

Adeno-associated Virus Inhibits Human Papillomavirus Type 16: A Viral Interaction Implicated in Cervical Cancer¹

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ABSTRACT

Human papillomavirus (HPV) infection, in particular that by HPV type 16, is positively associated with cervical/genital cancer. In contrast, human adeno-associated virus (AAV) infection is negatively associated with these same cancers. AAV has also been found to inhibit the oncogenic properties of a variety of DNA viruses, including bovine papillomavirus type 1, a relative of HPV-16. Taken together, these findings suggested the possibility that AAV and HPV-16 might interact, with AAV inhibiting HPV-16's oncogenic phenotype. Here this hypothesis is addressed using tissue culture assays measuring HPV-16-directed phenotypes. It is found that the cotransfection of AAV type 2 Rep78-positive plasmids resulted in the inhibition of HPV-16 sequence containing plasmids in oncogenic transformation/focus formation, G418-resistant colony formation, and chloramphenicol acetyltransferase expression assays. These data are consistent with the hypothesis that AAV's negative association with cervical cancer is at least partially due to its ability to inhibit HPV-16 expression.

INTRODUCTION

Certain anogenital HPVs,³ particularly type 16, are strongly associated with cervical cancer, as integrated and expressed HPV DNA is found in 70 to 90% of such tissues (1, 2). AAV is another virus which has been isolated from the anogenital area (3, 4). Seroepidemiological studies have found that cervical cancer patients have a lower prevalence of anti-AAV antibodies than people without cancer (5, 6). Thus, these epidemiological findings are consistent with the hypothesis that AAV infection plays a protective role against the development of cervical cancer. One possibility is that AAV may limit HPV-16's role in cervical carcinogenesis. Furthermore, in animal and tissue culture models, AAV interacts with and inhibits the oncogenic properties of a variety of DNA virus types (7-17), including BPV type 1 (7), a close relative of HPV-16. Genetic analysis of the AAV genome has indicated that the Rep78 gene product was responsible for this inhibition (7, 15). AAV is a helper dependent human parvovirus (dependovirus) which requires that the cell be coinfecting with an adeno- or herpes virus in order for productive AAV infection to take place (16, 17). Thus, AAV's life cycle is dependent upon its interaction with other virus types, and AAV might generally be considered to be a parasite of its helper viruses. In this study, we demonstrate that in tissue culture AAV does interact with and inhibit HPV-16-induced oncogenic transformation and HPV-16-directed gene expression.

MATERIALS AND METHODS

Cells and Plasmids. Swiss albino 3T3 (CCL 92; American Type Culture Collection) and C127 mouse fibroblasts were maintained (fed twice weekly) in

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³ The abbreviations used are: HPV, human papillomavirus; AAV, adeno-associated virus; BPV, bovine papillomavirus; CAT, chloramphenicol acetyltransferase; Neo, neomycin resistance gene; nt, nucleotide.

Delbecco's modified Eagle's medium with 7% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). Human HeLa cells (human cervical cancer, CCL2; American Type Culture Collection) were similarly maintained with the exception that 4% iron-supplemented calf serum was used. The plasmids pAT/HPV-16 (18), pSV2-Neo (19), dl52-91/Neo (20), dl52-91 (21), dl10-37 (21), ins96 (20), ins11 (21), ins32 (21), and ins42 (21) have been described previously. The AAV plasmids are derived from AAV type 2 (22). The pHPV-16/marker chimeric plasmids, pL67R, pL67N, and pL67C, containing the EJ-H-*ras* (23), neomycin resistance (19), and CAT (24) gene coding sequences, respectively (Fig. 1), were generated as follows. First, a *Hind*III linker was inserted into the *Nco*I site (nt 863, blunt end produced with T4 DNA polymerase plus dNTPs) of a cloned HPV-16 genome (pAT/HPV-16) to generate pHPV-16/863d3. Next, the *Sph*I-*Hind*III fragment from pSV2-CAT was replaced with the analogous fragment from pHPV-16/863d3 to generate pL67C. The 1.3-kilobase *Sph*I-*Hind*III HPV-16 fragment, which replaced the SV40 early promoter, contained the intact E6 and E7 open reading frames, the P97 promoter, and 500+ base pairs of upstream sequences. Finally, the *Hind*III-*Bam*HI sequences of pL67C, containing the CAT coding sequences, were replaced with either the coding sequences of Neo (from pSV2-Neo) or EJ-H-*ras* to give pL67N and pL67R, respectively. The coding sequences of EJ-H-*ras* inserted into pL67R were from nt 1244 (a *Bss*HI site converted to a *Hind*III site) to nt 6460 (*Bam*HI). The normal promoter of the *ras* gene is located more than 800 base pairs upstream from the *Bss*HI site (25-27).

Transformation Assays. The focus formation (loss of contact inhibition) and G418 resistance assays were carried out by calcium phosphate transfection as described by Chen and Okayama (28). G418 selection, when indicated, was initiated 2 days post-transfection using 400 µg/ml for murine 3T3 cells, or 800 µg/ml for HeLa cells. For the focus formation of Swiss albino 3T3 cells, the cells were reseeded at 2 weeks post-selection, allowed to reach confluence, and then fixed (4% formaldehyde) and stained (methylene blue) at 3 weeks postconfluence. For the focus formation of C127 cells, the cells were fixed (4% formaldehyde) and stained (methylene blue) at 2 weeks post-transfection (no G418 selection). For the G418-resistant colony formation of HeLa cells, the resistant colonies were fixed and stained at 2.5 weeks post-transfection.

Stable CAT Assay. HeLa cells were calcium phosphate transfected with 2 µg of the CAT-containing plasmid (L67C) and 2 µg of the indicated Neo-containing plasmid. After calcium phosphate transfection, the HeLa cells were selected with 800 µg/ml G418. The transfections resulted in approximately 200 G418-resistant colonies using either pSV2-Neo or dl52-91/Neo as the selectable plasmid. Extracts were then made from the cells and assayed for CAT activity at 4 weeks post-selection as described by Gorman *et al.* (24) with the exceptions that the extracts were equalized for protein content by spectroscopic analysis at 280 nm, and the reactions were allowed to proceed overnight.

RESULTS

AAV Inhibits HPV-16-induced Focus Formation of Swiss Albino 3T3 Cells. To observe the potential inhibition of HPV-16 oncogenic transformation by AAV, the assay developed by Phelps *et al.* (29) and Noda *et al.* (30) was used. This assay involves the calcium phosphate transfection and G418 selection of HPV-16 DNA stably introduced into contact-inhibited 3T3 mouse fibroblast cells. Focus formation by these G418-selected cells is then observed. In our assay, 2 G418 Neo plasmids were used. pSV2-Neo (19) has a SV40 early promoter-Neo transcription cassette, and dl52-91/Neo (20) has the same transcription cassette placed in an AAV Rep78-positive background. Swiss albino 3T3 cells, at 80% confluence, were transfected with 2 µg of one of the Neo plasmids plus 2 µg of pAT/HPV-16 (18). The plasmid pAT/HPV-16 contains the full length HPV-16 genome

with the late region disrupted. The cells were selected with 400 μg G418 for 2 weeks, reseeded, then fixed with 4% formaldehyde/phosphate-buffered saline and methylene blue stained at 3 weeks after

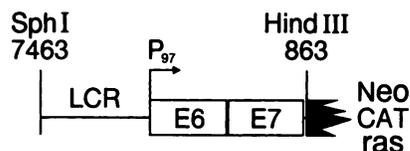
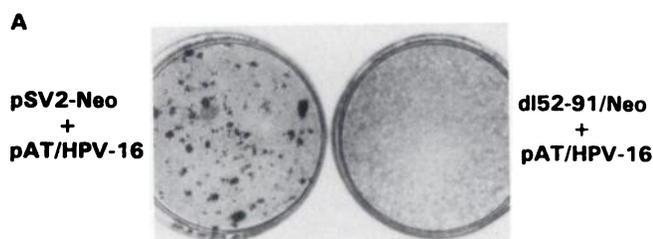


Fig. 1. Structure of the HPV-16 chimeric plasmids, pL67R, pL67N, and pL67C. Represented are the pHVP-16/marker chimeric plasmids, pL67R, pL67N, and pL67C, containing the EJ-H-*ras*, neomycin resistance, and chloramphenicol acetyltransferase gene coding sequences, respectively. Open boxes, indicated open reading frames; bent arrow, P97 promoter; black box with ragged right side, position of the marker coding sequences (ras, Neo, or CAT). The HindIII site at nt 863 was originally an NcoI site. The HPV-16 sequences extend 5' to the SphI site at nt 7463. See "Materials and Methods" for details of the constructions.



B

	Exp1	Exp2	Exp3
pSV2-Neo + pAT/HPV-16	24	39, 19	50
dl52-91/Neo + pAT/HPV-16	1	0, 0	0
pSV2-Neo alone	ND	0	ND
dl52-91/Neo alone	ND	0	ND

Fig. 2. Inhibition of HPV-16 induced oncogenic focus transformation of Swiss albino 3T3 cells by AAV. A, representative plates are shown. B, quantitated results from 3 experiments. Swiss albino 3T3 cells which take up and express the transfected DNAs were selected with G418 (400 $\mu\text{g}/\text{ml}$). Plasmid dl52-91/Neo contains the AAV *rep* region which expresses the Rep78 product. pSV2-Neo does not. Six-cm plates of 80% confluent 3T3 cells were transfected with 2 μg each of the marker plasmid and pAT/HPV-16. The plasmid pAT/HPV-16 contains the full length HPV-16 genome with the late region disrupted. The cells were selected for 2 weeks, reseeded, then fixed with 4% formaldehyde and methylene blue stained at 3 weeks after reaching confluence. Note that there is significant transformation (appearance of foci) in the plates transfected with pSV2-Neo + pAT/HPV-16. In contrast, there is little or no transformation present when dl52-91/Neo is used in place of pSV2-Neo.

reaching confluence. In 3 experiments, shown in Fig. 2, transfection of pAT/HPV-16 with pSV2-Neo resulted in oncogenically transformed foci. In contrast, transfection of pAT/HPV-16 with dl52-91/Neo resulted in a low or zero level of transformation. These data strongly suggested that the AAV Rep78 gene, present in dl52-91/Neo but not pSV2-Neo, limited the number of foci generated by HPV-16.

AAV Inhibits Focus Formation of C127 Cells by an HPV-16/*ras* Chimeric Plasmid and the Inhibition Maps to the AAV Rep78 Gene Product. To further study and verify the inhibition of HPV-16 by AAV, a fast focus formation assay was developed which did not require G418 selection. An HPV-16/*ras* chimeric genome, plasmid pL67R (Fig. 1), was constructed, which was able to generate foci on contact inhibited C127 (murine fibroblasts) cell monolayers at 2 weeks post-transfection. Briefly, pL67R contains the HPV-16 P97 promoter, 500+ bases of upstream sequences, the intact E6 and E7 genes, and replaces the E1-to-L2 genes with activated EJ-H-*ras* coding sequences (23) (see "Materials and Methods" for construction details). This positioning allows *ras* to be expressed from an upstream HPV-16 promoter (probably P97). The EJ-H-*ras* coding sequence was chosen for insertion because HPV-16 E7 and *ras* are known to cooperate in oncogenic transformation (29, 31). The *ras* coding sequence fragment inserted had no transforming activity on its own and did not contain any identified promoter sequences (25-27). To test the ability of AAV to inhibit HPV-16 expression, 5 μg of the pL67R were cotransfected into C127 cells with 10 μg of either AAV plasmid dl10-37 (Rep78⁻) or ins96 (Rep78⁺, wild type AAV) (20). As shown in Fig. 3, A and B, the cotransfection of the Rep78⁺ plasmid ins96 resulted in a 94% inhibition in focus formation (average of 3 experiments). In a genetic analysis of the AAV genome similar to that undertaken in previous studies (7, 32, 33), it was found that AAV genomes with mutations within *rep* at map units 11, 32, and 42 were defective for inhibition, while a large deletion mutation outside of *rep*, from map units 52 to 91, was not defective (21) (Fig. 3C). These data suggest that Rep78, the product from the full length *rep* open reading frame, was responsible for the inhibitory effect.

AAV Inhibits G418-resistant Colony Formation by an HPV-16/Neo Chimeric Plasmid. In a plasmid construction similar to that of pL67R, the Neo coding sequences (19) were ligated downstream of HPV-16 E7 to give the plasmid pL67N (Fig. 1; see "Materials and Methods" for details of construction). The pL67N chimeric plasmid was able to stably convert HeLa cells to G418 resistance upon transfection. Two μg of pL67N plasmid were calcium phosphate

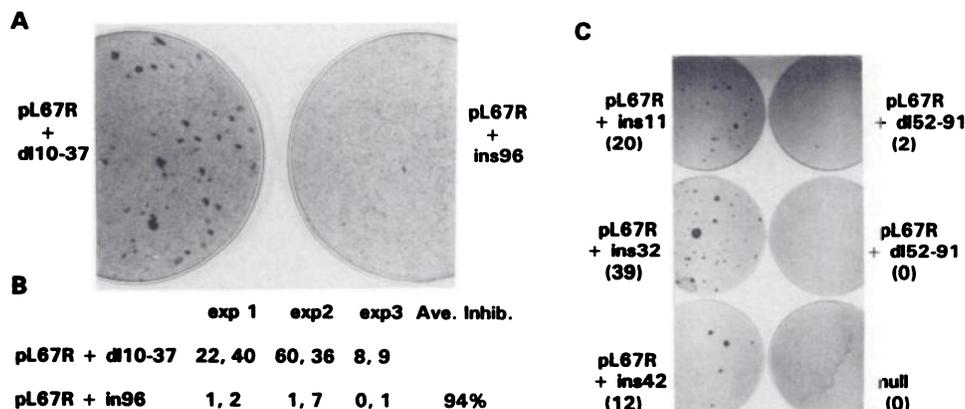


Fig. 3. AAV inhibits oncogenic focus transformation by a chimeric HPV-16/*ras* genome, pL67R. A, representative plates are shown. B, quantitated results from 3 focus formation experiments. Near confluent 10-cm plates of C127 cells were calcium phosphate transfected with 5 μg of pL67R plasmid plus 10 μg of the indicated AAV plasmid. The cells were fed twice weekly with Delbecco's modified Eagle's medium/10% fetal bovine serum for 2 weeks, then fixed with formalin and stained with methylene blue. As shown, the cotransfection of the wild type AAV plasmid, ins96, exhibited a strong inhibitory effect on focus formation by pL67R. C, mapping the oncogenic transformation inhibitor to the AAV Rep78 product. Experimental plates are pictured with the number of foci generated shown (parentheses). The experiment was carried out as in B; 5 μg of pL67R plasmid were transfected with 10 μg of the indicated AAV plasmid. Two different dl52-91 plasmid preparations were used. Mutations at map units 11, 32, or 42, within the AAV genome, knock out AAV's ability to inhibit transformation. All 3 of these mutations affect the Rep78 ORF which extends from map units 7 to 47. In contrast, dl52-91, with an intact Rep78 gene, is able to inhibit pL67R transformation.

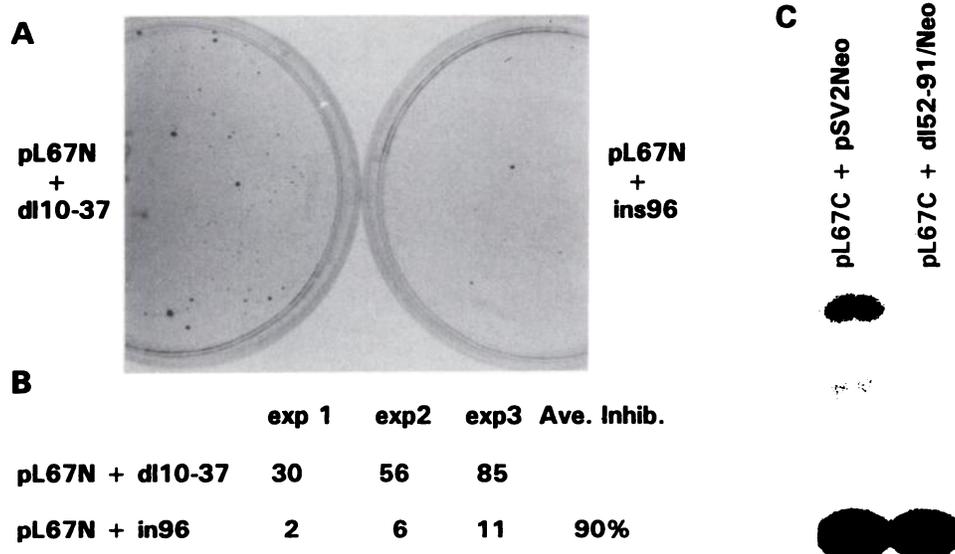


Fig. 4. AAV inhibits G418-resistant colony formation by an HPV-16/Neo chimeric genome, pL67N, and CAT expression by an HPV-16/CAT chimeric genome, pL67C. **A**, representative plates are shown. **B**, quantitated results from 3 G418 selection experiments. Seventy % confluent 10-cm plates of HeLa cells were calcium phosphate transfected with 2 μ g of L67N, HPV-16/neo chimeric plasmid, plus 4 μ g of the indicated AAV plasmid. The cells were selected with 800 μ g/ml G-418 for 1 week, and then 400 μ g/ml thereafter. The cells were fed twice weekly with Delbecco's modified Eagle's medium/4% iron-supplemented calf serum. At 2.5 weeks, the cells were fixed with formalin and stained with methylene blue. As shown, the wild type AAV plasmid, ins96, exhibited a strong inhibitory effect on G-418 colony formation by the pL67N. **C**, representative CAT experiment is shown. It shows AAV inhibition of CAT expression from an HPV-16/CAT chimeric genome, pL67C, in stably transfected HeLa cells. The presence of the Rep78 gene resulted in an inhibition of 79.4 \pm 13.6% (SD) inhibition of CAT expression by pL67C in 3 experiments. Seventy % confluent 10-cm plates of HeLa cells were calcium phosphate transfected with 2 μ g of pL67C plus 2 μ g of either pSV2-Neo or the Rep78-positive AAV plasmid, dl52-91/Neo. The cells were selected with 800 μ g/ml G418 in Delbecco's modified Eagle's medium/4% iron supplemented calf serum. Extracts of the bulk G418-resistant cells were equalized for protein concentration by absorbance at 280 nm. The extract from roughly 10^6 cells were then used in an overnight CAT reaction. As shown, the level of CAT activity is significantly lower in the cells transfected with dl52-91/neo.

cotransfected into HeLa cells with 4 μ g of either AAV plasmid dl10-37 (Rep78⁻) or ins96 (Rep78⁺). The cells were initially selected with 800 μ g/ml G418 starting 2 days post-transfection, and then shifted to 400 μ g/ml G418 after 1 week of selection. The cells were fixed and stained at 2.5 weeks. As shown in Fig. 4, **A** and **B**, the cotransfection of the Rep78⁺ plasmid ins96 resulted in a 90% inhibition in G418-resistant colony formation.

AAV Inhibits CAT Expression by an HPV-16/CAT Chimeric Plasmid. In the plasmid pL67C (Fig. 1), a third marker gene, CAT (24), was also ligated into HPV-16 where Neo and *ras* had been placed (see "Materials and Methods" for details). A stable CAT assay was then carried out in which HeLa cells were cotransfected with 2 μ g of pL67C plasmid plus either 2 μ g of pSV2-Neo plasmid or the AAV Rep78⁺/Neo plasmid, dl52-91/Neo. Two days after transfection, the HeLa cells were then selected with 800 μ g G418. Approximately 200 G418-resistant colonies resulted from these transfections. Extracts from the bulk G418-resistant cells were equalized for protein content by spectroscopic analysis at 280 nm and assayed for CAT activity by standard methodologies (24). In 3 experiments, the level of CAT activity in the cells transfected with dl52-91/Neo (Rep78⁺) was lower than the pSV2-Neo transfected cells by 79.4 \pm 13.6% (SD; Fig. 4C).

DISCUSSION

Seroepidemiological studies by 2 different laboratories have demonstrated that AAV infection is negatively associated with cervical cancer, a cancer which is strongly associated with HPV-16. The data presented in this study demonstrate that AAV Rep78 inhibits HPV-16 oncogenic transformation and HPV-16 directed gene expression in tissue culture. Thus, AAV is able to inhibit multiple papillomavirus types (HPV-16 and BPV-1), as it is known to also inhibit multiple adenovirus types (34). These data, and earlier AAV/BPV interaction studies by others (35) and ourselves (7, 33), are consistent with AAV limiting HPV-16's role in cervical carcinogenesis *in vivo*. In further

support of this hypothesis, we have recently generated preliminary data showing that AAV inhibits oncogenic transformation of primary human keratinocytes by the HPV-16/*ras* chimeric plasmid, pL67R.⁴

Although we have not formally shown on what level AAV Rep78 regulates HPV-16, inhibition of transcription seems a possible explanation. The HPV-16 oncogenes E6 and E7 are expressed from the P97 promoter (36), as are possibly the marker genes (*ras*, Neo, and CAT) placed downstream of E7. Thus, this promoter may be the target of inhibition by the AAV Rep78 protein. Alternatively, the marker genes may be expressed from another, though as yet not fully characterized, promoter present within the E7 open reading frame (37). It should be noted that the Rep78 protein is highly multifunctional, with one of its functions being as a transcription factor for regulating AAV and heterologous gene expression (38-40). On the other hand, there is evidence that the AAV (Rep78) may also regulate genes on a post-transcriptional level (41). Certainly, Rep78 is one of the most interesting and multifunctional proteins yet identified. In any case, AAV infection appears to have a beneficial effect on human health, as it is associated with a lower risk of cervical cancer. Possibly, AAV might be used as a protective agent against the development of cervical cancer since it is nonpathogenic. Finally, it appears reasonable that the assays used in this study represent a model system by which to explore some of the viral factors which determine the incidence of cervical cancer.

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⁴ P. L. Hermonat, unpublished data.

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