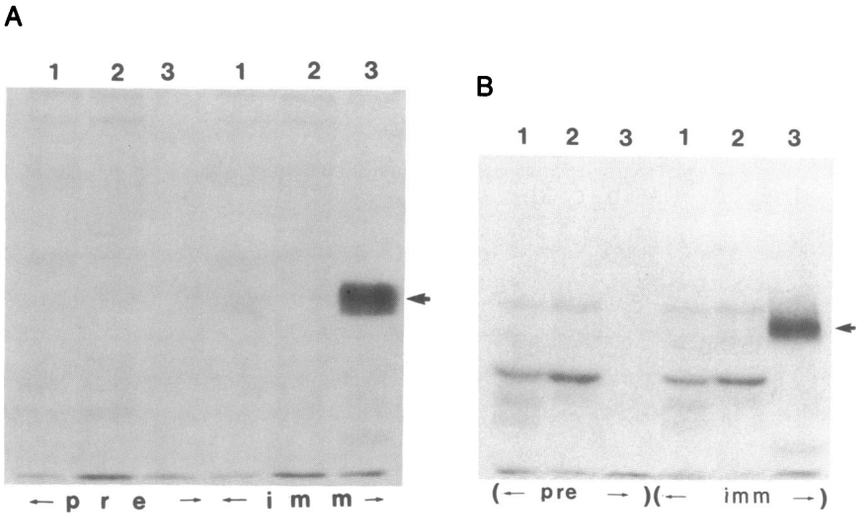

FIG. 4. Immunoprecipitates of culture medium from ^{32}P-labeled cells. Immunoprecipitations were performed with antiserum raised to the transformation-related secreted phosphoprotein (11). These are autoradiograms of 7.5% (wt/vol) Laemmli (26) polyacrylamide slab gels. pre, Precipitations performed with preimmune (control) serum; imm, precipitations performed with immune serum. (*A*) Lanes: 1, T24 cells; 2, MeNNG-T24 cells in culture 3–4 weeks after explantation; 3, MeNNG-T24 nude mouse tumor. (*B*) Lanes: 1, J82 cells; 2, MeNNG-J82 cells in culture 3–4 weeks after explantation; 3, MeNNG-J82 nude mouse tumor. The arrows indicate the position of the M_r 67,000 band.

membrane, has been shown to lose tumorigenicity when serially passaged *in vitro* (33).

The EJ human bladder carcinoma line, which bears the same c-Ha-*ras* mutation as T24 (15), is unlike T24 (ATCC) in that it is tumorigenic in nude mice (14). T24 and EJ cell lines may have been derived originally from the same cell population (14) but, in any event, they now provide important examples of malignant and benign cell lines, both with the same activated c-Ha-*ras* genes. Since the NIH 3T3 transfection assay has identified the EJ c-Ha-*ras* genes as the only activated oncogene associated with this cell line (15), this transfection assay apparently has failed so far to identify very critical genetic disparities between EJ and T24 that are ultimately responsible for their respective differences in tumorigenicity. These important genetic differences have yet to be elucidated, and the significance of the mutated T24/EJ c-Ha-*ras* gene is still uncertain. Several experimental approaches have indicated that the presence of this gene is insufficient for malignant transformation of normal human cells. Transfection of normal human fibroblasts with EJ c-Ha-*ras* neither immortalized nor rendered these cells tumorigenic (34). Furthermore, the tumorigenicity of EJ cells was lost upon fusion of these cells with normal human fibroblasts, despite the unaltered expression of the activated c-Ha-*ras* gene product (35). Consistent with these observations are our findings that T24 cells with the activated form of the c-Ha-*ras* gene are not tumorigenic but can be malignantly transformed with carcinogen.

In conclusion, the activated T24 c-Ha-*ras* gene, despite its ability to transform NIH 3T3 cells (12, 13), is not sufficient to transform T24 cells. Moreover, malignant transformation of J82 cells with chemical carcinogen, without activation of the c-Ha-*ras* gene (Fig. 2*B*), again suggests that the presence or absence of this oncogene is unrelated to the tumorigenicity of these bladder carcinoma cell lines. The detection of only 411-bp fragments in digests of T24 DNA (Fig. 2*A*) implies either a homozygous mutation of the c-Ha-*ras* gene or deletion of the normal c-Ha-*ras* allele. In either case, our data indicate that, even in the absence of any normal c-Ha-*ras* allele, the presence of an activated Ha-*ras* protooncogene is not sufficient for T24 cell transformation. Finally, T24 and J82 cell lines illustrate that data derived from human tumor cell lines should not be interpreted without regard for existing cellular characteristics. In the case of these cell lines and perhaps many other nontumorigenic human tumor lines as well, the original malignant phenotype has undoubtedly been lost as a result of years of *in vitro* culture, and these major phenotypic changes may be the result of significant genomic modifications. Although these cell lines may still be useful for some purposes, they are not valid as cancer cell models. Even tumorigenic human tumor lines are likely to have undergone some changes since their original derivation and, in particular, we have shown that cultivation of human tumor cells on plastic substrata can lead to a rapid loss of expression of a transformation-related secreted phosphoprotein that is expressed *in vivo*. Studies involving human tumor cell lines should include appropriate consideration of these various potential complications.

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