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Genetics

Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements

(E7 protein/epidermal growth factor/breast cancer/transfection)

VIMLA BAND, DEBORAH ZAJCHOWSKI, VICTORIA KULESA, AND RUTH SAGER*

Division of Cancer Genetics, Dana-Farber Cancer Institute, Boston, MA 02115

Contributed by Ruth Sager, October 27, 1989

ABSTRACT **Human papilloma virus (HPV) types 16 and 18 are most commonly associated with cervical carcinoma in patients and induce immortalization of human keratinocytes in culture. HPV has not been associated with breast cancer. This report describes the immortalization of normal human mammary epithelial cells (76N) by plasmid pHPV18 or pHPV16, each containing the linearized viral genome. Transfectants were grown continuously for more than 60 passages, whereas 76N cells senesce after 18–20 passages. The transfectants also differ from 76N cells in cloning in a completely defined medium called D2 and growing in a minimally supplemented defined medium (D3) containing epidermal growth factor. All transfectants tested contain integrated HPV DNA, express HPV RNA, and produce HPV E7 protein. HPV transfectants do not form tumors in a nude mouse assay. It is concluded that products of the HPV genome induce immortalization of human breast epithelial cells and reduce their growth factor requirements. This result raises the possibility that HPV might be involved in breast cancer. Furthermore, other tissue-specific primary epithelial cells that are presently difficult to grow and investigate may also be immortalized by HPV.**

Breast cancer is a very frequent lethal malignancy of women in North America and western Europe, but the underlying molecular genetics and pathobiology are poorly understood. A major deterrent to research has been the lack of suitable cell culture systems in which primary tumor cells could be grown and compared with the normal mammary epithelial cells of origin and in which the stepwise process of mammary tumorigenesis could be investigated (1). The recent development of a medium, DFCI-1, in our laboratory should alleviate this problem (2); newly isolated cell lines from primary tumors are now being studied (ref. 3; V.B. and R.S., unpublished data).

The tumor-derived cells that we have examined to date have already undergone several steps in tumorigenesis. It is important to define and characterize the very early stages. One way to do so experimentally is to start with normal cells and immortalize them, since immortalization is a crucial event in oncogenesis, and immortal nontumorigenic cell lines would provide excellent starting material for further studies. However, human cells are extremely difficult to immortalize in culture (4, 5), although two immortalized mammary epithelial cell lines were recovered after long-term exposure to benzo[*a*]pyrene (6). The usefulness of these cell lines is diminished by the difficulty in determining the molecular basis of carcinogen-induced genetic changes and by the rarity of cell lines recovered.

This report describes the successful and efficient immortalization of normal mammary epithelial cells by plasmids containing the human papilloma virus (HPV) genome. This finding is of particular interest since HPV has been found only in a restricted set of cell types and body locations—namely, on genital or oral skin and mucosal surfaces associated with benign or malignant epithelial lesions (7, 8). HPV plasmid transfection has been reported to immortalize human keratinocytes, a cell type from which HPV-associated epithelial tumors are derived, as well as human fibroblasts (8–11). However, HPV has not been found in breast tissue, nor has it been associated with breast cancer (12).

The results reported here merit follow-up with a systematic search for mammary tumor DNAs that cross-hybridize with HPV. Second, the immortalized but nontumorigenic cells provide starting material in a search for further genetic changes involved in tumorigenesis. In addition, these cells provide material for investigating the molecular basis of immortalization, especially since proteins encoded by the E6 and E7 genes of HPV have been implicated in the immortalization of human keratinocytes (13, 14) as well as rodent cells (15).

MATERIALS AND METHODS

Cells and Growth Media. 76N cells used in this study were obtained from a reduction mammoplasty specimen (2).

DFCI-1 (D). This medium consists of α-MEM/Ham's nutrient mixture F-12 (1:1, vol/vol) supplemented with epidermal growth factor (EGF) (12.5 ng/ml), 10 nM triiodothyronine, 10 mM Hepes, 50 μM freshly made ascorbic acid, 2 nM estradiol, insulin (1 μg/ml), 2.8 μM hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, transferrin (10 μg/ml), 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) (all from Sigma), 15 nM sodium selenite (Amend Drugs and Chemical, New York), cholera toxin (1 ng/ml) (Schwarz/Mann), 1% fetal calf serum (J.R. Scientific, Woodland, CA, or HyClone), bovine pituitary extract (35 μg/ml) (Hammond Cell/Tech, Alameda, CA). The pH is 7.4 at 6.5% CO_2/93.5% air.

D2. DFCI-1 medium minus fetal calf serum and bovine pituitary extract.

D3. D2 minus EGF, hydrocortisone, insulin, triiodothyronine, and cholera toxin.

Determination of Cell Growth. To measure growth in different media, cells were grown at least three passages in the respective medium. Cells were washed once with solution A (a balanced salts solution) (16) and treated with trypsin (0.025% trypsin/0.01% EDTA; Sigma). Trypsin digestion was stopped with 0.0375% soybean trypsin inhibitor (Sigma) in solution A, and cells were washed and counted in a Coulter counter. Approximately 5×10^4 cells were plated per 35-mm dish (Falcon) and grown at 37°C in a humidified atmosphere with 6.5% CO_2/93.5% air. The population doubling times were estimated from the linear portion of the curve.

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Abbreviations: HPV, human papilloma virus; EGF, epidermal growth factor.

*To whom reprint requests should be addressed.

Transfection and Selection. 76N cells cultured in DFCI-1 medium were plated at 5×10^5–10^6 cells per 100-mm dish 18 hr prior to transfection. Cells were treated with trypsin 3 hr before transfection. Plasmids containing 8 μg of total HPV-18 (9) or HPV-16 (17) viral DNA (linearized with *Eco*RI or *Bam*HI) were introduced by calcium phosphate coprecipitation with 2 μg of pSV2neo (to provide a selectable marker) as described (18). After glycerol shock [4 min; 15% (vol/vol) glycerol], fresh medium was added and selection in G418 (GIBCO) (75 μg/ml) began 48 hr later and continued for at least 2 weeks. Surviving colonies were either picked singly or pooled from individual dishes. Each pool is numbered for an individual transfection dish. For example, cell line 18-2P-1 is a clone (−1) derived from a pool (2P) of colonies on plate 2 transfected with HPV-18.

RNA Isolation and Analysis. Total RNA was prepared from 50–60% confluent cell monolayers. Northern blot hybridizations were carried out and quantitated by densitometric scanning of the autoradiograms as described (19).

Southern Analysis of Genomic DNA. High molecular weight DNA was isolated from cultured cells. DNAs were digested with *Eco*RI, *Bgl* I, or *Bam*HI, processed for Southern blotting, and hybridized under conditions previously described (19) to entire genomic HPV-18 or HPV-16 sequences.

Dot Blot Analysis. Quantitation of HPV copy number in transfectants was determined by dot blot analysis. DNA (2.5 μg) was denatured in 0.4 M NaOH at 65°C for 1 hr and 2-fold serial dilutions were spotted onto Zeta probe nylon filters (Bio-Rad) using a Schleicher & Schuell dot blot manifold. Hybridization with the indicated probes was essentially as described earlier (19).

Immunoprecipitation of HPV Proteins. Cells were metabolically labeled for 5 hr at 37°C with 500 μCi per 100-mm plate each of [^{35}S]methionine and [^{35}S]cysteine (1 Ci = 37 GBq) (Amersham) in a medium lacking cysteine and methionine. Cells were then washed with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% glucose and were then solubilized with 1 ml of 2% Triton X-100 in 50 mM Tris·HCl pH 7.6/0.15 M NaCl containing 1 mM phenylmethylsulfonyl fluoride and 8 mM iodoacetamide. The nuclear fraction was removed by centrifugation at $7500 \times g$ for 20 min at 4°C. Lysates were precleared three times with Pansorbin (fixed *Staphylococcus aureus* Cowan I strain) and immunoprecipitated with specific antibodies against HPV-18 E7 (L. Gissman and H. zur Hausen, personal communication) or HPV-16 E7 (14). The immunoprecipitates were resolved on a SDS/12% polyacrylamide gel (20).

Tumorigenicity of HPV Transfectants. Cells (10^7 per mouse) were injected subcutaneously into the mammary fat pad of female nude mice (3). Tumor growth was monitored weekly for 4–6 months.

RESULTS

Growth Properties and Phenotype of HPV-18 and -16 Transfectants. Cotransfection of pHPV-16 or -18 with pSV2neo into 76N cells and subsequent selection in DFCI-1 (D) medium containing G418 resulted in the generation of many colonies that were morphologically different from normal 76N cells. The majority of colonies consisted of polygonal cells, which tended to pile up, resembling mammary tumor cells cultured in the same medium (2, 3). In confluent monolayer culture (Fig. 1), the transfectants were more variable in size and more loosely packed than 76N cells, and some mitotic cells were seen. The frequency was one G418-resistant (G418^r) colony per 10^5 treated cells with both HPV-18 and -16. Every G418^r colony tested contained HPV DNA. No colonies were recovered from cells transfected with pSV2neo and a carrier plasmid pSP65. Individual clones from both HPV-16- and HPV-18-treated dishes, as well as pooled transfectants, were recovered.

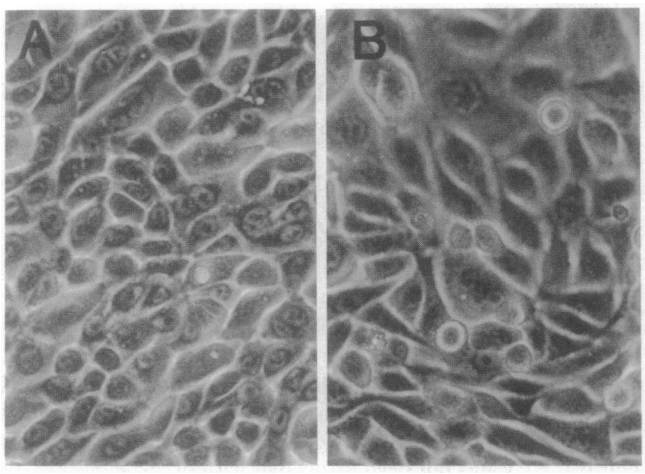

FIG. 1. Morphology of normal and HPV-immortalized human mammary epithelial cells. (*A*) Normal 76N cells. (*B*) HPV-18 transfected cells.

All transfectants—four clonal isolates and seven pools of HPV-18, and two clones and one pool of HPV-16—have been grown continuously in D medium for >60 passages, representing >120 population doublings. In contrast, 76N cells grow actively to about passage 16 and senesce at passages 18–20 under the same conditions. None of the transfectants grew in medium containing methylcellulose (data not shown). When tested for tumorigenicity, none of the transfectants produced tumors in 4–6 months, whereas ZR-75-1 cells (21) tested as controls always produced tumors of 1–2 cm within 1–2 months.

The average cellular DNA content was determined by cytofluorography (2). Most of the transfectants, whether clonal or pooled, were slightly hypodiploid, perhaps missing a single chromosome. However, HPV-16 (16AP and 16-2-1) and two HPV-18 pooled transfectants (18-4P and 18-6P) were in the triploid to tetraploid range as compared to the diploid DNA content of the parental 76N cell strain.

Loss of Growth Factor Requirements by Transfectants. Normal 76N cells do not plate in DFCI-1 (D) medium when seeded at low cell density. On the other hand, HPV transfectants plated with an efficiency of 15–20% in D medium and also in a completely defined medium (D2). 76N cells at high

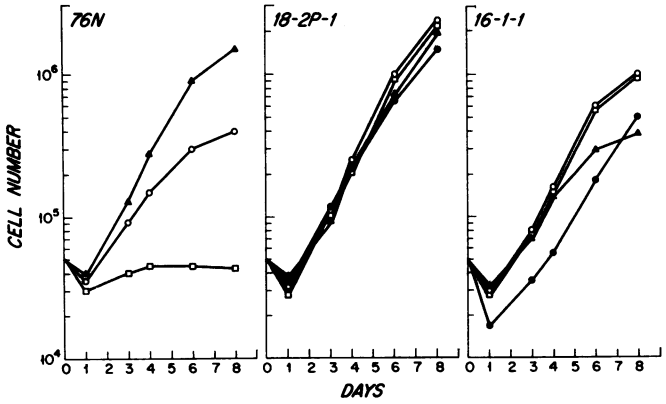

FIG. 2. Growth of normal and HPV-transfected 76N cells in different media. Cells were seeded (5×10^4 per 35-mm culture dish), and at the indicated times, cells were harvested and counted in a Coulter counter. Each data point represents the mean of triplicate determinations. ▲, DFCI-1; ○, D2 medium; ●, D3 plus EGF; □, D3 plus EGF plus hydrocortisone plus insulin. Cells were grown in medium for at least three passages before plating for the growth curve. 76N cells did not grow at all in D3 plus EGF, and, therefore, this experimental condition was not included in the figure.

cell density grow well in D2 medium but the HPV transfectants grow better under these conditions (Fig. 2). Transfectants have been in continuous culture for >10 passages in D2 medium with no decrease of growth rate.

To determine which growth factors are required by HPV transfectants, individual supplements were tested in a minimal medium called D3. Neither 76N nor transfectants showed any growth in unsupplemented D3 medium. However, when supplemented with EGF alone, this medium supported growth of the transfectants but not of 76N (Fig. 2). 76N cells, however, did not grow at all in D3 plus EGF and grew poorly even when further supplemented with insulin and hydrocortisone (Fig. 2).

Viral DNA and RNA. Southern analysis of total DNA from a few representative clones and pooled populations demonstrated that the bulk of the HPV DNA is integrated into genomic DNA, as shown by the high molecular weight bands observed upon *Eco*RI (for HPV-18 transfectants) or *Bam*HI (for HPV-16 transfectants) digestion (Fig. 3 *A* and *B*). Analysis of undigested DNA showed no evidence of episomal HPV DNA in the HPV-18 transfectants (data not shown), although the possibility of their presence at low abundance in the transfectants with high HPV copy number cannot be excluded since visualization is obscured by the high signal from the integrated DNA.

The identical pattern of HPV-18 hybridizing bands in the pooled and cloned populations of 18-2P (Fig. 3 *A* and *B*, lanes 5 and 6) after digestion with *Bgl* I is consistent with the conclusion that the predominant HPV-18 transfectant in this

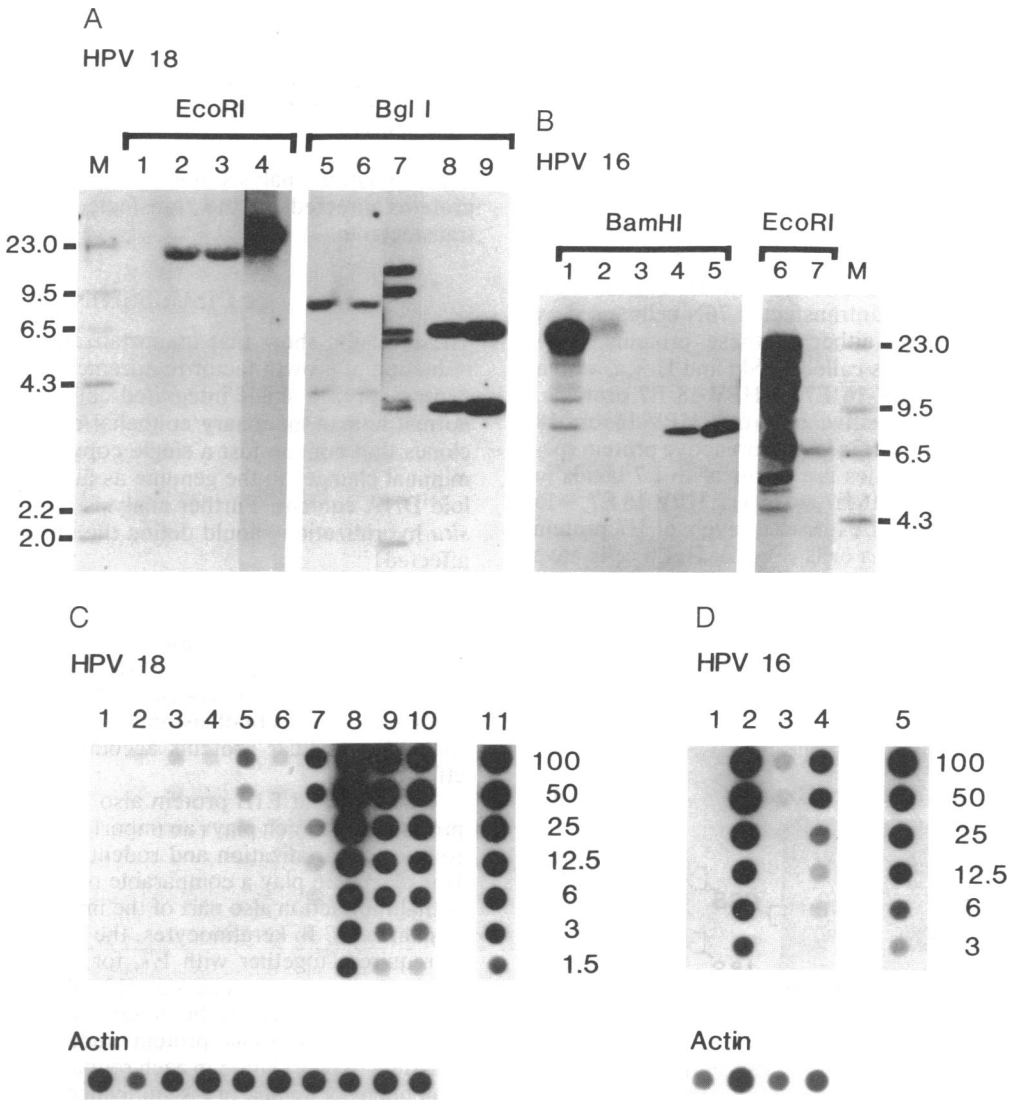

FIG. 3. (*A* and *B*) Southern analysis of genomic DNA from HPV transfectants. Ten micrograms of DNA was digested with *Eco*RI, *Bgl* I, or *Bam*HI, processed for Southern blotting, and hybridized to entire genomic HPV-18 or HPV-16 sequences excised by *Eco*RI digestion of pHPV18 and *Bam*HI digestion of pHPV16. (*A*) Analysis of DNA from 76N (lane 1) and HPV-18 transfectants 18-2P (lanes 2 and 5), 18-2P-1 (lanes 3 and 6), and 18-5P (lanes 4 and 7) hybridized to HPV-18 sequences. Lanes 8 and 9, one and five copies of HPV-18 DNA. Lane M, *Hin*dIII-digested λ DNA markers. (*B*) Analysis of DNA from 76N (lane 3) and HPV-16 transfectants 16AP (lanes 1 and 6) and 16-1-1 (lanes 2 and 7) hybridized to HPV-16 sequences. Lanes 4 and 5, one and five copies of HPV-16 DNA, respectively. (*C* and *D*) Quantitation of HPV copy number in transfectants by dot blot analysis. (*C*) High molecular weight DNA (2.5 μg) from 76N (lane 1), HPV-18 transfectants 18-2P, 18-2-1, 18-2P-1, 18-4P, 18-4-1, 18-5P, 18-6P, 18-7P, and 18-8P (lanes 2–10) were spotted onto nylon filters in serial 2-fold dilutions and analyzed by hybridization to HPV-18 sequences. A standard curve with 1.5–100 copies of pHPV18 (per 2.5 μg of total DNA) is provided in lane 11. Hybridization to a β-actin probe is shown for a representative row (2.5 μg); the actin signal intensities relative to the modal average are 1.0, 0.3, 0.7, 1.0, 0.8, 0.9, 1.0, 0.6, 1.1, 0.7, and 1.2 for lanes 1–10. (*D*) DNA (1.25 μg) from 76N (lane 1) and HPV-16 transfectants 16AP, 16-1-1, 16-2-1 (lanes 2 and 3) were processed as described above and hybridized with HPV-16 sequences. A standard curve with 3–100 copies of pHPV16 (per 1.25 μg of total DNA) is provided in lane 5. Actin hybridization to a representative row (1.25 μg) is shown below; relative signal intensities are 0.5, 2.0, 0.6, and 1.0 for lanes 1–4.

pooled population is actually 18-2P-1. In contrast, the complex banding observed in 18-5P (lane 7) can be explained by multiple integration events in a mixed population of transfectants. Similar conclusions can be drawn for the data obtained upon *Eco*RI digestion of the HPV-16 transfection pool, 16AP (HPV-16, lane 6). Dot blot analysis (Fig. 3 *C* and *D*) showed that some cloned cell lines had an average of only one copy of viral DNA per cell (HPV-18, lanes 2–4), whereas other pooled populations had as many as 200 copies (HPV-18, lane 8; HPV-16, lane 2).

Northern analysis (Fig. 4) revealed the presence of HPV-16 or HPV-18 RNA transcripts in all of the transfectants, whereas no detectable hybridization to the RNA isolated from the 76N recipient cell population was observed. The major HPV-18 transcripts are approximately 5.0, 4.8, and 1.7 kilobases, although the relative abundance of these RNA species varies in individual transfectants. The RNA transcripts detected in the two HPV-16 transfectants are approximately 4.5, 3.0, 2.0, and 1.6 kilobases. It is apparent that HPV RNA abundance hardly varies over a 200-fold range of HPV DNA copy number.

Expression of HPV E7 Protein. To look for the expression of E7 proteins encoded by HPV DNA, immunoprecipitation analysis was carried out in metabolically labeled HPV-16 or -18 transfectants using specific antisera against E7 proteins of HPV-16 (14) or HPV-18 (L. Gissman and H. zur Hausen, personal communication). Untransfected 76N cells, used as controls, did not express either of these proteins. Two cervical carcinoma cell lines called CaSki and HeLa, which are known to express HPV-16 E7 or HPV-18 E7 proteins, respectively, served as positive controls. HPV-16 or -18 transfectants expressed specific immunoreactive protein species whose SDS gel mobilities are identical to E7 bands in positive control cells (HPV-16 E7, ≈19 kDa; HPV-18 E7, ≈15 kDa). HPV-18 transfectants expressed levels of E7 protein comparable to those in HeLa cells (Fig. 5). HeLa cells have been reported to have 10–15 copies of HPV integrated in their genome (23). HPV-16 transfectants, on the other hand, expressed a much smaller amount of E7 protein compared to CaSki cells (Fig. 5). CaSki cells are reported to contain ≈600 copies of HPV integrated in their genome (23). Thus, immunoprecipitation analysis demonstrates the expression of E7 proteins directed by the transfected HPV genome in the transfectants.

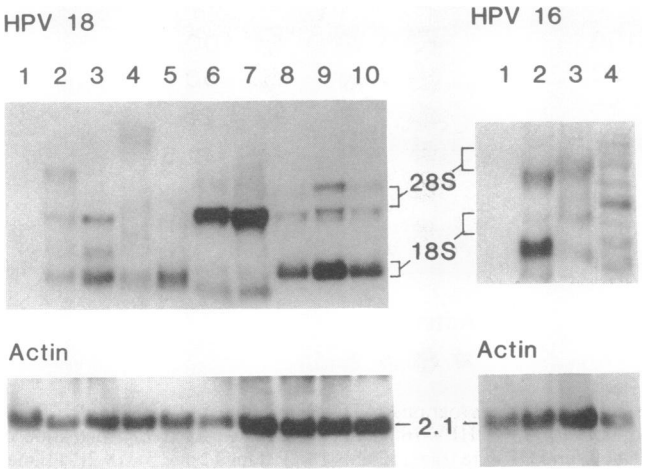

FIG. 4. HPV mRNA expression in cloned and pooled transfectants. (*Left*) Total cellular RNA isolated from 76N (lane 1) and HPV-18 transfectants 18-8P, 18-7P, 18-6P, 18-5P, 18-4-1, 18-4P, 18-2P-1, 18-2-1, and 18-2P (lanes 2–10) was analyzed by Northern blot and hybridization to a probe for the entire HPV-18 genomes. Hybridization to a chicken β-actin (22) probe is shown below. Location of the ribosomal RNAs (28S, 4850 base pairs; 18S, 1740 base pairs) is indicated. (*Right*) Total cellular RNA from 76N (lane 1) and HPV-16 transfectants 16-1-1 (lane 2), 16-2-1 (lane 3), and 16AP (lane 4) was analyzed by hybridization to viral HPV-16 sequences.

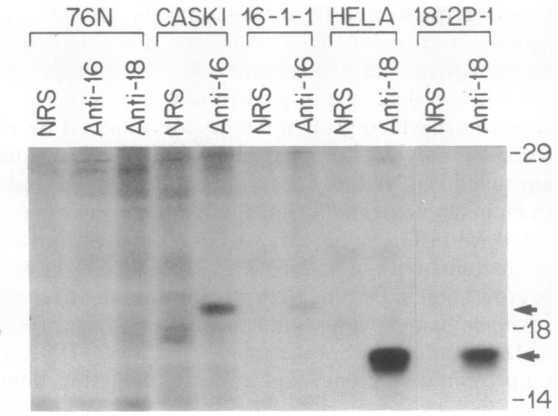

FIG. 5. Immunoprecipitation analysis of HPV-16 or -18 transfectants with specific antisera against E7 proteins. NRS, normal rabbit serum control. Arrows indicate the ≈19-kDa HPV-16 E7 and the ≈15-kDa HPV-18 E7 proteins. Molecular size markers are as indicated in kDa.

DISCUSSION

These results show that immortalization and a substantial reduction of growth factor requirements can be achieved by expression of a single integrated copy of HPV-18 or -16 in normal human mammary epithelial cells. Furthermore, the clones that contain just a single copy of HPV-18 also show minimal changes in the genome as judged by their hypodiploid DNA content. Further analysis by cytogenetics and *in situ* hybridization should define the chromosomal region(s) affected.

These results raise further questions. First, with respect to senescence and immortalization, the efficient immortalizing ability of HPV contrasts with the rare success of either simian virus 40 (24) or adenoviral (25) DNAs to immortalize human cells. Since proteins encoded by each of these viruses form complexes with retinoblastoma protein (26–28), it is likely that other cellular proteins account for the differences in efficacy.

T antigen and E1B protein also bind to the cellular gene product p53, which plays an important but not yet delineated role in immortalization and rodent transformation (29, 30). Does HPV E6 play a comparable role with p53 protein, and is that interaction also part of the immortalization process in human cells? In keratinocytes, the E6 protein from HPV-16 is required, together with E7, for immortalization, but no other viral genes are necessary (13). Whether the same is true in breast cells needs to be determined.

The retinoblastoma protein undergoes phosphorylation and dephosphorylation in each round of the cell cycle but the functional or regulatory significance of this process is not known (33–35). The fact that the same viral and cellular proteins are involved both in cell cycle events and in immortalization suggests that senescence is determined by cell cycle controls. Since senescence is a dominant trait in human cells (31), loss of senescence may be a consequence of mutation of cellular genes identifiable by the binding of their protein products to virus-encoded proteins or viral DNA.

In earlier studies, we showed that immortalization and tumor forming ability could be experimentally separated in diploid human fibroblasts (36). Thus, cells that were doubly transfected first with simian virus 40 viral DNA and then with activated Ki-ras formed very small tumors in nude mice, and then ceased to grow. Similar cells from the same transfected

clones senesced in culture after about the same number of doublings as in the nude mice. Thus, these double transfectants were not immortalized despite the expression of simian virus 40 T antigen and phosphorylated viral p21 proteins. This result clarifies the independent role of senescence in tumor suppression.

Another question is raised by the loss of most growth factor requirements in HPV-transfected breast cells. With the use of our defined media for growth of normal breast cells, we were able to identify EGF as the sole growth factor required by these cells. EGF has been associated with events that control cell cycle progress early in the G_1 phase, whereas the time of action of insulin-like growth factor 1 (or insulin) is later as cells approach the G_1/S boundary (32). The fact that the insulin requirement is lost in our HPV transfectants suggests that its regulatory role has been superseded by components of the HPV–retinoblastoma complex, and that HPV genes may play no role in earlier G_1 events.

The immortalized breast cells are not tumorigenic but have undergone significant preneoplastic changes. Thus, they represent valuable starting material for experimental induction of further events in mammary cell oncogenesis. The same is true for other epithelial cells if they could be immortalized by HPV.

Finally, our results pose the question: what is the molecular basis of host cell specificity in response to HPV? It is particularly surprising that mammary cells, not a known HPV target, can become preneoplastic under the aegis of HPV proteins. Our results raise the possibility that this virus might be associated with breast cancer.

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