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Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells

(intermediate filaments/immortalization/tumor and normal antigens/breast cancer)

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ABSTRACT **Keratin 5 (K5) mRNA and protein are shown to be expressed in normal mammary epithelial cells in culture and are absent from tumor-derived cell lines. To extend these findings, the full complements of keratins in normal, immortalized, and tumor cells were compared. It is shown here that normal cells produce keratins K5, K6, K7, K14, and K17, whereas tumor cells produce mainly keratins K8, K18, and K19. In immortalized cells, which are preneoplastic or partially transformed, the levels of K5 mRNA and protein are lower than in normal cells, whereas the amount of K18 is increased. Thus, K5 is an important marker in the tumorigenic process, distinguishing normal from tumor cells, and decreased K5 expression correlates with tumorigenic progression.**

One of the most frequent lethal malignancies in women is breast cancer. Recent advances in tissue culture methodology have made it possible to grow both normal and tumor-derived mammary epithelial cells under similar conditions (1). But there are as yet few markers to distinguish between these two classes. By employing subtractive hybridization techniques, we have isolated a cDNA clone encoding keratin 5 (K5), which is preferentially expressed in normal mammary epithelial cells.

Keratins have a number of distinct advantages for use as marker proteins. They are abundant, highly antigenic, intermediate filament proteins which can reliably identify cells of epithelial origin (2, 3). The keratin family, containing at least 19 members, represents the largest and most diverse class of intermediate filaments (2). A number of studies have sought to exploit this diversity to analyze expression differences between normal and tumor cells of various tissue origins (2, 4–11, 35, 36). Initial studies with keratins extracted from mammary glands showed differences between normal and tumor tissues (2).

Immunohistochemical characterization has demonstrated that within the normal duct, basal and lumenal cells can be discriminated by the keratins they express. In these studies, basal cells, lying between the lumenal cells and the basement membrane, were characterized by expression of keratins K5 and K14, which are typical of myoepithelial cells in stratified epithelium, and lumenal cells were characterized by expression of simple epithelial keratins K8, K18, and K19 (12–15).

These studies were performed *in situ*. Keratin expression may not be maintained after disruption of tissue architecture in cell culture. Medium constituents such as vitamin A, cAMP-elevating agents, epidermal growth factor, and other factors are known to affect keratin production (13, 16–18, 37). The development of a medium capable of supporting the growth of both tumor and normal breast epithelial cells (1) has allowed us to make a comparison of their keratin profiles independent of medium effects. We have analyzed K5 as a potential marker for normal cells and have reviewed the array of keratins produced by cultured tumor and normal cells.

MATERIALS AND METHODS

Cell Culture. Normal cell strains 70N, 76N, and 81N were derived from reduction mammoplasty tissue in this laboratory (1). Strains 172, 184, and 239 were derived from reduction mammoplasties and provided by M. Stampfer (19). The MCF-7 line was obtained from the Michigan Cancer Foundation and T-47D, ZR-75-1, SK-BR-3, MDA-MB-231, and BT-20 cells were obtained from the American Type Culture Collection. 21N, 21NT, and 21PT were derived from mastectomy samples from a patient with infiltrating ductal and intraductal carcinoma (V.B. and R.S., unpublished results), while 21MT-1 and 21MT-2 were metastatic cell lines derived from a pleural effusion from the same patient (1, 20). Normal human mammary epithelial cells (76N) were transfected with the viral DNA of human papilloma virus (HPV) strain 16 or 18 to generate immortal lines (21). A normal human mammary epithelial cell strain (184) was treated with benzo[*a*]pyrene and an immortal subclone (184B5) was isolated (22). 184B5 cells were infected with Kirsten sarcoma virus (infected cells are named 184B5KSV) and injected into nude mice. The cell line 184B5KSVTu2 was established from a tumor explanted from one of the nude mice (22, 23). All cells were cultured in DFCI-1 medium (1) at 37°C in a humidified atmosphere of 6.5% CO_2.

cDNA Library Production. Total RNA was isolated from 184 cells by lysis with guanidinium isothiocyanate, centrifugation over a 5.7 M CsCl cushion, and purification of the resulting RNA pellet (24). Poly(A)$^+$ RNA was purified by oligo(dT) chromatography using standard protocols (ref. 25, pp. 197–198). cDNA was made using the reverse transcriptase of Moloney murine leukemia virus (BRL) according to the supplier's instructions. cDNAs were blunt ended with T4 DNA polymerase, *Eco*RI linkers were ligated onto the cDNA, and the cDNA was cloned in the *Eco*RI site of λgt10.

Subtraction and Screening. The first-strand cDNA of 184 mRNA was hybridized with a 5-fold mass excess of 184B5KSVTu2 poly(A)$^+$ RNA to R_0t = 6000 mol (of nucleotide)·sec. Nonhybridizing nucleic acid was separated from DNA·RNA hybrids by chromatography over hydroxylapatite (26) and subjected to a further round of hybridization and hydroxylapatite chromatography. The final product (the "subtracted probe") was labeled by the random primer method (27) and used to screen approximately 60,000 independent plaques of a 184 λgt10 library. One hundred plaques with the highest relative signal were isolated, minilysates

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Abbreviations: K5, K6, etc., keratin 5, keratin 6, etc.; HPV, human papilloma virus; NEPHGE, nonequilibrium pH gradient gel electrophoresis.

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were prepared (25), and the DNA insert was excised with *Eco*RI, isolated by low melt agarose gel electrophoresis, and random-primed for expression analysis. The expression levels and sizes of the mRNAs corresponding to these inserts were determined by Northern analysis of 184 and 184B5KSVTu2 total RNA.

Northern Blot Analysis. Twenty micrograms of total RNA was denatured, fractionated by electrophoresis in 1.3% agarose/2.2 M formaldehyde slab gels, and electroblotted to Zetabind nylon filters (Bio-Rad). Filters were prehybridized 2–4 hr at 42°C in 1 M NaCl/50% (wt/wt) formamide/10% dextran sulfate/1% $NaDodSO_4$ containing sonicated salmon sperm DNA at 250 μg/ml. Hybridization with ^{32}P-labeled probes (27) was for 20–24 hr at 42°C. Filters were washed 30 min in two changes of 2× SSC (0.30 M NaCl/0.03 M sodium citrate, pH 7.0)/0.2% $NaDodSO_4$ at ambient temperature, followed by 1 hr in two changes of 2× SSC/1% $NaDodSO_4$ at 65°C and exposure to Kodak XAR-5 x-ray film.

Sequencing. The K5 cDNA was cloned in pGEM-3 and nested deletions were obtained by unidirectional digestion using exonuclease III (Erase-a-Base System, Promega). These clones were sequenced by using Sequenase DNA polymerase and dideoxynucleotides (United States Biochemical). The sequence of our clone was compared with that of K5 (28) using LOCAL (Molecular Biology Computer Research Resource), a high-speed sequence comparison program. A 250-base-pair (bp) *Xba* I–*Eco*RI fragment in the 3′ untranslated region was used as a specific probe for K5 mRNA (28).

Keratin Extraction and Analysis. Mammary epithelial cells were plated at 5×10^5 cells per 60-mm culture dish and grown until approximately 80% confluent. Medium was removed and 2 ml of DFCI-1 salts minus methionine containing [^{35}S]methionine at 50 μCi/ml (1 Ci = 37 GBq) and 1% dialyzed fetal calf serum was added. Cells were incubated for 2 hr and washed with 137 mM NaCl/3 mM KCl/1.5 mM KH_2PO_4/8 mM Na_2HPO_4, pH 7.2 and 1 mM phenylmethylsulfonyl fluoride (PMSF), and the cells were lysed with 1 ml of 20 mM Tris·HCl, pH 7.5/0.6 M KCl/5 mM EDTA/1.0% Triton X-100 (extraction buffer). The insoluble material was pelleted in an Eppendorf micro centrifuge for 4 min and the pellet was resuspended in 1 ml of extraction buffer, pelleted again, rinsed twice with PBS and 1 mM PMSF, and lyophilized.

The pellet was suspended in loading buffer, an aliquot was removed to determine the radioactivity of the sample, and 100,000 cpm was loaded onto a denaturing 10% polyacrylamide gel or analyzed by two-dimensional gel electrophoresis (29, 30).

RESULTS

Isolation of K5 cDNA Clone. The cDNA produced from normal human mammary epithelial cells (184) was hybridized with a 5-fold excess of mRNA from the tumorigenic cell line (184B5KSVTu2) derived from 184 cells. The resulting cDNA (the "subtracted probe"), enriched for sequences expressed only in normal cells, was labeled and used to screen a 184 phage library of approximately 60,000 recombinant plaques. One hundred of the most intensely hybridizing plaques were subjected to a secondary screen using the subtracted probe. The insert DNA from positive clones was used in Northern analysis and 74 clones were found to have a corresponding message that was highly overexpressed in 184 cells relative to 184B5KSVTu2 cells. Thirty-six of these clones were found to encode fibronectin mRNA. Another clone was confirmed to encode K5 by sequence analysis and comparison with the known sequence for this keratin (28).

K5 mRNA Expression in Normal and Tumor Mammary Epithelial Cells. Total RNA was isolated from a number of normal and tumor-derived mammary cells to determine the level of expression of K5 mRNA. Northern blot analysis

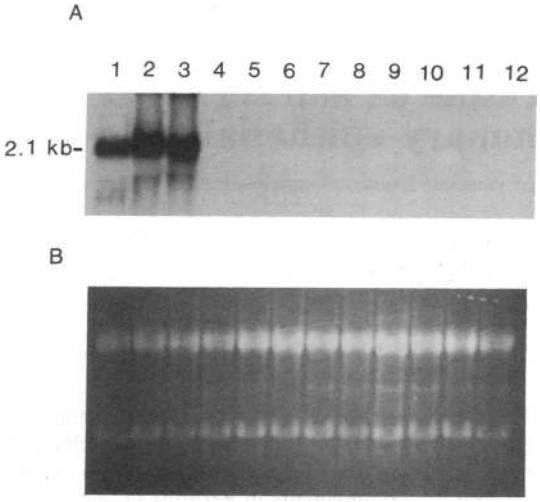

FIG. 1. (*A*) Northern analysis of normal and tumor mammary epithelial cells. Total RNA (20 μg) was analyzed on 1.3% formaldehyde/agarose gels, transferred to nylon, and hybridized to a K5-specific probe. The position of K5 message is indicated. Lanes: 1, 70N; 2, 76N; 3, 81N; 4, MCF-7; 5, T-47D; 6, ZR-75-1; 7, SK-BR-3; 8, MDA-MB-231; 9, 21NT; 10, 21PT; 11, 21MT-1; and 12, 21MT-2. (*B*) Ethidium bromide stain of gel before transfer.

using a probe specific for RNA encoding the carboxyl terminus of K5 hybridized with a single RNA species of 2.1 kilobases (kb) in cultured normal cells (Fig. 1), in agreement with the known size of K5 mRNA (28, 31). The message is abundantly expressed in the normal breast cell strains 70N, 76N, and 81N. No detectable expression was noted in estrogen receptor-positive lines MCF-7, T-47D, and ZR-75-1, in estrogen receptor-negative lines SK-BR-3 and MDA-MB-231, or in the 21T series. Upon long exposure slight production of K5 mRNA was noted in 21PT cells and 184B5KSVTu2 cells, though the expression relative to 184 cells was reduced approximately 50-fold (data not shown).

Keratin Production in Normal Mammary Epithelial Cells. K5 mRNA was abundant in all normal strains tested. To analyze the production of K5 protein in mammary epithelial cells and to examine the overall complement of keratins produced by normal cells, proteins were metabolically labeled and high-salt Triton-insoluble proteins were isolated. Extracts from six independent isolates were analyzed by one-dimensional gel electrophoresis, and the cells reproducibly expressed the same qualitative and quantitative array of keratins shown in Fig. 2. Keratins K5, K6, K14, and K17 were produced in abundant quantities while intermediate levels of K7 were detectable. In addition, a protein of approximately 45 kDa that could not be identified as an intact keratin or other intermediate filament protein was present in 70N cells (lane 4). Samples were analyzed by two-dimen-

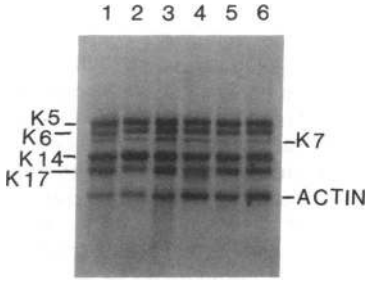

FIG. 2. $NaDodSO_4$/PAGE analysis of normal cell keratin production. Metabolically labeled keratins were analyzed on 10% polyacrylamide gels. The positions of the labeled keratins and actin are marked. Lanes: 1, 184; 2, 81N; 3, 76N; 4, 70N; 5, 172; and 6, 239.

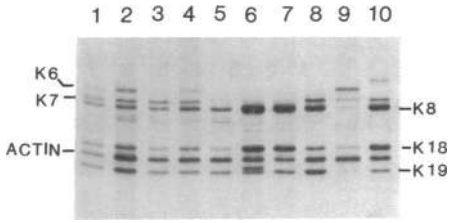

FIG. 3. Keratins produced by tumor cells. NaDodSO$_4$/PAGE analysis of established lines and the 21 series. Major keratins and actin are indicated. Lanes: 1, 21NT; 2, 21PT; 3, 21MT-1; 4, 21MT-2; 5, T-47D; 6, ZR-75-1; 7, MCF-7; 8, SK-BR-3; 9, MDA-MB-231; and 10, BT-20.

sional gel electrophoresis to confirm the identity of the proteins noted (an example is shown in Fig. 4).

Keratin Production in Tumor-Derived Mammary Epithelial Cells. For comparison with the normal cell strains, established tumor cell lines were analyzed (Fig. 3). Both estrogen receptor-positive lines (T-47D, ZR-75-1, and MCF-7; lanes 5–7) and receptor-negative lines (SK-BR-3, MDA-MB-231, BT-20, and 21T series; lanes 8–10 and 1–4) produced large amounts of K8, K18, and K19. These keratins were not detected in the normal cells by gel electrophoresis (see Fig. 2). Detectable amounts of K7 were found only in estrogen receptor-negative lines. An unidentified intense band from ZR-75-1 cells migrating slightly slower than K19 was observed.

A two-dimensional analysis of keratin production in normal, primary tumor-derived, and metastatic cells isolated from the same patient (21T series) is presented in Fig. 4. The keratin profile of 21N, a normal cell strain isolated from a mastectomy sample and analyzed before cells underwent selection (21), was the same as 76N and all other normal cell strains derived from reduction mammoplasties, showing high abundance of diagnostic keratins K5, K6, K14, and K17. A comparison between tumor cells isolated from the site of two primary tumors (21NT and 21PT) and metastatic lines (21MT-1 and 21MT-2) demonstrated no striking differences. The 21 series, except for 21MT-1, also made K6, unlike the established tumor cell lines.

Immortalization Results in Decreased Production of K5. One-dimensional gel electrophoresis was used to analyze metabolically labeled proteins from 10^5 cells to determine the level of K5 protein in the cells used initially to isolate the K5 cDNA clone (Fig. 5). K14 was the most abundant high-salt Triton-insoluble protein, followed in abundance by K5 in normal 184 epithelial cells (lane 1). An immortalized subclone, 185B5 (lane 2), produced significantly less keratins overall as well as proportionately less K5 (approximately a 10-fold drop in K5 protein based on densitometric scanning). The tumorigenic line, 184B5KSVTu2 (lane 4), produced 40-fold lower levels of K5. In the entire series, K5, K6, K7, K14, and K17 continued to be expressed, yet at approximately 10-fold lower levels after immortalization and tumorigenic transformation. K5 decreased the most of all the keratins analyzed, and a large decrease in its cognate partner K14 was also noted. To discern whether the decreased expression of K5 was unique to benzo[*a*]pyrene-immortalized cells or was caused by immortalization in general, normal mammary epithelial cells that were immortalized by HPV transfection were also examined.

Normal human mammary epithelial cells (76N) which contain integrated copies of HPV 18 or 16 DNA have reduced growth factor requirements and are immortal (21). A number of these lines have been analyzed for their production of keratins as well as expression of K5 mRNA (Fig. 6). Fig. 6*A* demonstrates that K5 mRNA expression is decreased in HPV-immortalized lines. This decrease is seen in both HPV 16- and HPV 18-transfected lines, thus paralleling the reduction seen in the benzo[*a*]pyrene-immortalized 184B5 line. K5 protein levels also decrease upon immortalization (Fig. 6*C*) along with levels of the cognate partner of K5, K14. The other keratins produced do not show a similar, consistent decrease in expression. Additionally, levels of K18 are increased upon HPV transfection.

DISCUSSION

We have shown that keratin production can be used to assess the probable origin of cells isolated in tissue culture, since normal and tumor cells produce unique arrays of keratins. K5 is a particularly useful marker because its mRNA is abundant and the protein is expressed in cultured normal mammary epithelial cells but is absent from tumor cells.

K5 mRNA levels were high in all normal cells tested. The development in this laboratory of DFCI-1, which supports the growth of both tumor and normal cells, has permitted a

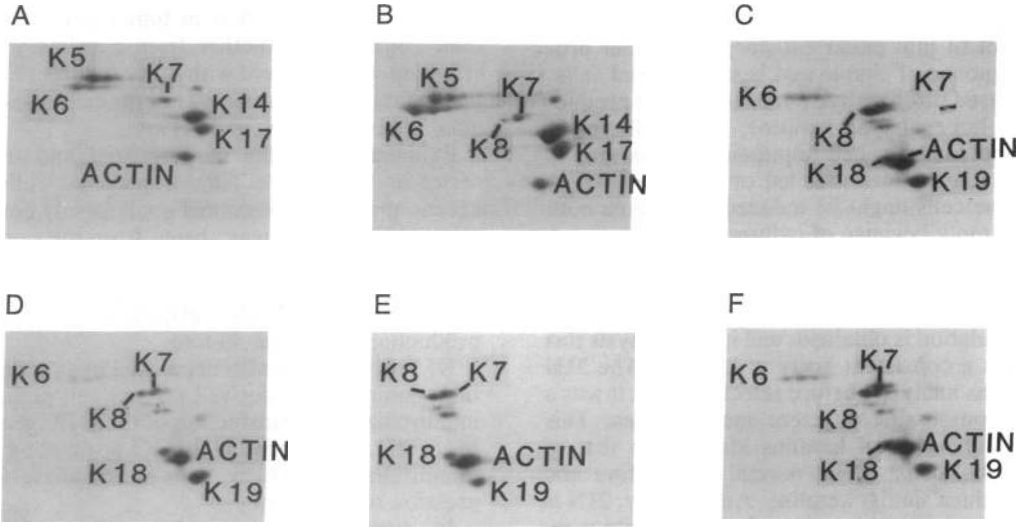

FIG. 4. Analysis of 21 series by two-dimensional electrophoresis. Cells were labeled with [^{35}S]methionine and extracted as described in the text, and proteins were analyzed by nonequilibrium pH gradient gel electrophoresis (NEPHGE) with a gradient of pH 3.5 to 10 (30). The top of the first dimension is toward the right with second dimension migration from top to bottom in 10% acrylamide. Actin and major keratins are indicated. (*A*) 76N. (*B*) 21N. (*C*) 21NT. (*D*) 21PT. (*E*) 21MT-1. (*F*) 21MT-2.

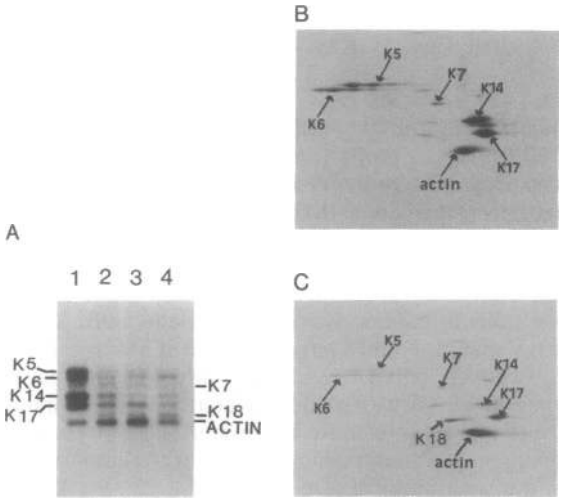

FIG. 5. One- and two-dimensional polyacrylamide gel electrophoresis of 184 series. (*A*) One-dimensional $NaDodSO_4$/PAGE analysis of extracted [^{35}S]methionine-labeled keratins obtained from 10^5 cells grown under identical conditions. Lanes: 1, 184; 2, 184B5; 3, 184B5KSV; and 4, 184B5KSVTu2. (*B*) Two-dimensional NEPHGE analysis of 184. (*C*) Two-dimensional NEPHGE analysis of 184B5KSVTu2.

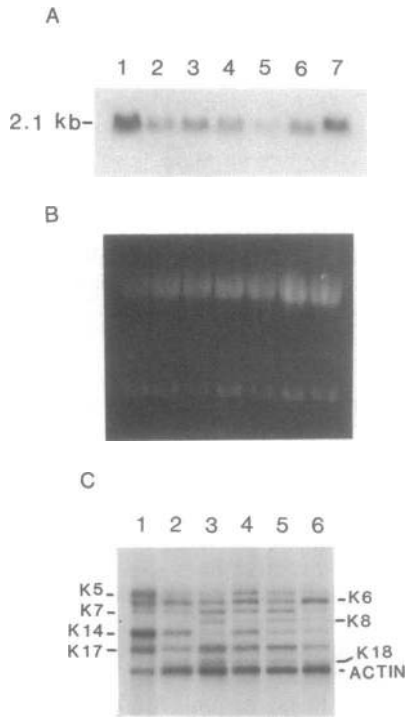

FIG. 6. Keratin expression of HPV-immortalized normal epithelial cells. (*A*) Total RNA (20 μg) from the parental and pooled or cloned HPV transfectants was analyzed. The blot was hybridized to the K5-specific probe. Location of K5 mRNA is indicated. Lanes: 1, 76N; 2, 18-2P; 3, 18-4P; 4, 18-5P; 5, 18-6P; 6, 18-2-1; and 7, 16-1-1. (*B*) Ethidium bromide stain of gel. (*C*) One-dimensional analysis of keratin protein. Metabolically labeled keratins were analyzed by $NaDodSO_4$/PAGE. The positions of keratins and actins are marked. Lanes: 1, 76N; 2, 18-2P; 3, 18-2P-1; 4, 18-4P; 5, 7P; and 6, 16-1-1.

comparative analysis of these cell types grown under the same conditions. In this way we have obviated differential media effects on keratin production (13, 16–18). The overall pattern of keratin protein expression is very different in normal and tumor cell lines. Dominant keratins produced in normal cells were K5, K6, K7, K14, and K17, while in tumor cells K8, K18, and K19 were the major intermediate filament proteins. Thus, the keratins produced in cultured normal mammary epithelial cells include a keratin produced by simple epithelia (K7) (4, 33), keratins produced by stratified or complex epithelial cells (K5 and K14) (34), and keratins produced in hyperproliferative situations (K6 and K17) (32). K6 expression, absent from normal breast tissue (2), is probably elevated during growth in culture.

Production of the above types of keratins usually distinguishes different epithelial cell types. Cells are considered to be simple epithelia on the basis of their production of K7, K8, or K18. The basal or myoepithelial cells are commonly diagnosed by their production of K5 and K14. As we show here, cells isolated from reduction mammoplasties and a mastectomy do not fit into either category, but rather produce keratins diagnostic of simple and basal epithelial cells. Due to their increased proliferative potential and the production of basal and lumenal type keratins, these cells might represent a pluripotent stem cell population that *in vivo* is capable of differentiating into lumenal or basal cell types. Alternatively, these cells might be induced to produce both types of keratin simply because of culture *in vitro*.

A primary organoid culture of cells derived from a reduction mammoplasty contains a number of epithelial cells presenting diverse morphologies. Following selection (21), a homogeneous population is obtained, and in our analysis this cell type produces a consistent array of keratins. The 21N sample in Fig. 4 was analyzed before selection; thus it was a mixture of cells possessing different morphologies. This sample produced an array of keratins identical to that of postselection normal cells. Thus, normal cells before and after selection produce similar keratins. Additionally, 21N is derived from a mastectomy sample rather than from a reduction mammoplasty as are the other normal mammary epithelial cells analyzed. Therefore, in terms of keratin production, normal cells from reduction mammoplasties and mastectomies make the same types and amounts.

The 21 series offers an opportunity to compare a normal cell population, two primary tumor cell lines, and a metastatic cell line from the same patient (refs. 1 and 20 and V.B. and R.S., unpublished results). The normal cells derived from the biopsy sample, 21N, produce the same keratins as other normal cells derived from reduction mammoplasties. The primary tumor lines (21NT and 21PT) produce high levels of those keratins normally associated with tumor cells (K8, K18, and K19) as do the metastatic lines 21MT-1 and 21MT-2. Thus, keratin production in tumor cells is independent of their cell source (whether from a primary site or pleural effusion). In agreement with other reports (2, 4), we find that the established mammary tumor cell lines produce high amounts of K8, K18, and K19.

Examination of the immortalized and transformed 184 series as well as the HPV-transfected cells revealed that keratin production remained qualitatively constant, with the exception that K18 was absent from the normal parent and expressed in the immortalized and tumorigenic derivatives. On the other hand, keratin production in the tumor-derived cells decreased 10-fold relative to normal cells and K5 production decreased 40-fold.

K5 mRNA levels also decreased in an independent reduction mammoplasty-derived normal cell population that was immortalized by transfection of the HPV genome (21). Thus in general, we have found that K5 production decreases upon immortalization, which is an intermediate step in the progression of malignancy.

In summary, we have shown that K5 is an important marker of the tumorigenic process in mammary epithelial cells. K5 was not expressed in any of the tumor cell lines tested, but it was highly expressed in the normal cells. K5 mRNA was decreased in normal cells that were immortalized

by transfection with HPV 16 or 18 or by treatment with benzo[*a*]pyrene. The putative ability of keratins to alter tumorigenic potential is an important question that remains to be elucidated.

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