JOURNAL OF VIROLOGY, Dec. 1991, p. 7078–7083
0022-538X/91/127078-06$02.00/0

Vol. 65, No. 12

Tumorigenic Transformation of Murine Keratinocytes by the E5 Genes of Bovine Papillomavirus Type 1 and Human Papillomavirus Type 16

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Received 8 July 1991/Accepted 16 September 1991

To examine the biological properties of the bovine papillomavirus type 1 (BPV) and human papillomavirus type 16 (HPV16) E5 genes, each was cloned separately into a retroviral expression vector and helper-free recombinant viruses were generated in packaging cell lines. The BPV E5 retroviruses efficiently caused morphologic and tumorigenic transformation of cultured lines of murine fibroblasts, whereas the HPV16 E5 viruses were inactive in these assays. In contrast, infection of the p117 established line of murine epidermal keratinocytes with either the BPV or the HPV16 E5 retrovirus resulted in the generation of tumorigenic cells. Pam212 murine keratinocytes were also transformed to tumorigenicity by the HPV16 E5 gene but not by the gene carrying a frameshift mutation. These results establish that the HPV16 E5 gene is a transforming gene in cells related to its normal host epithelial cells.

The papillomaviruses are small DNA viruses that induce tumor formation in their natural hosts, including humans (for a review, see reference 27). The true papillomaviruses, such as human papillomavirus type 16 (HPV16), induce the formation of papillomas consisting exclusively of epithelial cells. In contrast, the fibropapillomaviruses, such as bovine papillomavirus type 1 (BPV), induce the formation of skin fibropapillomas consisting of proliferating dermal fibroblasts as well as epidermal keratinocytes. The fibropapillomaviruses also differ from the true papillomaviruses in their ability to efficiently transform rodent fibroblast cell lines in culture (reviewed in reference 9).

The ability of BPV to transform fibroblasts in vitro is due in large part to the viral E5 protein, which is well conserved among the various fibropapillomaviruses (10, 17, 24, 29, 32). The BPV E5 protein is a very hydrophobic, 44-amino-acid, membrane-associated protein that associates with a 16-kDa subunit of the vacuolar H^+-ATPase (5, 14, 15, 30). In addition to transforming fibroblasts in the absence of other transfected genes, the E5 gene can stimulate the transforming ability of cotransfected genes encoding cell surface receptors, and it stabilizes heterologous epidermal growth factor receptors at the cell surface (19). We have recently shown that the BPV E5 protein activates the cellular platelet-derived growth factor (PDGF) β receptor in transformed fibroblasts and have proposed that the PDGF receptor is an important cellular mediator of the fibroblast-transforming activity of the fibropapillomavirus E5 proteins (26).

HPV16 and related viruses can immortalize and affect the differentiation of primary human keratinocytes, an activity that has been mapped to their E6 and E7 genes (9). Transfected BPV DNA also affects the differentiation of murine keratinocytes (28), but the role of specific BPV genes in keratinocytes has not been determined. The frequent retention and expression of the HPV E6 and E7 genes in the human carcinomas associated with HPV16 and HPV18 infection suggest that these genes may play a role in keratinocyte transformation in vivo (34). In addition to the E6 and E7 proteins, many of the HPVs are predicted to encode short, hydrophobic E5 proteins that are only distantly related to the fibropapillomavirus E5 proteins (4, 16). The weak transforming activity of HPV16 and HPV18 in cultured rodent fibroblasts resides primarily in the E7 gene, but it has been speculated that other viral genes, including E5, may also affect growth control in these cells (2, 9, 31). In the experiments described here, we have tested the transforming activity of the BPV and HPV16 E5 genes in keratinocytes, which are one of the natural target cell types for BPV and the only natural target cell for HPV transformation in vivo. Our results demonstrate that the E5 genes from both BPV and HPV16 can induce tumorigenic transformation of established epidermal keratinocytes, whereas only the BPV E5 gene can efficiently transform fibroblasts.

To introduce papillomavirus E5 genes into cells, the coding region plus minimal 5′ and 3′ flanking sequences were cloned into a retrovirus vector under the transcriptional control of the viral long terminal repeat (LTR) (Fig. 1). These clones were introduced into ψCRE and PA317 packaging cell lines (8, 20) to generate replication-defective, helper-free retroviral stocks which were used to infect tester cells in the presence of 4 μg of Polybrene per ml. It was possible to monitor successful infection and stable integration of a provirus because the viruses contained a neomycin resistance gene that conferred resistance to 600 μg of G418 per ml (200 μg/ml for infected p117 cells).

Activity of E5 genes in fibroblasts. NIH 3T3 fibroblasts and C127 cells were used for the analysis of the E5 genes in murine fibroblast cell lines. Most of the genetic and biological analysis of the BPV E5 protein has been performed in C127 cells, a flat, nontumorigenic cell line derived from a murine mammary carcinoma (12). Although it has not been established whether these cells are of fibroblastic (stromal) or epithelial origin, they contain abundant PDGF β receptors, are efficiently transformed by the fibropapillomaviruses but not by the true papillomaviruses, and upon BPV trans-

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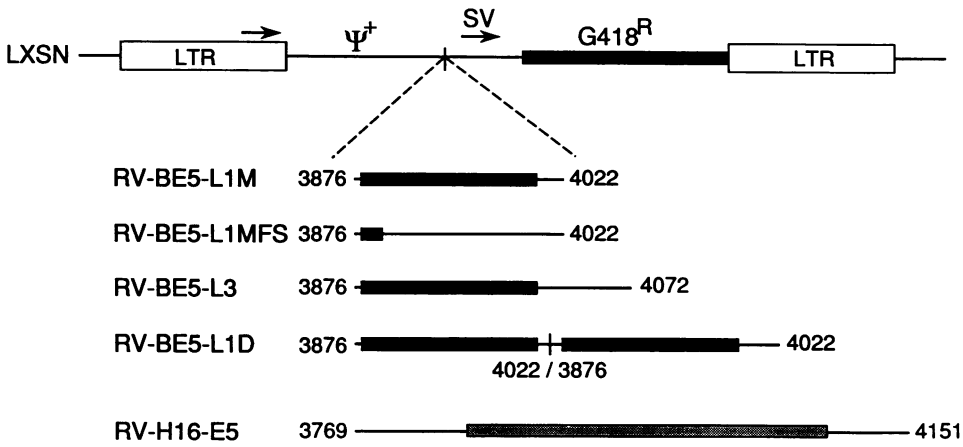

FIG. 1. Maps of the recombinant retroviruses used in these experiments. The top line shows the structure of the original vector, pLXSN (21), which was converted into pLXSN-Sal by insertion of a unique *SalI* site at the position of the short vertical line. Genes inserted at this site are transcribed from left to right from the promoter in the left LTR. The gene encoding resistance to G418 is transcribed from the simian virus 40 early promoter. The bacterial plasmid vector sequences joining the two LTRs are not shown. The maps below the top line indicate the inserted BPV and HPV16 E5 genes, represented by the dark and stippled bars, respectively, and the extent of each insert is indicated by the nucleotide numbers of the parental papillomavirus genomes. The BPV E5 coding region extends from BPV nucleotide 3876 to 4022, and the HPV16 E5 coding region extends from HPV16 nucleotide 3849 to 4098. Standard techniques were used to construct these plasmids, and details are available from the authors upon request. L1M-FS contains a frameshift mutation after the third codon of the BPV E5 gene; L1D contains a head-to-tail tandem dimer insert. Two independent isolates of the HPV16 E5 retrovirus plasmid (pRV-H16E5-1 and pRV-H16E5-6) were generated and analyzed.

formation can give rise to fibrosarcomas in animals. Thus, they are representative of the fibroblastic host cell of the fibropapillomaviruses. C127 cells were infected with approximately 1,000 G418-resistant CFU of various recombinant retroviruses, passaged once, and maintained at confluence without biochemical selection. Recombinant virus stocks containing the BPV E5 gene induced the appearance of numerous transformed foci, whereas no foci appeared after infection with virus stocks of the vector without insert, the vector containing a BPV E5 gene with a frameshift mutation, or two independent isolates of viruses containing the HPV E5 gene (Table 1). Similarly, the HPV16 E5 retroviruses did not induce foci in NIH 3T3 fibroblasts (data not shown). These results indicate that the cloned BPV insert contained sufficient genetic information to induce stable transformation of rodent fibroblasts, whereas the HPV E5 gene displayed no such activity.

TABLE 1. Transforming activities of E5 retroviruses in cultured fibroblasts

Virus	E5 gene	No. of foci in C127 cells	Morphologic transformation (no. of transformed cell lines/no. tested) of[a]:	
			C127 cells	NIH 3T3 cells
RV-LXSN-Sal	None	0	0/20	0/20
RV-H16E5-1	HPV16	0	0/11	0/6
RV-H16E5-6	HPV16	0	0/8	0/6
RV-BE5-L1M	BPV	401	ND[b]	
RV-BE5-L1M-FS	BPV (mutant)	0	0/20	
RV-BE5-L1D	BPV	339	20/20	
RV-BE5-L3	BPV	146	18/20	

[a] All cell lines were derived from individual G418-resistant colonies.
[b] ND, not done.

In a less stringent assay for transformation, C127 and NIH 3T3 cells were infected at a low multiplicity of infection with the E5 retroviruses, G418-resistant colonies were generated, and the behavior of these cells was examined. Many of the colonies induced by viruses carrying the BPV E5 gene contained morphologically transformed cells, whereas infection with the control viruses or with viruses containing the HPV E5 gene did not result in morphologic transformation (data not shown). Stable C127 and NIH 3T3 cell lines were established from individual drug-resistant colonies picked at random, and their morphologies were assessed (Table 1). The great majority of colonies generated with the BPV E5 viruses gave rise to morphologically transformed cell lines, whereas none of the G418-resistant cell lines derived by infection with the control viruses or the HPV E5 viruses displayed morphologic alterations. The cell lines generated with the BPV E5 gene were also growth transformed as judged by a number of additional criteria, including anchorage independence, higher saturation density, shorter doubling time, and growth in low serum, whereas cell lines generated with the HPV16 E5 gene were indistinguishable from parental cells (data not shown).

To assess the tumorigenicity of fibroblasts infected with the E5 retrovirus, several of the G418-resistant cell lines established from individual colonies or from plates containing at least 50 G418-resistant colonies were injected subcutaneously into 4- to 6-week-old BALB/c nude mice (10^6 cells per injection site). As shown in Table 2, rodent fibroblasts transformed by the BPV E5 gene induced tumor formation at the majority of injection sites. In contrast, cells infected with the vector alone were not tumorigenic. C127 and NIH 3T3 cell lines established by infection with independent stocks of the HPV16 retroviruses also failed to form tumors. Thus, in contrast to cells established with the BPV E5 retroviruses, fibroblasts infected with HPV E5 retroviruses did not display evidence of growth transformation.

TABLE 2. Tumorigenicity of E5 retroviruses

Virus	E5 gene	No. of tumors/no. of injection sites[a] with:			
		C127	NIH 3T3	p117	Pam 212
RV-LXSN-Sal	None	0/12[b]	0/8	0/7	ND[c]
RV-BE5-L3	BPV	15/18[b]	ND	9/10[b]	ND
RV-H16E5-1	HPV16	0/10	0/8	2/3	ND
RV-H16E5-6	HPV16	0/10	0/20[d]	7/9[b]	8/8[b]
RV-H16E5-fs1	HPV16 (mutant)	ND	ND	ND	1/8[b]

[a] Tumor formation was scored after 3 weeks (for C127 cells) or 4 to 5 weeks (for all other cell types). A single pooled cell line was tested in each case, unless otherwise indicated.
[b] Aggregate data for two independently derived pooled cell lines.
[c] ND, not done.
[d] Aggregate data for one pooled cell line and two independent clonal cell lines.

Biochemical analysis of the transformed cell lines documented the expression of the E5 genes. Total RNA was analyzed for E5-specific sequences by Northern (RNA) analysis. As shown in Figure 2, cells infected with either the BPV or HPV E5-containing retroviruses expressed abundant E5 RNA that was absent from parental cells or G418-resistant cells established with the vector alone. The 7-kDa BPV E5 protein was also detectable by immunoprecipitation from metabolically labelled cells (data not shown). We have not documented that the HPV16 E5 protein was expressed in these cells because satisfactory antibodies have not yet been described (16). However, the results presented in the next section indicate that these viruses are able to express biologically active HPV16 E5 protein.

Activity of E5 genes in keratinocytes. The activity of recombinant retroviruses expressing the E5 genes was also assessed in keratinocytes, one of the normal host cell types of BPV1 and the sole normal host cell of HPV16. In vivo tumorigenicity studies were used to assess the ability of the E5 genes to transform these cells because morphologic transformation and focus formation are difficult to detect in cultured keratinocytes. p117 murine epidermal keratinocytes, a nontumorigenic cell line (11, 22), were propagated in minimal essential medium containing low levels of calcium (0.05 mM) and supplemented with growth factors as described previously (22) and were infected with retrovirus stocks prepared in the same medium. Pooled populations of G418-resistant p117 cells were established following infection at the same multiplicity of infection with the control virus and viruses encoding the BPV and HPV16 E5 genes. An assay of the culture medium of G418-resistant p117 cells demonstrated the absence of viruses able to transduce G418 resistance (data not shown). BPV E5 RNA and protein were readily detected in these cells, as was HPV16 E5 RNA (Fig. 2 and data not shown). Subcutaneous injection of p117 cells infected with either the BPV or the HPV16 E5 viruses resulted in the appearance of tumors at most injection sites, whereas cells infected with the control virus were nontumorigenic (Table 2). Parental p117 cells and p117 cells infected with a variety of other retrovirus vectors were also nontumorigenic (11) (data not shown). These results were obtained with cell lines established from multiple independent infections with retroviruses transducing either the BPV or HPV16 E5 gene. Histologic examination of these tumors revealed several features consistent with their being squamous cell tumors, including well-developed intercellular bridges, dys-

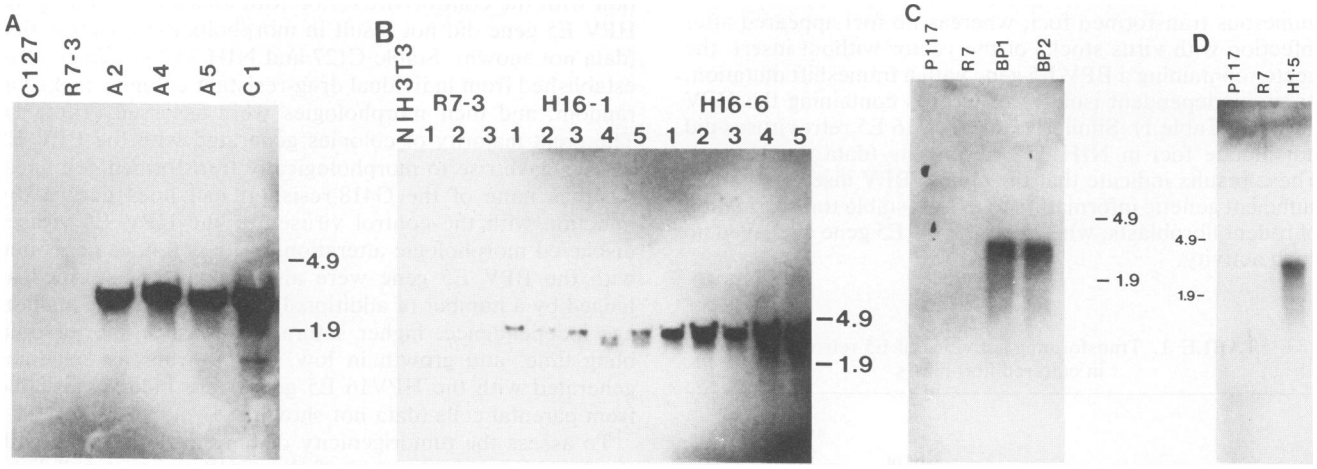

FIG. 2. E5 RNA in mouse fibroblasts and keratinocytes. Total RNA was prepared (7) from normal cells or G418-resistant cell lines established either from individual, independent drug-resistant colonies or from pooled colonies for the p117-derived cell lines induced by infection with retrovirus stocks. Ten micrograms of each sample was electrophoresed in a 1% agarose gel containing formaldehyde, transferred to nitrocellulose, and hybridized to a uniformly ^{32}P-labelled probe by using random priming of purified restriction fragments (13). Bands were visualized by autoradiography. The positions of 28S (4.9) and 18S (1.9) rRNA are indicated. (A) C127 cells and derivatives, identified as follows: C127, normal C127 cells; R7-3, cell line isolated following infection with control virus RV-LXSN-Sal; A2, A4, A5, and C1, cell lines isolated following infection with RV-BE5-L3. (B) NIH 3T3 cells and derivatives, identified as follows: NIH 3T3, normal NIH 3T3 cells; R7-3 (lanes 1 to 3), cell lines isolated following infection with control virus RV-LXSN-Sal; H16-1 (lanes 1 to 6), cell lines isolated following infection with RV-H16E5-1; H16-6 (lanes 1 to 6), cell lines isolated following infection with RV-H16E5-6. (C and D) p117 cells and derivatives, identified as follows: p117, normal cells; R7, pooled G418-resistant p117 cells established following infection with control virus RV-LXSN-Sal; BP-1 and BP-2, two independent pools of G418-resistant p117 cells infected with RV-BE5-L3; H1-5, pooled G418-resistant p117 cells infected with RV-H16E5-1. The probe used was BPV nucleotides 3881 to 4450 (panels A and C) or HPV16 nucleotides 3769 to 4151 (panels B and D). Rehybridization of the filters with an actin probe demonstrated that all samples from G418-resistant cell lines contained similar amounts of intact RNA (data not shown).

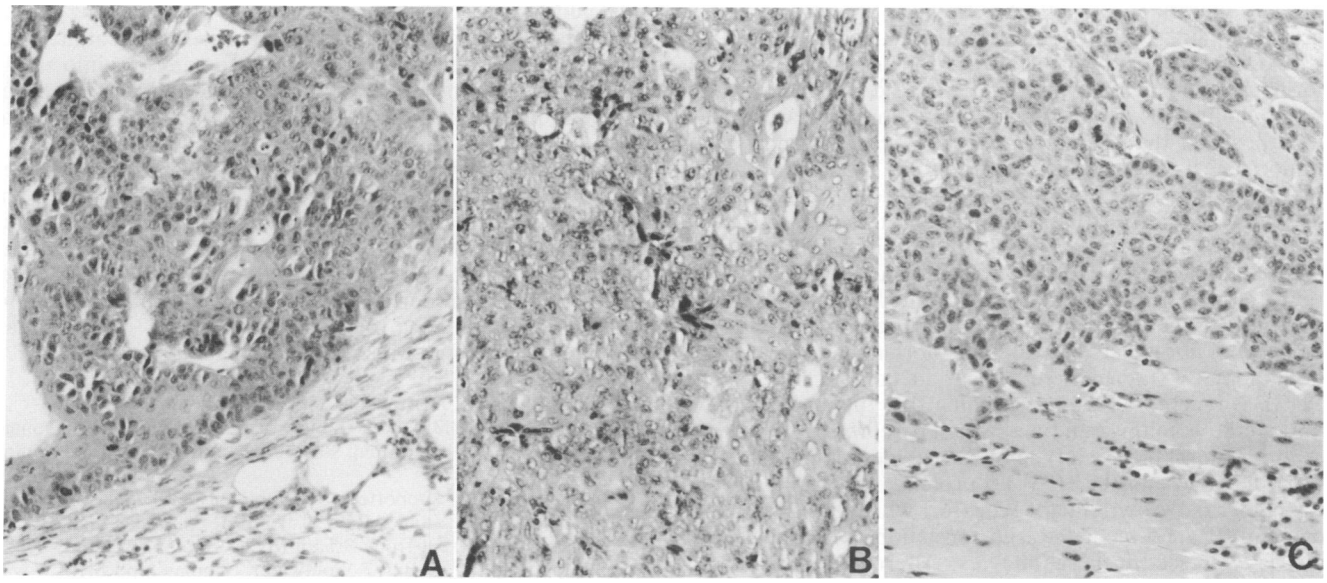

FIG. 3. Histologic appearance of tumors formed by p117 cells expressing the E5 genes. (A and B) HPV16 E5 tumors. Note the squamous differentiation with presence of basal and spinous layers in panel A (magnification, ×200). Note scattered horn pearls and moderate cytological atypia in panel B (magnification, ×400). (C) BPV E5 tumor. Note the tumor cells growing between muscle fibers and the presence of numerous mitotic figures (magnification, ×200).

keratotic cells, and horn pearls (Fig. 3). In general, the tumors induced by the BPV E5 viruses appeared to be squamous cell carcinomas. They grew more rapidly and to a larger size than the HPV16 E5 tumors, appearing histologically to be less differentiated and more invasive, infiltrating underlying connective tissue and muscle in five of nine tumors examined. These tumors also showed frequent mitotic figures and atypical nuclei. The tumors induced by the HPV16 E5 retroviruses displayed features consistant with their being benign papillomas or well-differentiated squamous cell carcinomas. They were well delimited and circumscribed, and some of them were cystic with numerous papillas and keratinized centers. These results establish that introduction of either the BPV or HPV16 E5 gene can induce tumorigenic transformation of the p117 keratinocyte cell line.

To confirm these results with a second, unrelated keratinocyte cell line and to verify that the transforming activity of the HPV16 E5 retrovirus was due to the E5 gene, we introduced a frameshift mutation into the E5 gene so that only the first two codons are translated in frame. The transforming activities of the wild-type and mutant HPV16 E5 retroviruses were tested in recloned Pam212 cells, an established line of murine keratinocytes (33), which grow considerably better that the p117 cells and do not require specialized growth medium. As shown in Table 2, the Pam212 cells infected with the wild-type HPV16 E5 gene efficiently induce tumors, whereas the frameshift mutant displayed minimal activity (comparable to that of uninfected cells) (data not shown). The HPV E5-induced Pam212 tumors appeared to be more aggressive than the HPV E5-induced p117 tumors, displaying a heterogeneous appearance with areas of squamous differentiation, cells appearing to form ductlike structures, and relative lack of differentiation within the same tumor. Infiltration into surrounding muscle was readily apparent. These results indicate that the ability of the HPV16 E5 retrovirus to transform murine epidermal keratinocytes to tumorigenicity is a consequence of the expression of the HPV16 E5 gene.

Discussion. The results reported here demonstrate that HPV16 and BPV E5 genes can transform keratinocytes to tumorigenicity. The HPV16 E5 gene, which has not previously been established to be a transforming gene, now emerges as a possible contributor to the neoplastic proliferation induced by HPV16 in vivo. In the case of the fibropapillomaviruses, our results suggest that the E5 genes may play a role in inducing both fibroblastic and epithelial proliferation during natural infection.

p117 cells have proven useful in assessing the differential effects of various oncogenes on skin carcinogenesis in vivo (11). Although these cells are consistently nontumorigenic upon subcutaneous injection into nude mice (11, 22), they have undoubtedly undergone significant genetic and/or physiological alterations during their derivation. For example, they form benign papillomas after being skin grafted onto mice (22), are TGF-β resistant (23), and are likely to have suffered an activating *ras* mutation, which is a common event during chemical carcinogenesis of mouse skin (1). Thus, they are a sensitive indicator cell line to detect additional changes that can drive a minimally transformed keratinocyte to outright tumorigenicity. However, the tumorigenic conversion induced by papillomavirus E5 genes in p117 keratinocytes is not a peculiarity of this cell line, since the HPV16 E5 gene also transformed Pam212 cells, an unrelated murine keratinocyte cell line. Our experiments do not indicate whether papillomavirus E5 genes are sufficient to induce keratinocyte transformation or whether other changes are also required. The possibility exists that the E5 protein may not be sufficient for immortalization and transformation in vivo, but rather that it may elicit its effects in cooperation with cellular genetic events or with other viral genes, particularly the E6 and E7 genes. We are currently testing the effects of papillomavirus E5 genes alone and in combination with the HPV E6 and E7 genes in normal,

primary keratinocytes. If the HPV16 E5 gene exerts a growth stimulatory effect in normal human epithelial cells, it would suggest that this gene plays a role in human papilloma formation and carcinogenesis. Since the E5 gene is only infrequently present in HPV-associated human carcinomas, it is possible that it acts at an early stage during human tumorigenesis and that it is not required later on.

Our results indicate that papillomavirus E5 genes can activate biochemical pathways in keratinocytes to cause tumorigenic transformation. On the basis of our previous studies, we have proposed that the activated PDGF β receptor is an important intracellular intermediate that transduces the transforming activity of the fibropapillomavirus E5 proteins in fibroblasts (18, 26). The normal and transformed keratinocytes studied here appear to contain little if any PDGF β receptors (25a), suggesting that this mechanism is not operative in this cell type, which is representative of the second natural host cell type of BPV. One possible target of both the BPV and the HPV E5 proteins in keratinocytes is the epidermal growth factor receptor, which has been implicated in BPV E5-mediated transformation of fibroblasts (19) and is expressed in keratinocytes. Alternatively, the interaction between the BPV E5 protein and the ATPase subunit, which has been documented in epithelial cells, may play a role in keratinocyte transformation (14, 15). Biochemical and physiological analysis of E5-transformed keratinocytes may identify the signal transduction pathways activated in these cells.

We were unable to document the fibroblast-transforming activity of the HPV 16 E5 gene by using expression vectors, host cells, and transformation assays which readily detected the transforming activity of the BPV E5 gene. Thus, the BPV and the HPV16 E5 genes can both induce tumorigenic transformation of cultured murine epidermal keratinocytes, but only the viruses carrying the BPV E5 gene display significant transforming activity in fibroblasts. This difference parallels the different tissue tropisms of the two papillomavirus types, with BPV inducing fibropapillomas (consisting of both mesenchymal and epithelial proliferative components) and HPV16 inducing papillomas (consisting exclusively of epithelial cells). Therefore, it is tempting to speculate that differential transforming activities of the E5 proteins contributes to the tissue tropisms of these virus types during natural infection. However, Chen and Mounts (6) have recently reported that the HPV6c E5a gene is able to induce morphologic transformation in NIH 3T3 and C127 cells, even though HPV6c normally induces purely epithelial lesions in humans. We do not know whether this difference between the HPV16 and HPV6c E5 genes reflects differences in the assay systems employed or intrinsic differences between the E5 genes of the two virus types.

In addition to suggesting a role of the papillomavirus E5 genes in keratinocyte transformation, the studies reported here conclusively show that the structure of the BPV E5 protein is entirely specified by the recognized E5 coding region and that this protein is sufficient to induce tumorigenic transformation. We previously reported that efficient tumorigenesis or induction of anchorage independence by the full-length BPV genome in C127 cells requires the BPV E6 and E7 genes in addition to the E5 gene (25). We speculate that overexpression of the BPV E5 gene from the viral LTR accounts for the increased transforming activity of the gene in the experiments reported here. Full transformation of C127 cells by a retrovirus containing the BPV E5 gene but not the E6 or E7 genes has been reported by Bergman et al. (3), but in that case the upstream reading frames were deleted by a not-well-characterized event occurring during virus propagation in animal cells. Because the great majority of colonies expressing the BPV E5 gene develop into stably growth-transformed cells, rare cellular events do not appear to be required for stable E5-mediated fibroblast transformation.

The papillomaviruses have emerged as leading candidates for human tumor viruses and as useful model systems for determining the viral basis of cell transformation. It is becoming clear that these viruses have multiple transforming genes that may play relatively discrete roles in tumorigenesis. The results reported here suggest that the E5 proteins play a role in causing the pathogenic effects of the human papillomaviruses as well as the fibropapillomaviruses.

C.L. and S.R.C. made equal contributions to this project.

We thank A. D. Miller for the gift of pLXSN and M. McDonald and D. Devore for performing some of the initial molecular cloning steps.

This work was supported by Public Health Service grants CA37157, CA16038, and AR39190 from the National Institutes of Health and by a grant from Bristol-Myers Squibb Company. C.L. was supported by a Barry Goldwater Scholarship, S.R.C. was supported by a fellowship from the Ministerio Español de Sanidad, R.K. was supported by a Deutsche Forschungsgemeinschaft Fellowship, B.H.H. was supported by the Medical Scientist Training Program, and D.J.R. was supported by the Howard Hughes Medical Institute.

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