

TRANSFORMATION OF HUMAN KIDNEY PROXIMAL TUBULE CELLS BY *ras*-CONTAINING RETROVIRUSES

Implications for Tumor Progression

By D. M. NANUS, S. A. D. EBRAHIM, N. H. BANDER,* F. X. REAL,
L. M. PFEFFER,† J. R. SHAPIRO, AND A. P. ALBINO

*From the Memorial Sloan-Kettering Cancer Center, *Cornell University Medical Center, and
†The Rockefeller University, New York, New York 10021*

Numerous studies have implicated various roles in carcinogenesis for dominant-acting oncogenes, activated by mutation, chromosomal rearrangement, insertion of a nearby promoter element, or gene amplification [1-5]. Other studies have indicated that tumorigenicity behaves as a recessive trait, in that dominant elements (suppressor genes) must first be inactivated (e.g., by gene or chromosomal deletion, rearrangement or mutation) in order for a cell to become neoplastic (6-8). Presumably, normal progenitor cells would require a specific series of gene activations and/or inactivations to complete the transformation process. Moreover, depending upon the tumor type and the differentiation program of the cell, it is likely that this series of changes would differ. The determination of a contributing role for oncogenes in the sequence of steps required for malignant transformation would require, therefore, an ability to directly test the effects of oncogenes on normal differentiated diploid progenitor cells of a particular tumor type (9). Human renal cells provide a suitable system for examining the temporal sequence of oncogene-related effects for several reasons. First, cells from both renal cancers and normal human kidney can be cultured, thereby enabling a direct comparison of the normal and malignant phenotypes (10-13). Second, immunofluorescent staining with mAbs generated against glycoproteins, glycolipids, and blood group antigens of normal and malignant renal cells demonstrate that ~90% of cultured normal human kidney cells and virtually all renal cell carcinomas (as distinguished from transitional cell carcinomas of the kidney) are of proximal tubule derivation (10, 14-20). Thus, transformation experiments with normal proximal tubule (PT)¹ cells would presumably directly relate to events occurring in vivo. Third, specific quantitative and qualitative changes in proximal tubular antigens that occur upon neoplastic transformation of the PT cell

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¹ *Abbreviations used in this paper:* CALLA, common acute lymphoblastic leukemia; CB, complete buffer; CM, conditioned serum-free medium; DAB, diaminobenzidine; EGF, epidermal growth factor; MuLV, murine leukemia virus; NRK, normal rat kidney; PT, proximal tubule; TGF, transforming growth factor; TLC, thin-layer chromatography.

in vivo are well documented (16, 20-22). Therefore, the contributing role of oncogenes in the phenotypic alterations associated with transformation can be dissected. *Ki-ras* and *Ha-ras* oncogenes encode related 21,000 dalton proteins that have transforming potential (23). We have previously shown that viral *ras* oncogenes induce in normal human melanocytes a wide range of transformation-related phenotypic traits characteristic of melanoma cells (24, 25). In this report, we have examined the biologic, antigenic and genetic effects of introducing either the viral *Ki-ras* or the viral *Ha-ras* oncogenes into primary cultures of normal human renal PT cells.

Materials and Methods

Tissue Culture. PT cell cultures and renal carcinoma cell lines (denoted as SK-RC) were derived as described previously (10). Cultures were maintained in Eagle's MEM supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml streptomycin, 100 U/ml penicillin, and 7.5% FCS.

Virological Techniques. Viral stocks were isolated from NIH/3T3 cells infected with 4070A amphotropic murine leukemia virus (MuLV) (24), or from nonproducer NIH/3T3 cells, containing either viral Kirsten-*ras* (*Ki-ras*) or Harvey-*ras* (*Ha-ras*) gene sequences, superinfected with 4070A amphotropic helper MuLV (26). Viral pseudotypes were collected as fresh 24-h cell-free supernatant fluids and frozen at -70°C until use. Approximately 4×10^5 PT cells (passage 1) were pretreated for 60 min with DEAE/dextran (25 $\mu\text{g}/\text{ml}$), washed, then incubated with virus at a multiplicity of infection of 1 focus forming unit/cell. Transforming activity of amphotropic pseudotype virus produced by infected cells was determined by focus assays on NIH/3T3 cells. Nonpseudotype virus production by PT cells infected with the amphotropic parent 4070A-MuLV was quantitated by titrating cell supernatants on NIH/3T3 cells and determining the expression of the core p30 *gag* gene protein in the cytoplasm as described (24). The ability of viral-infected cells to form growing colonies in soft-agar (27) and infectious centers on NIH/3T3 cells (28) was determined as described (24).

Serological Reagents. The specificity of mouse mAbs to human antigens is as follows: S4/URO-2: recognizes a glycoprotein (gp) of 160 kD, gp160, on the glomerulus and PT portion of the nephron (13); T43/URO-10: recognizes a gp of 85 kD (gp85) on the PT (29); F23/URO-3: recognizes a gp of 140 kD (gp140) on the PT (15); S27/URO-4, S23/URO-4a and S6: recognize different epitopes of the adenosine deaminase binding protein, a gp of 120 kD (gp120) on the PT and the loop of henle (LH) (16); AJ8: recognizes the common acute lymphoblastic leukemia antigen (CALLA; a gp of 100 kD) on the glomerulus and PT (30); F31/URO-8: recognizes a lipid on the PT cell (16); 10.32: recognizes the Tamm-Horsfall protein, a gp of 90 kD (gp90) on the LH and DT [14]; CAM 5.2: recognizes cyokeratin polypeptides with molecular weights of 40, 45, and 52 kD, found in epithelial cells (31); P12 (SSEA-1): recognizes the Lewis X blood group antigen on the PT (17, 32); R24: recognizes GD₃ ganglioside (33); 3F8: recognizes GD₂ ganglioside (34); and BDID₂C₃: recognizes villin (35). Non-mouse antibodies used were rat mAB Y13-259 (36), which reacts with the 21-kD (p21) protein encoded by the viral *Ha* or *Ki-ras*, and polyclonal rabbit anti-Rauscher-MuLV group-specific p30 serum (37).

Immunoperoxidase Staining. Immunoperoxidase staining of antigens was performed as previously described (31). Briefly, cells were seeded into Lab-Tek tissue culture chamber/slides (Miles Scientific, Naperville, IL) and incubated at 37°C in a CO₂ chamber for 24-48 h. The slides were fixed with methanol at -20°C for 10 min, quenched for 15 min in 1% hydrogen peroxide in PBS, washed several times with PBS, and incubated with suppressor serum (10% normal horse serum; Cappel Laboratories, Cochranville, PA) for 20 min. The suppressor serum was removed and sections of cells on each slide were incubated with appropriately diluted primary antibody overnight at 4°C . The avidin-biotin method used a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) as described (38). 5 mg of diaminobenzidine (DAB) tetrahydrochloride in 100 ml of PBS with 100 μl of 0.3% hydrogen peroxide was used as chromogen. The DAB solution was filtered and incubated with the slides for 5-10 min. After treatment, slides were washed with distilled H₂O, counterstained with

hematoxylin, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Slides were examined with an epifluorescent microscope and reactivity was graded from 0 to 3+ by two independent observers.

Immunofluorescence Assay. Detection of villin with BDID₂-C₃ antibody was performed as previously described (35). Briefly, cells were allowed to replicate on Lab-Tek slides as described above, fixed on the culture slide with periodate-lysine-formaldehyde, and labeled with BDID₂-C₃ antibody (in 0.1 M sodium cacodylate, pH 7.4). The sections were incubated with rabbit anti-mouse IgG secondary antibody conjugated to fluorescein (Cappel Laboratories) diluted at 1:1,000, and examined with a microscope equipped with epifluorescence and a 100-W mercury lamp. Reactivity was graded from 0 to 3+ by two independent observers.

Serological Assays. The protein A and anti-mouse Ig hemadsorption assays were performed as described (24). Indicator cells were prepared by conjugating either protein A (Pharmacia Fine Chemicals, Piscataway, NJ) or the Ig fraction of rabbit anti-mouse heavy chain (Dako Corp., Santa Barbara, CA) to human 0+ erythrocytes with 0.01% chromium chloride. Assays were performed in microtest plates (model 3040; Falcon Labware, Oxnard, CA). Target cells (plated 1-2 d previously) and serial antibody dilution were incubated for 1 h at room temperature. Target cells were then washed and indicator cells were added for 45 min. Target cells were washed again to remove nonadherent indicator cells. Titers were defined as the antibody dilution showing 20% positive (rosetted) target cells as evaluated under light microscopy.

Immunoprecipitation Analysis. Cells (in a near confluent 150-cm² flask) were radiolabeled by metabolic incorporation of [³⁵S]methionine (1,000 Ci/mmol; New England Nuclear, Boston, MA) using 500 μCi in 10 ml of methionine-free MEM containing 7.5% dialyzed FCS for 16 h. Labeled cells were extracted as described (39). 0.05 ml of protein A-agarose beads (Boehringer Mannheim Biochemicals, Indianapolis, IN) were incubated with 0.02 ml of goat anti-rat Ig (Dako Corp.) and 0.08 ml of complete buffer (CB: 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% NP-40, 0.1% SDS) for 1 h with rotation at 4°C, and washed five times with CB. The beads were resuspended in 0.05 ml CB and incubated with 5 μl of undiluted ascites fluid Y13-259 (anti-p21) for 1 h at 4°C, then washed five times with CB. Immunoprecipitation was carried out by incubating a portion of the cell extract (10⁷ cpm) with the beads and CB to a total volume of 0.3 ml for 16 h at 4°C. The beads were washed six times with CB and four times with CB/high salt (2.1 gm NaCl in 100 ml CB). Labeled components were detected by SDS-PAGE and fluorography as described (39).

Radiolabeled Binding Assay for Gangliosides. Cells were trypsinized and resuspended in Tris-MEM (490 ml unsupplemented MEM, 10 ml 1 M Tris, pH 8.0, 2 gm BSA, 40 mg sodium azide) at a concentration of 1-2 × 10⁶ cells/ml. 96-well plates (Dynatech Laboratories, Alexandria, VA) were preincubated with Tris-MEM (0.1 ml/well) for 30 min at room temperature. The media was discarded and the wells were allowed to air-dry. A cell suspension of 5 × 10⁴ to 1 × 10⁵ cells/0.05 ml Tris-MEM was deposited in each well, together with 0.05 ml of antibody diluted serially, and incubated for 1 h at 4°C. All incubations were performed in triplicate. After the incubation, the plates were centrifuged at 1,200 rpm for 5 min and washed three times with Tris-MEM. Cells were resuspended in 0.05 ml Tris-MEM together with 100,000 cpm of ¹²⁵I-labeled protein A (in 0.05 ml Tris-MEM) for 1 h at 4°C, washed five times, and allowed to air-dry overnight at 37°C. Individual wells were placed in Omni plastic vials and quantitated in a gamma counter (Packard Instrument Co., Downers Grove, IL).

Thin-Layer Chromatography. Cells were radiolabeled by metabolic incorporation of [³H]glucosamine (New England Nuclear) 30-60 Ci/mmol, by using 500 μCi in 10 ml of MEM with 10% FCS for 72 h. Radiolabeled cells were extracted successively with chloroform/methanol 2:1, 1:1, and 1:2 and the acidic glycolipids were isolated by florisil and DEAE-sephadex chromatography as described (40). Radiolabeled gangliosides were separated by thin layer chromatography in chloroform/methanol/2.5-N-ammonium hydroxide (60:35:8) and detected by spraying the plate with Enlightening (New England Nuclear), air-drying, and exposing to XAR-5 film (Eastman Kodak Co., Rochester, NY).

Growth Factor Analysis. Conditioned serum-free medium (CM) was collected from 90% confluent monolayers of cells growing in 150-cm² flasks after 72 h, dialyzed in acetic acid, lyophilized, and reconstituted in 1% BSA/PBS as described (41, 42). The concentration of protein in each sample was determined using a colorimetric protein assay (Bio-Rad Labora-

tories, Richmond, CA). Other reagents include epidermal growth factor (EGF) (culture-grade; Collaborative Research, Inc., Bedford, MA) and transforming growth factor β (TGF- β) (generously provided by M. B. Sporn, National Institute of Health, Bethesda, MD). NRK cells, clone 49F (American Type Culture Collection, Rockville, MD), were grown in MEM as described above. Soft-agar colony-forming activity was determined using NRK clone 49F cells grown in 0.35% agar in the presence or absence of added growth factors, CM, or both (41, 42). The number and size of colonies were determined at day 14 by counting microscopic fields using a grid.

Cytogenetic Analysis. Karyotypic analysis was performed as previously described (43). Briefly, mitotic cells were arrested by exposure to colcemid (0.05 $\mu\text{g}/\text{ml}$) for 2 h at 37°C before harvesting. Cell suspensions were treated with a hypotonic solution (0.075 M KCl) for 20 min at 37°C, fixed with Carnoy's fixative (methanol/acetic acid, 3:1), and washed three to five times. Air-dried slides were stained with quinacrine mustard. 30–50 metaphases were photographed, counted, and analyzed; 12–20 metaphases were karyotyped. Chromosome identification followed the International System for Human Cytogenetics Nomenclature (ISCN) (44).

IFN Binding to Cells. Recombinant DNA-derived IFN- α Con₁, designed as a consensus of the known IFN- α subtypes (Amgen, Inc., Thousand Oaks, CA) (45) was coupled to ¹²⁵I by using limiting amounts of chloramine T. Iodinated IFN- α (~150 Ci/gm), separated from free ¹²⁵I on a column of G-50 Sephadex, retained >90% of its antiproliferative and antiviral activity on sensitive cell lines (46). For analysis of ¹²⁵I-IFN- α binding to cells, monolayer cell lines were washed with PBS, and harvested by incubation with 1 mM EDTA in PBS for 5 min at 37°C. Cells were resuspended at 2×10^6 cells/ml in Dulbecco's modified Eagle's MEM supplemented with 10% FCS and containing 20 mM Hepes and incubated with varying concentrations of ¹²⁵I-IFN- α (1–100 pM). After incubation for 100 min at 15°C, duplicate 200- μl aliquots of the binding mixture were layered over 150 μl of a mixture of di-*n*-butyl/dinonyl phthalate (2:1) in a 400- μl microcentrifuge tube and centrifuged (10,000 *g*, 1 min). The supernatant medium was aspirated, the top of the tube was aspirated with 200 μl of distilled water, and the oil was aspirated in the final rinse. The tip of each tube was cut off, and the cell-bound radioactivity was assayed in a gamma counter. The specific binding of ¹²⁵I-IFN- α is determined by the difference of ¹²⁵I-IFN binding in the absence and presence of 10 nM unlabeled IFN- α . Data are plotted according to the procedure of Scatchard (47) and analyzed by a linear nonreiterative least square curve fitting program.

IFN Effect on Proliferation. Cell lines were plated at 10^5 cells/25-cm² flask in Dulbecco's MEM. After 1 d the cells were refed with medium containing IFN- α at varying concentrations (100–3,000 U/ml). Control cultures received no IFN. At 6 d after IFN- α addition, cells were washed once with PBS and harvested by trypsinization for 5 min at 37°C. Cell counts were performed on a Coulter counter (Coulter Electronics, Hialeah, FL). The ratios of the cell number on day 6 to that on day 0 in IFN-treated cultures were expressed as a percentage of the ratio in untreated control cultures.

Results

Biological Characteristics. 2 wk after infection of passage 1 kidney cultures with amphotropic retroviruses containing either the Kirsten-*ras* (*Ki-ras*) or the Harvey-*ras* (*Ha-ras*) oncogenes, islands of rapidly proliferating cells appeared possessing morphologies similar to those seen in renal cancers. Cultured renal cancers display three main morphological types (10): (a) well-defined spindle shaped cells that grow in mounds several layers deep; (b) islands of compact epithelial cells with irregular, sharply defined edges; and (c) globular shaped cells with pseudopod-like structures. In contrast, cultured normal kidney cells demonstrate either crescentic, round or polygonal configurations (48). *Ki-ras*-infected cells (termed PT-Ki) appeared similar to type 1 renal cancers that have a well-defined spindle shaped morphology. *Ha-ras*-infected cells (termed PT-Ha) grew as islands of compact epithelial cells similar to type 2 renal cancer cells. Sister kidney cultures infected with control 4070A amphotropic

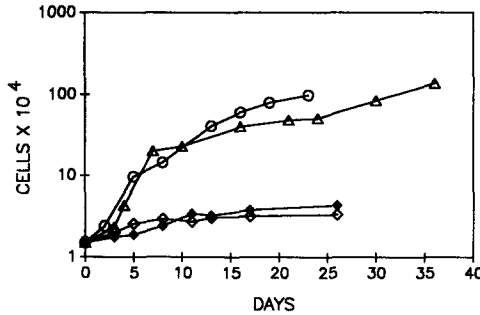


FIGURE 1. Growth kinetics of PT cells. Growth rates of PT-Ki cells at passage 12 (O); PT-Ha cells at passage 6 (Δ); PT cells infected with 4070A-MuLV at passage 3 (\blacklozenge); and uninfected PT cells at passage 3 (\diamond).

MuLV, which does not contain an oncogene, maintained a characteristically normal morphology.

Infected PT-Ki and PT-Ha cells proliferated vigorously beyond passage 3, becoming the dominant cell in the culture by 4 wk after infection. Fig. 1 compares the growth potential of PT-Ha and PT-Ki cells with uninfected kidney cells and 4070A-infected cells. The increased proliferative capacity of PT-Ki and PT-Ha cells, sustained in culture for >24 mo, is in sharp contrast to normal kidney cells, which, though capable of rapid growth between passages 0-2, manifest a sharp decline in growth rate after passage 2 and senesce by passage 4 or 5 (i.e., by 8-10 generations) (10).

Uninfected kidney cells exhibit other biological traits characteristic of short-term cultures of normal cells, including contact inhibition and anchorage-dependent growth. In contrast, renal cancer cells can replicate indefinitely in culture, do not exhibit contact inhibition, and form colonies in semi-solid medium with high efficiencies (10; Bander, N. H., unpublished results). Table I compares the biological characteristics of early passage (i.e., <17 passages) PT-Ki and PT-Ha cells with those of renal cancers and early passage normal kidney cells. Similar to renal cancer cells, PT-Ha and PT-Ki cells (a) are apparently immortalized as they do not senesce by passage 5 (as do normal kidney cells) and have an unlimited growth capacity, (b) are not contact inhibited, growing to high saturation densities and in multiple cell layers,

TABLE I
Biologic Characteristics of Cultured PT Cells

Biological trait	Normal kidney	PT-Ki cells	PT-Ha cells	Renal cancers
Proliferative capacity*	-	+	+	+
Contact inhibition	+	-	-	-
Growth factor production†	-	+	+	+
Unlimited growth‡	-	+	+	+
Anchorage independence§	-	-	-	+
Karyotype	Diploid	Aneuploid	Aneuploid	Aneuploid

+ , presence of phenotypic trait; - , absence of phenotypic trait.

* Defined as the ability to proliferate beyond passage 5 and to be subcultured at a low-seeding density (i.e., <100 cells/cm²).

† Growth factor production determined by NRK colony forming assay.

‡ Defined as having an immortalized phenotype.

§ Defined as the ability to form colonies in semi-solid media.

(*c*) produce growth factors (see below), and (*d*) have an aneuploid karyotype (see below). One notable dissimilarity is that, during early passages, PT-Ki and PT-Ha cells are still anchorage dependent. At later passages, however (see below), PT-Ki cells acquire the capacity for anchorage-independent growth in soft agar with a high efficiency. Kidney cells infected with control 4070A virus maintained all the phenotypic characteristics of uninfected normal kidney, manifesting a decline in growth rate at passage 3 and senescing by passage 5.

Viological Characteristics. Infectious center analysis at passage 5 indicated that 100% of PT-Ki and PT-Ha cells were infected and releasing transforming virus particles. Supernatants from PT-Ki and PT-Ha cultures contained between 10^3 and 10^5 infectious virus particles per milliliter. A Northern blot analysis demonstrated the presence of messenger RNA transcripts of either Ki-*ras* or Ha-*ras* oncogenes in Ki- or Ha-infected cells respectively (not shown). The presence of viral *ras*-encoded p21 protein in PT-Ki and PT-Ha cells was confirmed by immunoprecipitation studies (see Fig. 2) and Western blotting (not shown). Control MuLV-infected kidney cultures assayed for cytoplasmic viral p30 *gag* gene protein showed that 100% of the cells were infected.

Proximal Tubule Cell Origin of Infected Kidney Cells. 90% of the cells in passage 0 normal kidney cultures are derived from the PT portion of the nephron and are of epithelial origin (10–12, 48). The remaining 10% of cultured cells originate either from other divisions within the nephron (i.e., glomerulus, distal tubule, or collecting duct) or from connective tissue. The precise derivation of PT-Ki and PT-Ha cells was defined by determining the expression of a series of cell surface and cytoplasmic kidney cell markers. First, as summarized in Table II, PT-Ki and PT-Ha cells strongly immunostained for low molecular weight cytokeratins, confirming an epithelial origin (31). Second, PT-Ki and PT-Ha cells expressed villin, a 95-kD molecular mass protein found only in intestinal epithelium and renal proximal tubule epithelium (49, 50). Third, as determined by immunorsetting assays, PT-Ki and PT-Ha cells expressed a number of surface antigens characteristic of the PT portion of the human nephron. These antigens include (*a*) adenosine deaminase binding protein (ADA bp) [16]; (*b*) cell surface glycoproteins recognized by mAbs S4/URO-2, F23/URO-3, and T43/URO-10 (13, 15, 29); (*c*) the CALLA recognized by mAb AJ8 (which is also expressed by lymphocytes) (16, 30); and (*d*) the Lewis X blood group antigen, a carbohydrate structure found on the PT portion of the nephron (17), as well as

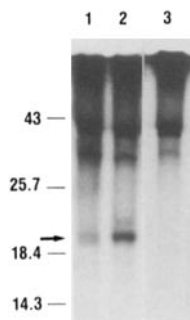


FIGURE 2. Immunoprecipitation of *ras* p21 protein. Autoradiograms of immunoprecipitates obtained with mAb Y13-259 (anti-p21) and extracts of [35 S]methionine-labeled PT-Ha and PT-Ki cells as analyzed by SDS-PAGE. (Lane 1) PT-Ha cells; (lane 2) PT-Ki cells; (lane 3) uninfected normal PT cells. Arrow shows p21 protein.

TABLE II
PT Phenotype of Infected Kidney Cells

	Cultured kidney cells	PT-Ki cells	PT-Ha cells
Keratins*	3 +	3 +	3 +
Villin†	1 +	1 +	1 +
ADAbp	10,000	1,000	1,000
S4/URO-2	100	100	10
F23/URO-3	100	1,000	1,000
T43/URO-10	100,000	100,000	100,000
AJ8 (CALLA)	1,000	100	100
Le ^x (SSEA-1)	100	100	1,000
Tamm-Horsfall protein	—	—	—

Expression of adenosine deaminase binding protein (ADAbp), and the antigens S4/URO-2, F23/URO-3, T43/URO-10, and AJ8 (CALLA), the Lewis X blood group antigen (Le^x, SSEA-1), and the Tamm-Horsfall protein was determined by erythrocyte rosetting assays. Values represent reciprocal of serum titers $\times 10^3$, at which 20% of the cells were positive. (—) Indicates no positive cells.

* Keratin expression determined by immunoperoxidase staining and scored 0 to 3 +.

† Villin expression was determined using immunofluorescence staining and scored 0 to 3 +.

on the surface of erythrocytes and various epithelia of the body (51). The Tamm-Horsfall protein, expressed by the distal tubule portion of the nephron (16), was not expressed by PT-Ki, PT-Ha, or normal kidney cells. Based on the pattern of antigen expression, infected PT-Ki and PT-Ha kidney cells were judged to be of PT origin, and not of glomerular, distal tubule, collecting duct, or connective tissue origin. In addition, there were no qualitative changes in the expression of any of these antigens.

Antigenic Alterations in Infected PT Cells. In contrast to the series of antigens discussed above, cultured renal carcinomas express antigenic determinants apparently related to the transformation process. Two of these determinants are the disialogangliosides GD₂ and GD₃, each of which is expressed by a distinct subset of renal cancers but not by normal kidney (Albino, A. P., unpublished results). GD₃ could be detected on the cell surfaces of PT-Ki and PT-Ha cells at the earliest time point analyzed (i.e., 2 wk after infection) with immunorosetting assays (see Table III). The induction of GD₃ expression in these cells was confirmed by direct isolation and analysis on thin-layer chromatography (TLC) (Fig. 3). Radiolabeled binding studies indicated that the quantity of GD₃ in PT-Ki and PT-Ha cells increased 5–10-fold as compared with uninfected PT cells or PT cells infected with control 4070A virus. GD₂, whose structural precursor is GD₃ (52), was not detectable in either PT-Ki or PT-Ha cells at early passages (i.e., <17). However, by later passages (i.e., 24), GD₂ could be detected in PT-Ki cells by direct binding of radiolabeled anti-GD₂ antibody, but not by the less sensitive TLC method. PT-Ha cells did not express detectable levels of GD₂ even at similarly late passages. We noted (and further discuss below) that the expression of GD₂ in PT-Ki cells appeared concurrently with the acquisition of an anchorage independent phenotype. PT-Ha cells also did not convert to an anchorage-independent mode of growth. In contrast to GD₃ and

TABLE III
Lipid Content of Normal and Transformed Kidney Cells

	Normal PT cells	PT-Ki cells	PT-Ha cells	Renal carcinomas
GM ₃ ganglioside	+	+	+	+
GD ₃ ganglioside	-	+	+	10%*
GD ₂ ganglioside	-	-	-	10%
F31/URO-8 [†]	- [‡]	-	-	70%

Ganglioside content was determined by immunorsetting and/or TLC. +, antigen expressed; -, antigen not expressed.

* Increased expression of GD₃ ganglioside is present in 10% of renal cancers (see text).

[†] F31/URO-8 is a lipid expressed by over 70% of renal cancers.

[‡] Cells from the convoluted PT.

GD₂, expression of GM₃ ganglioside in PT-Ki and PT-Ha cells appeared to be unaltered.

A third transformation-related antigen that is differentially expressed by kidney cancers and normal kidney cells is an undefined lipid recognized by mAb F31/URO-8. This lipid moiety is expressed on the cell surfaces of 70% of cultured renal cancers (16, 21), but undetectable on cultured cells derived from the convoluted proximal tubule (see Table III). The F31/URO-8 antigen was undetectable on the cell surfaces of PT-Ki or PT-Ha cells.

Growth Factor Production. A fraction of cultured and noncultured renal cancers produce growth regulatory factors collectively referred to as transforming growth factors (TGFs) (53, 54; Nanus, D. M., unpublished results). To determine whether transformed PT-Ki or PT-Ha cells generate TGFs, CM derived from these cells were tested in the NRK soft-agar growth assay. TGFs induce normal rat kidney (NRK) fibroblasts to form colonies in soft-agar, thereby providing an assay to detect and quantitate the mitogenic activity of these factors (42). Furthermore, combinations of various growth factors apparently act synergistically, causing the number and size of NRK colonies to increase (42, 55). As summarized in Table IV, CM from PT-Ha, PT-Ki (early and late passages), and 1/3 renal cancers cell lines induced colony-formation of NRK cells in soft agar, thereby indicating the presence of TGFs in the supernatants. Table V shows that the CM from passage 24 PT-Ki cells induced the formation of large colonies (>100 μ m) in either the absence or presence of up to 100 ng of EGF per milliliter. This latter result indicates that the CM from these cells contained more than one biologically distinct growth factor, the precise biochemical nature of which remains to be elucidated.

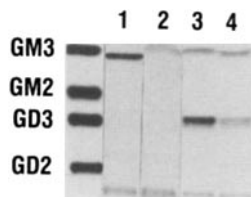


FIGURE 3. Analysis of gangliosides by TLC. (Lanes 1 and 2) Two independent uninfected PT cell cultures; (lane 3) PT-Ki cells; (lane 4) PT-Ha cells. Marker lane shows migration of purified gangliosides.

TABLE IV
Transforming Growth Factor Activity in Renal Cell Lines

Source of conditioned media*	Number of NRK-49F colonies [†]
Normal kidney 1	<2
Normal kidney 2	<2
SK-RC-21	<2
SK-RC-46	<2
SK-RC-42	61
PT-Ha (early passage)	150 [§]
PT-Ki (early passage)	130 [§]
PT-Ki (late passage)	125 [§]

* 1,700 μ g of protein from processed conditioned media was added to each well.

[†] 3×10^3 cells were seeded in soft agar as described in Materials and Methods. Colonies were allowed to form for 14 d. Values represent the mean of duplicate plates.

[§] Colony size >100 μ m; all others have a colony size <100 μ m.

Karyotypic Analysis of PT Cells. Cytogenetic analysis of a short-term culture of uninfected PT cells indicates a normal diploid male karyotype (46, XY). In contrast, cytogenetic analyses of PT-Ha cells (passage 3) and PT-Ki cells (passage 4) demonstrated nonrandom abnormalities involving chromosome 21 in both lines. Fig. 4 shows that the karyotypic anomaly in PT-Ki cells was a translocation between the q portions of chromosomes 1 and 21, specifically der(21)t(1;21)(q21;q22), which was observed in 92.5% of cells analyzed (see Table VI). Fig. 5 shows that PT-Ha cells were monosomic for chromosome 21, an abnormality which was observed in 50% of cells analyzed (see Table VI).

Progression of Infected PT Cells. Serially passaged PT-Ki and PT-Ha cells were reg-

TABLE V
Transforming Growth Factor Activity in Conditioned Medium from PT-Ki Late Passage Cells

Growth factor added	Amount ng/ml	Number of NRK-49F colonies	
		Growth factors minus conditioned media [†]	Growth factors plus conditioned media [†]
None		<2	38 [§]
EGF	0.05	<2	30 [§]
EGF	0.5	<2	72 [§]
EGF	5.0	25	190 [§]
EGF	10.0	26	138 [§]
EGF	100.0	22	133 [§]
TGF- β	0.5	<2	96 [§]
EGF plus TGF- β	5.0	133 [§]	ND
TGF- β	0.5		

* 3×10^3 cells were seeded in soft agar as described in Materials and Methods. Colonies were allowed to form for 14 d. Values represent the mean number of colonies from duplicate plates.

[†] 1,700 μ g of protein from processed conditioned media was added to each well.

[§] Colony size >100 μ m; all others have a colony size <100 μ m.

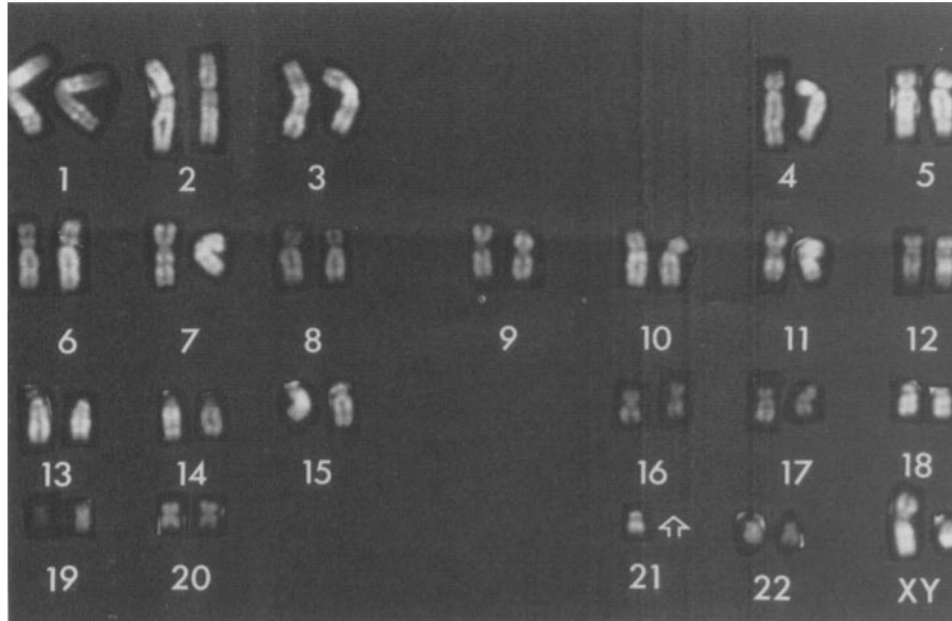


FIGURE 4. Q-banded karyotype of PT-Ha cells. Arrow indicates loss of one copy of chromosome 21 (45,XY,-21).

ularly monitored to identify temporal and progressive alterations in their antigenic, biologic, and chromosomal phenotypes. A comparison of the biological and antigenic phenotypes of early passage (i.e., <17) PT-Ki and PT-Ha cells indicated that these cells possessed all of the measured characteristics of PT cells and renal cancers with the exception of anchorage independence.

We noted that by passage 24 (~8 mo in culture), PT-Ki cells spontaneously acquired an anchorage-independent phenotype, and could form colonies in soft agar

TABLE VI
Chromosomal Rearrangements in PT-Ki and PT-Ha Cells

Cell line	Cell passage	Stem line	Karyotypic deviations	Percent of cells with der(21)
PT-Ki	3	sl	46,XY, - 21, + der(21)t(1;21)(q21;q22)	92.5
PT-Ki	24	sl	46,XY, - 21, + der(21)t(1;21)(q21;q22)	100
PT-Ki	57	sl	47,XY, - 3, - 21, + 20, + der(3)t(3;?)(q27::?) + der(21)t(1;21)(q21;q22)	100
				Percent of cells with monosomy 21
PT-Ha	3	sl	45,XY, - 21	50
PT-Ha	24		45,XY, - 21	14.2

Stem line (sl) refers to the most frequent karyotypes (with respect to chromosome number and/or chromosome structure) of a tumor cell population.

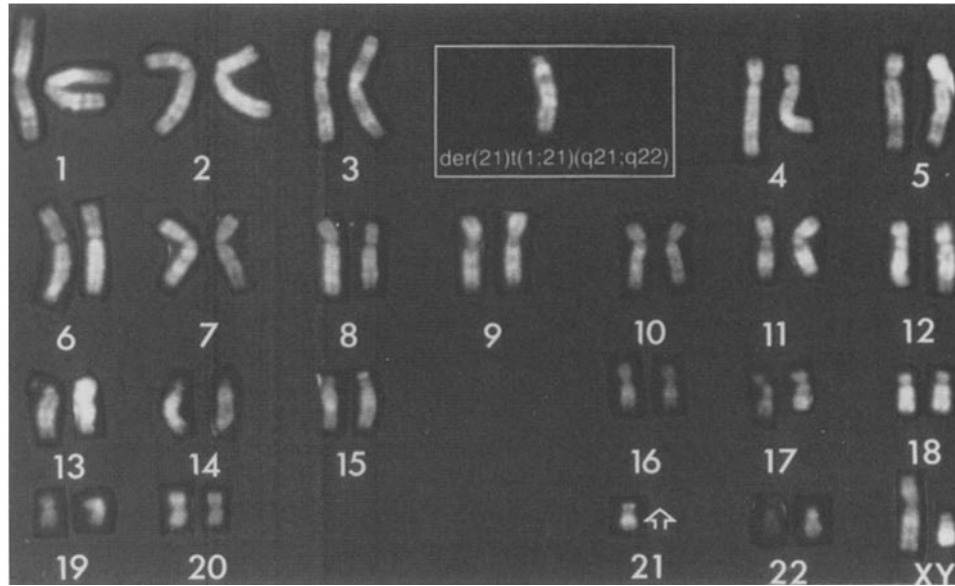


FIGURE 5. Q-banded karyotype of PT-Ki cells. Arrow shows loss of chromosome 21 and boxed figure indicates the involvement of chromosome 21 in conjunction with chromosome 1 (46,XY,-21,+der(21)t(1;21)(q21;q22)).

with an efficiency of $\sim 10\%$. Karyotypic analysis of these cells revealed that 100% of the cells still displayed der(21)t(1;21)(q21;q22) observed in early passage cultures; but, in addition, other secondary chromosomal abnormalities were observed. Since the acquisition of anchorage independence occurred as a relatively discrete temporal event, we attempted to correlate the development of this phenotype with a specific secondary chromosomal abnormality. PT-Ki passage 24 cells were cloned in soft agar and five individual clones were isolated, grown to mass culture and their karyotypes analyzed (Table VII, reference 56). Aside from the der(21)t(1;21)(q21;q22), which persisted in 100% of the cells from each of the 5 clones, no common secondary chromosomal abnormality (which included numerical and structural alterations in

TABLE VII
Chromosomal Deviations in Soft-agar Clones of PT-Ki Cells

Cell line	Stem line	Karyotypic deviations	Percent of cells with der(21)
Clone 1	sl	45,Y,dic(X;15)(qter→pter), -21,dup(7)(q21;q31), + der(21)t(1;21)(q21;q22)	100
Clone 2	sl	45,X,-Y,-21 + der(21)t(1;21)(q21;q22)	100
Clone 3	sl	46,XY,-21,19q+, + der(21)t(1;21)(q21;q22)	100
Clone 4	sl	46,XY,-21,18p+, + der(21)t(1;21)(q21;q22)	100
Clone 5	sl	44,X,-Y,-18,-21,19q+, + der(21)t(1;21)(q21;q22)	100

Stem line (sl) refers to the most frequent karyotypes (with respect to chromosome number and/or chromosome structure) of a tumor cell population.

chromosomes [1, 5, 7, 8, 15, 18, 19, X and Y; and see reference 56] appeared to correlate with the acquisition of anchorage independence. The five soft agar clones and the parental PT-Ki cells were analyzed for other progressive alterations. Each of the clones and the parental cells continued to express a proximal tubule phenotype as well as GD₃ ganglioside. But, in addition, each of these cell lines also expressed GD₂ ganglioside (whose biosynthetic precursor is GD₃), as detected by direct serological and binding assays. Most interesting, however, was the observation that upon further subculturing and subsequent chromosomal analysis (i.e., at passage 57), PT-Ki cells manifested, in addition to der(21)t(1;21) (q21;q22), a rearrangement of chromosome 3 and an additional copy of chromosome 20 in 100% of the cells. Chromosome 3 has been implicated as having a fundamental role in the pathogenesis of renal carcinoma and it is postulated that a recessive oncogene is present on the short arm (57, 58).

Analysis of PT-Ha cells at passage 24 revealed no new antigenic or biologic alterations. Chromosomal analyses of these cells at passage 24 showed that monosomy 21 persisted in 14.2% of cells (Table VI).

Analysis of Genes on Chromosome 21. To date, 17 genes have been assigned to chromosome 21, including the proto-oncogenes *ets2*, *bce1*, and *erg*, and the gene coding for the receptors for type I interferons (α and β) (59, 60). We have begun an analysis designed to detect the presence of abnormalities in these genes. First, high molecular weight DNA from PT-Ki, PT-Ha, and renal cancer cell lines was analyzed by Southern blotting for the presence of rearrangements in the *ets2* gene. Compared with normal diploid proximal tubule cells, each of the cell lines tested had a normal genomic organization of the *ets2* gene, indicating no rearrangement or amplification in the regions comprising this gene (data not shown).

Second, alterations in type I interferon (IFN- α) receptors were analyzed by determining the receptor number and the affinity of these receptors for interferon. Binding data obtained at 15°C were plotted according to the method of Scatchard (47), which yielded linear plots. Both PT-Ki and PT-Ha cell lines possess similar numbers of high affinity IFN- α binding sites ($K_d \sim 100$ pM; 1,500 and 1,250 receptors/cell for PT-Ki and PT-Ha, respectively). These numbers of IFN- α receptors and the affinity of IFN- α binding are comparable to values obtained with several cell types, both malignant and normal (Pfeffer, L. M., unpublished results). We have found that a subset of cultured renal cancers are sensitive to the antiproliferative action of IFN (Nanus, D. M., et al., unpublished results), and thus, analyzed the sensitivity of infected PT cells to IFN action. Both the PT-Ki and PT-Ha cell lines were relatively resistant to the antiproliferative action of IFN- α , with no significant inhibition observed at IFN concentrations below 15 pM. At 150 pM IFN, there was only 40% inhibition of cell proliferation, as determined by the fold-increase in the treated culture relative to that in untreated control cells. The level of inhibition of cell proliferation in the transformed cell lines is similar to the inhibition that has been observed in several lines of normal human kidney cells and in diploid human fibroblasts (Pfeffer, L. M., N. H. Bander, and A. P. Albino, unpublished results).

Discussion

Our objective was to define the biologic, antigenic, and genetic effects of introducing the *Ki-ras* or *Ha-ras* oncogenes into normal human PT cells, the normal cellular coun-

terpart of renal cell carcinomas. Our goal is to use PT cells (*a*) as a model of transformation, ascertaining the temporal sequence of events involved in the etiology of renal carcinoma, and (*b*) as a system for defining the relationship of these events with the presence of specific oncogenes. We report there that the introduction of the *Ki-ras* or *Ha-ras* oncogenes initiates a complex series of events which propels the PT cell towards transformation in a temporal sequence, resulting in cells resembling *in vivo* renal carcinomas.

PT cells appear to undergo two distinct phases in response to infection with *ras*-containing retroviruses. In the first or early stage (i.e., between passages 1-3), *ras*-infected PT cells manifest distinct morphological alterations and undergo a burst of proliferation and outgrowth. Subsequently, these cells no longer senesce by passage 5, as do normal PT cells and PT cells infected with a non-oncogene-containing retrovirus. In addition, *ras*-infected PT cells at this stage are not contact inhibited and appear to be immortalized, in that they are capable of indefinite proliferation. However, these cells are still anchorage dependent.

Concomitant with the biologic alterations observed in the early phase, PT cells containing a *Ki-ras* or *Ha-ras* oncogene also begin expressing GD₃ ganglioside, whereas untransformed PT cells do not. Induction of GD₃ expression occurred within 2 wk of viral infection and correlated with the capacity of PT cells to proliferate. GD₃ expression is apparently associated with the process of malignant transformation, as suggested by studies showing increased quantities of GD₃ in (*a*) rat fibroblasts transfected with the adenovirus E1 transforming gene (61); (*b*) melanocytes transformed *in vitro* (24) and *in vivo* (62), and (*c*) malignant glial cells (63). GD₃ appears to be involved in both cellular adherence and membrane permeability (64), and, furthermore, large concentrations may be necessary for sustaining a high metabolic activity and/or rapid cellular proliferation (65).

If GD₃ has a fundamental role in the development of the transformed phenotype in renal cancers, its precise effect appears to be complex; for even though *ras*-transformed PT cells express high levels of GD₃, our results show that only 10% of renal cancers express detectable levels of GD₃. These results suggest several interpretations, as follows: (*a*) GD₃ is directly involved in the pathogenesis of most renal cancers, but is only discernible, by the methods used, in a small subset of transformed renal cells; or (*b*) GD₃ has a necessary role in only a minority of renal cancers; or (*c*) qualitative alterations in GD₃ expression may occur early in the transformation process and are subsequently downregulated during progression; or (*d*) GD₃ is simply one of many glycolipid alterations to occur in transformed cells, and whose sporadic appearance indicates a less important and more indirect role (52).

PT cells transformed with *ras* oncogenes produce at least several, as yet undefined, TGFs. Alterations in the mechanisms that govern endogenous growth factor production in these cells is a relatively rapid event, occurring early in the transformation process (i.e., by passage 17). The production of growth factors by PT cells may be involved in the transition from cells with a limited growth potential to cells possessing a dramatically increased proliferative capacity. Most renal carcinomas have an increased expression of TGF- α and - β in comparison to normal kidney (53, 54). Therefore, while the precise role of TGFs in initiation or maintenance of renal cancers remains to be determined, our results suggest that growth factor secretion and presumably autocrine and/or paracrine growth stimulation may be a critical early event.

Perhaps the most crucial effect of the insertion of *ras* oncogenes into PT cells is the resulting chromosomal instability, leading to the acquisition of advantageous genetic alterations. Cultured PT cells have a normal diploid karyotype, which is maintained during the short life-span of these cells in culture. However, PT cells which undergo outgrowth and alterations in proliferative capacity upon introduction of *ras* oncogenes display consistent defects in chromosome 21. Early in the outgrowth phase PT-Ki cells exhibit a specific 1:21 translocation while PT-Ha cells lose an entire copy of chromosome 21. It is unlikely that *ras* oncogenes are specifically causing these genetic abnormalities as retroviral proviral genomes integrate in a generally random manner (66); moreover, it is difficult to envision how a proviral genome could cause the specific expulsion of an entire chromosome. Presumably, p21 protein triggers a cascade of effects that affect a wide range of cellular functions, differentiation pathways, and corresponding phenotypes. While our data do not suggest the precise nature of this cascade on the normal management of genetic information, they do suggest that a gene(s) or genetic element regulating (directly or indirectly) the proliferation of PT cells may be present on chromosome 21, and that its disruption, by either translocation or deletion, provides a strong selective advantage, initiating or contributing to the uncontrolled growth of these cells. We presume that this proliferation-related gene resides at or near the site of translocation of chromosome 21 with chromosome 1 observed in PT-Ki cells. To identify this breakpoint we have begun an analysis using polymorphic probes to specific portions of chromosome 21. Of the genes identified on this chromosome to date, we have analyzed the *ets* oncogene for gross rearrangements and the type I interferon receptor for alterations in expression and binding affinity, and have found no detectable perturbations in either of them.

In the second or late phase of PT cell transformation with *ras* oncogenes, PT-Ki cells underwent detectable biologic and genetic progression, spontaneously acquiring the capacity for anchorage-independent growth, and exhibiting additional chromosomal alterations (Table VII). None of these secondary genetic abnormalities appeared to correlate with the newly acquired anchorage independent phenotype, and therefore, the precise genetic alterations governing anchorage independence may not be detectable by karyotyping. Furthermore, despite the generation of abnormalities in other chromosomes, the marker chromosome 21 defects were uniformly maintained in 100% of all cells examined, again indicating a strong selective advantage of the genetic lesions involving chromosome 21.

The most common nonrandom chromosomal abnormalities reported in both familial and nonfamilial renal carcinomas involve a translocation and/or deletion of the short arm of chromosome 3, and/or trisomy or tetrasomy of chromosome 7 (57, 67). Less frequent clonal chromosomal aberrations described, however, include trisomies 8 and 12, isochromosome 1, loss of sex chromosomes (67-69), and structural and numerical aberrations of chromosome 21 (58, 68). Secondary chromosomal abnormalities in late passage PT-Ki cells also involved chromosomes 1, 7, and 3. The prevailing supposition is that genetic abnormalities on the short arm of chromosome 3 are primarily responsible for the induction, maintenance, and metastases of renal cancers. We suggest that chromosome 21 may have an early disruptive role in at least a subset of renal cancers that can be augmented or superseded by progressive genetic abnormalities involving chromosome 3. It is possible that defects in chromosome 3 in renal

cancers may be more responsible for the events inducing maintenance and metastases rather than initiation.

Our results with PT-Ha cells are more difficult to interpret. 50% of early passage cells had monosomy 21, which is usually a lethal genotype (70). By passage 24, <15% of these cells had this abnormality, yet the PT-Ha cells still maintained a transformed phenotype. The simplest interpretation of these results is that either the karyotypically abnormal cells are providing a growth stimulus, or, more likely, that the majority of cells with an apparent normal karyotype have one or more subchromosomal defects (possibly on chromosome 21), which induces the cells to proliferate. Further analysis of these cells may distinguish between these possibilities.

Normal diploid human cells have been difficult to transform with activated *ras* oncogenes as the sole transforming agent (71, 72). However, in systems where cells have either a genetically determined chromosomal abnormality, as in Bloom's syndrome (73), or have undergone extensive aneuploidy as a consequence of prolonged passage in tissue culture (74), introduction of an activated *ras* oncogene can induce the full range of transformation characteristics. While the precise complement of *ras* oncogenes and host genes is unknown, these studies suggest that a combination of an activated *ras* oncogene and aneuploidy may satisfy the minimal requirements for transformation. A second effect of *ras* oncogenes appears to be a generalized induction of chromosomal instability in diploid cells initiating a transformation cascade leading to neoplastic transformation. Studies using normal human bronchial epithelial cells transfected with a v-Ha-*ras* oncogene showed that after 3 mo of subculturing, transformed foci of immortalized cells and neoplastic cells arose (74). Karyotype analyses of these cells at first passage after transformation revealed aneuploidy with multiple chromosomal aberrations, but no specific, nonrandom chromosomal changes. Specific genetic changes that contributed to the transformed phenotype could not be deciphered. We have previously shown that the insertion of a *ras* oncogene into cultured human melanocytes results in a series of morphologic and antigenic alterations and a cellular phenotype characteristic of malignant melanoma cells (24). Further, we have noted that an early response to *ras* oncogenes is the development of hyperdiploid chromosome number; with prolonged passage and the generation of specific chromosomal abnormalities, these *ras*-containing melanocytes acquired the fully transformed features of melanoma (Albino, A. P., unpublished data). Our studies with PT cells support the interpretation that Ki- and Ha-*ras* oncogenes destabilize normal chromosomal structure by unknown mechanisms. If this instability results in biologically advantageous karyotypic abnormalities (and it is likely that these will differ for each differentiated cell type), then the cell acquires altered characteristics that release it from a normally disciplined and confined milieu. One plausible consequence of this release can be increased proliferative capacity and a potential for biologic and genetic progression driving the cell towards a transformed, and ultimately, neoplastic phenotype. If this assumption is correct, it may be possible to define those complementing genetic lesions that may be directly or, more likely, indirectly induced by the transforming and destabilizing potential of *ras* oncogenes.

In summary, it is clear that viral *ras* oncogenes have a pronounced effect on proximal tubule cells, affecting alterations not only in morphology and antigen expres-

sion, but also in cellular mechanisms that appear to be necessary for progression to a full malignant phenotype (e.g., anchorage independence and chromosomal instability). Moreover, these alterations reflect events associated with renal carcinomas and, more importantly, indicate a temporal association or sequence. What is the evidence that activated *ras* genes are involved in the pathogenesis of renal cancers in vivo? At present there is a lack of consistent *ras* perturbations in renal cancers. Slamon et al. (75) reported a two to fourfold increase of Ha-*ras* m-RNA transcripts in four and of Ki-*ras* transcripts in three of seven renal carcinomas as compared with autologous normal kidney. Others have also detected expression of c-Ha-*ras*, c-Ki-*ras*, and N-*ras* in a limited number of renal carcinomas (76, 77). It is possible that *ras* oncogenes are involved in the etiology of renal cancers but will require more sophisticated approaches to discern; or that there are multiple pathways by which a PT cell becomes neoplastic and there may exist a putative gene whose transforming potential is mimicked, or can be substituted for, by *ras* oncogenes; or that the products of *ras* oncogenes and this unknown gene may intersect some common genetic element which, if perturbed, could induce transformation-related events. In all probability, it is likely to be complex. If *ras* destabilizes normal chromosome structure, and if this leads to the activation or deactivation of a proliferation related gene by direct or indirect mechanisms, the stage may be set for the evolution of cells with imprecise genetic instructions, ultimately acquiring a neoplastic phenotype.

Summary

Normal human kidney proximal tubule cells into which a *ras* oncogene was inserted undergo a series of transformation-related alterations that are characteristic of renal carcinomas. These include changes in morphology, growth potential, anchorage dependence, antigen expression, growth factor production, and chromosomal stability. Further, there are spontaneous progressive alterations in vitro in the karyotype and antigenic profile of the transformed cells. Cytogenetic analyses suggest that alterations of chromosome 21 may play an early and pivotal role in the development of transformed proximal tubule cells.

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