JOURNAL OF VIROLOGY, Dec. 1991, p. 6671–6676
0022-538X/91/126671-06$02.00/0
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Vol. 65, No. 12

Loss of p53 Protein in Human Papillomavirus Type 16 E6-Immortalized Human Mammary Epithelial Cells

VIMLA BAND,[1]† JAMES A. DE CAPRIO,[2] LAURIE DELMOLINO,[1]† VICTORIA KULESA,[1] AND RUTH SAGER[1]*

Division of Cancer Genetics[1] and Division of Neoplastic Disease Mechanisms,[2] Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Received 29 April 1991/Accepted 16 September 1991

We have shown previously that introduction of the human papillomavirus type 16 (HPV16) or HPV18 genome into human mammary epithelial cells induces their immortalization. These immortalized cells have reduced growth factor requirements. We report here that transfection with a single HPV16 gene E6 is sufficient to immortalize these cells and reduce their growth factor requirements. The RB protein is normal in these cells, but the p53 protein is sharply reduced, as shown by immunoprecipitation with anti-p53 antibody (pAB 421). We infer that the E6 protein reduces the p53 protein perhaps by signalling its destruction by the ubiquitin system. The HPV-transforming gene E7 was unable to immortalize human mammary epithelial cells. Thus, cell-specific factors may determine which viral oncogene plays a major role in oncogenesis.

Human papillomavirus types 16 and 18 (HPV16 and HPV18) have been associated with cervical carcinoma, although most of the 65 or more known HPV types are restricted to small benign epidermal lesions, such as warts and papillomas (14). New HPV types and new associations with oral as well as genital carcinomas are being reported (14), but no evidence for an association of HPV with breast cancer has yet been found. Indeed, it has been postulated that the narrow host range of HPV, apparently limited to squamous epithelial cells, may reflect a specific cellular interaction provided by these cells.

Introduction of viral DNA to induce early neoplastic changes has provided useful in vitro models of oncogenesis (18, 19, 24). Such models have not been established for human mammary epithelial cells. Following the report that human keratinocytes transfected with plasmids containing HPV18 or HPV16 DNA were immortalized (18, 27), similar experiments were undertaken in this laboratory with normal human mammary epithelial cells (76N) derived from reduction mammoplasty. Mammary epithelial cells senesce after approximately 60 population doublings in culture (3), similar to human fibroblasts. Surprisingly, the mammary epithelial cells were readily immortalized by transfection with the HPV16 or HPV18 genome (4). The immortalized cells express a number of early neoplastic traits, including loss of growth factor requirements and chromosome changes, but remain nontumorigenic (4, 33).

The HPV genome contains seven early genes, of which E6 and E7 have been associated with transformation in rodent cells (5) and more recently have been shown to be sufficient for HPV-induced immortalization of human keratinocytes (24). HPV16 E7 alone in a retroviral vector induced immortalization of human keratinocytes at a low frequency, but transfection with E6 and E7 together was more effective (12). The two transforming gene products E6 and E7 are known to bind to tumor suppressor gene products p53 and retinoblastoma (RB), respectively. In the in vitro reticulocyte system, E7 protein complexes with RB (25). RB forms complexes with other viral transforming gene products, simian virus 40 (SV40) large T antigen, and adenovirus E1A proteins (7, 35). Recently, E6 protein, another transforming gene product of HPV, has been shown to bind p53 protein in the in vitro reticulocyte assay (34). This interaction leads to degradation of p53 protein through the ubiquitin-dependent protease system (28). This has been suggested as a possible mechanism for the transforming capability of the E6 gene.

We were interested in identifying the roles of E6 and E7 in human mammary epithelial cell immortalization and their reduced growth factor requirements. Using mutants of HPV16, which were created by inserting translation termination linkers into open reading frames (ORFs) of various early genes (24), we show here that (i) E6 alone is sufficient to cause immortalization of mammary epithelial cells, (ii) E6-immortalized cells show the same reduced growth factor requirements as do whole genome-transfected cells, (iii) cells immortalized by E6 alone or E6 and E7 have normal RB protein, and (iv) E6-immortalized cells have markedly less immunoprecipitable p53 protein than do parent cells.

MATERIALS AND METHODS

Cells and cell culture. Strains 76N, 81N, and 70N were derived from reduction mammoplasties as described previously (3). These cells were grown in DFCI-1 medium.

Media. DFCI-1 medium consists of α-minimal essential medium–Ham's nutrient mixture F-12 (1:1, vol/vol) supplemented with 12.5 ng of epidermal growth factor (EGF) per ml, 10 nM triiodothyronine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 50 μM freshly made ascorbic acid, 2 nM estradiol, 1 μg of insulin per ml, 2.8 μM hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 10 μg of transferrin per ml, 2 mM L-glutamine, 50 μg of gentamycin per ml, 15 nM sodium selenite (all from Sigma), 1 ng of cholera toxin per ml (Schwartz/Mann), 1% fetal calf serum (Hyclone defined), and 35 μg of bovine pituitary extract per ml (Hammond Cell/Tech, Alameda, Calif.). The pH is 7.4 at 6.5% CO_2–93.5% air.

DFCI-1 medium minus fetal calf serum and bovine pitu-

* Corresponding author.
† Present address: Division of Radiation and Cancer Biology, New England Medical Center, Boston, MA 02111.

itary extract is designated D2. This medium, in addition, has 0.05% bovine serum albumin (BSA) (tissue culture grade; Sigma). D2 medium minus EGF, hydrocortisone, insulin, triiodothyronine, and cholera toxin is designated D3.

Determination of cell growth. To measure growth in different media, cells were grown for at least three passages in the respective medium. Cells were washed with calcium- and magnesium-free phosphate-buffered saline (PBS) and released with 0.025% trypsin–0.01% EDTA. Trypsin digestion was stopped with 0.0375% soybean trypsin inhibitor (Sigma) in PBS, and cells were washed and counted in a Coulter counter. Approximately 5×10^4 cells were plated per 35-mm-diameter dish (Falcon) and grown at 37°C in a humidified atmosphere with 6.5% CO_2.

Transfection and selection. Normal cell strains (76N, 81N, and 70N) cultured in DFCI-1 medium were plated at 10^6 cells per 100-mm-diameter dish 18 h prior to transfection. Linearized plasmid DNA (8 μg) was transfected into cells by calcium phosphate coprecipitation with 2 μg of linearized pSV2neo (to provide a selectable marker), as described previously (4). After 6 h, cells were treated with 15% (vol/vol) glycerol for 4 min, and then fresh medium was added. After 48 h of transfection, selection in 50 μg of G418 (GIBCO) per ml was started and continued for 10 days. The surviving cells were then shifted to the D2 medium.

RNA isolation and analysis. Total RNA was isolated from 50 to 60% confluent cell monolayers, and Northern (RNA) blot hybridizations were carried out as described previously (4).

Immunoprecipitation of HPV16 E7 protein. Cells were metabolically labeled for 6 h at 37°C with 500 μCi (each) of [^{35}S]cysteine and [^{35}S]methionine in 4 ml of medium lacking cysteine and methionine per 100-mm-diameter plate. Cells were washed with PBS containing 0.1% BSA and 0.1% glucose and were 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 7,500 × *g* for 20 min at 4°C. Lysates were precleared three times with Pansorbin-fixed *Staphylococcus aureus* Cowan 1 strain (Pansorbin; Calbiochem) and immunoprecipitated with specific rabbit antibodies against HPV16 E7 (19), and immune complexes were collected with protein A-Sepharose 4B (Pharmacia). The immunoprecipitates were resolved on SDS–12% polyacrylamide gels (15).

Immunoprecipitation of HPV16 E6 protein. Cells were metabolically labeled with [^{35}S]cysteine (400 μCi per 100-mm-diameter dish) for 3 h in cysteine-deficient medium supplemented with 10% dialyzed fetal calf serum and then processed as described above. Immunoprecipitation was carried out with affinity-purified polyclonal rabbit antisera against HPV16 E6 as described previously (1).

Immunoprecipitation of RB and p53 proteins. Cells were labeled with [^{35}S]methionine (200 μCi/ml) for 3 h, and protein lysates were prepared in 120 mM NaCl–20 mM Tris (pH 8.0)–0.5% Nonidet P-40. Aliquots of the lysate were immunoprecipitated with monoclonal antibody pAB 419 (anti-SV40 T antigen, control) (13), pAB 421 (anti-p53), or RB-PMG3-245 (anti-RB; PharMingen), as previously described (7, 10, 22). For testing the binding function of RB to the SV40 large T antigen, 10 μg of unlabeled lysate of baculovirus-infected Sf9 cells expressing SV40 large T antigen was mixed with [^{35}S]methionine-labeled lysates from cells, as described above. The mixture was incubated at 4°C for 30 min, and immunoprecipitation with the SV40 large T antibody was performed (7).

TABLE 1. Immortalization of mammary epithelial cells by HPV16 mutants[a]

Plasmid	Disrupted ORF(s)	Designation	No. of immortalized colonies (no. of G418-resistant colonies)[b]
p1427	E6, E6*[c]	ΔE6E6*	0 (0), 0 (0), 0 (0)
p1428	E6	ΔE6	0 (0), 0 (0)
p1429	E1	ΔE1	1 (5), 3 (4)
p1433	E2, E4	ΔE2E4	0 (5), 2 (4)
p1466	E7	ΔE7	0 (14), 1 (4), 2 (19)

[a] HPV genes are expressed from the HPV LCR promoter.

[b] The numbers of immortalized colonies obtained after growth of initially G418-resistant colonies in D2 medium are given. The numbers in parentheses indicate the numbers of G418-resistant colonies picked. Colony frequencies were 4×10^{-6} to 6×10^{-6}. Transfection with constructs containing the mutant E6 gene produced some G418-resistant single cells which, however, failed to grow and make colonies. Thus, they could not be further tested in D2 medium. Results for two or three experiments are shown.

[c] E6* is an internally spliced version of E6 (24).

Western immunoblot analysis of p53. Western blotting was done as described previously (8). In brief, cells were lysed in a buffer containing 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM Na orthovanadate, 10 μg of aprotinin, 10 μg of leupeptin, and 10 μg of phenylmethylsulfonyl fluoride per ml for 20 min on ice. The lysates were cleared by centrifugation at 14,000 × *g* for 15 min. Protein (200 μg per lane) was loaded on SDS–7.5% polyacrylamide gels. pAB 1801 (Oncogene Science) was used at 0.03 μg/ml in Tris-buffered saline (10 mM Tris [pH 8.0], 120 mM NaCl) and 5% BSA. Secondary antibody was goat anti-mouse alkaline phosphatase (Promega). The blot was developed with a nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) mixture (Bethesda Research Laboratories, Inc.).

RESULTS

E6 alone is sufficient for immortalization of normal mammary epithelial cells. To determine which early genes are required for immortalization of human mammary cells, we used a series of HPV mutants as described by Munger et al. (24). The mutants were created by inserting translation termination linkers (TTLs) into the ORFs of the early genes of HPV16. As shown in Table 1, interruption of the E1, E2, E4, and E7 ORFs (ΔE1, ΔE2, ΔE4, and ΔE7) did not eliminate the ability of the HPV genome to immortalize 76N cells. In contrast, interruption of HPV E6 ORF (ΔE6) completely eliminated the immortalizing ability of the HPV genome. These results show that the E6 ORF is essential for immortalization of 76N cells.

Since ΔE7 constructs showed a reduced efficiency of immortalization compared with those of ΔE1, ΔE2, and ΔE4, we wished to know whether E6 alone was sufficient or whether E7 improved immortalization efficiency. For this purpose, we used constructs expressing E6 alone, E7 alone, or both ORFs under the control of either the HPV long control region (LCR) or the β-actin promoter (24). As shown in Table 2, transfection with plasmids in which E6 and E7 are expressed under the control of the HPV LCR (designated E6E7) resulted in immortalization of 76N cells at a lower frequency than that with the actin promoter. Actin-driven constructs in which the E6 ORF was interrupted (i.e., E7A and E7E6*A [Table 2]) produced no immortal colonies.

TABLE 2. Immortalization of 76N cells by HPV16 E6 but not E7

Plasmid	Promoter-intact ORFs[a]	Designation	No. of immortalized colonies (G418-resistant colonies)
	LCR-E6E7	E6E7	1 (4)
p1321	Actin-E6E7	E6E7A	5 (6)
p1434	Actin-E7	E7A	0 (0)
p1435	Actin-E7E6*	E7E6*A	0 (0)
p1436	Actin-E6E6*	E6A	9 (10)

[a] LCR-E6E7, expression of E6 and E7 ORFs is under control of the HPV16 LCR. In all other plasmids, the expression of HPV genes is under the control of the human β-actin promoter (24). E6* is an internally spliced version of E6 (24).

In contrast, constructs in which the actin-driven E6 ORF was intact (E6A) immortalized 76N cells effectively. Normally, HPV LCR-transfected cells go through a crisis (nongrowing) period after G418 selection. It usually takes about 30 to 60 days before cells start growing again. Constructs containing the actin promoter produced colonies that did not go through a crisis period compared with constructs with an HPV LCR promoter. These results suggest that in mammary epithelial cells the actin promoter is more effective than the LCR in promoting E6-induced functions, since E6A immortalized mammary epithelial cells more rapidly and with better efficiency. These immortalization experiments with E6A and E6E7A constructs were confirmed in two other normal mammary epithelial cell strains, 81N and 70N, with similar results (data not shown). Thus, transfection with HPV16 E6 alone is sufficient to immortalize normal mammary epithelial cells. These immortalized cells are grown in culture for more than 1 year.

Reduced growth factor requirements of HPV16 E6-immortalized cells. HPV16-immortalized mammary epithelial cells have reduced growth factor requirements compared with normal parent cells (4). E6A- and E6E7A-immortalized cells also demonstrate the same reduced growth factor requirements (4) (Fig. 1). The HPV transfectants tested to date have lost requirements for serum and pituitary extract, as well as all growth factors in DFCI-1 medium except EGF (4).

Transfectants containing intact E6 but disrupted E7 do not produce E7 protein. In order to demonstrate that E7 mutants do not produce any E7 protein, we immunoprecipitated cell lysates with polyclonal rabbit anti-E7 antiserum. This antiserum recognizes epitopes throughout the E7 ORF (11). As shown in Fig. 2, this antiserum recognized E7 protein of approximately 20 kDa in Caski cells, a cervical carcinoma cell line (containing approximately 600 copies of HPV16 DNA), as well as in 76N cells transfected with the HPV16 whole genome (Fig. 2A, lanes labeled 16-1-1 and 16-2-1) (4) or with mutant genomes (ΔE1, ΔE2, and ΔE4) that contain an intact E7 ORF (Fig. 2A). In contrast, all five clones established by transfection with a construct expressing E6 and no E7 (16E6A1 through A5 and 16ΔE7-2) showed no detectable E7 protein compared with E6E7 transfectants (16E6E7A2) (Fig. 2B). These clones do express E7 mRNA transcripts (not shown), as expected from a TTL insertion that blocks translation.

Expression of HPV16 E6 RNA transcripts in E6 transfectants. An HPV16 E6-specific probe containing 94 to 559 nucleotides of the HPV16 genome (obtained from P. Howley) was used to examine expression of the E6 gene in transfectants. E6-specific RNA transcripts were observed (Fig. 3) in all transfectants examined. As expected, the normal parent cells did not show any HPV transcripts.

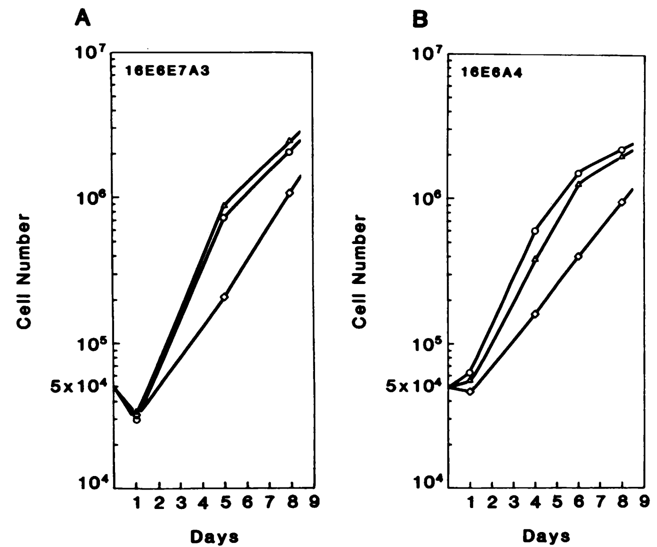

FIG. 1. Reduced growth factor requirements of HPV16 E6E7A3 (A)- and E6A4 (B)-immortalized cells. Cells were seeded in defined medium at 5×10^4 per 35-mm-diameter culture dish, harvested, and counted at the times indicated. To determine growth factor requirements, the cells were assayed in defined medium D2 (△), D3 plus EGF (□), and D3 plus EGF, hydrocortisone, and insulin (○).

Expression of HPV16 E6 protein in E6 transfectants. Cells immortalized with HPV16 E6 or HPV16 E6E7 constructs were examined by immunoprecipitation analysis with affinity-purified polyclonal rabbit antibodies (1) against HPV16 E6 (Fig. 4). All of these transfectants showed a 18-kDa E6 protein (Fig. 4, second lanes in each group except lane 76N) that was specifically immunoprecipitated with anti-E6 antibody. As expected, normal parent cells (Fig. 4, lane labeled 76N) did not express this protein.

Retinoblastoma and p53 proteins in E6 and E6E7 transfectants. As E6 and E7 proteins are known to form complexes with p53 and RB respectively, we examined the RB and p53 proteins in these transfectants. Figure 5 demonstrates that E6- and E6E7-immortalized cells have a normal functional RB protein, as judged by the presence of full-length protein that is able to bind SV40 large T antigen and its ability to become phosphorylated.

In contrast, the E6E7- and E6-immortalized mammary epithelial cells contained very low levels of PAb 421-immunoprecipitable p53 protein compared with 76N parent cells. Similarly, exogenously added SV40 large T antigen coprecipitated little, if any, p53 from the E6- or E6E7-immortalized cells compared with the parent cell strain 76N (in Fig. 5B, compare lanes 2 and 3 with lane 1).

To study the steady-state levels of p53 protein in normal parent cells as well as E6 transfectants more quantitatively, we performed Western blot analysis. As shown in Fig. 6, normal parent cells (lane labeled 76N) and Va2 cells (an SV40-transfected fibroblast cell line [26]), used as a positive control, expressed p53 protein reactive with the antibody pAB 1801. Under similar conditions, all E6 transfectants (lanes labeled E6A1, E6A4, E6E7A2, and ΔE7-2) and Siha, a cervical carcinoma cell line, showed undetectable levels of p53 protein, indicating the possibility of degradation of p53 by E6.

p53 RNA expression in E6 transfectants. To demonstrate that the reduction in the level of p53 protein in E6 transfec-

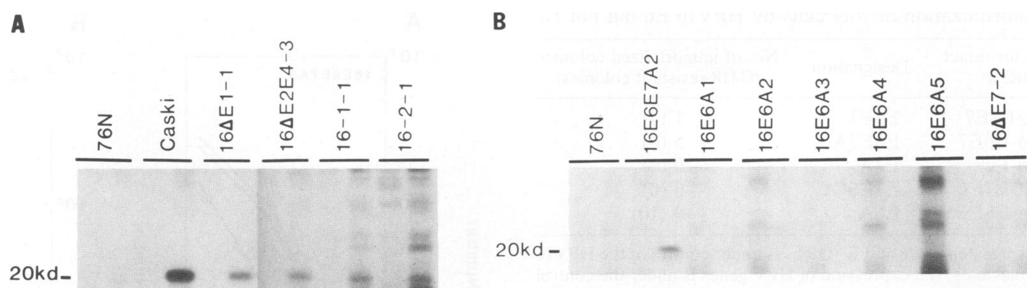

FIG. 2. Expression of HPV16 E7 protein in transfectants. (A) E7 protein (20 kDa) in the Caski (a cervical carcinoma) cell line used as an positive control and in all other transfectants having intact E7 ORFs. (B) Cells transfected with disrupted E7 ORFs. In each case, the first lane represents immunoprecipitation with normal rabbit serum and second lane represents immunoprecipitation with E7 antiserum.

tants is not at the RNA level, we measured p53 mRNA levels in normal parent and E6-immortalized cells by Northern blot analysis. Figure 7 shows that p53 mRNA levels are equal in parent cells and E6 transfectants (all lanes except the two labeled Siha and Va2). The size of the p53 RNA transcript is similar to that seen with Va2 and Siha used as controls.

DISCUSSION

The results presented in this paper demonstrate that immortalization and a substantial reduction of growth factor requirements of human mammary epithelial cells can be achieved by a single HPV oncogene, E6. This conclusion is based on the use of plasmid constructs in which ORFs of various genes were destroyed by inserting translation termination linkers (24). When genes other than E6 (E1, E2, E4, and E7) were rendered nonfunctional, the constructs still retained the immortalizing ability for mammary epithelial cells. In contrast, plasmids in which the E6 ORF was interrupted failed to immortalize breast cells.

Importantly, constructs in which only HPV E6 and E7 sequences were present and the E7 ORF was destroyed (E6A) still retained the immortalizing capacity. Cells immortalized with the E6 plasmids expressed E6-specific RNA and an 18-kDa protein immunoprecipitable with E6-specific antibodies. Furthermore, these transfectants lacked any detectable E7 protein as assessed with polyclonal rabbit antisera known to recognize epitopes throughout the E7 ORF (11). These antibodies did immunoprecipitate E7 protein in transfectants in which an intact E7 ORF was included in the transfected construct. Together, these results demonstrate that the E7 protein is not required for immortalization.

The possible involvement of an aberrant product of the E7 gene not detectable with these antibodies, while unlikely, has not been formally ruled out. Use of plasmid constructs containing only E6 sequences should resolve this question. The result that transfection with HPV16 E6 alone can immortalize mammary cells is quite different from the results obtained with keratinocytes (12, 24) in which both E6 and E7 oncogenes are required for optimal immortalization. Mammary cell immortalization with E6 alone is particularly surprising since mammary epithelial cells, in contrast to squamous epithelial cells, are not a known target of HPV infection. For example, in cervical carcinomas, integrated copies of both E6 and E7 are regularly found (1, 31). However, the E6 gene of bovine papillomavirus type 1 has been shown to transform C127, a mouse mammary cell line (29).

E7-mediated oncogenic transformation has been ascribed to its ability to bind with the RB protein. Phosphorylation of the RB gene product is modulated during the cell cycle and cellular differentiation (6). Most, if not all, mutations in RB that have been identified in human tumors affect its ability to bind the SV40 T antigen and to become phosphorylated (6, 17, 30). However, neither the phosphorylation pattern of RB protein nor its binding to T antigen was altered in HPV E6-immortalized cells even when E7 was simultaneously transfected. These results indicate that alterations of RB

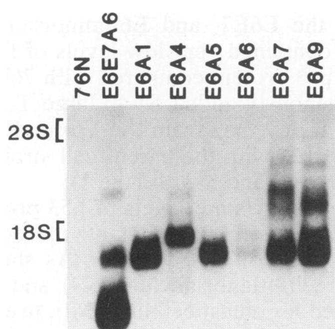

FIG. 3. HPV16 E6 mRNA expression in transfectants containing intact E6 or E6E7 ORFs. Total cellular RNA isolated from normal parent cells (the first lane), E6E7-transfected clone (the second lane), and various E6-transfected clones (the third through the eighth lanes) was analyzed by Northern blot hybridization with an E6-specific probe containing nucleotides 94 to 559 of the HPV16 genome. Locations of the ribosomal RNAs (28S, 4,850 bp, and 18S, 1,740 bp) are indicated.

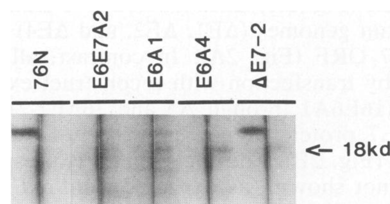

FIG. 4. Immunoprecipitation analysis of normal and E6-transfected mammary cells with affinity-purified rabbit antibodies against HPV16 E6 protein. For each cell line, the first lane represents immunoprecipitation with normal rabbit serum and the second lane represents that with anti-E6 antiserum. The arrow indicates the 18-kDa HPV16 E6 protein, which is present in all E6 transfectants (the 4th, 6th, 8th, and 10th lanes) but not in normal parent 76N cells (second lane).

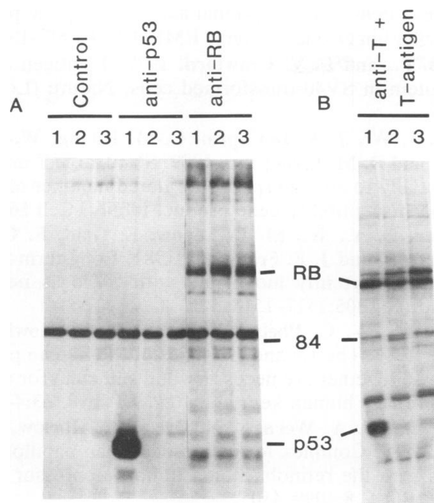

FIG. 5. Expression of RB and p53 proteins in normal mammary cells and transfectants. (A) Autoradiograph of SDS–7.5% polyacrylamide gel. Four plates (p100) of 76N (lanes 1), E6A1 (lanes 2), and E6E7A2 (lanes 3) were each labeled with [^{35}S]methionine, and protein lysates were prepared. Aliquots of the lysate were immunoprecipitated with monoclonal antibody PAB 419, PAB 421, or RB-PMG3-245. Bars indicate the positions of RB and p53, determined with prestained molecular weight markers (Sigma). (B) Unlabelled lysate (10 μg) of baculovirus-infected Sf9 cells expressing SV40 large T antigen was mixed with [^{35}S]methionine-labelled lysates from 76N (lane 1), ΔE7 transfectant (lane 2), and E6E7A2 (lane 3) prepared as described above. The mixture was incubated at 4°C for 30 min, the SV40 large T antibody was added, and an immunoprecipitation was performed. Bars indicate the positions of the coprecipitated RB and p53; 84 is a molecular weight marker. p107 (a band just above the RB protein) is also present (9).

protein are unlikely to mediate the immortalization in mammary epithelial cells.

The presence of normal RB protein, both phosphorylated and unphosphorylated in the immortalized cells, further suggests that RB is not functioning as a senescence gene in these cells; its function is not lost during immortalization. The same is true in many tumors in which RB expression is normal (16). In contrast, p53 protein may have a senescence function in these mammary cells, in view of its reduced expression in the HPV-immortalized cells. An important immortalizing role of E6 may lie in its ability to signal the degradation of p53 protein (28).

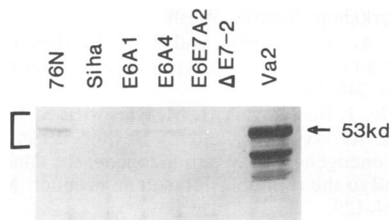

FIG. 6. Western blot analysis of p53 protein expression in normal mammary cells and E6 transfectants. Each lane was loaded with 200 μg of protein lysates from the normal parent cell line 76N, cervical carcinoma cell line Siha, known to contain HPV16 E6 protein (1), transfectants containing only intact E6 (lanes labeled E6A1, E6A4, and ΔE7-2), transfectants containing intact E6 and E7 (lane labeled E6E7A2), and an SV40 T antigen-transfected fibroblast cell line, Va2, known to have stable p53 expression, which was used as a positive control. The proteins were transferred to nitrocellulose, reacted with anti-p53 monoclonal antibody pAB 1801, and visualized with alkaline phosphatase-conjugated goat anti-mouse antibody with nitroblue tetrazolium chloride and BCIP for color development.

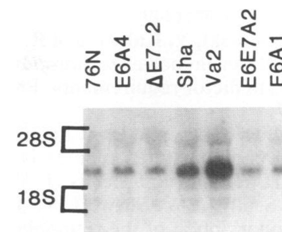

FIG. 7. p53 mRNA expression in normal and E6-transfected cells. Total cellular RNA (20 μg) isolated from 76N, intact E6 ORF containing transfectants (lanes labeled E6A4, ΔE7-2, and E6A1), intact E6 and E7 ORFs containing transfectant (lane labeled E6E7A2), the cervical carcinoma cell line Siha known to express HPV16 E6 protein, and an SV40 T antigen-transfected fibroblast cell line, Va2, was resolved on agarose gels transferred to a zeta probe and hybridized with a human 1.8-kb p53 cDNA probe (2), as described in Materials and Methods. All E6 transfectants expressed levels of p53 mRNA similar to that expressed in the parent cell strain 76N.

These results showing that E6 alone can immortalize mammary epithelial cells raise several questions. (i) E6 protein may interact with multiple cellular proteins, only one of which is p53. For example, E6 is a possible transcription factor (20), but the identities of cellular genes that may be targets of E6 regulation are as yet unknown. Thus, E6 may have multiple effects on cells in which it is expressed. (ii) HPV-immortalized cells, both keratinocytes (32) and mammary cells (4, 33) have undergone some chromosomal changes. They may include nonrandom changes that contribute to the immortal phenotype. (iii) The additional genetic changes associated with progression to neoplasia have not yet been identified.

Of particular interest is the fact that neither SV40 viral infection nor transfection is itself tumorigenic in human cells, nor does SV40 contribute to clinical cancer (21, 23). Yet HPV does participate in the development of genital and oral squamous carcinomas (1). Does the difference lie primarily in the destruction of p53 by HPV E6 but not by the SV40 T antigen? Because of their responsiveness to HPV E6, human mammary epithelial cells may be uniquely well suited for use in pursuing answers to these questions.

ACKNOWLEDGMENTS

We thank P. Howley and K. Munger for constructs and advice, D. Galloway for E7 antiserum, E. Androphy for E6 antiserum, and S. Budd for preparing the manuscript.

This research was supported by National Cancer Institute grant CA39814 to R.S.

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