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Rhesus Papillomavirus Type 1 Cooperates with Activated *ras* in Transforming Primary Epithelial Rat Cells Independent of Dexamethasone

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Rhesus Papillomavirus type 1 (RhPV-1) was recently cloned from a rhesus monkey lymph node metastasis of a penile squamous cell carcinoma. In this paper, we demonstrate that RhPV-1 cooperates with the activated *ras* oncogene to transform primary cells at a level comparable to human papillomavirus type 16. The viral DNAs were cloned such that their expression was under the control of their natural promoter elements. Unlike human papillomavirus type 16, RhPV-1 DNA cooperated with *ras* independently of the hormone dexamethasone. However, dexamethasone did have a positive influence on the ability of some RhPV-1 cotransformed cells to grow in soft-agar assays. The transformed cells are highly tumorigenic in vivo in nude mice.

Human papillomaviruses (HPV) are small, epitheliotropic DNA viruses. More than 60 genotypes associated with specific anatomical sites have been isolated to date. A subset of these is associated with the development of anogenital tumors. Some (HPV-16, -18, and -33) are closely associated with anogenital dysplasia and neoplasia (1, 5, 15, 19), while others (HPV-6 and HPV-11) are usually restricted to condylomata and low-grade cervical intraepithelial lesions (23, 24). Another group (HPV-5 and HPV-8) is associated with epidermodysplasia verruciformis, an autosomal recessive disease characterized by disseminated flat warts that, when exposed to sunlight, can develop into cutaneous squamous cell carcinoma (13). Clinical and experimental evidence strongly suggests that HPV infection by itself is insufficient to produce malignancy, implicating the requirement of other factors in the progression from a benign to a malignant state (24, 25). Moreover, HPV-related malignancies appear to develop after a long latency and are dependent on the HPV type, host, and environmental factors. This model is consistent with the concept of multistage carcinogenesis.

Viral DNA is found integrated into the cellular chromosomal DNA in the majority of HPV-related malignancies (6), often with deletions or rearrangements within the E1 and/or E2 open reading frame (ORF) (1, 15, 19). Interruption of the viral genome may be important to the transcriptional activity of the virus since the E2 gene products are capable of *trans*-activating as well as *trans*-repressing early viral gene expression by binding to elements found within the upstream regulatory region (URR) (2, 8, 11, 22). Conversely, the E6 and E7 ORFs are unaltered and efficiently expressed in tumors (15, 18). Furthermore, in various in vitro systems E6 and E7 ORFs have been implicated as oncoproteins (3, 7, 12, 16).

The early-region genes from HPV-16 and HPV-18 DNAs can extend the life span (establish) of human cervical and foreskin keratinocytes (4, 9, 17). Moreover, HPV-16, -18, -31, and -33 DNAs when under the control of heterologous transcriptional elements cooperate with either Ha-*ras* or v-*fos* oncogenes to fully transform transfected primary rodent cells (3, 21). HPV-16 under homologous control requires the presence of dexamethasone for Ha-*ras* cooperation, but not when cotransfected with c-*myc* (3, 12). The requirement in vitro for HPV to cooperate with another oncogene to transform cells emphasizes the importance of factors in addition to HPV that are important in the development of HPV-related cancer. For instance, HPV transcription mediated through the URRs has been shown to respond to a complex system of regulatory factors which include the products of the E2 gene (2, 11, 22) and steroid hormone responsive elements (8), as well as a large number of epithelial cell transcription factors (2, 8).

We have recently reported the cloning of a novel papillomavirus from a rhesus monkey lymph node metastasis of a penile squamous cell carcinoma (rhesus papillomavirus type 1 [RhPV-1]) (10) and have shown epidemiological evidence that RhPV-1 is transmitted in rhesus monkeys in a fashion analogous to what is believed to occur in humans and that this virus is associated with the development of malignancies (14). Specifically, RhPV-1 appears to be sexually transmitted since 70% of the animals in a mating cohort were found to have RhPV-1 DNA. Moreover, 10% of these animals were found to have invasive cancers of the genital tract which contained RhPV-1 DNA, in some cases in an integrated state. In the present study we have utilized an in vitro assay of primary baby rat kidney (BRK) cells to investigate the potential transforming capability of RhPV-1 DNA in vitro. We demonstrate that RhPV-1 cooperates with Ha-*ras* in fully transforming these cells independently of the hormone dexamethasone.

Transfection experiments utilized the RhPV-1 genome under its own transcriptional control. The RhPV-1 DNA was cloned into the *Bam*HI site of pUC19 (10) to produce the vector pRhPV-1 (Fig. 1), interrupting the viral DNA within the L2 ORF, therefore allowing its early-region ORFs to be under the transcriptional control of the viral URR promoter elements. pRhPV-1 was transfected into primary BRK cells alone or together with the Ha-*ras* oncogene contained in pEJ6.6 (20). Adenovirus E1a (pCE; obtained from N. Jones of the Imperial Cancer Research Fund) and HPV-16 and

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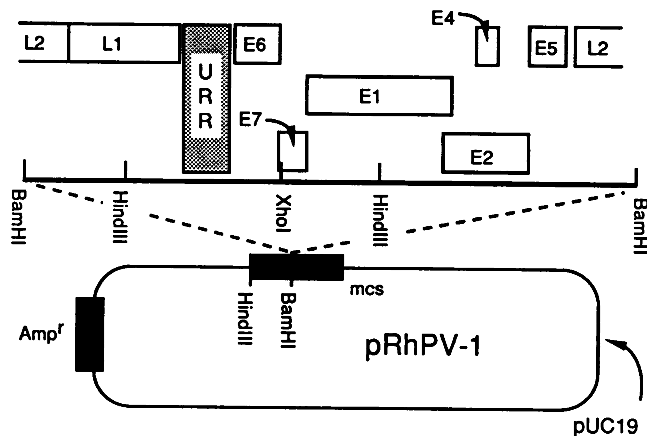

FIG. 1. Structure of pRhPV-1. pRhPV-1 was cloned into the *Bam*HI site of pUC19 in such a manner as to break the papillomavirus DNA within the L2 ORF. The ORFs, indicated by boxes, are in codon register (complete sequence in reference 14a). Restriction sites are indicated, and msc refers to multiple cloning site. The shaded box represents the URR.

HPV-11 DNAs were used for comparison of transforming frequencies and growth properties. The prototype HPV-16 and HPV-11 plasmids were kindly provided by H. zur Hausen and L. Gissmann. Primary BRK cells from Fischer rats were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cells were transfected by the DNA-calcium phosphate coprecipitation method. Aliquots (0.5 ml) of transfection cocktail containing 3 μg of the indicated plasmids were added to 60-mm dishes, which were passed onto 90-mm dishes after 2 days, at which time the selection with hormone was started. Following transfection, cells were grown in either the presence or the absence of 10^{-6} M dexamethasone. Transformation was monitored by the appearance of rapidly growing, dense foci 3 to 4 weeks posttransfection. Foci were isolated, expanded as single-cell clones, and subsequently analyzed for establishment, growth rate, anchorage independence, and tumorigenicity. The results from several repeated experiments are shown in Table 1. As expected, pEJ6.6 DNA alone never

TABLE 1. Cotransformation of primary BRK cells by various papillomaviruses and Ha-*ras*

Transfected plasmid	No. of foci per plate					Cloning efficiency (%)[a]
	10^{-6} M dexamethasone			Without dexamethasone		
	Expt 1	Expt 2	Expt 3	Expt 2	Expt 3	
pEJ6.6[b]	0	0	0	0	0	
pCE[c]/pEJ6.6	87	78	29	74	29	100 (10/10)
pRhPV-1	0	1	0	0	0	0 (0/1)
pRhPV-1/pEJ6.6	5	9	14	9	21	75 (18/24)
HPV-16	0	1	1	0	0	0 (0/2)
HPV-16/pEJ6.6	4	6	5	0	0	67 10/15
HPV-11	0	0	0	0	0	
HPV-11/pEJ6.6	0	0	0	0	0	

[a] Relative percentage of foci which could be expanded into continuous cell lines. Number of cell lines expanded/number tested is shown in parentheses.
[b] pEJ6.6 plasmid contains the Ha-*ras* oncogene derived from the EJ bladder carcinoma cell line.
[c] pCE plasmid contains the adenovirus type 5 Ela gene.

produced foci, whereas the DNA combination pCE/pEJ6.6 produced easily expandable foci with or without dexamethasone.

When either RhPV-1 or HPV-16 DNA was transfected alone, occasional foci were detected, but all attempts to clone and expand these failed. However, the transfection of BRK cells in the presence of dexamethasone with the combination of pEJ6.6 and either pRhPV-1 or HPV-16 DNA produced similar numbers of foci after 10 days that were easily established into continuous cell lines. The combination HPV-16/pEJ6.6 produced foci only in the presence of hormone, whereas RhPV-1/pEJ6.6 produced foci independently of exogenous dexamethasone. Cotransfection utilizing the nononcogenic HPV-11 DNA produced no foci. After repeated experiments it was noticed that the focus-forming frequency of RhPV-1/pEJ6.6 was reproducibly ~30% less in the presence of dexamethasone, suggesting a slight inhibiting effect on transformation (Table 1). Several of the above expanded foci were characterized in more detail.

The presence of pRhPV-1 DNA in the cell lines was verified by Southern blot hybridization analysis (Fig. 2A). Cellular DNA was isolated by sodium dodecyl sulfate lysis and proteinase K digestion. DNA (10 μg) was then digested with excess restriction endonuclease (*Hin*dIII), electrophoresed through 0.8% agarose gels, and transferred onto nylon membranes by Southern blotting. The hybridization probe consisted of the entire RhPV-1 plasmid labeled with ^{32}P by the random primer method. Bands of 1.5, 2.9, and 5.4 kb were detected in all lines derived from cotransfection of pRhPV-1/pEJ6.6, confirming the presence and integrity of the viral DNA. The viral DNA was present in high copy number. To demonstrate the integrated state of the viral DNA, uncleaved DNA was electrophoresed in a two-dimensional gel and transferred to a nylon membrane as described previously (10) (Fig. 2B). RhPV-1 DNA-related sequences were only found to comigrate with chromosomal DNA, indicating the integrated state of the viral DNA in all four clones tested. Expression of RhPV-1 was determined by Northern (RNA) blot analysis. RNA was isolated from cells by the guanidinium isothiocyanate-cesium chloride method (12). Total cellular RNA (20 μg) was electrophoresed through 1% agarose gels and transferred to nylon membranes. Northern blots utilized a ^{32}P-labeled *Hin*dIII fragment probe (6,690 to 1,540 bp), which hybridizes to the E6 and E7 ORFs. The analysis indicated the presence of several RNA bands that hybridize to a probe derived from the E6/E7 region of the viral genome, verifying that these lines were expressing RhPV-1-related sequences (data not shown).

The growth properties of established cell lines were evaluated by counting cell numbers over a period of 78 h. For growth rate analysis, 5×10^4 cells were seeded in 60-mm dishes and grown in Dulbecco modified Eagle medium in the presence of 0.5 or 10% fetal calf serum with or without 10^{-6} M dexamethasone and counted daily. Cell lines derived from either pRhPV-1 or HPV-16 cotransformants grew at similar rates and to similar saturation densities in both low- and high-serum-containing media. However, their growth rates were much slower in low serum (0.5%). In comparison, pCE/pEJ6.6-derived lines (which grew at roughly similar rates and densities in both high- and low-serum levels) grew more rapidly than RhPV-1 lines based on time to confluency in low serum, but not in high serum (data not shown). Freshly explanted primary BRK cells, as expected, grew well in high serum and were growth arrested in low serum.

To analyze further the effects of serum starvation and confluency on the growth parameters of these cells, a flow

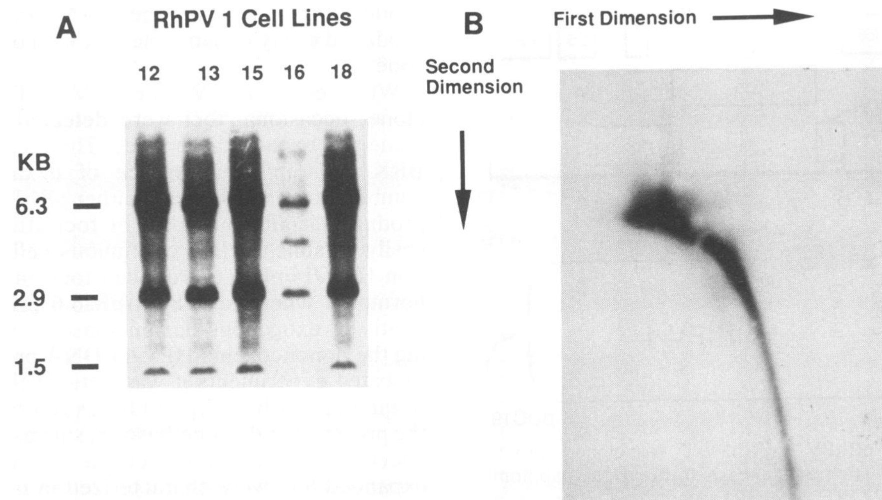

FIG. 2. (A) Southern blot analysis of RhPV-1 DNA sequences in transformed BRK monoclonal cell lines derived from cotransfection with pEJ6.6 (Ha-*ras*). Cellular DNA was digested with *Hin*dIII and hybridized to a ^{32}P-labeled probe made from the entire pRhPV-1. The expected band sizes corresponding to the RhPV-1 plasmid are 1.5, 2.9, and 6.3 kb, which result from the viral *Hin*dIII sites at bp 1541 and 6692 and one *Hin*dIII site in the vector multiple cloning site. (B) Two-dimensional gel analysis of uncut transformed cell line DNA demonstrating the integrated state of RhPV-1 DNA.

cytometric bromodeoxyuridine (BrdU) labeling assay was used. Growth of cells was determined by the increase in the cell number over time and by the proportion of cells in the S and G2+M phases of the cell cycle at various states of culture confluency. Cell cycle analysis was performed by flow cytometry, using a FACS-Star plus flow cytometer (Becton Dickinson, Mountainview, Calif.), by methods described previously (22). Cells were labeled with 10 μM BrdU for 30 mins, followed by fixation, denaturation, neutralization in 100 mM sodium tetraborate, and staining with the monoclonal anti-BrdU antibody (Becton Dickinson). Labeling reactions with primary antibody or secondary reactions with phycoerythrin-labeled goat antimurine antibodies (Becton Dickinson) were incubated at room temperature for 40 min, followed by two washes with phosphate-buffered saline (PBS). All cell lines were also stained with nonspecific anti-human immunoglobulin G antibody staining by fluorochrome-labeled immunoglobulin G1 murine sera to determine the background and autofluorescence (Becton Dickinson). The values in Table 2 represent the percentage of total cells counted which incorporated BrdU, therefore indicating the proportion of cells in the S, G2, and M phases of the cell cycle. Two different experiments were performed: one measured the effect of confluency, while the other measured the effect of serum levels on the growth of the transformed cells

TABLE 2. Growth analysis of transformed cell lines

Cell line[a]	% (±2% SE) of total cells which incorporated BrdU					
	Confluency		Serum			
	50%	100%	0%	2.5%	5%	10%
Rh-r1	15	16	15	18	18	18
BRK	24	10	0	11	16	25
HeLa	24	21	9	24	24	24
H16-r	18	18	13	15	17	17

[a] Rh-r lines were derived by cotransfection of pRhPV-1/pEJ6.6; pCE-r, by cotransfection of pCE/pEJ6.6; and H16-r, by cotransfection of HPV-16/pEJ6.6.

compared with their normal counterparts (BRK cells). The difference between experiments demonstrate that the papillomavirus-transformed cells continue to grow despite confluency and serum deprivation, whereas the nontransfected BRK cells grow rapidly only in high serum and low confluency, thus exhibiting contact inhibition and growth arrest in conditions of confluency and serum deprivation. Therefore, the papillomavirus-transformed cells display growth properties common to fully transformed cells.

The level of morphological transformation of the various cell lines was further analyzed by anchorage-independent growth and tumorigenicity assays (Table 3). Growth in soft agar was measured in the presence or absence of dexamethasone. pCE/pEJ6.6 cell lines grew well in both conditions, producing large colonies in soft agar, whereas HPV-16/pEJ6.6 cells grew only in the presence of dexamethasone. By contrast, the pRhPV-1/pEJ6.6-derived lines demonstrated a spectrum of results. Several lines (Rh-r1, -12, and -13) produced colonies regardless of the presence or absence of hormone, whereas others (Rh-r4, -15, and -18) displayed a dependence on dexamethasone for soft-agar growth. The pRhPV-1/pEJ6.6 cell lines that failed to grow in soft agar in the absence of hormone mimic HPV-16/pEJ6.6-derived lines; however, unlike HPV-16-transformed cell lines, they grew well without dexamethasone in normal medium growth curves (see above).

Cell line tumorigenicity was determined in nude BALB/c *nu*/*nu* mice, bred and housed in pathogen-free conditions. Cells grown in culture were resuspended by washing with PBS (0.15 M NaCl, 0.014 M KH_2PO_4, 0.086 M K_2HPO_4) followed by trypsinization. The cell suspension was washed twice with PBS, and the volume was adjusted to a density of 10^6 cells per ml. All animals received 0.1 ml of cell suspension, injected subcutaneously in both flanks with a 19-gauge needle. Tumors were either quick frozen in liquid nitrogen for subsequent nucleic acid extraction or fixed in buffered Formalin, thin sliced, and stained for histological analysis. Mice injected with cells cotransfected with pRhPV-1/pEJ6.6 DNA produced massive, rapidly growing tumors within 10

TABLE 3. Analysis of soft-agar growth and tumorigenicity of transformed cell lines

Cell line	Soft-agar growth[a]		Tumorigenicity (no. of animals with tumors)	
	10^{-6} M dexamethasone	Without dexamethasone	Expt 1 (n = 5)	Expt 2 (n = 3)
pCE-r	+++	+++	5	3
Rh-r1	++	++	ND[b]	3
Rh-r4	+	−	ND	3
Rh-r12	++	++	5	3
Rh-r13	+++	+++	5	3
Rh-r15	+	−	ND	3
Rh-r18	+++	−	5	3
H16-r	++	−	ND	3
BRK	−	−	0	0
NIH 3T3	ND	−	ND	3[c]
Rh/3T3	ND	+++	ND	3[d]
H16/3T3	ND	+++	ND	3[d]

[a] Percentage of cells which grew in soft agar are indicated: −, <1%; +, <20%; ++, 20 to 40%; +++, >40%.
[b] ND, not done.
[c] Tumor was a small benign nodule, noninvasive.
[d] Tumor aggressively invaded the surrounding muscle tissue.

days in 100% of the animals tested. Mice injected with pCE/pEJ6.6 also produced tumors, but these grew slower than the RhPV-1-derived tumors. Primary BRK cells did not produce any tumors. Histopathology analysis revealed that in most cases the pCE and pRhPV-1 tumors were undifferentiated carcinomas, although some level of epithelial differentiation was observed in some cases. DNA was extracted from these tumors and analyzed for papillomavirus DNA. As expected, tumor cells maintained RhPV DNA without noticeable rearrangement (data not shown).

Cotransfection in the absence of dexamethasone of NIH 3T3 cells with RhPV-1 and SV2Neo produced foci of cells following G418 selection. These foci were expanded and found to contain RhPV-1 DNA (data not shown). The RhPV-1/NIH 3T3 cell lines displayed anchorage independence in soft-agar assays. Moreover, normal NIH 3T3 cells produced a small benign nodule in tumorigenicity studies in nude mice, whereas the RhPV-1/NIH 3T3-transformed cells produced tumors which aggressively invaded the surrounding muscular tissue.

We reported previously that RhPV-1 DNA was isolated from a rhesus monkey lymph node metastasis of a penile squamous cell carcinoma (10), and the initial characterization of this virus suggests that it behaves biologically like the human oncogenic HPVs rather than the nononcogenic ones. Similar to HPV-16 in human tumors, RhPV-1 DNA was found to be integrated within cellular DNA in a head-to-tail fashion with ~100 integrated copies in the lymph node metastatsis. In comparison, the nononcogenic HPV-6 and HPV-11 DNAs frequently associated with benign lesions typically maintain only episomal viral DNA, and when they are found in more neoplastic lesions, their DNAs are usually present in both integrated and episomal states. Similar to HPVs epidemiologically, RhPV-1 was found within a mating cohort in ~70% of the individuals. Ten percent of these individuals had invasive cancers, while a further 36% had evidence of low-grade lesions (CIN1) or abnormal appearing cells (koilocytosis) within their genital tracts. The RhPV-1 genome has been sequenced, and some interesting characteristics have been noted (14a). In summary, initial epidemiological, nucleotide sequence, and structural data suggest that RhPV-1 DNA is more closely associated with human oncogenic subtypes than with nononcogenic ones.

The present study broadens this analysis by comparing RhPV-1 DNA with the oncogenic and nononcogenic HPV DNAs in in vitro transformation assays. The ability of viral DNAs to cooperate with other oncogenes in the transformation of primary BRK cells has been used to study several HPV types. To date, only the oncogenic HPV-16, -18, -31, and -33 DNAs have been shown to cotransform primary cells in culture efficiently (3), whereas the nononcogenic types HPV-6 and HPV-11 normally do not transform at detectable levels (although exceptions do exist [12b]). However, Storey et al. (21) demonstrated that, if the E7 gene products from the nononcogenic HPV types 6 and 11 were expressed by a heterologous promoter, they could cooperate with Ha-*ras* to transform BRK cells. In our analysis, the cotransforming frequencies of RhPV-1 and the relatively oncogenic human types (here represented by HPV-16) are comparable. Moreover, like its human counterparts, RhPV-1 DNA cannot transform (establish) primary BRK cells on its own, but requires the cooperation of a second oncogene such as Ha-*ras*. The RhPV-1/Ha-*ras* combination, like HPV-16/Ha-*ras*, when transfected produces fully transformed cells which demonstrate anchorage-independent growth and tumorigenicity in athymic nude mice. Moreover, NIH 3T3 cells transfected with RhPV-1 displayed a transformed phenotype, as determined by increased growth in soft agar and increased tumorigenicity as compare with normal NIH 3T3 cells. This is similar to results found previously for the oncogenic HPV-16.

The transformation assays used in this work generally are thought to measure two different capabilities of an oncogene: the primary BRK assay measures the ability of an oncogene to immortalize and cooperate with *ras*, whereas the NIH 3T3 assay measures its capacity to alter morphologically a cell's transformed state (*ras*-like function). Curiously, oncogenic papillomaviruses including RhPV-1 can display either phenotype one at a time but are incapable of displaying them simultaneously (i.e., transforming primary cells by themselves). Recent evidence in our lab demonstrates that oncogenic papillomaviruses can indeed transform primary BRK cells alone, but depend heavily on the growth factor content of the media (12b).

One feature differs in the transformation process between HPV-16 and RhPV-1. HPV-16 DNA requires the presence of glucocorticoids to transform primary BRK cells cooperatively, presumably due to the increased viral transcription from induction of a glucocorticoid receptor element (GRE) within the URR. Conversely, RhPV-1 DNA cooperates with Ha-*ras* in the absence of hormone; in fact, dexamethasone may slightly inhibit the frequency of transformation. Growth assays (in both media and soft agar) have shown, however, that all lines derived from RhPV-1 transformation grow more rapidly in the presence of dexamethasone (12a). Moreover, some cell lines (Rh-r4, Rh-r15, and Rh-r18) absolutely required the hormone for soft-agar growth. A comparison of the putative RhPV-1 GRE with other known inducible GRE elements found in HPV DNA and with the consensus sequence for a GRE element is shown in Fig. 3. The putative RhPV-1 GRE differs by 3 bp from the consensus sequence, although two of these (residues 1 and 12) are found in other HPV GRE elements known to be responsive to glucocorticoids. Only the change at residue 14 is unique to RhPV-1. Subcloning of the RhPV-1 GRE into a chloramphenicol

Consensus	G G T A C A N N N T G T T C T
RhPV 1	T G T A C A A A A T G C T T T
HPV 16	T G T A C A T T G T G T C A T
HPV 11	G G T A C A T A T T G C C C T
HPV 6	G G T A C A T A T T G C A C T

FIG. 3. Comparison of GRE elements in RhPV-1 and other HPVs with the consensus GRE element. RhPV-1 residues that differ from the consensus sequence are underlined; the highlighted residue is unique to RhPV-1.

acetyltransferase expression vector to determine its responsiveness to dexamethasone as well as other hormones is in progress. It is not known whether dexamethasone increases the transforming potential of papillomaviruses by simply increasing E6/E7 transcription, by modulating differential promoter usage, or by inducing other novel low-copy viral transcripts. Recent evidence within our lab suggests that slight changes within the URR may have dramatic effects on the transforming capabilities of HPV-11 isolates (12b). Moreover, the host- and tissue-specific nature of individual types of papillomaviruses is certainly due in part to the URR's complex regulation of viral transcription. Clearly, by focusing our attention on the differences and similarities among the URRs of various papillomavirus types, we will gain insight into the control of viral transcription and thus viral transformation.

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