

Cooperative Transformation and Coexpression of Bovine Papillomavirus Type 1 E5 and E7 Proteins

JOANNA BOHL,¹ BRUCE HULL,² AND SCOTT B. VANDE POL^{1*}

Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106,¹ and Department of Surgery, School of Veterinary Science, Ohio State University, Columbus, Ohio 43210²

Received 10 July 2000/Accepted 28 September 2000

Productively infected bovine fibropapillomas were examined for bovine papillomavirus type 1 (BPV-1) E7 localization. BPV-1 E7 was observed in the cytoplasm of basal and lower spinous epithelial cells, coexpressed in the cytoplasm of basal cells with the E5 oncoprotein. E7 was also observed in nucleoli throughout the basal and spinous layers but not in the granular cell layer. Ectopic expression of E7 in cultured epithelial cells gave rise to localization similar to that seen in productive fibropapillomas, with cytoplasmic and nucleolar expression observed. Consistent with the coexpression of E7 and E5 in basal keratinocytes, BPV-1 E7 cooperated with E5 as well as E6 in an anchorage independence transformation assay. While E5 is expressed in both basal and superficial differentiating keratinocytes, BPV-1 E7 is only observed in basal and lower spinous epithelial cells. Therefore, BPV-1 E7 may serve to modulate the cellular response of basal epithelial cells to E5 expression.

Papillomaviruses are causative agents for a variety of epithelial neoplasms in vertebrates. Typically, virus-induced epitheliomas are benign lesions containing episomal viral DNA in proliferative basal epithelial cells, producing virus within superficial differentiated epithelial cells. The role of virus-encoded oncogenes (E5, E6, and E7) in the maintenance of viral DNA within proliferative basal epithelial cells and/or the role of these oncogenes in the transition to vegetative viral DNA amplification and virus production has yet to be fully defined. The separation of papillomavirus oncogenes into at least three separate polypeptides (E5, E6, and E7) may reflect a requirement for separate regulation of expression and activity of each product in the virus life cycle. One area of uncertainty in papillomavirus biology has been the location of each oncoprotein within the stratified epithelium of a virus-induced epithelioma.

While bovine papillomavirus type 1 (BPV-1) encodes E5, E6, and E7 oncoproteins, only mutation of BPV-1 E5 decreases transformed cell focus formation by BPV-1 viral DNA. Mutations in the BPV-1 E6 or E7 genes have little effect upon focus formation or viral DNA replication, although there is a decrease in anchorage-independent growth and tumorigenicity (12, 15, 20). The modest oncogenic potency of BPV-1 E6 and BPV-1 E7 expressed from the wild-type BPV-1 genome is due to repression by two separate repressor mechanisms that, when mutated, reveal cooperative transformation by BPV-1 E6 and BPV-1 E7 (35). Since both BPV-1 E6 and BPV-1 E7 are thought to be translated from the same mRNA transcript, it was unproven if BPV-1 E7 had a direct role in transformation together with BPV-1 E6. Correlating with this observation, BPV-1 E6 strongly transforms murine C127 cells when expressed from retroviral long terminal repeats, whereas E7 did not (27).

The E6 proteins of human papillomavirus type 16 (HPV-16) (16E6) and BPV-1 E6 bind to the cellular targets E6AP, ERC-55, and paxillin through interaction with homologous peptide sequences found on the target proteins (5, 8, 36), and transformation by E6 can be repressed by competitive binding of E6 to peptides that interact with E6 (3). The cancer-associated HPV E6 oncoproteins bind p53 together with the cellular ubiquitin ligase E6AP, resulting in the degradation of p53 through ubiquitin-mediated proteolysis *in vitro* (13). While BPV-1 E6 interacts with E6AP, targeted degradation of p53 by BPV-1 E6 has not been observed (21).

The BPV-1 E5 oncoprotein interacts with the platelet-derived growth factor (PDGF) receptor to stimulate ligand-independent intracellular activation of the PDGF receptor (23). BPV-1 E5 also modulates epidermal growth factor (EGF) receptor downregulation (6, 16), resulting in enhanced surface expression of the EGF receptor. It is as yet uncertain if E5 transformation is exclusively through activation of receptor tyrosine kinases or if additional activities contribute. BPV-1 E5 interacts with a 16-kDa vacuolar ATPase (10), resulting in the alkalization of intracellular compartments, which may contribute to its transforming ability (22, 26, 29, 30, 32). Interactions between HPV E5 oncoproteins and receptor tyrosine kinases have varied between different HPV types (7), but HPV E5 oncoproteins have been found to synergize with HPV E6 and E7 in the immortalization of keratinocytes (31).

There are striking differences between BPV-1 E7 and HPV E7 oncoproteins, most notably in the absence of a LXCXE interaction motif in BPV-1 E7 for the retinoblastoma family of tumor suppressors that is found in all HPV E7 oncoproteins. While HPV E7 proteins interact with the retinoblastoma protein *in vitro*, BPV-1 E7 does not (19).

Since BPV-1 E7 has not been described as having a strong independent transforming activity, it may act to enhance or modulate transforming activity of BPV-1 E5 or BPV-1 E6. In order to determine if BPV-1 E5 or BPV-1 E6 might be coexpressed with BPV-1 E7, we determined the predominant localization of BPV-1 E7 within productively infected bovine

* Corresponding author. Mailing address: Institute of Pathology, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106. Phone: (216) 368-1679. Fax: (216) 368-1300. E-mail: sbv@pop.cwru.edu.

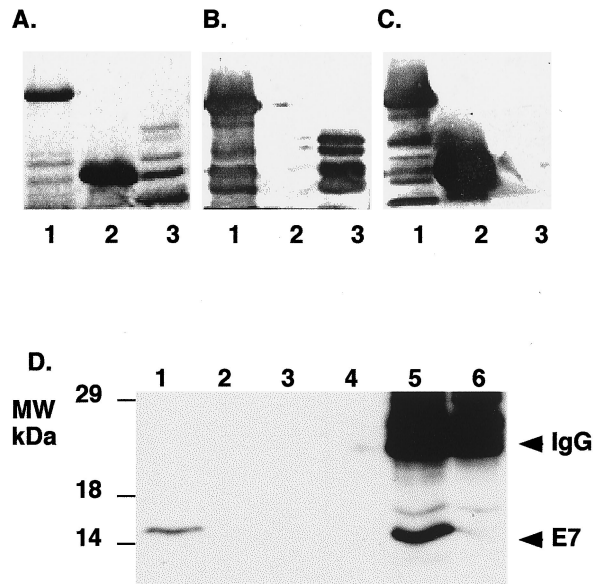


FIG. 1. Specificity of antibodies to E7. Antiserum to bacterially expressed histidine-tagged full-length E7 and antiserum to a synthetic peptide comprising the C-terminal 10 amino acids were raised in rabbits. Antibodies were extensively adsorbed to fixed, detergent-extracted bovine MDBK cells and then to fixed and detergent-extracted normal bovine epidermis to remove antibodies cross-reactive to bovine skin. The adsorbed antiserum raised against full-length E7 was affinity purified by binding and elution from a glutathione *S*-transferase (GST) fusion to the first 80 amino acids of E7. The anti-peptide antibody was affinity purified by binding and elution to immobilized E7 amino acids 80 to 127. (A, B, and C) Lanes 1, 2, and 3 show bacterially expressed GST fusion proteins of full-length E7 amino acids 1 to 127, 80 to 127, and 1 to 80, respectively. In panel A, the proteins are Coomassie blue stained, while in panels B and C the proteins were analyzed by Western blot with affinity-purified antibodies directed to the amino terminus of E7 (B) and the carboxy terminus of E7 (C). (D) Analysis of E7 in fibropapillomas. Approximately 50 mg of fibropapillomas or uninfected bovine tissue were frozen sectioned and thawed into 1.0 ml of boiling 1% SDS. The sonicated, clarified lysate was diluted into 9 ml of phosphate-buffered saline (PBS) containing 1% Triton X-100. E7 was immunoprecipitated overnight with 10 μ l of rabbit antibody to E7 and 50 μ l of protein A-Sepharose. An SDS-15% polyacrylamide gel electrophoresis gel was analyzed by immunoblot with affinity-purified rabbit antibody to the E7 carboxy terminus and detected with peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) and enhanced chemiluminescence. Lanes 1 and 2, 25 μ g of protein from Cos-1 cells transfected with E7 or empty control plasmid, respectively; lanes 3 and 4, 10 and 50 μ g of bovine MDBK cell lysate, respectively; lanes 5 and 6, anti-E7 immunoprecipitates from bovine fibropapilloma (lane 5) or uninfected bovine tissue (lane 6). Arrowheads indicate the positions of E7 and rabbit IgG light chains.

fibropapillomas and found E7 coexpressed with E5 in the cytoplasm of basal epithelial cells. Correlating with this observation, BPV-1 E7 cooperated with both E5 and E6 in the transformation of rodent cells to anchorage-independent growth.

Location of E7 in fibropapillomas. Rabbit polyclonal antibodies specific to nonoverlapping segments of the E7 amino terminus or carboxy terminus were prepared and analyzed for specificity against bacterially expressed fragments of E7 (Fig. 1A to C). These antibodies immunoprecipitated and identified on a Western blot a 15-kDa protein from a bovine papilloma

that comigrated with E7 derived from transiently transfected Cos-1 cells (Fig. 1D).

Immunolocalization of E7 showed two distinct patterns of expression in naturally occurring fibropapillomas (Fig. 2). E7 was principally localized to the cytoplasm of basal and parabasal epithelial cells (Fig. 2c). E7 cytoplasmic staining was strongest in the basal cells (Fig. 2c and 3j) but was not strictly localized to those cells, since staining into the lower spinous epithelial cells three to five cell layers up from the basement membrane was observed. Sporadic cytoplasmic staining of epithelial cells within mid-spinous cells was seen (Fig. 3j). Normal bovine epithelium within the same tissue section but adjacent to the fibropapilloma showed no similar immunostaining (Fig. 2e and f). While the most intense staining for E7 was observed in basal cell cytoplasm, scattered and less-intense punctate nuclear staining was observed throughout the basal and spinous cell layers (best seen in Fig. 4d and e). No E7 staining was observed in the superficial granular cell layer. E7 within the keratinized layer of fibropapillomas could not be assessed due to staining of the keratinized layer by the secondary goat anti-rabbit antibody (Fig. 2f). E7 staining of the basal and lower spinous cells was observed in four fibropapillomas from two separate animals; similar, although weaker, epithelial staining was also observed in an experimentally induced BPV-1 fibropapilloma (generously provided by Alison McBride, National Institute of Allergy and Infectious Diseases, Bethesda, Md.; data not shown). Two separate affinity-purified rabbit polyclonal antibodies to E7 gave the same pattern of E7 localization. In Fig. 2c, 4d, and 4e (see below), E7 immunostaining was performed with an antibody specific to the amino terminus of E7, while in Fig. 3j (see below), E7 was localized with an antiserum specific to the C terminus of E7. E7 staining of the fibroma was not consistently higher than background staining of adjacent normal-appearing dermis.

Comparison of E5 and E7 expression in fibropapillomas. BPV-1 E5 was localized utilizing a rabbit anti-BPV-1 E5 antiserum previously used to localize BPV-1 E5 in bovine fibropapillomas (4) and a second rabbit antibody (generously provided by Richard Schlegel). With both antibodies BPV-1 E5 was localized to basal cells and the superficial granular cell layer (Fig. 3d). Unlike staining of E7 where the basal cell layer was uniformly stained (Fig. 2c), prominent E5 staining was patchy along the basal cell layer, being more intense at the base of dermal papilla (Fig. 3f). Sporadic E5 staining within mid-spinous cell layers was observed but was much less intense than basal E5 staining (Fig. 3f and h). In tissue culture, E5 is expressed in the membranes of the Golgi and the endoplasmic reticulum. The resolution of our photomicrographs on frozen sections could not sublocalize E5 within the cytoplasm. In Fig. 3h an apparent expansion of E5-expressing cells above the basal layer is due to tangential sectioning of this portion of the fibropapilloma. In comparison to E5, E7 was expressed only basally and within the lower spinous cell layer (compare E7 in Fig. 3j to E5 in Fig. 3f and h). While there was clear BPV-1 E5 staining in the granular cell layer where viral capsid proteins are synthesized and virions are assembled, BPV-1 E7 immunostaining was not observed in the granular layer (not shown). We have not as yet been able to observe E6 in either BPV-1 or BPV-2 fibropapillomas by immunofluorescent staining.

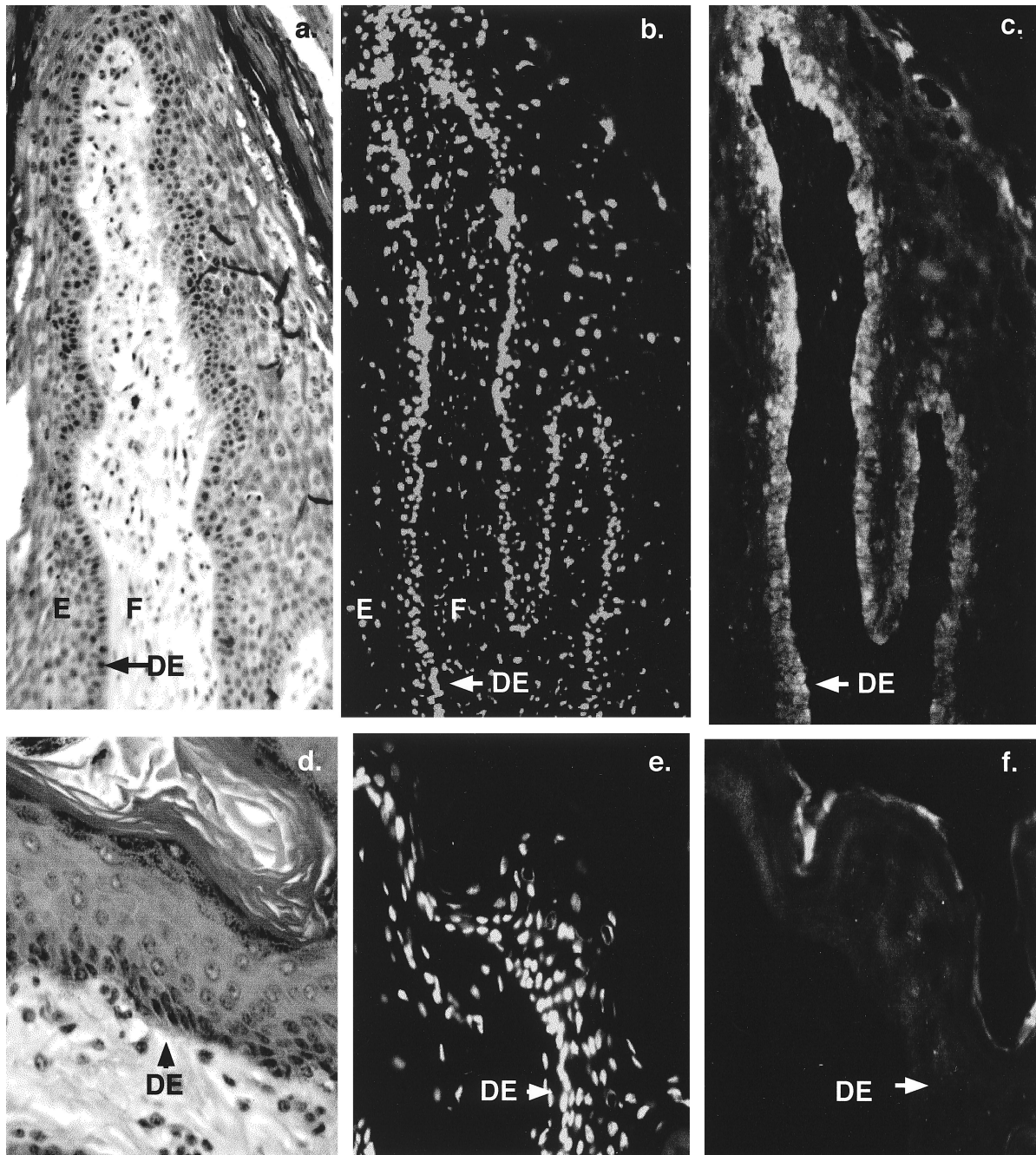


FIG. 2. Immunolocalization of E7 in bovine fibropapillomas. Bovine fibropapillomas were collected at the Ohio State University veterinary surgical clinic from domestic cows. Four warts from two cows were analyzed by immunostaining. Frozen sections were air dried, stored at -85°C , and fixed for 6 min in PBS containing 3% freshly prepared formaldehyde. Alexa-468-labeled goat anti-rabbit antibodies (Molecular Probes) were extensively adsorbed with a fixed extract of a bovine fibropapilloma and fixed human HaCat cells to remove antibodies reactive to fibropapilloma or HaCat cells. Panels a and d are shown for orientation and were taken from regions of the same fibropapilloma similar to those of panels b, c, e, and f but are not adjacent sections. In all of the sections, arrow DE indicates the dermal-epidermal junction. E, epithelioma; F, fibroma. (a) Low-power ($\times 100$) hematoxylin-and-eosin-stained section, shown for orientation. (b) Low-power photomicrograph ($\times 100$) showing DAPI-stained nuclear DNA. (c) Low-power photomicrograph ($\times 100$) of the same field as in panel b, showing BPV-1 E7 cytoplasmic expression at the dermal-epidermal junction. (d) High-power ($\times 400$) hematoxylin-and-eosin-stained normal bovine skin, shown for orientation for panels e and f. (e) DAPI-stained normal bovine skin adjacent to the fibropapilloma in the same tissue section as panel c. (f) E7 immunostained normal bovine skin, showing the same field as that shown in panel e.

Figures 2 and 3 show immunostaining for BPV-2 fibropapillomas. Fibropapillomas associated with BPV-1 and BPV-2 were similar in both histological appearance and immunostaining for E5 and E7. Sequencing of viral DNA isolated from the

BPV-2 fibropapilloma shown in Fig. 2 revealed three amino acid differences within the E7 gene compared to the prototype BPV-2 sequence (T6N, S62P, and H84N).

In addition to the predominant cytoplasmic localization of

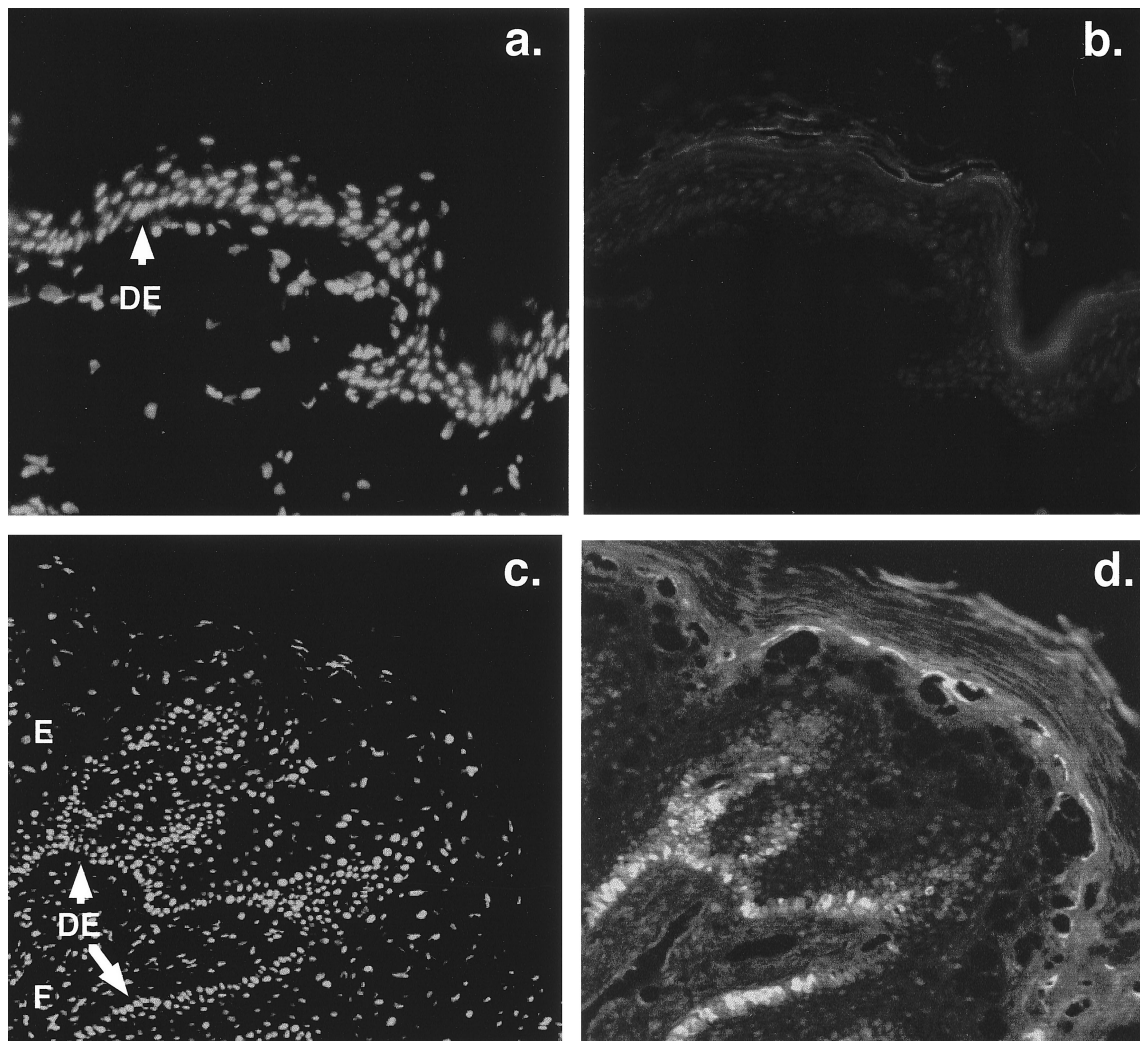


FIG. 3. Comparison of E5 and E7 localization in fibropapillomas. Panel pairs a and b, c and d, e and f, g and h, and i and j are identical views stained with DAPI (a, c, e, g, and i), antibody to E5 (b, d, f, and h, gifts of Daniel DiMaio and Richard Schlegel), or antibody to E7 (j, C-terminal peptide antibody affinity purified to the E7 carboxy terminus), respectively. Annotations are as in Fig. 2. Slides were treated with 0.2% SDS after fixation but prior to immunofluorescence to enhance immunostaining signal. (a and b) High-power ($\times 400$), same views of normal bovine skin adjacent to fibropapilloma stained with DAPI (a) or immunostained with antibody to E5 (b). (c and d) Same $\times 100$ views of bovine fibropapilloma stained with DAPI (c) or immunostained with antibody to E5 (d). (e and f) Same $\times 200$ views of fibropapilloma, stained with DAPI (e) or immunostained with antibody to E5 (f). (g and h) Same $\times 200$ views of fibropapilloma, stained with DAPI (g) or immunostained with antibody to E5 (h). The arrow in panel h indicates sporadically positive E5-expressing cells in the spinous layer. (i and j) Same $\times 200$ views of fibropapilloma, stained with DAPI (i) or immunostained with antibody to E7 carboxy terminus (j).

E7 in fibropapillomas, focal nuclear staining of E7 was also observed (not seen in Fig. 2 and 3). Figure 4 shows a section of fibropapilloma immunostained for both the nucleolar protein fibrillar and E7. Fibrillar was exclusively localized to foci within DAPI (4',6'-diamidino-2-phenylindole)-stained nuclei (Fig. 4a and b). Focal E7 staining within the spinous cell layer typically, but not invariably, colocalized with fibrillar staining (Fig. 4c and d), with 46% of the 200 nuclei counted demonstrating focal E7 nuclear expression and with 12% of the spinous cells showing only focal nuclear E7. Figure 4e demonstrates focal E7 staining within DAPI-stained nuclei in a separate fibropapilloma than that shown in Fig. 2 or 3. E7 nucleolar staining can vary depending upon the processing of the samples. Both fibrillar and E7 nucleolar staining were

reduced by sodium dodecyl sulfate (SDS) denaturation of fibropapilloma tissue sections prior to immunostaining, while E7 cytoplasmic staining was unchanged. The section in Fig. 3j was treated with SDS prior to immunostaining with antibodies to E7, while sections in Fig. 4 were not treated with SDS.

Location of E7 in transiently transfected cells. In order to compare the experimental expression of BPV-1 E7 with that seen in the fibropapilloma, BPV-1 E7 was transiently expressed in human HaCat epithelial cells. The expression patterns of E7 in HaCat cells were similar to that seen in the fibropapilloma. Figure 5a shows the DAPI-stained nuclei of five cells, two of which express E7 (Fig. 5b). In the upper cell of Fig. 5b, E7 is predominantly cytoplasmic with a fibrillar pattern, although some E7 is within the nucleus in a punctate

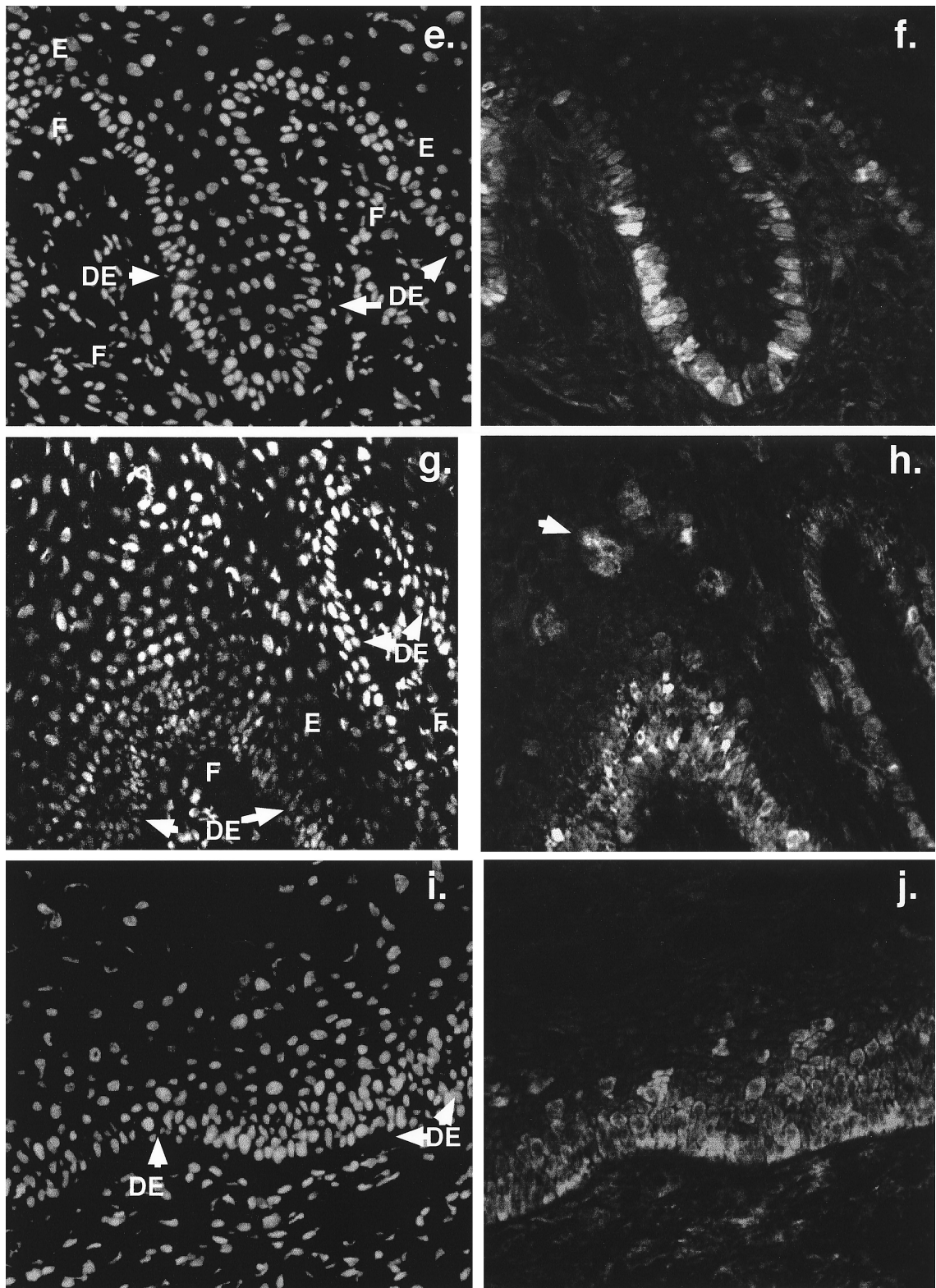


FIG. 3—Continued.

distribution. In the fibropapilloma, strong cytoplasmic expression is observed in basal and parabasal epithelial cells (Fig. 3j and 4e). The lower E7-expressing cell in Fig. 5b demonstrates E7 that is predominantly nuclear with focal concentrations, as

is observed in some spinous cells of the fibropapilloma (Fig. 4c and e). Comparison of Fig. 5c and d demonstrates that focal concentrations of E7 in the HaCat cell nucleus can colocalize with the nucleolar protein fibrillarin. However, the E7 concen-

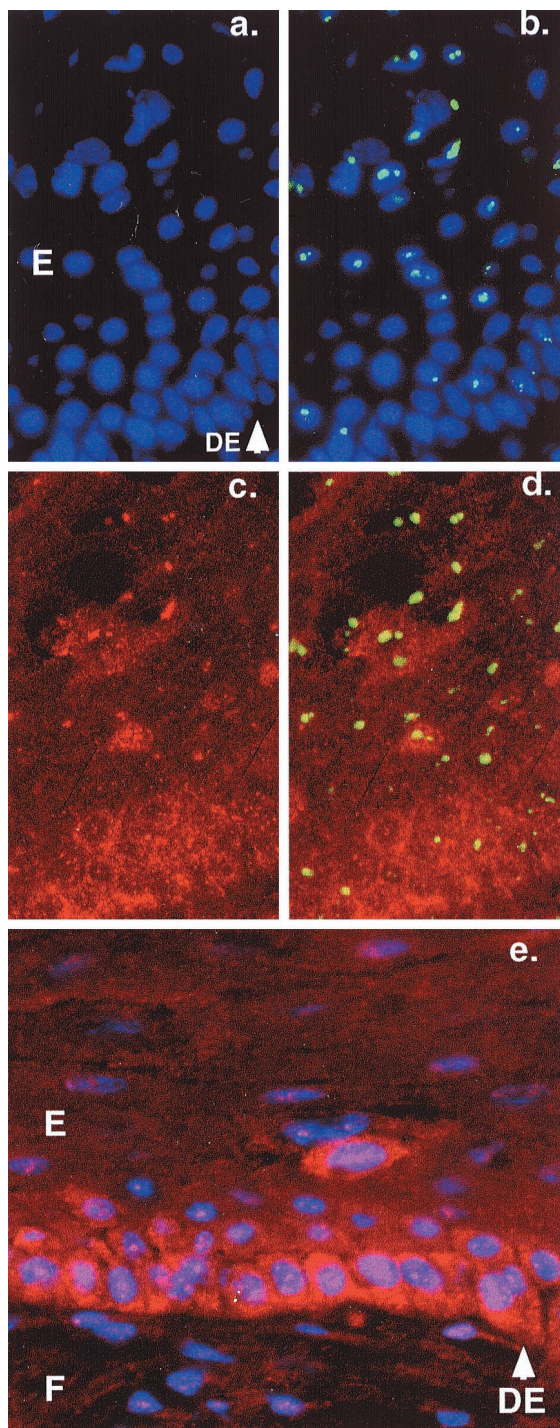


FIG. 4. Localization of E7 within the cytoplasm and nucleolus of fibropapilloma epithelial cells. (a and b) Same $\times 400$ views of DAPI-stained cell (a, blue) or cell stained with DAPI plus monoclonal antibody 72B9 to fibrillarin (24) (green, b). (c and d) Same views as in panels a and b but stained for E7 (red, c) or E7 plus fibrillarin (fibrillarin is green, d). (e) A different bovine fibropapilloma than that shown in panels a to d or Fig 1 and 2 was immunostained with amino-terminal specific antibody to E7 (red), and the nuclei were counterstained with DAPI (blue), showing basal and parabasal E7 cytoplasmic staining, together with focal E7 nuclear staining.

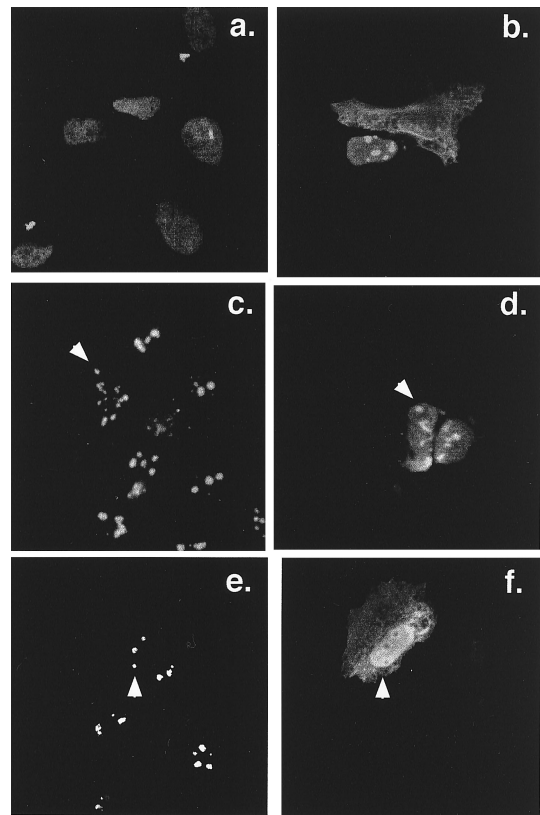


FIG. 5. Localization of BPV-1 E7 within the cytoplasm and nucleolus of epithelial cells. HaCat cells lipofected with a plasmid expressing BPV-1 E7 were fixed 24 h posttransfection and examined by indirect immunofluorescence with rabbit polyclonal antibodies to E7 and mouse monoclonal antibody to fibrillarin. (a and b) Same views of cells stained with DAPI (a) or immunostained for E7 (b). (c and d) Same views immunostained for fibrillarin (c) and E7 (d). (e and f) Same views immunostained for fibrillarin (e) and E7 (f).

tration within nucleoli was not uniformly observed in HaCat transfections, since some E7-expressing cells showed either no nucleolar concentration of E7 or even occasionally perinucleolar concentration of E7 (Fig. 5e and f). The percentage of E7-expressing cells with nucleolar localization-transfected cells varied from 18 to 54% in three separate transfections. The variable localization of E7 suggests that localization of E7 is subject to regulation but was not related to cell density, time posttransfection, or the intensity of E7 expression, nor were cells expressing nuclear versus cytoplasmic E7 expression grossly altered in morphology. E7 localization was not altered by treatment of transfected HaCat cells with leptomycin B, a drug that blocks Crm1-mediated nuclear export of proteins that contain leucine-rich export sequences (9), so it is unlikely that E7 localization is regulated by a typical nuclear export signal (data not shown). Despite numerous attempts, we have been unsuccessful in obtaining cell lines that stably express E7 that can be observed by immunofluorescence.

Synergistic transformation by BPV-1 E5 and E7. Since BPV-1 E7 and BPV-1 E5 are coexpressed within the basal cell layer of fibropapillomas, they might cooperate in controlling cell proliferation. To investigate this possibility, we examined the influence of E7 upon transformation by E5 and E6 of

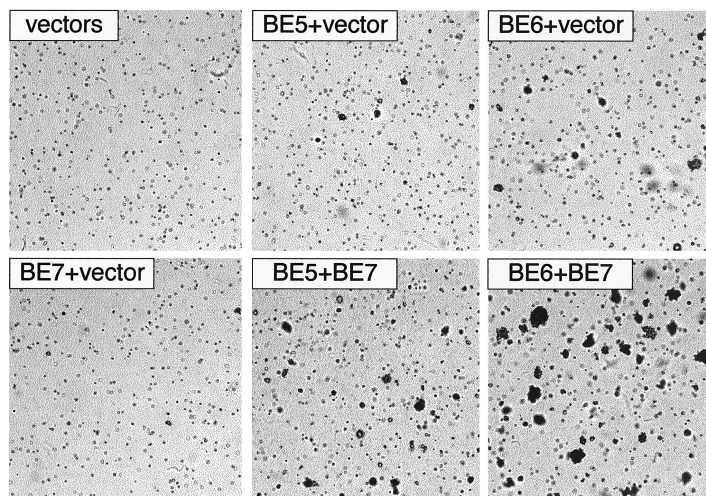


FIG. 6. E7 synergizes with E6 and E5 in transformation. C127 cells retrovirally transduced with the indicated BPV-1 oncogenes were assayed for anchorage-independent colony formation as described in the legend to Table 1. Colonies were photographed 14 days after seeding into agar.

anchorage-dependent rodent cells. BPV-1 E6 and BPV-1 E7 open reading frames were cloned into retroviral expression plasmids with either neomycin or puromycin resistance genes to allow for positive selection of two separately transduced genes. BPV-1 E5 was cloned as an *Eco*RI-to-*Bam*HI fragment of BPV-1 containing a translation termination linker within the E2 and E4 open reading frames. This plasmid is predicted to express only the intact E5 polypeptides.

Murine C127 cells were transduced with replication-defective retroviruses expressing BPV-1 E5, BPV-1 E6, BPV-1 E7, or dually infected combinations so that every resulting cell line expressed both puromycin and neomycin resistance at the same cell culture passage history. C127 cells transduced with either E5 or E6 had a typical transformed appearance, while cells transduced with E7 alone were unchanged in appearance (data not shown). Pooled drug-resistant colonies ($>10^4$ colonies per infection) were tested for anchorage-independent colony formation. While both E5- and E6-expressing retroviruses induced anchorage independence in the absence of E7, coexpression of E7 together with E5 or E6 increased both the frequency and the size of colony formation (Fig. 6). Results from separate experiments using independently transduced cells are shown in Table 1 (group A). E7 enhanced focus formation by E5 and E6 by ca. 4- to 10-fold. Disruption of the E5 open reading frame with a translation stop codon eliminated anchorage-independent colony formation (Table 1, group B). Similarly, disruption of the E7 open reading frame eliminated synergistic transformation with both E6 and E5. E7 had a small direct oncogenic activity independent of E5 or E6, but the resulting colonies were infrequent and much smaller than those produced by either E6 or E5 alone. No significant differences in E6 or E5 protein expression levels were observed by Western blot or immunoprecipitation when coexpressed with E7 (data not shown).

The separation of papillomavirus oncogenes into separate polypeptides (E5, E6, and E7) allows for the separate regulation of expression and activity of each product within the differentiated layers of an epithelioma. This prompted our examination of fibropapillomas for E5 and E7 proteins. Exami-

nation of mRNA expression in both human and animal epitheliomas has been performed, but few studies have examined protein expression. While we were able to localize E7 and E5 in this study, we were unable to localize E6 in fibropapillomas, indicating a more-restricted expression of E6 than E7 in fibropapillomas.

E7 protein has not been previously localized in HPV-induced epitheliomas or in animal papillomaviruses that are similar to HPV papillomaviruses. E7 has been localized in BPV-4, a BPV that is quite different from BPV-1, BPV-2, and HPV viruses. While BPV-4 contains an E7 protein with an LXCXE retinoblastoma protein binding motif, it does not encode an E6 protein (14). In BPV-4 alimentary epitheliomas, E7 was detected within the nucleus of basal and parabasal cells and within the cytoplasm of differentiated spinous and granular cells (1). In contrast, in the present study we observe uniform BPV-1 E7 expression in the cytoplasm and nucleus of basal and lower spinous epithelial cells. Nuclear staining that colocalized with the nucleolus was sporadically observed in basal and spinous epithelial cells of fibropapillomas and was also observed within transfected HaCat epithelial cells in tissue culture.

It is difficult to correlate the expression of E7 protein to viral mRNAs. In BPV-1-induced fibropapillomas, in situ hybridizations to basal epithelial cells detected only mRNA from the P2443 and P3080 promoters that are thought to make E5 and the E2 repressor, respectively (2). From these in situ hybridization studies it is unclear which promoter(s) is responsible for the synthesis of either E1 (necessary for plasmid maintenance) or for E7 in basal cells. While it is presumed that E1, E6, and E7 proteins are made in basal cells from transcripts originating at promoter P89, this is as yet unproven. It is possible that an additional viral promoter upstream of E7 may be responsible for the expression of E7 protein observed in this study.

Subcellular HPV E7 localization within cultured cells has varied with different studies, with both nuclear and cytoplasmic distribution observed, depending upon the expression system. A recent study using new monoclonal antibodies to HPV-16 E7

TABLE 1. BPV-1 E7 cooperates in the transformation of C127 cells with BPV-1 E5 and BPV-1 E6

Group ^a	Neo virus ^b	Puro virus ^c	No. of anchorage-independent colonies ^d		
			Expt 1	Expt 2	Expt 3
A	Vector	Vector	0	0	0
	Vector	E7	2	1	0
	E6	Vector	29	33	13
	E6	E7	110	153	140
	E5	Vector	10	4	5
	E5	E7	43	31	26
B	Vector	Vector	0	0	0
	E6	Vector	ND	ND	14
	E6	E7	ND	ND	71
	E6	E7oc2	ND	ND	8
	E5	Vector	2	10	1
	E5	E7	21	82	21
	E5tt1	E7	0	0	ND
	Vector	E7	0	5	ND
	E5	E7oc2	ND	6	2

^a Groups: A, experiments using independently transduced cells expressing the intact E5, E6, and E7 genes; B, experiments involving disruption of the E5 and E7 open reading frames.

^b For Neo virus, BPV-1 E5 or BPV-1 E6 was transduced by replication-defective murine retroviruses also containing the neomycin resistance gene. BPV-1 E5 was expressed from a 2,348-nucleotide *EcoRI*-to-*Bam*HI fragment of BPV-1 containing a translation termination linker that disrupts the E2 and E4 genes at nucleotide 3459 of BPV-1, thereby leaving only the E5 gene intact. E5tt1 contains a translation termination linker within the E5 gene, causing translation arrest after amino acid 3 of E5 (nucleotide 3885 of the BPV-1 genome). Vector, cells transduced by empty retroviruses and selected for drug resistance.

^c For Puro virus, BPV-1 E7 was isolated by PCR using the E7 mutant E7SA as a template (20), mutating a splice acceptor within the E7 open reading frame but not altering the amino acid coding sequence, and was cloned into the retroviral expression plasmid pBabe Puro (18). E7oc2 contains a stop codon at amino acid 22 of E7.

^d Murine C127 cells were sequentially transduced with both puromycin and neomycin retroviruses. Pooled cells from >10⁴ infectious events from each retrovirus were passaged three times and assayed for anchorage-independent colony formation. Low-power (×50) photomicrographs were taken at 14 days post-seeding into agarose, and four separate fields were counted. Shown are the average number of colonies per photographed field. The highest percentage of colony formation was observed with the combination of E6 and E7, where efficiencies ranged from 15 to 22% of seeded cells in the experiments shown. ND, not done.

localized E7 to nucleoli and to the cytoplasm in HPV-transformed CaSki cells (38). In overexpression experiments, HPV E7 cytoplasmic expression can be prominent, and E7 has been shown associate with actin stress fibers in the cytoplasm when expressed by vaccinia virus (25). We have not observed actin colocalization of BPV-1 E7 either in transfected HaCat cells or in vaccinia virus expression systems, nor does cytochalasin D alter the localization of BPV-1 E7 in these transfection systems (data not shown). The absence of a retinoblastoma protein binding motif on BPV-1 E7 indicates that BPV-1 E7 and HPV-16 E7 are in some ways functionally dissimilar, but it is possible the BPV-1 E7 and HPV E7 proteins may share as-yet-unknown cytoplasmic functions and nucleolar functions despite their limited primary sequence homology.

BPV-1 E5 was the first papillomavirus oncoprotein whose expression pattern in a productive infection was described (4). As documented in that study and here, E5 is strongly expressed in the basal and granular cell layers and is sporadically expressed in the spinous cell layer. Although all early region transcripts in BPV-1 include the E5 open reading frame, the

genetic evidence indicates that E5-dependent transformation results from a spliced message originating from the P2440 promoter and spliced from nucleotides 2505 to 3225 (11). Correlating with this observation, only a spliced message from nucleotides 2505 to 3225 could be specifically localized to basal epithelial cells in BPV-1 fibropapillomas (2). It is unclear which messages may give rise to E5 expression observed in the spinous and granular cell layers. A recent study of the oncogenic HPV-31 E5 showed a pattern of E5 expression similar to that of BPV-1 (17).

The cytoplasmic coexpression of BPV-1 E7 with BPV-1 E5 in bovine fibropapillomas observed in this study prompted us to reexamine cooperative transformation between E7 and E5 and between E7 and E6. BPV-1 E5 and BPV-1 E6 synergy in transformation has been previously described (37) and was not explored here. We observed clear synergy in transformation between E5 and E7 and between E6 and E7. Synergy in transformation between BPV-1 E5 and BPV-1 E7 has not been previously reported. In the experiments described in the present study each oncoprotein was expressed from separately transduced retroviruses, and care was taken to prevent the dominance of the culture by infrequent but highly transformed clones of cells. It is possible that prior studies failed to identify the activity of BPV-1 E7 due to the overgrowth of the culture by cells highly transformed by either E5 or E6. If that were the case, cells fully transformed by E6 or E5 might mask the contribution to anchorage-independent colony formation by E7.

The E5 immunofluorescence signal in fibropapillomas was much stronger than that of E7 despite high-titer antibodies to E7, suggesting low levels of E7 in fibropapillomas. But localization of neither E5 nor E7 within transformed murine C127 cells could be unambiguously observed utilizing the same antibodies, indicating even lower expression levels in transformed C127 cells (data not shown). E5 and E7 are clearly biologically active within transformed C127 cells, as shown by the transformation assay in Fig. 6. Thus, our inability to observe E7 above the lower spinous cell layer in epithellomas or the inability to observe E5 within most cells of the spinous cell layer should not as yet be interpreted as strong evidence for an absence of biological function for these oncoproteins in these regions of a fibropapilloma.

The colocalization of E7 with E5 within the cytoplasm of basal epithelial cells and the failure to detect E7 in the differentiated granular layer where E5 is expressed suggest the possibility that E7 may act to modulate the response of basal keratinocytes to E5 expression. As noted in the introduction, E5 activates receptor tyrosine kinases and stimulates mitogen-activated protein kinase activity. In neuronal and epithelial cell culture systems, transient ERK activation results in cell proliferation, while sustained ERK activation can result in terminal differentiation (33, 34). Similar to these observations, sustained *ras* signaling in primary fibroblasts results in terminal differentiation to a senescent phenotype (28, 39). If keratinocytes might differentiate as a result of sustained E5 expression, modulation of E5-induced signaling might be necessary to sustain cellular proliferation within basal epithelial cells. We hypothesize that the coexpression of E7 and possibly E6 may act to modulate cellular responses of epithelial cells to sustained high-level expression of E5.

This study was supported by NIH grant CA69292 to S.B.V.P.

We thank Alison McBride for frozen sections of a BPV-1 bovine fibropapilloma and Daniel DiMaio and Richard Schlegel for rabbit antibody to E5.

REFERENCES

- Anderson, R. A., L. Scobie, B. W. O'Neil, G. J. Grindlay, and M. S. Campo. 1997. Viral proteins of bovine papillomavirus type 4 during the development of alimentary canal tumours. *Vet. J.* **154**:69–78.
- Barksdale, S. K., and C. C. Baker. 1993. Differentiation-specific expression from the bovine papillomavirus type 1 P2443 and late promoters. *J. Virol.* **67**:5605–5616.
- Bohl, J., K. Das, B. Dasgupta, and S. B. Vande Pol. 2000. Competitive binding to a charged leucine motif represses transformation by a papillomavirus E6 oncoprotein. *Virology* **271**:163–170.
- Burnett, S., N. Jareborg, and D. DiMaio. 1992. Localization of bovine papillomavirus type 1 E5 protein to transformed basal keratinocytes and permissive differentiated cells in fibropapilloma tissue. *Proc. Natl. Acad. Sci. USA* **89**:5665–5669.
- Chen, J. J., Y. Hong, E. Rustamzadeh, J. D. Baleja, and E. J. Androphy. 1998. Identification of an alpha helical motif sufficient for association with papillomavirus E6. *J. Biol. Chem.* **273**:13537–13544.
- Cohen, B. D., D. J. Goldstein, L. Rutledge, W. C. Vass, D. R. Lowy, R. Schlegel, and J. T. Schiller. 1993. Transformation-specific interaction of the bovine papillomavirus E5 oncoprotein with the platelet-derived growth factor receptor transmembrane domain and the epidermal growth factor receptor cytoplasmic domain. *J. Virol.* **67**:5303–5311.
- Conrad, M., D. Goldstein, T. Andersson, and R. Schlegel. 1994. The E5 protein of HPV-6, but not HPV-16, associates efficiently with cellular growth factor receptors. *Virology* **200**:796–800.
- Elston, R. C., S. Naphine, and J. Doorbar. 1998. The identification of a conserved binding motif within human papillomavirus type 16 E6 binding peptides, E6AP and E6BP. *J. Gen. Virol.* **79**(Pt. 2):371–374.
- Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida. 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**:308–311.
- Goldstein, D. J., M. E. Finbow, T. Andersson, P. McLean, K. Smith, V. Bubb, and R. Schlegel. 1991. Bovine papillomavirus E5 oncoprotein binds to the 16K component of vacuolar H⁺-ATPases. *Nature* **352**:347–349.
- Hermonat, P. L., and P. M. Howley. 1987. Mutational analysis of the 3' open reading frames and the splice junction at nucleotide 3225 of bovine papillomavirus type 1. *J. Virol.* **61**:3889–3895.
- Howley, P. M., Y. C. Yang, B. A. Spalholz, and M. S. Rabson. 1986. Papillomavirus transforming functions. *Ciba Found. Symp.* **120**:39–52.
- Huibregtse, J. M., M. Scheffner, and P. M. Howley. 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol. Cell. Biol.* **13**:775–784.
- Jackson, M. E., W. D. Pennie, R. E. McCaffery, K. T. Smith, G. J. Grindlay, and M. S. Campo. 1991. The B subgroup bovine papillomaviruses lack an identifiable E6 open reading frame. *Mol. Carcinog.* **4**:382–387.
- Jareborg, N., A. Alderborn, and S. Burnett. 1992. Identification and genetic definition of a bovine papillomavirus type 1 E7 protein and absence of a low-copy-number phenotype exhibited by E5, E6, or E7 viral mutants. *J. Virol.* **66**:4957–4965.
- Martin, P., W. C. Vass, J. T. Schiller, D. R. Lowy, and T. J. Velu. 1989. The bovine papillomavirus E5 transforming protein can stimulate the transforming activity of EGF and CSF-1 receptors. *Cell* **59**:21–32.
- Mayer, T. J., and C. Meyers. 1998. Temporal and spatial expression of the E5a protein during the differentiation-dependent life cycle of human papillomavirus type 31b. *Virology* **248**:208–217.
- Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18**:3587–3596.
- Munger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.* **8**:4099–4105.
- Neary, K., and D. DiMaio. 1989. Open reading frames E6 and E7 of bovine papillomavirus type 1 are both required for full transformation of mouse C127 cells. *J. Virol.* **63**:259–266.
- Ned, R., S. Allen, and S. Vande Pol. 1997. Transformation by bovine papillomavirus type 1 (BPV-1) E6 is independent of transcriptional activation by E6. *J. Virol.* **71**:4866–4870.
- Oelze, I., J. Kartenbeck, K. Crusius, and A. Alonso. 1995. Human papillomavirus type 16 E5 protein affects cell-cell communication in an epithelial cell line. *J. Virol.* **69**:4489–4494.
- Petti, L., L. A. Nilson, and D. DiMaio. 1991. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. *EMBO J.* **10**:845–855.
- Reimer, G., K. M. Pollard, C. A. Penning, R. L. Ochs, M. A. Lischwe, H. Busch, and E. M. Tan. 1987. Monoclonal autoantibody from a (New Zealand black × New Zealand white)F₁ mouse and some human scleroderma sera target an M_r 34,000 nucleolar protein of the U3 RNP particle. *Arthritis Rheum.* **30**:793–800.
- Rey, O., S. Lee, M. A. Baluda, J. Swee, B. Ackerson, R. Chiu, and N. H. Park. 2000. The E7 oncoprotein of human papillomavirus type 16 interacts with F-actin in vitro and in vivo. *Virology* **268**:372–381.
- Schapiro, F., J. Sparkowski, A. Adduci, F. Supryniewicz, R. Schlegel, and S. Grinstein. 2000. Golgi alkalization by the papillomavirus E5 oncoprotein. *J. Cell Biol.* **148**:305–316.
- Schiller, J. T., W. C. Vass, and D. R. Lowy. 1984. Identification of a second transforming region in bovine papillomavirus. *Proc. Natl. Acad. Sci. USA* **81**:7880–7884.
- Serrano, M., A. W. Lin, M. E. McCurrach, D. Beach, and S. W. Lowe. 1997. Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**:593–602.
- Sparkowski, J., J. Anders, and R. Schlegel. 1995. E5 oncoprotein retained in the endoplasmic reticulum/cis Golgi still induces PDGF receptor autophosphorylation but does not transform cells. *EMBO J.* **14**:3055–3063.
- Sparkowski, J., M. Mense, J. Anders, and R. Schlegel. 1996. E5 oncoprotein transmembrane mutants dissociate fibroblast transforming activity from 16-kilodalton protein binding and platelet-derived growth factor receptor binding and phosphorylation. *J. Virol.* **70**:2420–2430.
- Stoppler, M. C., S. W. Straight, G. Tsao, R. Schlegel, and D. J. McCance. 1996. The E5 gene of HPV-16 enhances keratinocyte immortalization by full-length DNA. *Virology* **223**:251–254.
- Straight, S. W., B. Herman, and D. J. McCance. 1995. The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. *J. Virol.* **69**:3185–3192.
- Tombes, R. M., K. L. Auer, R. Mikkelsen, K. Valerie, M. P. Wymann, C. J. Marshall, M. McMahon, and P. Dent. 1998. The mitogen-activated protein (MAP) kinase cascade can either stimulate or inhibit DNA synthesis in primary cultures of rat hepatocytes depending upon whether its activation is acute/phasic or chronic. *Biochem. J.* **330**(Pt. 3):1451–1460.
- Traverse, S., N. Gomez, H. Paterson, C. Marshall, and P. Cohen. 1992. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem. J.* **288**(Pt. 2):351–355.
- Vande Pol, S., and P. Howley. 1995. Negative regulation of the bovine papillomavirus E5, E6, and E7 oncogenes by the viral E1 and E2 genes. *J. Virol.* **69**:395–402.
- Vande Pol, S. B., M. C. Brown, and C. E. Turner. 1998. Association of bovine papillomavirus type 1 E6 oncoprotein with the focal adhesion protein paxillin through a conserved protein interaction motif. *Oncogene* **16**:43–52.
- Yang, Y. C., H. Okayama, and P. M. Howley. 1985. Bovine papillomavirus contains multiple transforming genes. *Proc. Natl. Acad. Sci. USA* **82**:1030–1034.
- Zatsepina, O., J. Braspenning, D. Roberson, M. A. Hajibagheri, K. J. Blight, S. Ely, M. Hibma, D. Spitkovsky, M. Trendelenburg, L. Crawford, and M. Tommasino. 1997. The human papillomavirus type 16 E7 protein is associated with the nucleolus in mammalian and yeast cells. *Oncogene* **14**:1137–1145.
- Zhu, J., D. Woods, M. McMahon, and J. M. Bishop. 1998. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12**:2997–3007.