Expression of Keratin and Vimentin Intermediate Filaments in Rabbit Bladder Epithelial Cells at Different Stages of Benzo[*a*]pyrene-induced Neoplastic Progression

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ABSTRACT Rabbit bladder epithelium, grown on collagen gels and exposed to the chemical carcinogen benzo[*a*]pyrene, produced nontumorigenic altered foci as well as tumorigenic epithelial cell lines during 120-180 d in culture. Immunofluorescence studies revealed extensive keratin filaments in both primary epithelial cells and benzo[*a*]pyrene-induced altered epithelial foci but showed no detectable vimentin filaments. The absence of vimentin expression in these cells was confirmed by two-dimensional gel electrophoresis. In contrast, immunofluorescence staining of the cloned benzo[*a*]pyrene-transformed rabbit bladder epithelial cell line, RBC-1, revealed a reduction in filamentous keratin concomitant with the expression of vimentin filaments. The epithelial nature of this cell line was established by the observation that cells injected into nude mice formed well-differentiated adenocarcinomas. Frozen sections of such tumors showed strong staining with antikeratins antibodies, but no detectable staining with antivimentin antibodies. These results demonstrated a differential expression of intermediate filament type in cells at different stages of neoplastic progression and in cells maintained in different growth environments. It is apparent that the expression of intermediate filaments throughout neoplastic progression is best studied by use of an in vivo model system in parallel with culture studies.

The intermediate filaments of mammalian cells represent an immunologically diverse group of cytoskeletal elements and have been subdivided into five groups: keratins (10, 14, 31-34), vimentin (1, 2, 17, 18, 29), desmin (7, 8, 20, 22), neurofilaments (23, 27), and astrocyte filaments (3, 23, 27). There is a growing number of reports demonstrating the specificity of keratins to epithelium both in vivo (9, 28, 34) and in culture (10, 12, 33, 34). Recent immunofluorescence studies have shown that some epithelial cells in culture express both the epithelial (keratin) and mesenchymal (vimentin) types of intermediate filaments (11-13, 33), although the latter can often only be resolved after treatment with Colcemid (12, 13). Our unpublished observations indicate that some established human and mouse bladder epithelial cell lines express very limited filamentous keratin but abundant vimentin filaments. Expression of vimentin filaments in established tumorigenic epithelial cell lines appears common (11, 13, 14), but it is not clear whether the appearance of vimentin in cells of epithelial origin is correlated with the transformed state, proliferation, or adaptation to culture growth conditions.

In this study we describe a new system for the culture and neoplastic transformation of rabbit bladder epithelium after exposure to a chemical carcinogen, benzo[*a*]pyrene. Studies of intermediate filament expression in epithelial cells at different stages of neoplastic progression revealed an absence of vimentin in primary urothelial cultures and cells of altered epithelial foci but an extensive keratin cytoskeleton, as assessed by immunofluorescence and two-dimensional gel electrophoresis. The acquisition of tumorigenicity in this transformation system was accompanied by a reduction in keratin expression and the appearance of the mesenchymal vimentin filament.

MATERIALS AND METHODS

Tissue Culture

Bladders from New Zealand white rabbits were removed aseptically and the urothelium was dissected away from the underlying stroma under a binocular microscope. The separated urothelium was chopped with fine curved scissors in a few drops of nutrient medium (Dulbecco's modified Eagle's medium) supplemented with 10% calf serum. Explants were suspended in a minimal amount of

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nutrient medium and plated out onto previously prepared collagen gels (for methodology, see reference 25) in 60-mm petri dishes. Adherence of the explants to the gels was facilitated by incubation at 37°C for 60 min before further addition of the medium. Cells grown on cover slips and treated with colchicine were maintained in low serum (2%) for 48 h, followed by the addition of nutrient medium with 2% calf serum and 20 μg/ml colchicine. Cells were left in colchicine medium for 24 and 48 h before being processed for immunofluorescence. For cloning, trypsinized cells at a low density were plated in a 60-mm petri dish for 5 min. Singly isolated cells were identified and transferred by a micropipette to a 6.4-mm culture well (Costar Co., Cambridge, Mass.). After 10 h, the wells were reexamined for the presence of only one cell. Cells grown from such wells were then trypsinized and recloned by the same procedure. The established cloned cell line, RBC-1, has been continuously passaged for 50 times with more than 200 cell doublings.

Carcinogen Treatment

Benzo[*a*]pyrene (BP; Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide (DMSO) and diluted in nutrient medium to give a final concentration of 1 μg of BP/ml and 0.05% DMSO. Primary cultures were exposed to the carcinogen for 5 d, commencing 24 h after plating; control cultures were exposed to medium containing 0.05% DMSO for the same period. After treatment, all cultures were maintained in carcinogen-free medium.

Frozen Sections

Tissue was immersed in Tissue Tek II O.C.T. compound (Lab-Tek Products Div., Miles Laboratories, Inc., Elkhart, Ind.) on a small piece of cork, placed inside a bag, and frozen by placing in an acetone/dry ice mixture for 2 min. Frozen tissues were stored in isopentane at −20°C until used. Frozen sections were cut on an IEC cryostat (Damon Corp., IEC Div., Needham, Mass.) and stored in slide boxes at −20°C.

Antibodies and Immunofluorescence

The preparation and characterization of rabbit antikeratins antisera have been described in detail elsewhere (31–33). Rabbit antiserum directed against the mesenchymal intermediate-sized filament, vimentin, was provided by Drs. R. O. Hynes, S. Blose, and W. Gordon (4, 17, 18).

Tissue sections were hydrated in phosphate-buffered saline (PBS), covered with 20 μl of anti-intermediate filament serum, and incubated in an humidified chamber at 37°C for 60 min. These were then washed in PBS and overlaid with fluorescein-conjugated goat anti-rabbit IgG (Meloy Laboratories Inc., Springfield, Va.) at a dilution of 1:10 and incubated at 37°C for 60 min. After being rinsed again in PBS, sections were rinsed with water, mounted in Gelvatol (Monsanto Co., St. Louis, Mo.), and viewed in a Zeiss photomicroscope III using epifluorescence illumination (26). Cells on cover slips were briefly washed in PBS and then fixed in methanol at −20°C. The staining procedure was the same as for sections but the incubation times were reduced to 30 min. All photographs were taken using Kodak Tri-X film at EI 1600.

Radiolabeling of "Cytoskeletal" Elements and Resolution by Two-dimensional Gel Electrophoresis

Cells were labeled for 3 h with 200 μCi/ml of [^{35}S]methionine (400 Ci/mmol New England Nuclear, Boston, Mass.) in methionine-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed calf serum. After this incubation, cells were treated with Penman's cytoskeletal (CSK) extraction buffer containing 1% Triton X-100, 10 mM PIPES (pH 6.8), 100 mM CKl, 300 mM sucrose, 2.5 mM MgCl, 1 mM CaCl, 1 mM phenylmethanesulfonyl fluoride, and 100 kallikrein inhibitor units/ml of aprotinin (Sigma Chemical Co.) at 4°C for 3 min (5). The insoluble cellular framework, still attached to the dishes, was scraped with 0.2 ml of CSK buffer by use of a rubber policeman and lyophilized. These samples were then treated with DNase I and RNase A in the presence of 0.3% SDS according to Garrels (16). The samples were lyophilized, dissolved in IEF (isoelectric focusing) sample buffer, and analyzed by IEF and SDS polyacrylamide gel electrophoresis according to Garrels (16).

RESULTS

Tissue Culture System

Cells from rabbit urothelial explants migrated out over the collagen gel during the first few days in culture and formed an extensive epithelial sheet (Fig. 1 *a*) with little mesenchymal cell contamination. Few mitotic figures could be seen during the early culture period (days 1–3) when most of the cellular activity was migratory in nature. After 7 d in culture, the collagen gels began to break up, and many of the epithelial sheets attached and migrated out onto the plastic (Fig. 1 *b*). This was the first stage at which cells could be studied by use of immunofluorescence techniques. Cells at this stage were grown on glass cover slips by wounding the sheet and then placing the cover slip at the edge of the wound, enabling the epithelium to move onto the glass. The epithelial nature of these cells was confirmed by electron microscopy, which revealed desmosomal contacts between cells, and by immunofluorescence, which demonstrated a keratin cytoskeleton characteristic of the epithelial cell lining of the bladder (Fig. 2 *a* and *c*; also see references 31 and 34).

Cultures exposed to the chemical carcinogen BP produced actively growing epithelial foci in a majority of dishes (60%), between 21 and 40 d. These altered epithelial foci were characterized by small cuboidal cells growing as an organized sheet

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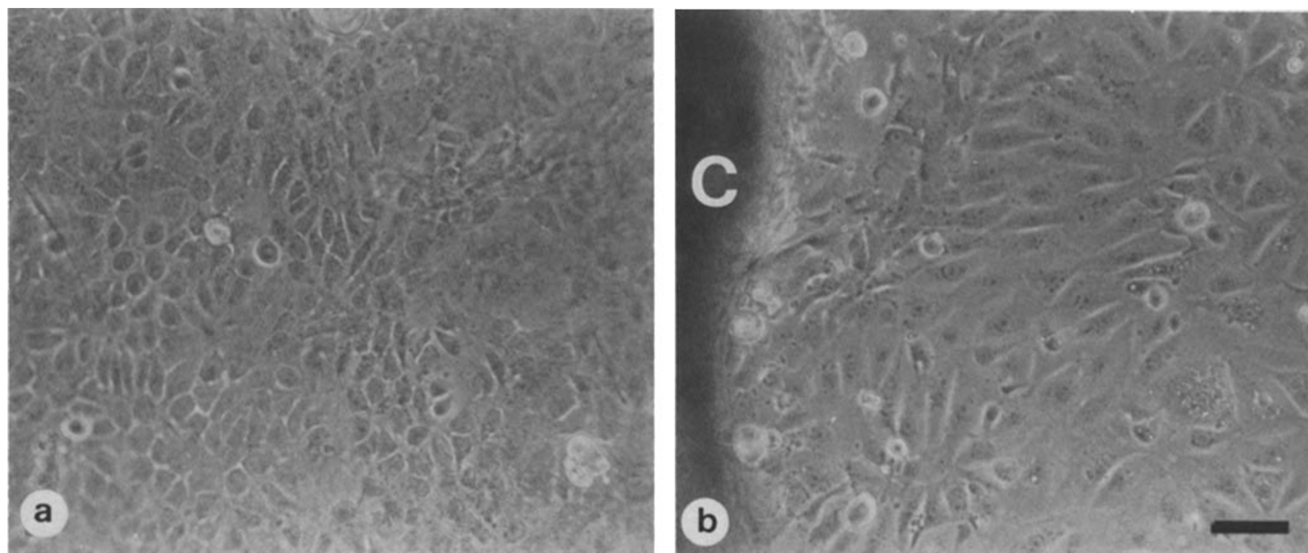

FIGURE 1 Phase-contrast microscopy of (*a*) rabbit bladder epithelial outgrowth on a collagen gel and (*b*) bladder epithelial cells migrating from the collagen gel and (*c*) onto the plastic substrate. Bar, 120 μm.

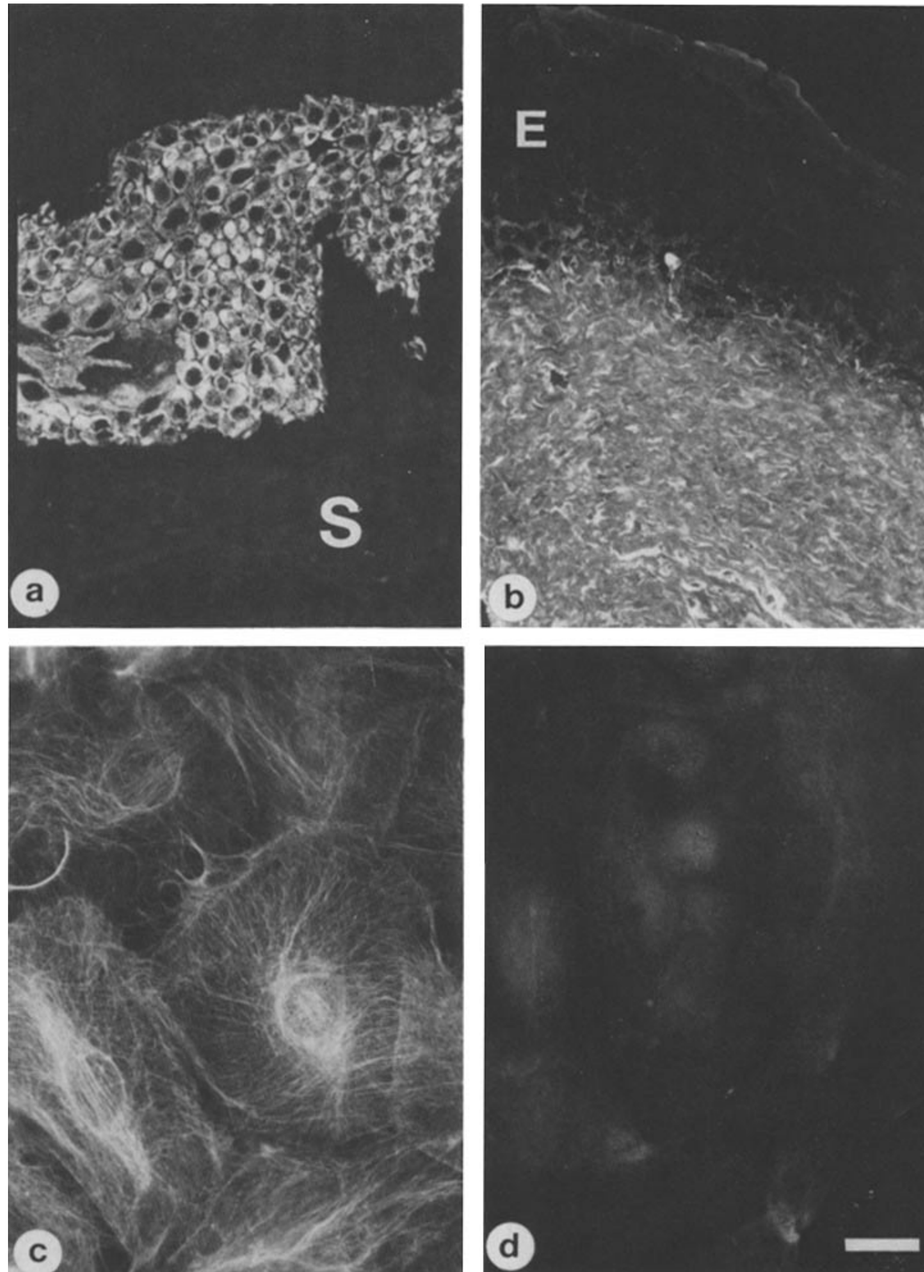

FIGURE 2 Immunofluorescence microscopy of rabbit bladder frozen sections (*a* and *b*) and epithelial cell cultures (*c* and *d*) as revealed after reaction to keratin (*a* and *c*) and vimentin (*b* and *d*). Note that in bladder frozen sections stained with keratin (*a*) the stromal element (*S*) shows no staining, whereas the urothelial layer stains intensely. In sections stained with vimentin (*b*) the reverse is found, with no staining in the epithelial layer (*E*) and fluorescence localized in the stroma. The same pattern is observed with epithelial cells in culture (*c* and *d*) where antikeratin antibody stains an extensive filamentous system but no fibrous element can be resolved with antibodies to vimentin even after exposure to colchicine (*d*). Bar, 160 μm (*a* and *b*) and 30 μm (*c* and *d*).

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with many mitotic figures. A small percentage (<5%) of control cultures produced epithelial foci, none of which, however, progressed to established cell lines. Most epithelial areas in untreated cultures had deteriorated and detached from the substrate within 30 d. Although the frequency of altered epithelial foci was greatly increased in carcinogen-treated cultures, only a low percentage (<20%) were established as cell lines. This system provided us with three well-defined stages associated with neoplastic transformation similar to the stages described in other epithelial transformation systems (15, 19, 30): normal epithelial outgrowths, altered epithelial foci, and established epithelial cell lines. By use of immunofluorescence and two-dimensional gel electrophoretic techniques, the expressions of keratin and vimentin were studied at different stages of neoplastic progression,

Immunofluorescence Staining of Intermediate Filaments in Nontumorigenic Urothelial Cells

Frozen sections of normal rabbit bladder were stained with antibody either to epidermal keratins or to vimentin and viewed by fluorescence microscopy. Bladder tissue reacted with keratin antibody showed a strong specific staining in the epithelial cell layer (Fig. 2*a*), confirming observations by Sun et al. (34); the lamina propria, blood vessels, and muscle layers

were completely negative in these sections. In contrast, incubating with antibodies to vimentin showed staining only in the stromal regions (Fig. 2*b*) but no fluorescence associated with the urothelium. Comparison of Fig. 2*a* and *b* shows the mutual exclusiveness of the two staining patterns (12). Primary epithelial outgrowths shared the same staining pattern as urothelium in vivo, with an extensive cytoskeletal network apparent after reaction with keratin antibodies (Fig. 2*c*). The keratin fibers of the bladder epithelium were very fine, with concentrated bundles surrounding the nucleus and radiating out to the cell periphery (31, 34). Exposure of these cells to colchicine did not affect the keratin filament system and did not facilitate the staining of any intermediate-sized filaments by antibodies to vimentin (Fig. 2*d*) as has been reported for some established cell lines (10, 14, 31).

Epithelial cells in altered foci were characteristically much smaller than cells in the primary outgrowth, with frequent mitotic figures occurring within the cell sheet. These cells showed the same staining pattern as the primary epithelial outgrowths having an extensive keratin cytoskeleton (Fig. 3*a* and *b*) that was most apparent in the better-spread cells at the leading edge of the sheet. No filamentous cytoskeletal elements could be discerned by use of antibodies to vimentin, even after exposure to colchicine (Fig. 3*c* and *d*). Cells at this stage could not be established as a continuous line, and subcutaneous injections of 10^6 cells into nude mice produced no tumors after 3 mo.

Two-dimensional Gel Electrophoretic Analysis

Confirmation that the absence of vimentin, observed in primary epithelial cell cultures and altered epithelial foci, was attributable to the absence of the protein rather than limited cross-reactivity of the antibody was demonstrated by two-dimensional gel electrophoresis (Fig. 4*A–C*). Extraction of [^{35}S]methionine-labeled cells with CSK extraction buffer (5), before electrophoretic analysis, facilitated the study of insoluble "cytoskeletal" components in different cell types. Primary cultures of bladder mesenchymal cells treated in this manner displayed major protein spots (Fig. 4*A* and *B*) (~55,000–57,000 daltons) that comigrated with purified vimentin (from hamster cell line BHK-21) in two-dimensional gel electrophoresis. In contrast, primary epithelial cultures showed no such major spots in this region (Fig. 4*C*) although two labeled proteins could be discerned after prolonged exposure and are thought to be attributable to contaminative vimentin from mesenchymal cells within the tissue explants. Cells from altered epithelial foci, which contained few mesenchymal cell contaminants, showed a total absence of labeling in the vimentin region (Fig. 4*D*), confirming our immunofluorescence observations of a lack of vimentin in these cells. Fig. 4*E* is the mixture of mesenchymal cells and altered epithelial foci.

Immunofluorescence Staining of Intermediate Filaments in Tumorigenic Urothelial Cells

Epithelial cell lines were established from BP-treated cultures between 120 and 180 d after plating. Established cell lines were distinct from the cells of altered epithelial foci from which they arose with regard to morphology, growth characteristics, and

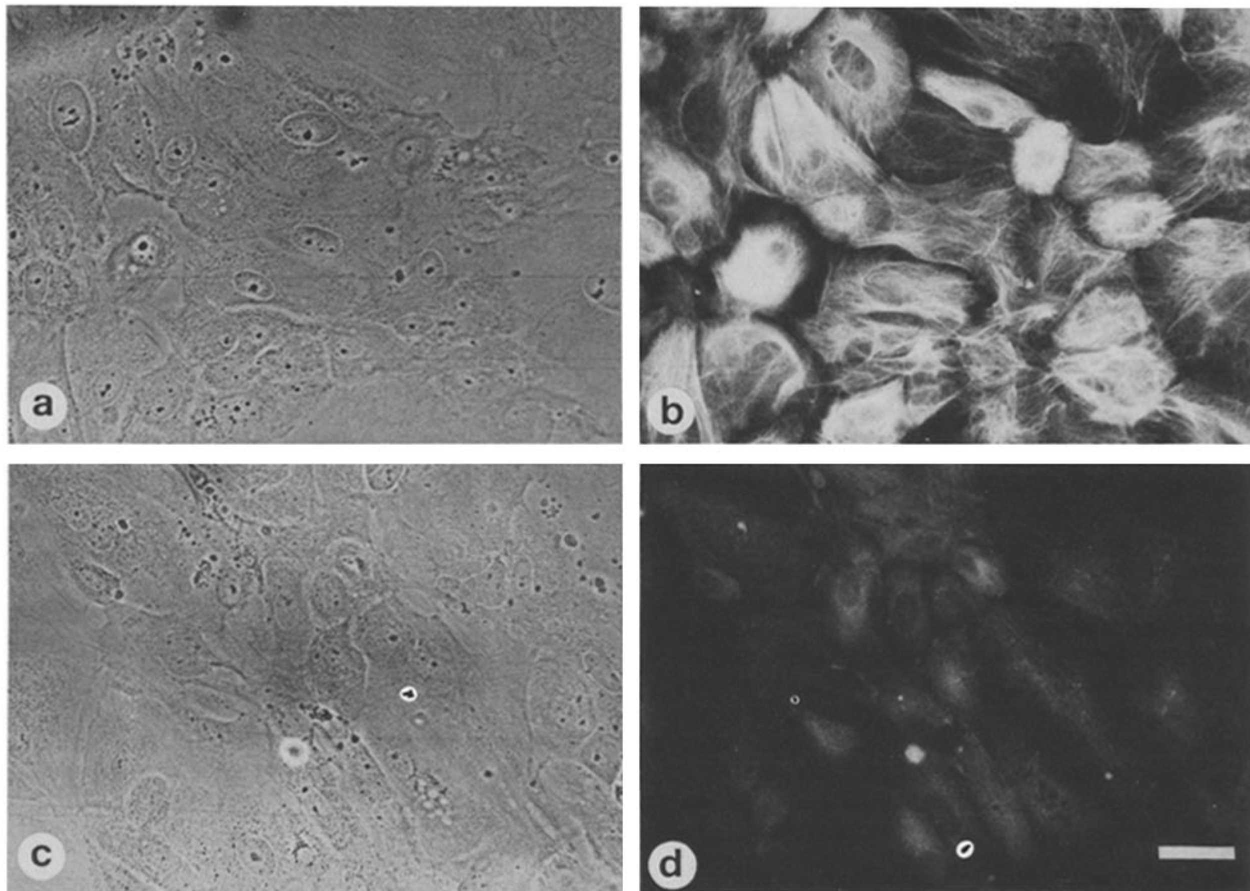

FIGURE 3 Phase-contrast (*a* and *c*) and fluorescence micrographs (*b* and *d*) of the same field. Cells of altered epithelial foci after immunofluorescence staining with antibodies to keratin (*a* and *b*) and vimentin (*c* and *d*). Cells stained with vimentin have been exposed to colchicine. Bar, 30 μm.

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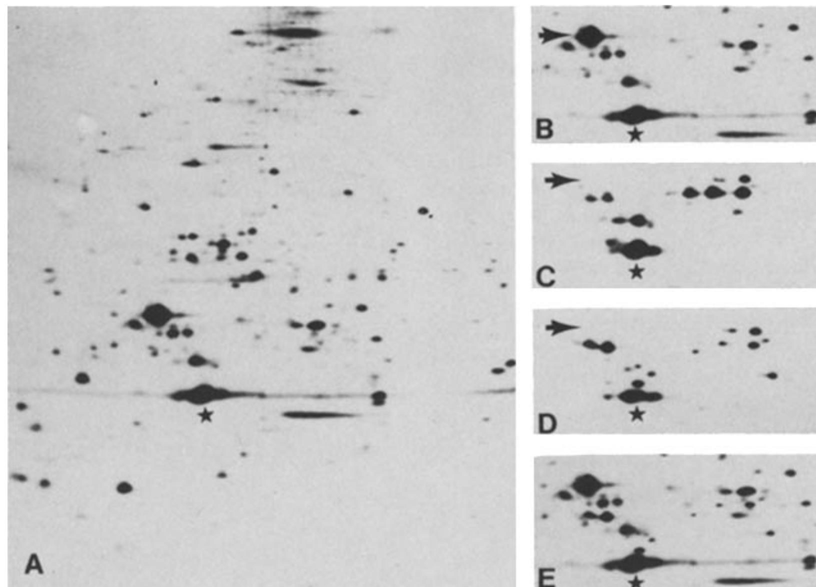

FIGURE 4 Two-dimensional gel analysis of [^{35}S]methionine-labeled detergent-insoluble cytoskeletal components from (*A* and *B*) rabbit mesenchymal cells, (*C*) primary rabbit bladder epithelium, and (*D*) bladder epithelium of altered epithelial focus. Arrows show the presence of vimentin in mesenchymal cells (*A* and *B*) and absence in primary epithelial cells (*C*) and cells of altered epithelial foci (*D*). The mixture of mesenchymal cells and altered epithelial foci is shown in *E*. Stars indicate actin.

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intercellular organization. Whereas cells of the foci were epithelial in morphology and showed no piling up but grew as a sheet, cell lines were more mesenchymal in appearance, piled up, and showed less dependency on cell-cell contact for growth. In these respects the rabbit cell lines were like the type II cells described in a previous mouse urothelial transformation system (30). The cloned cell line used in this study (RBC-1) was tumorigenic in nude mice, producing well-differentiated adenocarcinomas in five out of five mice after subcutaneous injection of 10^6 cells, thus confirming the epithelial origin of these cells.

The cloned BP-transformed rabbit epithelial cell line (RBC-1) in culture showed very limited filamentous keratin by immunofluorescence in ~90% of cells, with 10% of cells displaying no detectable keratin filaments (Fig. 5 *a*). Staining of these cells with vimentin antibody revealed a filamentous network concentrated around the nucleus, with few filaments extending into the cytoplasm in 100% of cells (Fig. 5 *b*). In many cells a brightly stained focus containing vimentin was observed in a juxtanuclear position (Fig. 5 *b*). When frozen sections of tumors in nude mice derived from these cells were reacted with antibodies to keratin, cells in acini stained very strongly (Fig. 6 *A*). The most intensive staining occurred between the apical regions of cells lining the acini, an area associated with tight-junctional complexes as revealed by electron microscopy (data not shown). Interestingly, differentiated cells in acini were negative for vimentin (Fig. 6 *B*), whereas cells in the non-acinus areas showed a strong staining reaction with this antibody (Fig. 6 *C*).

When tumors from nude mice were excised and cultured, a mixed cell population arose from the explanted tumor. The major cell type was similar to cells of the original inoculum, having a mesenchymal morphology, similar growth characteristics, and cell piling up. When these cells were reacted with antibodies to keratins and vimentin, they stained in the same manner as cells of the original inoculum and showed few keratin filaments but a characteristic nuclear ring of fluorescence after staining with vimentin. A cloned cell line of such type II tumor-derived cells, injected back into mice, produced a well-differentiated adenocarcinoma, confirming the epithelial origin of these cells.

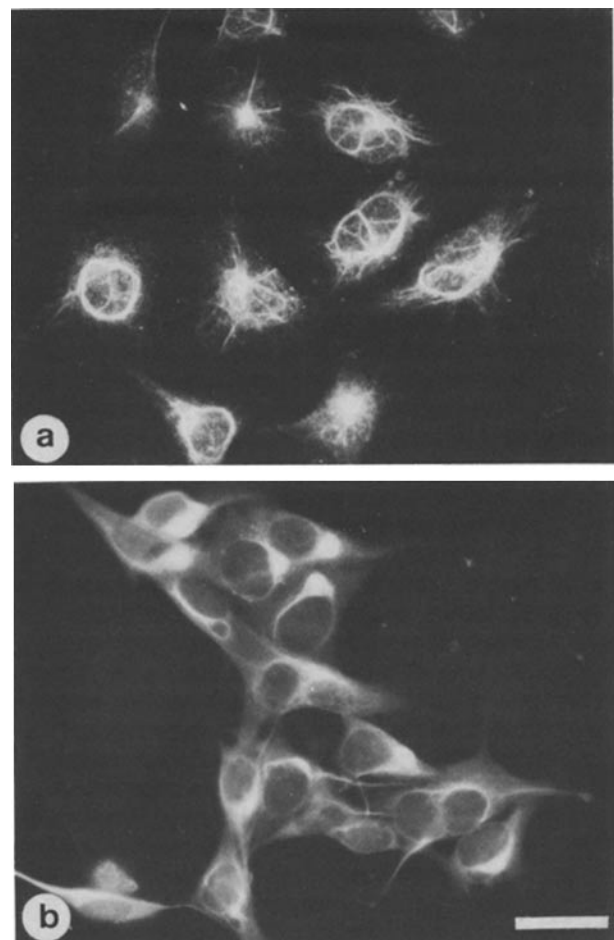

FIGURE 5 Immunofluorescence microscopy of tumorigenic rabbit bladder epithelial cell line (RBC-1) stained wth antibodies to keratin (*a*) and vimentin (*b*). Bar, 30 μm.

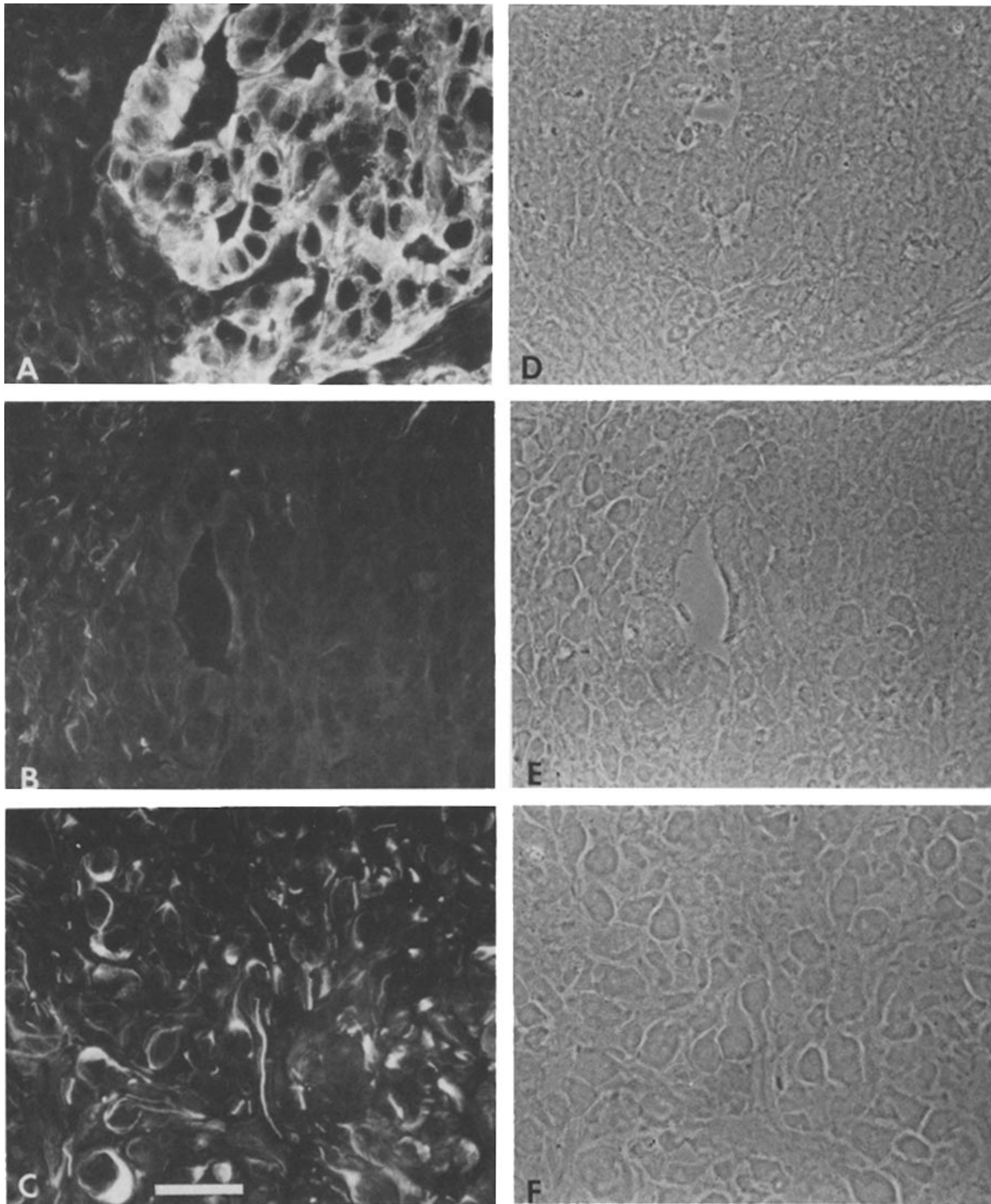

FIGURE 6 Immunofluorescence microscopy of frozen sections of rabbit bladder tumor from nude mouse; (*A*) acini in adenocarcinoma showing intense staining after incubation with keratin antibody; (*B* and *C*) neighboring section of tumor stained with antivimentin antibody showing lack of staining in differentiated acinus area (*B*) but extensive filamentous staining in nonacini area (*C*). Phase-contrast micrographs of the same field are shown in *D*, *E*, and *F*, respectively. Bar, 30 μm.

DISCUSSION

In this study we have reported a new system for the growth and neoplastic transformation of rabbit bladder epithelium after culture exposure to a chemical carcinogen. The different stages of neoplastic transformation identified are similar to those previously reported in other epithelial systems (19, 30), and the events differ markedly from events associated with mesenchymal cell transformation. Perhaps the most significant difference between the two transformation systems is the protracted latency period before altered foci are observed, a characteristic shared by other epithelial cell transformation systems (for review, see reference 15). The long lag period and the relatively low transformation frequency observed with epithelial cells still present major drawbacks to the general application of these systems for transformation studies.

Our results confirm earlier observations concerning the expression of keratin as the major intermediate-sized filament

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in primary cultures of epithelial cells (12, 14, 33, 34) and demonstrate a lack of vimentin-type filaments in these cells as assessed by two-dimensional gel analysis, a situation thought to exist in epithelial cells in vivo (9, 28, 34). The reduced expression of keratin in the rabbit tumorigenic cell line in this study has also been observed in some established human (24) and mouse epithelial cell lines (our unpublished observations) but does not appear to be a necessary prerequisite for epithelial cell transformation, because Franke et al. (14) and Sun et al. (34) have reported an extensive keratin cytoskeleton in many established carcinoma cell lines. In contrast, expression of vimentin filaments to varying degrees in established epithelial cell lines seems common (11, 13, 14, 32). That vimentin appears in subcultured hepatocytes (12) and in our long term cultured altered foci of rabbit bladder epithelial cells (data not shown) suggests that the appearance of vimentin is not correlated with the transformation state but with the in vitro proliferation or adaptation of cells to culture conditions.

In this study and in other reports (10, 13, 15, 32), keratin-positive cells have had a typical epithelial morphology and grown as small islands at low densities. In contrast, the epithelial cell line lacking an extensive keratin cytoskeleton in this study is typically mesenchymal in morphology and lacks the apparent dependence on cell-cell contact for optimal growth. The expression of keratin filaments and the changes observed in growth and morphology in cells derived from tumors argue strongly for a keratin role in the maintenance of epithelial cell shape, organization, and differentiation. The occurrence of a concentration of vimentin filaments in a perinuclear position in mesenchymal cells (4, 17, 18) and some epithelial cells (11) indicates that vimentin is closely associated with the cell nucleus (21) possibly providing mechanical support or participating in nuclear-spatial organization, a role perhaps performed by keratin in epithelial cells lacking any vimentin filamentous system.

The reduction in filamentous keratin expression observed in our cell lines may be a result of culture growth conditions and perhaps selection for a more rapidly proliferating, dedifferentiated epithelial cell type. Nonetheless, the system described in this study as well as those described by Doran et al. (6) provide a useful tool for studying differentiation and the role of intermediate-sized filaments in cellular organization. The results reported here show a differential expression in filament types in cells at different stages of neoplastic progression and in cells maintained in different environments. These observations add a cautionary note to tissue culture studies. It is apparent that the expression of cytoskeletal elements throughout neoplastic progression is best studied by use of an in vivo model system in parallel with culture studies.

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