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# Biological Properties of the Deer Papillomavirus E5 Gene in Mouse C127 Cells: Growth Transformation, Induction of DNA Synthesis, and Activation of the Platelet-Derived Growth Factor Receptor

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**We determined the biological activities of the 44-amino-acid deer papillomavirus (DPV) E5 protein in mouse C127 cells. The DPV E5 gene can induce focus formation, stable and acute morphologic transformation, and DNA synthesis, and it activates the platelet-derived growth factor (PDGF) β receptor as assessed by increased constitutive tyrosine phosphorylation of mature and precursor receptor forms. Thus, the DPV E5 protein has biological activities similar to those of the closely related E5 protein from bovine papillomavirus type 1. Moreover, like the bovine papillomavirus type 1 E5 protein, the DPV E5 protein shares a short region of sequence similarity with the B chain of PDGF. These results show that activation of the PDGF receptor is a general property of fibropapillomavirus E5 proteins, lending support to our previous proposal (L. Petti, L. Nilson, and D. DiMaio, EMBO J. 10:845–855, 1991) that activation of the PDGF receptor may play a central role in transformation of fibroblasts by E5 proteins.**

Papillomaviruses infect mainly the basal layers of squamous epithelia or mucous membranes, resulting in proliferation of the infected tissues (23). In lesions induced by the fibropapillomavirus subgroup of the animal papillomaviruses, proliferation of epidermal keratinocytes is accompanied by proliferation of the underlying dermal fibroblasts. The fibropapillomaviruses also efficiently induce morphologic and tumorigenic transformation of cultured rodent fibroblasts. The prototype fibropapillomavirus is bovine papillomavirus type 1 (BPV), which has been subjected to detailed genetic and physiological analysis (6). The primary BPV transforming gene for established lines of rodent fibroblasts is the E5 gene, which encodes a very hydrophobic 44-amino-acid protein that associates with a 16-kDa cellular protein (Fig. 1) (2, 3, 5, 9, 26, 27, 30). Detailed mutational analysis of the BPV E5 protein has identified three features that are essential for morphologic transformation and induction of cellular DNA synthesis in quiescent cells: (i) seven specific amino acids in the carboxyl-terminal third of the protein, (ii) a hydrophilic amino acid (normally glutamine at position 17) in an otherwise hydrophobic region, and (iii) the overall hydrophobicity of the middle portion of the molecule (10, 14, 15, 24, 29). Because the essential carboxyl-terminal amino acids are highly conserved among the E5 proteins of all of the fibropapillomaviruses and because this portion of the protein is able to induce cellular DNA synthesis in peptide injection experiments, the carboxyl-terminal third of the fibropapillomavirus E5 proteins appears to be the active site of the molecule (1, 10, 12, 14, 15, 20).

The small size and unusual composition of fibropapillomavirus E5 proteins and the genetic analysis of the BPV E5 protein suggest that E5 proteins transform cells by influencing the activity of a cellular protein involved in growth regulation. Martin et al. demonstrated that the BPV E5 protein could influence the metabolism of epidermal growth factor and colony-stimulating factor 1 receptors (18). We have recently shown that the BPV E5 protein activates the cellular platelet-derived growth factor (PDGF) β receptor and have proposed that the PDGF receptor is an important mediator of the transforming activity of the E5 protein in fibroblasts (22). The ability of fibropapillomaviruses to induce fibroblast proliferation in vivo and the existence of sequence similarity between the active site of the E5 proteins and PDGF (Fig. 1) support the view that the PDGF receptor is a natural target of fibropapillomavirus E5 proteins.

The deer fibropapillomavirus (DPV) can cause stable growth transformation of BALB/c 3T3 and NIH 3T3 cells, but the viral gene(s) responsible for DPV transformation has not been identified (11, 16). It has been reported that DPV is not able to transform mouse C127 cells, the standard host cell for BPV E5-mediated transformation (8, 12, 16). This is an unexpected result, because the 44-amino-acid DPV E5 protein shares with its BPV counterpart all three features required for C127 cell transformation, including the carboxyl-terminal conserved amino acids in the region of homology with PDGF, glutamine at position 17, and a very hydrophobic middle portion of the molecule (Fig. 1). Therefore, we decided to extend the analysis of fibropapillomavirus E5 proteins to a second example, the DPV E5 protein, and reassess the ability of the DPV E5 protein to transform C127 cells. We show here that the DPV E5 protein can in fact induce stable and acute transformation of C127 cells and that this protein, like the BPV E5 protein, activates the PDGF receptor.

## MATERIALS AND METHODS

**Cell culture.** Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (DME-10) and passaged at subconfluence. Transformed C127 cells were established by calcium phosphate-mediated DNA transfection as previously described, by using 100 ng of *Bam*HI-digested BPV-derived constructs or 1 μg of intact retroviral plasmids (7). Stable cell lines were

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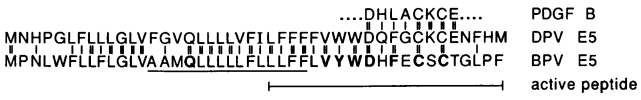


FIG. 1. Sequence comparison of the DPV and BPV E5 proteins and PDGF. The predicted amino acid sequences of the DPV and BPV E5 proteins and a related sequence from the human PDGF B chain (residues 174 to 181 of the primary translation product) are shown by the single-letter abbreviations. Identical amino acids are shown by double lines, and similar amino acids are shown by single lines. Specific amino acids required for efficient C127 transformation by the BPV E5 protein are shown in boldface, and the middle hydrophobic region that can be replaced by random hydrophobic sequences is underlined (14, 15). The minimal peptide that can efficiently stimulate cellular DNA synthesis in microinjection experiments is indicated (24).

established from individual or pooled foci 14 to 25 days after DNA transfer. Cultures were supplemented with G418 (500 μg/ml) as appropriate. For immunoprecipitation of the PDGF receptor, cells were grown to confluence in 10-cm-diameter plates and then incubated overnight in serum-free medium. As indicated, starved cells were incubated for 20 min in DME with no fetal bovine serum (DME-0) containing 10 ng of the recombinant human PDGF BB homodimer (PDGF, Inc.) per ml prior to extraction. For analysis of the PDGF receptor following infection with recombinant BPV-simian virus 40 (SV40), C127 cells were infected at 90% confluence in 60-mm-diameter dishes at a multiplicity of infection of approximately 1,500. After infection, the C127 cells were incubated in DME with 2% fetal bovine serum (DME-2) for another 48 h and then lysed for analysis.

**Plasmid constructions.** The DPV E5 gene (the coding region extends from DPV nucleotides [nt] 3914 to 4045) was cloned into the *Sal*I site of the Bluescribe vector (Pharmacia) following polymerase chain reaction amplification of DPV nt 3891 to 4076 by using pDEG98 (12) as the template and DPV-specific oligonucleotide primers containing *Sal*I recognition sites. The sequence of the DPV segment was confirmed by dideoxynucleotide sequencing. MLM-DE5-L3 was constructed by using the cloned fragment containing the DPV E5 gene to replace the small *Xho*I fragment of MLM-BE5-L3, a BPV clone containing *Xho*I sites at BPV nt 3876 and 4072, flanking the BPV E5 gene (the coding region extends from BPV nt 3879 to 4010). The small *Xho*I fragment containing the BPV E5 gene in MLM-BE5-L3 was deleted to generate MLM-L3-E5X. pBPV-E6fs2-DE5 and pBPV-E6fs2-E5X were constructed by replacing the small *Bst*EII-to-*Sal*I fragment of pBPV-E6fs2, a full-length BPV vector harboring a frameshift mutation in the E6 open reading frame (21), with the corresponding *Bst*EII-to-*Sal*I fragments of MLM-DE5-L3 (containing the DPV E5 gene) and MLM-L3-E5X (which lacks the E5 open reading frame), respectively.

The DPV E5 *Sal*I fragment was also cloned into the *Xho*I site of retroviral vector pLXSN (19), downstream of the viral left long terminal repeat, to create pRV-DE5. The correct orientation of the insert was confirmed by restriction mapping. The retroviral clone containing the BPV E5 gene, pRV-BE5-L3, has already been described (17).

Plasmids pPava1 and pPava2, which contain the SV40 late region and origin of replication and the 3′ transforming region of BPV, and pPavaE5d29, which contains a frameshift mutation in the E5 open reading frame, have been previously described (28, 29). pPava-DE5, in which the BPV E5 open reading frame is substituted by DPV E5, was constructed by replacing the *Bst*EII-to-*Bgl*II small fragment of pPava2 with the *Bst*EII-to-*Bam*HI fragment of pBPV-E6fs2-DE5. Viral stocks were prepared by excising the viral DNA from these plasmids and transfection of monkey cells expressing SV40 large-T antigen as previously described (28).

**Analysis of viral DNA and RNA.** Viral DNA and RNA were purified from cell lines (4, 13) and subjected to Southern and Northern (RNA) analyses using hybridization probes labelled with $^{32}P$ by using random oligonucleotide priming.

**Assays for cellular DNA synthesis.** The titers of the different BPV-SV40 recombinant virus stocks were determined by employing a virus-mediated quantitative transactivation assay which measures expression of the BPV-1 E2 gene (28). For DNA synthesis assays, confluent C127 cells in 24-well plates were infected in duplicate in DME-0 with equivalent amounts of virus (multiplicity of infection, approximately 1,500). After the cells were maintained for another 40 h in serum-free medium, they were incubated for 2 h in medium containing 1.5 μCi of [$^3H$]thymidine per ml. DNA synthesis was determined by measuring trichloroacetic acid-precipitable, hot-perchloric-acid-soluble label as previously described (29).

**Antibodies, immunoprecipitations, and immunoblotting.** Rabbit anti-PDGF receptor peptide serum PR4, which recognizes the carboxyl-terminal 13 amino acids of the receptor, was the gift of S. Courtneidge (European Molecular Biology Laboratory). Anti-phosphotyrosine monoclonal antibody 4G10, used for immunoblotting, was purchased from Upstate Biotechnology, Inc.

Immunoprecipitations were performed as described previously (22). Briefly, cell monolayers were washed twice with ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and then lysed with 1 ml of cold EBC buffer (50 mM Tris-HCl [pH 8.0], 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate) for 20 min on ice, followed by centrifugation in a microcentrifuge for 5 min. The protein concentrations of the supernatants were determined, equal amounts of extracted protein were treated with saturating amounts of PR4 antibody, and the mixture was incubated on ice for 2 to 4 h. An 80-μl volume of a 1:1 suspension of protein A-Sepharose (Pharmacia) in TBS-BSA (25 mM Tris-HCl [pH 8.0], 120 mM NaCl, 10% bovine serum albumin) was added, and the mixture was rotated for 30 to 60 min at 4°C. The protein A-Sepharose beads were collected by centrifugation and washed five times with 1 ml of cold NET-N buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride).

Immunoprecipitates were suspended in protein sample buffer, solubilized by boiling, and electrophoresed on discontinous 7.5% acrylamide–0.17% bisacrylamide gels as previously described (22). Gels were soaked for 5 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol, 0.1% sodium dodecyl sulfate) and transferred electrophoretically to nitrocellulose in the cold at 50 V and 400 to 600 mA overnight. Blots were stained with 10% Ponceau S (Sigma) to determine the migration of the size markers. Immunoblots were processed and probed with anti-phosphotyrosine antibody as previously described (22).

## RESULTS AND DISCUSSION

To investigate the transforming activity of DPV, cloned full-length DPV DNA was excised from bacterial vector sequences and transfected onto C127 cells. In agreement

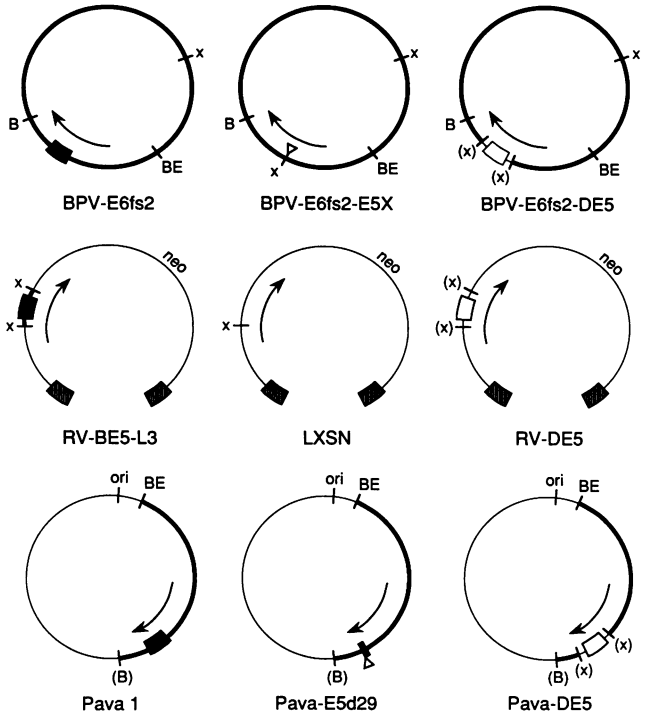


FIG. 2. Maps of the vectors used to express the E5 genes. The three rows show three sets of isogenic expression vectors. In each set, the map on the left shows the construct expressing the BPV E5 gene, the middle map shows the negative control vector without a functional E5 gene, and the map on the right shows the DPV E5 construct. The direction of E5 gene transcription is shown by the arrows. Thick lines represent BPV sequences. The BPV E5 gene is represented by solid boxes, the DPV E5 gene is represented by open boxes, and the retroviral vector LTRs are represented by hatched boxes. Triangles represent deletion mutations. Relevant restriction endonuclease recognition sites are shown by the letters B (*Bam*HI), BE (*Bst*EII), and X (*Xho*I). Parentheses indicate that the site has been destroyed by a cloning step. For simplification of the diagram, plasmid vector sequences are not shown. Rows: top, full-length BPV vectors, all containing the E6fs2 mutation (which generates an *Xho*I recognition site); middle, retroviral vectors (neo represents the G418 resistance gene on the vector); bottom, BPV-SV40 recombinant viral vectors (ori represents the SV40 origin of replication).

with earlier reports, no foci of transformed cells were observed, whereas numerous foci appeared in cells transfected in parallel with BPV DNA (data not shown). Because of the structural similarities between the BPV and DPV E5 proteins, including all of the features of the BPV protein known to be required for stable growth transformation for C127 cells, it was surprising that DPV was unable to transform C127 cells.

We reasoned that the inability of DPV DNA to transform C127 cells might be a consequence of inefficient expression of the DPV E5 protein in these cells rather than intrinsic structural differences between the DPV and BPV E5 proteins. To ensure adequate expression of the DPV E5 protein, the DPV E5 gene was inserted into the BPV genome, which efficiently induces BPV E5-dependent focus formation in C127 cells. DPV nt 3891 to 4076, which contain the E5 gene and minimal flanking sequences, were cloned in place of the BPV E5 gene in the full-length BPV genome (Fig. 2). To facilitate cloning, *Xho*I sites had been generated in the BPV vector at positions 3876 and 4072, thereby allowing precise

TABLE 1. Transforming activity of the DPV E5 gene

| Source of E5 gene | Relative transforming activity (% of BPV E5) | | |
|---|---|---|---|
| | Focus formation | | DNA synthesis[a] |
| | Full length[b] | LTR[c] | |
| BPV | 100 | 100 | 100 |
| DPV | 167 | 233 | 205 |
| None | <1 | <2 | 9 |

[a] BPV-SV40 recombinant viruses: Pava-1 (BPV E5), Pava-DE5 (DPV E5), and Pava-E5d29. Typical value for Pava-1, 71,000 cpm incorporated.
[b] Full-length vectors: E6fs2 (BPV), E6fs2-DE5 (DPV E5), and E6fs2-E5X. Typical value for E6fs2, 1,350 foci per μg of DNA.
[c] Retroviral vectors: pRV-BE5-L3 (BPV E5), pRV-L3-DE5 (DPV E5), and LXSN. Typical value for pRV-BE5-L3, 15 foci per μg of DNA. LTR, long terminal repeat.

replacement of the BPV E5 gene with the DPV sequence. We also introduced a frameshift mutation which inactivates the BPV E6 gene, a viral gene that displays weak activity in our focus formation assays in C127 cells.

After removal from bacterial vector sequences and transfection of C127 cells, viral DNA containing either the BPV or the DPV E5 gene in the full-length BPV genome induced the formation of numerous transformed foci (Table 1). Homologous viral DNA containing an E5 gene deletion did not induce foci. The foci induced by the construct containing the DPV E5 gene consistently appeared earlier and were larger than the BPV foci. Extrachromosomal viral DNA containing the DPV E5 gene and DPV E5-specific viral RNA was present in cells transformed with the DPV recombinant (data not shown). The ability of the DPV E5 gene to restore efficient focus-forming activity to a transformation-defective BPV E5 mutant genome strongly suggests that the DPV E5 gene is able to transform C127 cells stably.

To establish that the DPV E5 gene was capable of causing stable growth transformation of C127 cells in the absence of all other papillomavirus genes, it was subcloned into a Moloney murine sarcoma virus-derived retroviral expression vector that expressed the E5 gene from the proviral long terminal repeat and, in addition, encoded resistance to G418 (Fig. 2). Transfection of C127 cells with BPV or DPV E5 retroviral DNA induced numerous foci, whereas the vector without either E5 gene was transformation defective (Table 1). After normalization to the number of G418-resistant colonies generated by a portion of the transfected cells, DPV E5 retroviral DNA induced approximately twice as many foci as did BPV E5 retroviral DNA (data not shown). Cell lines established from individual DPV E5-induced foci appeared morphologically similar to cell lines transformed by the BPV E5 gene, displaying a refractile and spindle-shaped appearance and loss of contact inhibition compared with parental C127 cells (data not shown). Total RNA was extracted from several independent cell lines derived from individual transformed foci and was subjected to Northern analysis. Cells transformed by the DPV E5 gene expressed a readily detectable DPV E5 RNA that was absent from parental cells and cells transformed with the BPV E5 gene (Fig. 3 and data not shown). DPV E5 RNA comigrated with the BPV E5 RNA present in cell lines transformed by the BPV E5 gene (data not shown). These results establish that the DPV E5 gene, in the absence of any other papillomavirus gene, can cause stable growth transformation of C127 cells.

We have recently developed a BPV-SV40 recombinant virus that efficiently induces acute BPV E5-mediated trans-

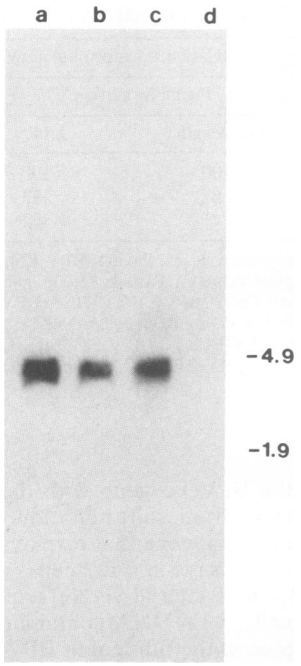


FIG. 3. Expression of DPV E5 RNA in transformed cells. Total RNA was prepared from transformed cell lines established from individual foci induced by transfection with retroviral vector pRV-DE5 (lanes a to c) or pRV-BE5-L3 (lane d), electrophoresed on a 1% agarose gel, and transferred to nitrocellulose. DPV E5-specific RNA was detected by autoradiography after hybridization to a $^{32}$P-labelled DNA fragment containing the DPV E5 gene. The positions of 4.9-kb 28S and 1.9-kb 18S rRNAs are indicated.

formation of C127 cells (28, 29). This allows analysis of the cellular response to E5 transformation in the absence of later and possibly nonspecific events that might occur during stable transformation. To compare the acute transforming activities of the BPV and DPV E5 genes, the BPV E5 gene in the BPV-SV40 recombinant virus was replaced with the DPV E5 gene (Fig. 2). C127 cells at 80 to 90% confluence were infected at high multiplicity with recombinant viruses carrying either the BPV or DPV E5 gene or with the virus bearing a frameshift mutation within the BPV E5 gene. Similar morphological transformation was observed within 24 h after infection with viruses carrying either E5 gene but not after infection with the frameshift mutant (Fig. 4). To assess the acute transforming ability of DPV E5 quantitatively in comparison with that of the BPV E5 protein, DNA synthesis assays were performed. C127 cells made quiescent by contact inhibition and serum starvation were infected at the same multiplicity with the recombinant viruses. After maintenance in serum-free medium for an additional 40 h, DNA synthesis was determined during a 2-h labelling period as incorporation of [$^3$H]thymidine into trichloroacetic acid-precipitable material. Table 1 shows that expression of either the DPV or the BPV E5 gene induced significant levels of cellular DNA synthesis, whereas the frameshift mutant did not lead to a significant increase in DNA synthesis. Thus, the DPV E5 protein efficiently induces both acute and stable transformation of C127 cells.

Our results establish that when the BPV and DPV E5 proteins are expressed in three different sets of isogenic contructs, both are potent transforming agents for C127 cells, inducing stable and acute morphologic transformation and cellular DNA synthesis in quiescent cells. These results confirm the importance of the conserved sequence motifs between the two proteins, including the features required for BPV E5 transformation. These results show that the apparent difference in the abilities of BPV and DPV to transform C127 cells is not due to structural differences between the two E5 proteins. We speculate that the inability of DPV DNA to induce focus formation in C127 cells is due to relatively poor expression of the DPV E5 gene from the DPV genome in these cells. This hypothesis is supported by the recent finding that replacement of the DPV transcriptional long control region with its counterpart from BPV allows efficient C127 cell focus formation by the chimeric DPV genome (16).

We have recently shown that the PDGF receptor is constitutively activated in rodent fibroblasts transformed by the BPV E5 protein (22). Activation was demonstrated by a number of criteria, including increased tyrosine phosphorylation of the 200-kDa mature receptor and a 165-kDa precursor form of the receptor in BPV E5-transformed C127 cells compared with untransformed cells. To test whether the PDGF receptor was tyrosine phosphorylated in C127 cells transformed by the DPV E5 gene, extracts were prepared from cell lines transformed with the retroviral constructs. After immunoprecipitation with anti-PDGF receptor serum and immunoblot analysis with an anti-phosphotyrosine monoclonal antibody, highly tyrosine-phosphorylated 165- and 200-kDa forms of the PDGF receptor were readily detected in cell lines transformed by either the DPV or BPV E5 gene but not in untransformed cells (Fig. 5a). Immunoblot analysis of total phosphotyrosine proteins did not reveal obvious novel phosphotyrosine-containing proteins in DPV E5-transformed cells, other than the PDGF receptor, and immunoblot analysis of whole-cell extracts probed with an anti-PDGF-receptor antibody demonstrated no significant difference in the amounts of the PDGF receptor between DPV E5-transformed and untransformed cell lines (data not shown). To test whether the DPV E5 protein induced tyrosine phosphorylation of the PDGF receptor in the acute transformation system, C127 cells were infected at high multiplicity with the BPV-SV40 recombinant virus carrying either the DPV or the BPV E5 gene or a mutant BPV E5 gene. At 48 h after infection, the cells were lysed and their PDGF receptor was immunoprecipitated with anti-receptor antibody and immunoblotted by using anti-phosphotyrosine antibody. Infection with viruses carrying either the DPV or the BPV E5 protein induced increased tyrosine phosphorylation of both forms of the receptor in comparison with cells infected with the E5 mutant virus (Fig. 5b), thereby establishing that acute expression of the DPV E5 gene induces rapid induction of tyrosine phosphorylation of the PDGF receptor. The ability of both the BPV and DPV E5 proteins to activate the PDGF receptor in both stable and acute transformation systems lends further support to the hypothesis that this activation plays a central role in growth transformation of fibroblasts by fibropapillomavirus E5 proteins (22). We note that the extent of amino acid sequence homology between PDGF B and the E5 proteins is greater for DPV than it is for BPV (22) and that the DPV E5 gene appears to be slightly more active than the BPV E5 gene, as assessed by several different transformation assays using different expression vectors.

Papillomas induced by BPV consist of both an epithelial and a fibroblastic component, whereas the epithelial component of DPV-induced papillomas is minimal. It is not known whether DPV exerts effects on keratinocytes growing in

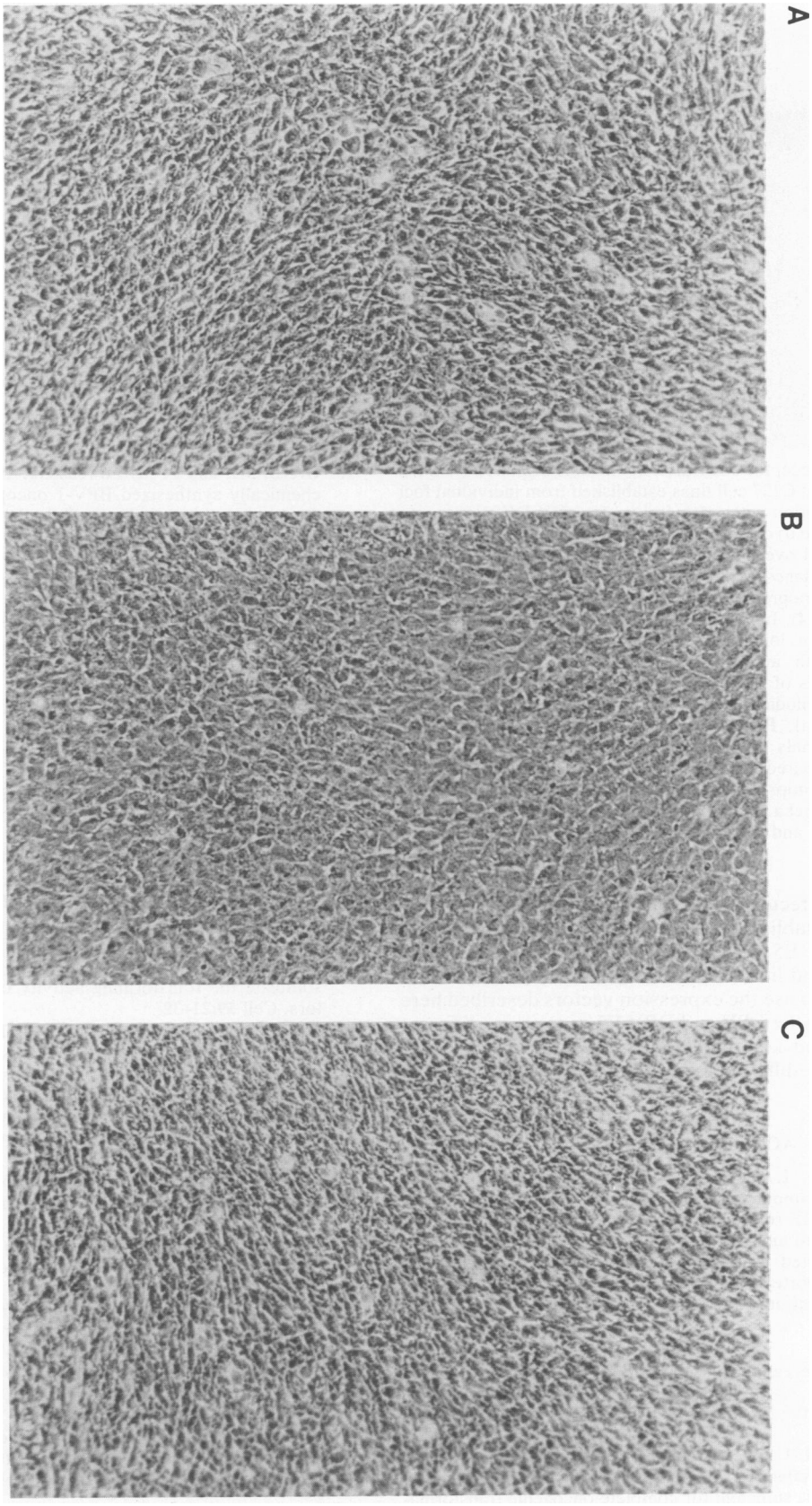


FIG. 4. Acute morphologic transformation of C127 cells by DPV E5. C127 cells approaching confluence in 35-mm-diameter wells were infected at the same multiplicity with high-titer stocks of Pava-1 (A), Pava-E5d29 (B), or Pava-DE5 (C) and maintained in DME-10. Cells were photographed by using phase-contrast microscopy 48 h after infection.

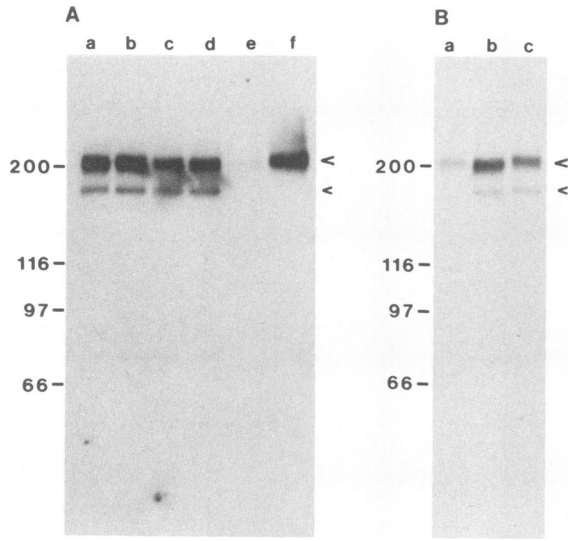


FIG. 5. PDGF receptor activation upon transformation by DPV E5. (a) Transformed C127 cell lines established from individual foci induced by transfection with retroviral vector pRV-DE5 (lanes a to c) or pRV-BE5 (lane d) or normal untransformed C127 cells (lanes e and f) were starved overnight and then incubated in the presence (lane f) or absence (lanes a to e) of 10 ng of PDGF per ml. The PDGF receptor was immunoprecipitated from cell lysates with anti-PDGF receptor serum (PR4). Immunoprecipitates from approximately 400 μg of protein were loaded in each lane, electrophoresed, and immunoblotted with anti-phosphotyrosine monoclonal antibody 4G10. The positions of coelectrophoresed molecular size markers are indicated (in kilodaltons). (b) C127 cells were infected with Pava-E5d29 (lane a), Pava-DE5 (lane b), or Pava-1 (lane c) as described in Materials and Methods. After 48 h in DME-2, cell extracts were prepared and the tyrosine-phosphorylated PDGF receptor was immunoprecipitated and detected by immunoblotting as described for panel a. The arrowheads to the right of both panels indicate the mature and precursor receptor forms.

culture, but transfected BPV DNA alters the differentiation program of an established line of murine keratinocytes, and the isolated BPV E5 gene can cause tumorigenic transformation of a second line of murine keratinocytes (17, 25). It may be possible to use the expression vectors described here to assess whether the BPV and DPV E5 genes have different effects on cultured keratinocytes and whether these genes contribute to the difference in tissue tropism observed in vivo.

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