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The Products of the E5, E6, or E7 Open Reading Frames of RhPV 1 Can Individually Transform NIH 3T3 Cells or in Cotransfections with Activated Ras Can Transform Primary Rodent Epithelial Cells

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Rhesus papillomavirus (RhPV) type 1 was recently shown to cooperate with the activated *ras* oncogene to transform primary rodent epithelial cells at a level comparable to HPV 16. In similar cotransfection studies, subgenomic portions of RhPV 1 driven by either their natural or a strong heterologous promoter were used in primary baby rat kidney cells to demonstrate that transforming properties of RhPV 1 could be localized individually to the E5, E6, and E7 open reading frames. Fully transformed cells were observed when either E5 or E7 were downstream of a strong heterologous promoter. Similarly, either E6 or E6 and E7 downstream of the native promoter fully transformed these cells as determined by immortalization, anchorage independent growth and tumorigenicity studies.

Human papillomaviruses (HPV) of the genital track are generally regarded as belonging to groups which are characterized as highly (*1–4*) or minimally oncogenic (*5*). These portrayals are based upon epidemiologic, molecular, and pathological correlations *in vivo*, transformation studies *in vitro*, and tumorigenicity studies in experimental animals. We have previously demonstrated that by these criteria a papillomavirus derived from a metastatic tumor of a Rhesus monkey (RhPV 1) falls into the highly oncogenic category which also contains HPV 16 and HPV 18 (*6–8*). One might, therefore, expect other parallels in function at a subgenomic level.

In various *in vitro* systems E6 and E7 ORFs have been implicated as oncoproteins (*9–27*). Each protein product binds to a cellular protein which controls cellular growth (*9, 10*). These early region genes from HPV 16 and HPV 18 DNAs can individually or in tandem immortalize human cervical and foreskin keratinocytes, or primary rat brain cells (*11–16*). E6 (*19*) and/or E7 have also been shown to be able to transform various established cell lines as determined by producing morphologically altered cells, acquiring anchorage-independent growth, and/or producing tumors in nude mice (*14, 17–19*). Under the control of homologous or heterologous transcriptional elements HPV 16, 18, 31, and 33 DNAs cooperate with either Ha-*ras* or v-*fos* oncogenes to fully transform primary rodent or human cells (*20–26*). Finally, CRPV E6 and E7 each were found to transform cells continuous cell lines (*27*).

E5, the major transforming protein of bovine papillomavirus (*28–29*), has been shown to activate epidermal growth factor receptors EGFR (*30*) and platelet-derived growth factor receptor (PDGR) (*31*) and binds to a 16-kDa vacuolar ATPase which is a component of gap-junction-like membrane complexes (*32*). CRPV E5 could not transform continuous cell lines (*27*); however, E5 of BPV 1, HPV 16, and HPV 6 could (*33–34*). It was also reported that HPV 16 E5 has some transforming potential in murine keratinocytes (*35*).

The complete RhPV 1 has been previously shown to transform cells only in the presence of activated Ha-*ras* (*8*). We sought to localize the regions of the genome responsible for this transforming potential by two complementary routes. First, each of the three ORFs, E5, E6, and E7, which have previously been shown to be critical for transformation by other papillomaviruses, was cloned into the eukaryotic expression vector pLNCX under the control of a heterologous strong promoter (pLNCE5, pLNCE6, and pLNCE7, respectively). pLNCX and pLNSX (*36*) contain a neomycin resistance gene following a murine LTR promoter as well as promoters from CMV or SV40, respectively, upstream of multiple cloning sites (Fig. 1). Oligonucleotide primers modified to contain a *Hin*dIII site (primer set 1, Table 1) downstream of the classic TATA box (nt 67) which bracketed the following methionine codon (nt 103) and the termination codon were used in a polymerase chain reaction (PCR) amplification of the E6 region from RhPV 1. The resulting PCR product was ligated into the *Hin*dIII site of pLNCX to generate pLNCE6 which ex-

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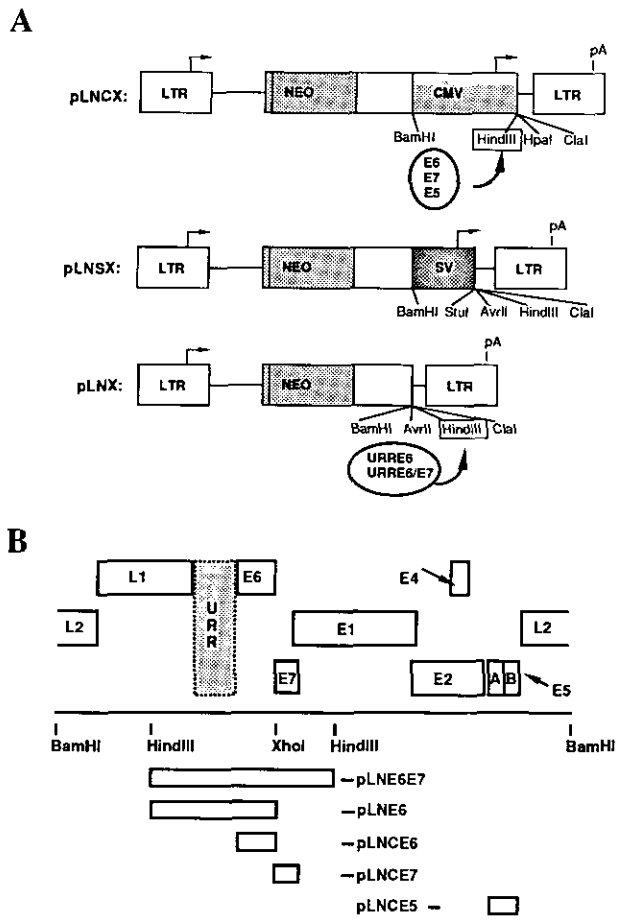

FIG. 1. Expression plasmids and RhPV gene insertions. (A) Shown schematically are the parent expression vectors, pLNCX, pLNSX, and pLNX. The portions of the RhPV 1 genome which were inserted into each respective vector is indicated in the circles. An ATG was inserted in-frame at the beginning of the original E5 ORF in pLNCE5 which naturally lacked a start codon. LTR, MoMLV retroviral long terminal repeat; NEO, neomycin resistance gene; CMV, cytomegalovirus promoter region; SV, SV40 promoter region; pA, splice acceptor and poly adenylation signals. (B) Shown above are the open reading frames of RhPV 1 and below key restriction endonuclease sites and those portions of the RhPV 1 genome which were used to produce the indicated expression vectors. Not shown are the subclones of pLNCE5 which give rise to the clones pLNCE5-2, pLNCE5A, and pLNCE5B.

presses the E6 ORF from a strong heterologous CMV promoter and includes codons for five amino acids of the E7 ORF. A similar method (primer set 2) was used to generate pLNCE7 which expresses the E7 ORF. A slight modification of this method was necessary for the expression of the E5 ORF. As the RhPV 1 E5 ORF as originally described (*37*) did not contain a methionine codon, one was incorporated to generate pLNCE5 (primer set 3). All PCR generated clones were completely sequenced for correct structure and orientation. After sequencing pLNCE5 it became apparent that there was a single nucleotide difference from our published sequence which consists of an insertion of a "T" between the two "G"s at nucleotides 4143 and 4144. Reexamination of our original genomic sequencing gels confirmed that this was the correct sequence. This gives rise to two shorter ORFs, one of which spans nucleotides 4044 to 4287 and contains a methionine codon at nucleotide 4167. Sequence comparisons of this ORF with HPV 16, 18, 6, and BPV 1 E5 proteins indicate that some homology with BPV 1 (six contiguous amino acids) exists upstream of this methionine, and some homology with the HPVs exists downstream of the methionine. However, comparisons are difficult due to the vast differences in sizes and compositions of various E5 open reading frames. As our original RhPV 1 E5 expression clone, pLNCE5, contained this entire region, we further pursued the identification and characterization of those sequences which represent the true RhPV 1 E5 gene. Thus, we created two new eukaryotic expression clones. One (pLNCE5-2, primer set 4) includes the region containing the entire new ORF and methionine while creating a new ATG to translate the upstream sequences. A subset of this region containing only those sequences including and downstream of the natural methionine (nt 4167) was cloned to produce pLNCE5A (primer set 5). Finally, one other small open reading frame (pLNCE5B, primer set 6) containing a natural methionine from nucleotides 4310 to 4442 was similarly cloned.

We also examined the transforming properties of the E6 and E6/E7 ORFs while under the control of their native promoter (pLNE6 and pLNE6E7, respectively). A new vector designated pLNX was generated which removed the SV40 promoter of pLNSX (Fig. 1) by deleting sequences between the *Bam*HI and *Stu*I and reconstituting the *Bam*HI site. To generate pLNE6E7 which contains the upstream regulatory region (URR), E6, E7, and a small part of the E1 regions, the 2.9-kb DNA fragment of pRhPV 1 (*8*) was ligated into the *Hin*dIII site of pLNX. The correct orientation and structure was confirmed by nucleotide sequence analysis and comparison with the known genomic sequence (*37*). The 2.9-kb RhPV 1 *Hin*dIII fragment was also treated with *Xho*I (at nucleotide 616), blunt-ended, attached to *Hin*dIII linkers, and ligated into *Hin*dIII-cleaved pLNX to generate pLNE6 which contains the RhPV 1 URR and the E6 ORF and has nucleotides coding for eight amino acids of E7, which are in a different reading frame. Sequence comparison of RhPV 1 with other HPVs (*38*) indicate that the possibility of initiation of a partial E7 gene product would not produce a complicating result as this would not contain critical features necessary for E7 transformation such as CD1 or CD2 regions, casein kinase II phosphorylation sites (*39*), or Cys–X–X–Cys motifs (*40*).

Transformation assays were performed using BRK cells in cotransfections with Ha-*ras* and test plasmid and were selected by G418 resistance (*8, 41*). Cells of

TABLE 1

OLIGONUCLEOTIDES USED FOR AMPLIFICATION

Primer set	Sequence[a]	RhPV nucleotides	ORF[b]	Plasmid
1	GCTCCTGAA*GCTT*TGG	75–90	E6	pLNCE6
	CCTCGAGGG*AAGCTT*AGGCCC	621–600		
2	*ATAATAAGCTT*GCAAGCATGATTGG	585–601	E7	pLNCE7
	CTGGTG*AAGCTT*CAGGGTCC	962–943		
3	*ATATAAGCTT*GAA*TG*GTGGTGTGCATTGG	3936–3956	E5	pLNCE5
	*AATTAAGCTT*GCGCCGCCGTCTCAGCTG	4440–4422		
4	TGCTA*AGCTT*GTAAA*TG*GCCACCATTCTGC	4030–4059	E5	pLNCE5-2
	AACTA*AGCTT*AACATGTCTATGTGCAG	4325–4299		
5	TTCC*AAGCTT*GCCTCAGTGTTGCTGC	4139–4164	E5A	pLNCE5A
	AACTA*AGCTT*AACATGTCTATGTGCAG	4325–4299		
6	GATG*AAGCTT*CTGCACATAGACATGTT	4289–4315	E5B	pLNCE5B
	GTGT*AAGCTT*TATTATCTGCGCCGCCG	4457–4431		
7	GCGAGCAGCGTGAGGAGA	158–175	E6	
	GCAAGAAACTACTGCCC	589–573		
8	TTGTTGCTGCTACGCTTG	4023–4040	E5	
	GCTGGTGGCTTTAAACGC	4399–4382		

[a] Upstream and downstream primer pairs. Changes (plain text italics) in the native sequence (bold) introduced *Hin*dIII restriction endonuclease sites (underlined). Natural and artificial ATG sites are double underlined.

[b] ORF, open reading frame.

some plates were fixed and stained 3 to 4 weeks later with 3% crystal violet in methanol. Representative colonies containing either flat or morphologically transformed cells were isolated in a cloning ring, and transferred to new plates. Cells which grew and could be passaged for 6 months beyond the senescence of control cells were then described as immortalized. In multiple experiments using different plasmid preparations, morphologically transformed colonies were observed for pLNCE5, pLNCE7, pLNE6, and pLNE6E7 in cotransfections (Table 2). We have now identified the minimal transforming region of E5 as the region from 4167 to 4293 (represented by pLNCE5A) using transformation assays (Table 2). Continuous cell lines were derived from individual colonies for each of the RhPV 1 constructs which produced morphologically transformed cells. In addition to morphologically transformed cell lines, some flat, but immortalized cell lines were derived from nontransformed colonies (Table 2). No continuous cell lines were derived from transfections with the pLNCE6 plasmid. Rounded morphology was observed in many of the colonies when Ha-*ras* was cotransfected with the adenovirus E1 a plasmid, genomic HPV 16, or RhPV 1 DNA into BRK cells (Table 2). Primary BRK cells senesced within a few weeks.

Southern blot analysis (*8, 42*) showed that the DNA for each construct was present in the cell lines. Hybridization probes consisted of the entire individual E5, E6, or E7 regions of RhPV 1 separated from the appropriate expression vectors and labeled with ^{32}P by the random primer method. After cleavage of cellular DNA with *Hin*dIII which separates the viral insert from the vector, bands were observed which comigrated with the insert as well as many bands which migrated more slowly indicating integration of the plasmid sequences (data not shown). mRNA was isolated from cells using a FastTrack kit (Invitrogen, San Diego, CA), electrophoresed through 1% agarose gels, and transferred to nylon membranes for Northern blot analysis. E5-specific bands were observed for genomic RhPV 1 and pLNCE5 transformed cell lines, and E7-specific bands were observed for RhPV 1, pLNE6E7, and pLNCE7 transformed cells. However, we were unable to detect E6 mRNA in any cell line at this level of detection. None of the probes detected any bands in normal BRK mRNA (data not shown). Discrete bands could be observed which were accompanied by a background smear in some cases (data not shown). Rehybridization of the blots with an actin gene probe produced a single tight band indicating that the mRNA had not been generally degraded during the preparation or analysis (data not shown). To increase sensitivity, each of the mRNA preparations was subjected to treatment in the presence or absence of reverse transcriptase followed by polymerase chain reaction (PCR) amplification. mRNA analysis utilized the primers indicated for E7 (primer set 2), E6 (primer set 7), or E5 (primer set 8 for pLNCE5) incubated with M-MLV reverse transcriptase (BRL,

TABLE 2

IMMORTALIZATION AND MORPHOLOGIC TRANSFORMATION

DNA	Colonies[a] Experiment 1	Experiment 2	Experiment 3	Experiment 4	Expansion[b]
ras[c] + pLNCX	0	0	0	0	1/6
ras + E1a[d]	>100	>100			**2/2**
ras + HPV16	1	>100	>100	>100	**5/6**
ras + genomic RhPV 1	67	113			**6/6**
ras + pLNCE5	32		11	13	**6/6**
ras + pLNCE5-2			7	11	
ras + pLNCE5A			18	21	
ras + pLNCE5B			0	0	
ras + pLNCE6	0	0			0/4
ras + pLNCE7	9	2			**4/6**
ras + pLNE6	12	2			0/1 **5/5**
ras + pLNE6E7	2	3			1/2 **4/4**

[a] Total *morphologically transformed* colonies per 10 μg of test plasmid in Experiments 1 and 2 and total *morphologically transformed* cells from four independent transfections of 5 μg in each of Experiments 3 and 4. Transfections that did not contain pLNCX or its derivatives contained the plasmid pSV2NEO.

[b] Number expanded over number attempted, bold numbers represent attempts at expanding morphologically transformed cells, plain text are attempts to expand flat colonies which occassionally appeared.

[c] ras is the pEJ6.6 plasmid containing the Ha-*ras* oncogene derived from the EJ bladder carcinoma cell line (Shih and Weinberg, 1982).

[d] E1a is the pCE plasmid containing the adenovirus type 5 E1a gene.

Gaithersburg MD) and nucleotide precursors according to the manufacturer's instructions followed by boiling for 5 min, addition of PCR buffers and *Taq* polymerase, and 40 cycles of amplification yielding products of 377, 432, and 376 bp, respectively. Following amplification, DNA was electrophoresed in a 1.2% agarose gel and transferred to a membrane. Bands of appropriate sizes were detected in cell lines containing E7, E6, or E5 genes, respectively, when hybridized to a mixed (Fig. 2), or gene-specific DNA probes (data not shown). No bands were observed in mRNA from normal BRK cells, in reagent controls, or in mRNA samples lacking reverse transcriptase which eliminated the possibility of DNA contamination of the mRNA samples (data not shown). When mRNA from the E5A transformed cell lines were tested by reverse transcriptase–PCR using primer set 5, an appropriately sized band of 187 nucleotides was observed (data not shown). No bands were observed when reverse transcriptase was omitted (data not shown) or if BRK mRNA was used (Fig. 2). Thus all expanded cell lines tested contained the appropriate gene(s) and these genes were expressed.

Morphologically transformed BRK cells were found to exhibit anchorage-independent growth confirming their transformed nature (Table 3) (*8*). Growth in soft agar was generally strong for most cell lines, but some variation was observed (Table 3). pLNCE5A transformed cells also grew in soft agar with high efficiency (data not shown). Positive controls included BRK cells

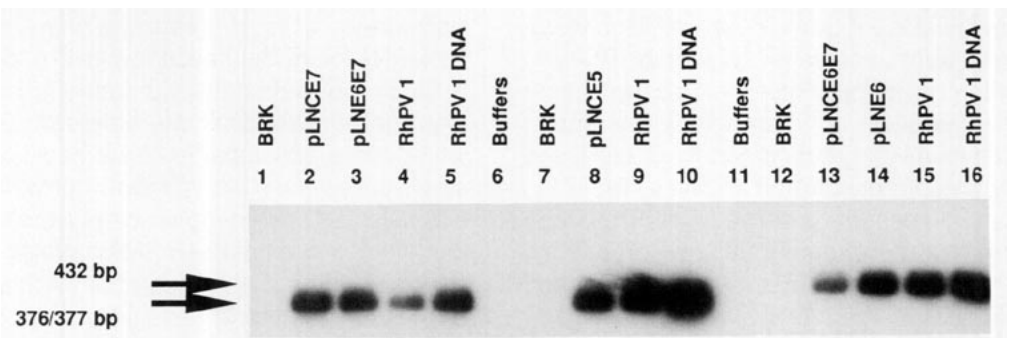

FIG. 2. Analysis of mRNA which was reverse transcribed and amplified from various RhPV 1 DNA-containing cells. mRNA was isolated from cell lines as indicated, treated with reverse transcriptase, subjected to PCR amplification for 40 cycles using E7, E5, or E6 primers sets, electrophoresed in 1.2% agarose, transferred to nylon membranes, and hybridized to a mixed ^{32}P-labeled probe made from the eluted E5, E6, and E7 ORFs of RhPV 1. Similar results were obtained when lanes 1–6, 7–11, and 12–16 were individually hybridized to the E7, E5, and E6 DNA probes, respectively (data not shown). No hybridization was observed in mRNA samples if reverse transcriptase was omitted (data not shown). Lanes labeled "RhPV 1 DNA" were genomic DNA template positive controls.

TABLE 3

ANCHORAGE INDEPENDENT GROWTH AND TUMORIGENICITY

DNA[a]	Clone name	Morphology[b]	Soft agar[c]	Tumorigenicity[d]
ras[e] + pLNCX	5A1	N	–	0/3
ras + E1a[f] + pSV2Neo[g]	3A1	T	++	
	3A2	T	++	
ras + HPV16 + pSV2Neo	3B1	T	+++	3/3
	3B2	T	+++	
	3B3	T	+++	
ras + genomic RhPV 1 + pSV2Neo	4A1	T	+++	3/3
	4A2	T	+++	3/3
	4B1	T	++	3/3
ras + pLNCE5	6A1	T	–	
	6A2	T	–	
	6A3	T	+	0/3
	6A4	T	+++	3/3
	6A5	T	+++	3/3
	6A6	T	+++	
ras + pLNCE7	8A2	T	+	
	8A3	T	+++	3/3
	8B1	T	+	3/3
ras + pLNE6	13A1	T	+++	2/3
	13A2	T	+++	
	13A3	T	+++	3/3
	13B1	T	++	
	13B2	T	+++	1/3
ras + pLNE6E7	16A1	T	+++	3/3
	16B1	T	+++	3/3
	16B2	T	++	1/3
	16B3	T	++	
	16B4	N	–	
Cells: Primary BRK			–	0/3

[a] Genes contained within expanded clones.
[b] T, transformed, N, not transformed in appearance.
[c] Percentage of cells which grew in soft agar are indicated: –<1%, +<20%, ++20–40%, +++>40%.
[d] Tumor growth after 16 days, results are reported as number of animals with tumors/number of animals tested.
[e] ras in the pEJ6.6 plasmid containing the Ha-*ras* oncogene derived from the EJ bladder carcinoma cell line.
[f] E1a is the pCE plasmid containing the adenovirus type 5 E1a gene.
[g] pSV2Neo, an expression plasmid for G418 resistance.

transformed in cotransfections with activated Ha-*ras* and genomic RhPV 1 or HPV 16 plasmids. Freshly explanted BRK cells did not grow in soft agar. Various cell lines that were immortalized but not morphologically transformed did not grow in soft agar. This included a single immortalized cell line derived from cotransfection of Ha-*ras* and the vector pLNCX (Table 3).

pLNCE5, pLNCE7, and pLNE6 were each transfected into NIH 3T3 cells along with a plasmid containing a neomycin resistance marker. Transformed colonies were observed with each of these plasmids. Clones were expanded and examined for DNA by Southern blot analysis. Each clone maintained the papillomavirus gene with which they were transformed and expressed the viral gene as determined by Northern blot analysis (data not shown). In addition, both the pLNCE5 and pLNCE7 clones grew in soft agar whereas NIH 3T3 cells alone did not. pLNCE5A was also found to transform NIH 3T3 cells with high efficiency, whereas pLNCE5B did not (data not shown).

Representative cell lines were injected into athymic nude mice to test for tumorigenicity (*8*). Tumors were observed in most animals which had been injected with cells previously observed to grow in soft agar (Table 3). The HPV 16 and genomic RhPV 1 cell lines produced tumors. Neither the BRK nor a single immortalized but morphologically flat and anchorage-dependent cell line arising from a pLNCX vector control transfection produced any tumors. Two of the three pLNCE5 cell lines produced tumors in all animals. Two

pLNCE7-containing cell lines produced tumors in all animals. Six of the nine animals injected with pLNE6-containing cells produced tumors. Seven of the nine animals injected with pLNE6E7-containing cells produced tumors. Passage number did not seem to be important in this observation (data not shown). Pathological analysis indicated tumors were undifferentiated carcinomas (data not shown).

We previously reported that RhPV 1 DNA was isolated from a rhesus monkey lymph node metastasis of a penile squamous cell carcinoma (*6*), that it appears to be transmitted sexually (*7*), and that the complete genome was capable of cooperating with activated *ras* in transforming primary epithelial cells *in vitro* thus demonstrating a tumorigenic potential at least equal to that of the sexually transmitted human oncogenic papillomavirus HPV 16 (*8*). We have expanded these studies by examining the oncogenic potential of three major RhPV 1 open reading frames which in other types of papillomaviruses have been shown to interact with cellular factors which control cellular proliferation as indicated above.

We observed in cotransfections with activated Ha-*ras* transformation of BRK cells by pLNE6E7, which contained the native RhPV 1 URR and the E6 and E7 ORFs. We also observed transformation with pLNE6 containing the native URR and the E6 ORF and with the E5 or E7 ORFs downstream of a heterologous promoter. It was determined that the minimal region of our original E5 clone which was necessary for transformation was represented by the region designated as E5A. We assume that all large clones which contained E5A as a subset were able to produce a protein from the native downstream methionine even though we had introduced artificial methionines upstream. All of the cell lines transformed by these plasmids maintained and expressed the appropriate viral genes. Most morphologically transformed cells could be expanded and could grow in soft agar and form tumors in nude mice which supports the hypothesis that each of these gene products maintains some oncogenic potential. One might also expect that if pLNE6 transformed cells, then the E6 downstream of a strong heterologous promoter would be more efficient. However, this could relate to the levels of RNA and protein produced by each construct. In any event, these studies indicate that under the appropriate conditions, the E5, E6, or E7 ORFs can individually provide sufficient properties in cotransfections with activated *ras* to fully transform primary epithelial cells. In partial confirmation, we have also observed that pLNCE5, pLNCE5A, and pLNCE7 were able to transform both NIH 3T3 cells and that the resulting cell lines could grow in soft agar. It has recently been reported that E6, but not E7, of HPV 33 in NIH 3T3 cells had a weak tumorigenic capability, although no anchorage independence was induced (*43*). Our results would seem to confirm that the E6 ORF alone can have transforming properties as others have shown for heterologously driven HPV 16 E6 as demonstrated by growth in soft agar of transfected keratinocytes and NIH 3T3 cells (*21*). However, our results go further by indicating that this potential exists in primary epithelial cells using the native promoter of RhPV 1 as demonstrated by expansion, morphologic transformation, anchorage-independent growth and tumorigenicity results. Our results with the RhPV 1 E7 gene parallel that found with human oncogenic papillomaviruses. The results of this study using RhPV 1 confirms the oncogenic potential of the E5 ORF from genital transforming papillomaviruses.

The current studies have thus demonstrated that the same three genes in RhPV 1 can even transform primary epithelial cells in cooperation with activated *ras*. This confirmed that RhPV 1 maintains characteristics of the oncogenic human papillomavirus types and further may suggest that all three of the genes studied here may also express sufficient oncogenic properties in the human papillomavirus counterparts.

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