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Adenoassociated Virus Type 2-Induced Inhibition of the Human Papillomavirus Type 18 Promoter in Transgenic Mice

Christian M. Walz,*,†,[1] Margarita M. Correa-Ochoa,†,‡ Martin Müller,*,† and Jörg R. Schlehofer*,[2]

*Deutsches Krebsforschungszentrum, Angewandte Tumorvirologie, F0100, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany; †Loyola University Medical Center, Stritch School of Medicine, Department of Obstetrics and Gynecology, Division of Research, South First Avenue, Maywood, Illinois; and ‡Escuela de Bacteriología y Laboratorio Clínico, Universidad de Antioquia, Colombia

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The epithelium of the *cervix uteri* has been reported to be frequently coinfected with both human papillomaviruses (HPV) and helper virus-dependent adenoassociated viruses (AAV). Seroepidemiological data suggest that AAV infection could inhibit cervical cancer that is caused by specific ("high-risk") types of papillomaviruses. *In vitro,* infection with AAV type 2 (AAV-2) or transfection of AAV-2 early (*rep*) genes has been shown to inhibit transformation by papillomaviruses. To analyze the effects of AAV on HPV *in vivo,* we studied the influence of AAV-2 infection on the promoter activity of high-risk HPV type 18 (HPV-18) in mice, transgenic for sequences of the upstream regulatory region (URR) of HPV-18 controlling transcription of the reporter gene, *lacZ.* Transgenic animals (or tongue cells thereof, explanted and grown in culture) were treated with dexamethasone to induce the HPV-18 promoter. Simultaneously they were (i) infected with AAV, (ii) inoculated with AAV virus-like particles (VLPs; empty capsids), or (iii) mock infected. Inoculation with AAV-2 or VLPs inhibited activation of the HPV-18 promoter. *In vitro,* in baby hamster kidney cells transfected with the HPV-18-*lacZ* construct, tissue extracts from AAV-infected animals suppressed the HPV-18 URR to a similar extent as AAV infection did. Down-regulation of the HPV-18 promoter was less efficient with extracts from animals inoculated with VLPs and was not observed with extracts from uninfected or dexamethasone-treated animals. This indicates that AAV induces cellular factor(s) *in vivo* capable of mediating down-regulation of the HPV-18 promoter also in cells *in vitro.* In contrast, promoters of the low-risk HPV types (HPV-6, HPV-11) were not influenced by AAV infection as opposed to promoters of the high-risk types (HPV-18 and HPV-16). © 2002 Elsevier Science (USA)

Key Words: AAV; HPV; AAV-induced factor; transgene; HPV promoter.

INTRODUCTION

Human papillomaviruses (HPV) induce a variety of proliferative epithelial lesions (papillomas) (for reviews, see Buckley, 1994; von Knebel Doeberitz, 1992). Certain HPV types specifically infect the mucosal epithelium of the anogenital region and a subset thereof (notably types 16 and 18) is causatively linked to genital cancer (Bosch *et al.,* 1995; Matsukura and Sugase, 1995; zur Hausen, 1999).

The noncoding, upstream regulatory region (URR; ≈1 kb) of the circular HPV genome is located between the "late" (L) and the "early" (E) region. The URR contains control signals for transcription and genome replication, an epithelial cell-specific enhancer, binding sites for transcription factors such as AP-1, OCT-1, SP-1, and for glucocorticoid receptor complexes (Gloss *et al.,* 1987; Hoppe-Seyler and Butz, 1992; Hoppe-Seyler *et al.,* 1991; Stanley, 1994; Strähle *et al.,* 1987; Thierry *et al.,* 1992). The glucocorticoid responsive elements of the URR play an important role in HPV-mediated oncogenesis as has been demonstrated in experimental, epidemiological, and clinical studies (Mittal *et al.,* 1993).

The helper virus dependent human adenoassociated virus (AAV, six serotypes), a member of the family of Parvoviridae, is thought to be nonpathogenic and has tumor suppressive properties (Berns and Giraud, 1996; Schlehofer, 1994; Schlehofer and Dupressoir, 2000). AAV seems to establish persistent infection in the human genital tract (Burguete *et al.,* 1999; Han *et al.,* 1996; Tobiasch *et al.,* 1994; Walz *et al.,* 1998; Venturoli *et al.,* 2001), notably in cells of the uterine cervix that is also a target for latent infections with HPV (cf., de Sanjose *et al.,* 1999; von Knebel Doeberitz, 1992; zur Hausen, 1994, 1999). It has been demonstrated that AAV inhibits bovine papillomavirus (BPV)- and HPV-induced transformation in cell culture (Hermonat, 1994). This inhibition was mediated by the AAV *rep* gene product (Rep-78) suppressing the papillomavirus promoter (Hermonat, 1989; Hermonat *et al.,* 1997; Zhan *et al.,* 1999). In addition, cotransfection of DNA of HPV type 16 and AAV type 2 (AAV-2) in human keratinocytes induced a cytopathic effect (Walz *et al.,* 1997). Furthermore, seroepidemiological data has been reported demonstrating that women with HPV-related cervical carcinoma less frequently have antibodies to

[1] Present address: Institute of Medical Psychology, Otto-von-Guericke University Medical Faculty, D-39120 Magdeburg, Germany.

[2] To whom correspondence and reprint requests should be addressed. Fax: +49-6221-424962. E-mail: j.schlehofer@dkfz.de.

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AAV when compared to age-matched control women (Georg-Fries *et al.,* 1984; Mayor *et al.,* 1976; Smith *et al.,* 2001; Sprecher-Goldberger *et al.,* 1971). These data point to an interaction of AAV and HPV in cervical tissue possibly interfering with HPV-associated tumor development (Rabreau and Schlehofer, 1995; Walz *et al.,* 1997).

To assess interaction of AAV and HPV *in vivo,* we investigated the influence of AAV infection on the URR of HPV-18 in transgenic mice (Cid *et al.,* 1993). In addition, the effect of tissue extracts from AAV-infected mice on the HPV-18 promoter was tested *in vitro.* Furthermore, we analyzed the influence of AAV on the URR of other HPV types *in vitro.* The data demonstrate that AAV inhibits promoter activity of the high-risk HPV types (HPV-16/18) *in vivo* and *in vitro* (but not of the benign HPV types 6/11) and that this inhibition is due to AAV-induced cellular factor(s) *in vivo.*

RESULTS

AAV down-regulates the HPV-18 promoter *in vivo*

To examine the influence of AAV on the HPV-18 promoter activity *in vivo,* we performed experiments with mice, transgenic for the HPV-18 upstream regulatory region controlling transcription of the *Escherichia coli* β-galactosidase reporter gene (HPV-18-URR-*lacZ*). It had been shown previously that after treatment with dexamethasone, these mice express high levels of β-galactosidase in the tongue (Cid *et al.,* 1993). We used this system of dexamethasone-induced HPV promoter activation throughout the experiments.

Dexamethasone-treated (supplemented with drinking water) or untreated animals were intravenously infected either with infectious AAV-2 (10^{10}–10^{11} particles per animal) or with an inoculum of AAV-2 VLPs (10^{10}–10^{11} particles per animal). Since dexamethasone-induced URR activity is maximal after 12 h of treatment, mice were sacrificed 12 h after infection/treatment, and β-galactosidase activity was measured in protein extracts from tongue tissue of the animals by spectrophotochemical analysis. As shown in Fig. 1, β-galactosidase activity in tongue tissue was about fivefold higher in animals treated with dexamethasone, demonstrating URR transcriptional activity mediated by the dexamethasone-inducible glucocorticoid receptor complex, as previously shown for these mice (Cid *et al.,* 1993; Michelin *et al.,* 1997). In AAV-2-infected mice, however, this β-galactosidase activity was not observed, indicating suppression of the basal activity as well as of the dexamethasone-induced HPV-18 promoter activity (Fig. 1A). Similarly, tongue extracts from animals inoculated with virus-like particles (VLPs) showed an inhibition of inducible β-galactosidase activity, albeit to a lesser extent than the effect of infectious virions. Controls, i.e., tongue extracts from nontransgenic animals or from animals that were not treated with dexamethasone, exhibited a low background β-galactosidase activity or none at all, respectively (Fig. 1A).

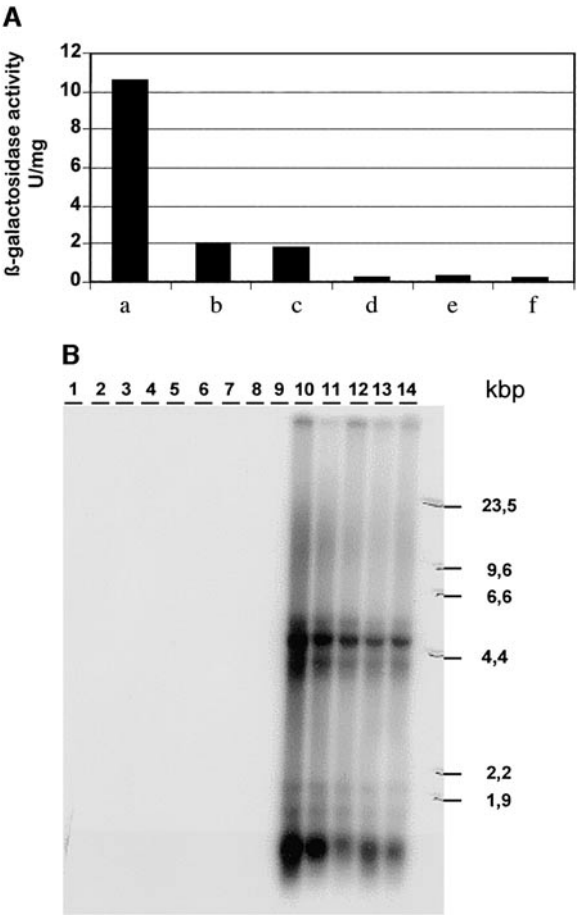
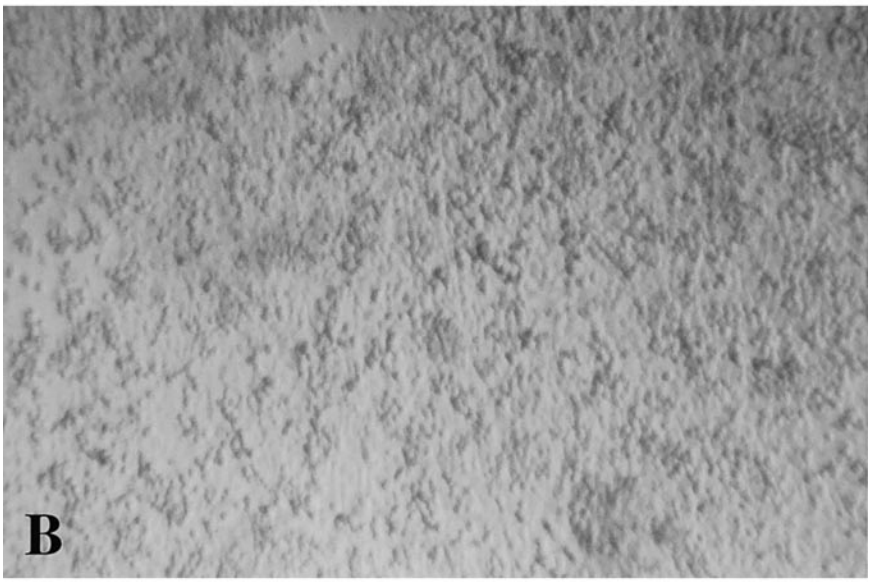


FIG. 1. (A) β-galactosidase activity (units per mg protein) in the tongue of HPV-18 URR *lacZ* transgenic mice. As negative controls, transgene-negative littermates were used. Each group of animals consisted of six male individuals. Expression from the URR in transgenic animals, treated with dexamethasone [(a) fivefold increased β-galactosidase activity above basal activity (b)]. Nontransgenic control animals did not show any difference of expression treated (e) or not treated (f) with dexamethasone. In animals treated with dexamethasone and infected with AAV-2 showed a nearly complete suppression of the basal and induced β-galactosidase activity (d). Inoculation of AAV VLPs into transgenic animals down-regulated the dexamethasone-induced β-galactosidase activity (c) less efficiently than infection with AAV virions (d). (B) Southern blot of genomic DNA (20 μg/lane) extracted from tongues of HPV18-URR *lacZ* transgenic mice, hybridized with a radioactively labeled AAV DNA probe. All animals were treated with dexamethasone. Lanes 1–4: mock-infected control animals; lanes 5–9: animals inoculated intravenously with AAV VLPs; lanes 10–14: animals infected intravenously with AAV-2 demonstrating AAV in tongue cells. The 4.6-kb band shows ds AAV DNA, the absence of bands of higher molecular weight (replicative intermediates) is indicative of lack of replication of AAV DNA. For each group, four animals were analyzed.

Southern blot analysis of tongues of infected animals gave no indication of AAV DNA replication or integration in the cellular genome, as expected after a 12-h infection (Fig. 1B). In addition to the tongue, genomic AAV DNA could be also detected by Southern blot analysis in various tissues of AAV-infected mice (blood, spleen, liver, kidney, heart, lung, gut, stomach), but not in brain tissue

+ dexamethasone



+ dexamethasone + AAV

FIG. 2. Inhibition of β-galactosidase activity in cells cultured from tongue tissue of HPV-18-URR-*LacZ* transgenic animals. Cells were treated with dexamethasone at a concentration of 4 μg/ml medium. Cells expressing the transgene from the HPV-18 URR) appear dark stained. (A) Uninfected, (B) AAV-2-infected (m.o.i. = 10^3 IU/cell) cells.

[data not shown; cf. also earlier results with AAV-infected nude mice (Walz *et al.,* 1992)].

AAV down-regulates the HPV-18 promoter *in vitro*

To further assess the influence of AAV on the HPV-18 URR, tongue cells from transgenic mice were isolated and cultured *in vitro.* Similar to the observations *in vivo,* β-galactosidase activity controlled by the HPV-18 URR was induced after dexamethasone treatment of the culture (Fig. 2), albeit not in all cells. [This was expected because of heterogeneity of the cells and since the transgene is usually not expressed in all cells (Cid *et al.,* 1993)]. Induction of β-galactosidase activity was abolished when tongue cells were infected with AAV (m.o.i. = 10^3 IU/cell) (Fig. 2), or inoculated with VLPs, in which case inhibition was less pronounced (data not shown).

AAV induces cellular factor(s) *in vivo* substituting for AAV in down-regulation of HPV-18 promoter *in vitro*

It is difficult to conceive that AAV or VLPs reach the majority of cells of the intravenously injected animals at the particle number used in the experiments. In addition,

without a helper virus, AAV is unlikely to replicate or express viral genes. Therefore we analyzed whether the observed strong inhibition of URR activity might be mediated by a factor induced in AAV-treated animals. To address this issue, extracts were prepared from tongue tissue from transgenic mice that had been (i) infected with AAV, (ii) inoculated with AAV empty capsids, or (iii) had not been treated with virus. Experiments were carried out in dexamethasone-treated as well as in untreated mice. Extracts were controlled to be free of infectious virions and of AAV DNA.

Extracts were added to BHK cells that had been transfected with HPV-18-URR-*lacZ,* and the cells were treated with dexamethasone for 12–16 h to activate the HPV-18 URR. Extracts from tissue from AAV-infected animals strongly suppressed the URR activity similar to the effect observed after AAV infection of the cells (Fig. 3). Extracts from animals inoculated with empty AAV capsids reduced β-galactosidase expression in the transfected BHK cells at a lower efficiency (Fig. 3). Similar results were obtained with extracts from AAV-infected or VLP-treated BHK cells (but not with cell culture supernatant from infected cultured cells), suggesting that AAV-mediated induction of cellular factor(s) influencing the HPV-18 URR occurs also *in vitro* (data not shown). Extracts from animals treated only with dexamethasone or extracts from mock-infected animals did not influence the expression of the transfected reporter gene in the cell cultures.

These data suggest that one or more cellular factors suppressing the HPV URR are induced *in vivo* after exposure to AAV-particles.

AAV-mediated down-regulation of the HPV promoter is restricted to high risk HPV types

To address the specificity of the HPV-18 URR in responding to AAV, we tested *in vitro* AAV effects on other HPV promoters. BHK cells were transfected with constructs containing the luciferase gene under the control of the URR of various HPV types (HPV-18, -16, -6b, -11) and cultivated with or without dexamethasone. The transfected cells were either infected with AAV (m.o.i. = 10^3–10^4 IU/cell) or transfected with AAV constructs expressing AAV *rep* genes. Infection with AAV virions or expression of AAV *rep* genes suppressed the URR of HPV types 16 and 18 induced with dexamethasone (Fig. 4). In these experiments, even the basal activity of the URR (without induction by dexamethasone) was suppressed. Suppression was more efficient with *rep*-constructs compared to AAV infection, and expression of the Rep-78 protein exceeded the down-regulating effects of the expression of all AAV *rep* genes (Fig. 4). With URR constructs from the nontumorigenic HPV types 6b and 11, the dexamethasone-induced activity was much lower than that observed with HPV-16 and -18, and a significant reduction of activity was not observed after AAV infection (Fig. 4).

DISCUSSION

Persistent infection of human genital tissue with AAV seems to be rather common (Bantel-Schaal and zur Hausen, 1984; Friedman-Einat *et al.,* 1997; Han *et al.,* 1996; Tobiasch *et al.,* 1994; Walz *et al.,* 1997, 1998; Venturoli *et al.,* 2001). The same tissue is frequently infected with HPV (de Sanjose *et al.,* 1999; Gomez *et al.,* 1995; von Knebel Doeberitz, 1992), certain types thereof (mainly types 16 and 18) being causatively linked to genital cancer (zur Hausen, 1994, 1999). Papillomaviruses have been identified as helpers for AAV replication (Walz *et al.,* 1997), and an inhibitory effect of AAV functions on transcriptional activity of the transformation-associated HPV genes, E6/E7, has been reported (Hermonat, 1994; Hermonat *et al.,* 1997; Zhan *et al.,* 1999). These findings suggest an interaction of the two viruses *in vivo* possibly interfering with HPV-associated tumor development (Rabreau and Schlehofer, 1995; Walz *et al.,* 1997).

To address this issue we performed experiments with mice carrying the *E. coli lacZ* gene under the control of the promoter region of HPV type 18 as a transgene (Cid *et al.,* 1993). In these mice, treatment with dexamethasone induces expression of β-galactosidase by interacting with the glucocorticoid responsive elements of the HPV-18 upstream regulatory region.

Intravenous injection of AAV-2 and of AAV-2 empty particles led to suppression of the dexamethasone-induced β-galactosidase activity in the HPV-18-URR-*lacZ* transgenic animals. This *in vivo* observation is in line with previous *in vitro* results showing down-regulation of HPV promoter by expression of AAV *rep* genes (Hermonat, 1994; Hermonat *et al.,* 1997). It has been shown that the AAV Rep78 major regulatory protein binds to the promoter of HPV-16 thereby directly inhibiting E6/E7 transcription (Zhan *et al.,* 1999). However, in the *in vivo* system presented here there is no evidence of expression of AAV Rep proteins after AAV infection, and down-regulation of the HPV-18 promoter was also observed with empty AAV particles. Paralleling the *in vivo* results, the HPV-18 URR was inhibited in tongue cells from transgenic mice explanted *in vitro,* after infection of the cell cultures with AAV. In this *in vitro* assay, treatment with dexamethasone did not induce β-galactosidase expression in all cells, although all cells contained the transgene. This is in line with earlier studies showing that not all cells of the transgenic animals can be induced to express the transgene to the same extent (Cid *et al.,* 1993).

In *in vitro* analyses with HPV types other than HPV-18 (HPV-16, HPV-6b, HPV-11), it was observed that down-regulation by AAV of the HPV URR was restricted to the "high-risk" types 16 and 18. In BHK cells transfected with HPV URRs of HPV-16 or -18, controlling the luciferase reporter gene, not only the dexamethasone-induced URR activity but also the basal level of the URR was reduced after infection with AAV. In this system, not only infection

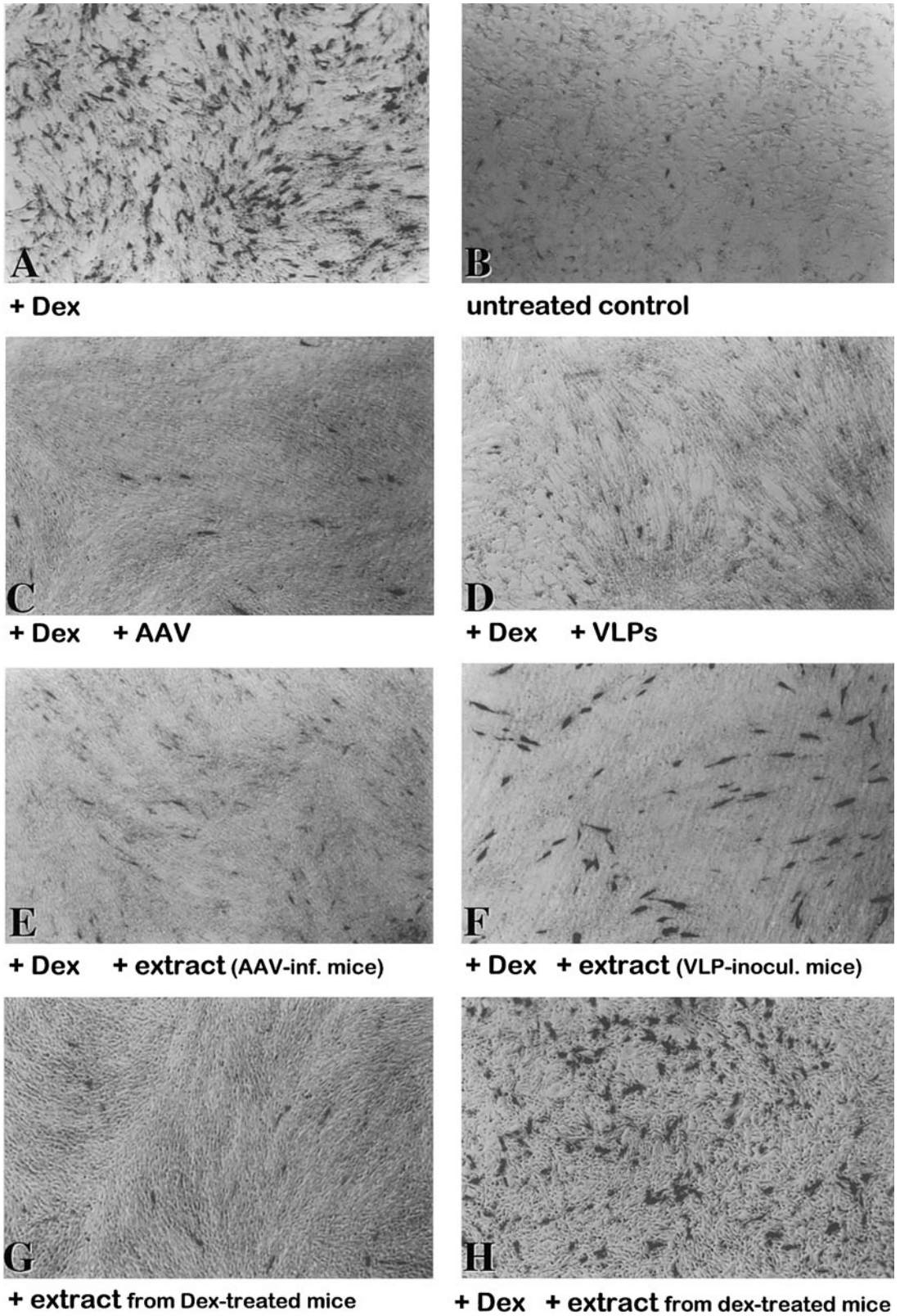


FIG. 3. *In vitro* β-galactosidase assays. BHK cells, transfected with the HPV-18-URR-*lacZ* construct were treated as indicated below and cells expressing the reporter gene were visualized (dark staining cells). All panels are at the same magnification. (A) Cells treated with dexamethasone; (B) untreated controls; (C) cells treated with dexamethasone and infected with AAV-2 (m.o.i. = 1×10^3 IU/cell); (D) cells treated with dexamethasone and inoculated with AAV VLPs (1×10^3–10^4 particles/cell); (E) cells treated with dexamethasone and inoculated with protein extract from the tongue of a transgenic mouse that had been treated with dexamethasone and infected with AAV-2; (F) cells treated with dexamethasone and inoculated with protein extract from the tongue of a transgenic mouse that had been inoculated with VLPs; (G) untreated cells inoculated with protein extract from the tongue of a control mouse that had been treated with dexamethasone; (H) cells treated with dexamethasone and inoculated with the same protein extract as in (G).

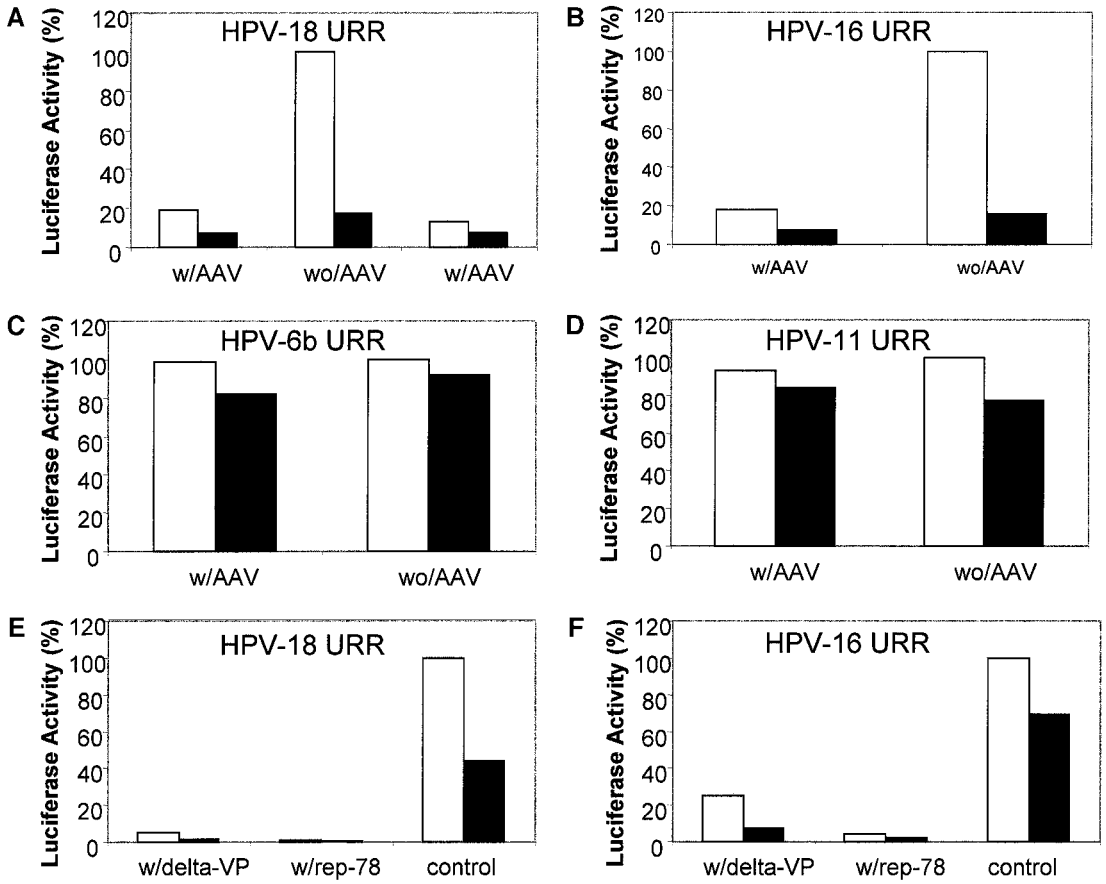


FIG. 4. Suppression of luciferase activity controlled by URRs of different HPV types after exposure to AAV. BHK cells were transfected with HPV URR constructs controlling luciferase expression, treated or not with dexamethasone (4 μg/ml), infected with AAV (m.o.i. = 10^3 IU/cell), or cotransfected with constructs expressing AAV rep genes. Light columns, luciferase expression in dexamethasone-treated cells, dark columns, without dexamethasone. w/AAV, AAV-infected; wo/AAV, uninfected cells; w/delta-VP, cotransfection with a construct expressing all AAV-2 *rep* genes under the control of the natural AAV promoter; w/rep-78, cotransfection with a construct expressing the Rep 78 protein under the control of the CMV promoter. (A) HPV-18 URR (2 AAV stocks were tested); (B) HPV-16 URR; (C) HPV-6b URR; (D) HPV-11 URR; (E) HPV-18 URR; (F) HPV-16 URR.

with AAV but also expression of AAV rep genes was able to suppress HPV promoter activity. This indicates that in addition to effects mediated by AAV virions or empty particles, AAV Rep proteins down-regulate promoters of high-risk HPV types, confirming results from others (Hermonat, 1994).

The results presented here show that infectious virions as well as empty AAV particles can mediate down-regulation of HPV-18 URR activity, *in vitro* and *in vivo.* As expected in the absence of AAV helper viruses, there was no indication of replication of AAV or expression of AAV proteins. In addition, at least *in vivo,* it is very unlikely that a large number of cells are reached by AAV particles. Therefore we assumed that the observed effects on HPV URR might be mediated by factors induced after exposure of cells to AAV particles. This is supported by our findings that protein extracts from tissue of AAV-infected animals were able to substitute for AAV infection in cell-culture experiments. Interestingly, protein extracts from animals inoculated with VLPs also showed an inhibition of expression of the reporter gene, but less efficiently than extracts from virion-infected animals. These observations suggest that components of virus particles interacting with structures or functions of cells can trigger synthesis of the presumed factor(s). Experiments with extracts from AAV-infected cell cultures indicate that such molecule(s) are also induced *in vitro,* albeit less efficiently than tissue extracts from infected animals.

It cannot be excluded that in experiments with infectious virions a transient expression of Rep proteins might act directly on the HPV URR (Hermonat *et al.,* 2000; Zhan *et al.,* 1999), thereby rendering AAV effects more efficient than the ones observed with empty particles. It remains to be determined whether the AAV-mediated cellular factors act directly on the HPV-18 promoter, whether they interfere with binding of dexamethasone to the glucocorticoid responsive elements of the URR, or whether they induce cellular functions down-regulating URR activity.

It is tempting to speculate that also in human cervical tissue, persistent AAV—possibly replicated through the helper activity of HPV—may down-regulate E6/E7 expression of oncogenic HPVs. This inhibition of HPV promoters may not only be achieved by direct interaction of

early AAV proteins with the HPV promoter but also via AAV-induced cellular factors acting on the activity of the HPV URR. Such AAV-mediated functions might explain epidemiological observations, indicating a somewhat protective effect of AAV on development of HPV-associated cervical cancer (Georg-Fries *et al.,* 1984; Mayor, 1993; Mayor *et al.,* 1976; Sprecher-Goldberger *et al.,* 1971; Smith *et al.,* 2001).

AAV infection and empty AAV particles have been shown to sensitize a variety of human tumor cells to irradiation or to the cytotoxic action of chemotherapeutic drugs, *in vitro* and also *in vivo* (i.e., tumors derived from human tumor cells inoculated in nude mice) (Hillgenberg *et al.,* 1999; Klein-Bauernschmitt *et al.,* 1996; Walz *et al.,* 1992). It is unexplained how a relatively low dose of viruses injected into animals achieved this sensitizing effect on the relatively big tumor mass. The finding of AAV-mediated induction of cellular factors presented here might hint to such factors also involved in rendering tumor cells more sensitive to genotoxic agents.

The present study focused on *in vivo* effects of AAV infection on HPV promoter activity. Future experiments are required to identify and characterize the molecular nature of AAV-induced cellular factor(s). Recently, a cytokine-like peptide (AiF, AAV-induced factor) has been described that is secreted from melanoma cell cultures after integration of AAV-2 DNA (Bantel-Schaal, 2001). This factor is described to influence cell adhesion and to modulate cell proliferation in opposite ways for tumor cells and fibroblasts. Though this factor seems to be induced upon integration of AAV DNA and has not yet been connected to papillomaviruses, it will be interesting to compare it with the factor(s) induced in the experiments described in this report.

MATERIAL AND METHODS

Animals

A homogeneous colony of male mice, heterozygous for the transgene, *E. coli* β-galactosidase gene, under the control of the HPV-18 upstream regulatory region, URR (pURR-18-*lacZ*) (Cid *et al.,* 1993), was used for *in vivo* experiments. The animals were conceived by injection of transgene-positive pronuclei of eggs of C57/BL6xC3H hybrids. The strongest expression of the reporter gene was found in the tongue, gonads, uterus, stomach, small intestine, kidney, hair follicles, and respiratory epithelium (Cid *et al.,* 1993). Heterozygous animals were crossbred to obtain homozygous breeder animals. (Animals were kept under conditions approved by the Loyola University of Chicago Medical Center Institutional Animal Care and Use Committee and the German Tierschutzgesetz.) The status of the transgene was analyzed by PCR (see below). To distinguish homozygous from heterozygous (PCR-positive) individuals, animals were crossbred with transgene-negative littermates. After obtaining homozygous animals, these were bred with negative littermates to obtain hemizygous male animals for the assays. For identification of mice carrying the transgene, DNA from tail biopsies was analyzed by PCR utilizing the following HPV-18 URR-specific primers (Cid *et al.,* 1993; Michelin *et al.,* 1997): 5′-TAC AAG CCA AGT ATG CAA TTA GC-3′; 5′-TAC GTG CCA GGA AGT AAT ATG TG-3′. Amplification was performed using a PTC-100 thermocycler (MJ Research Inc., Watertown, MA) with 1 cycle at 95°C for 5 min followed by 30 cycles of 40 s at 94°C; 40 s at 63°C, and 30 s at 75°C; and a final cycle at 75°C for 5 min. As a positive PCR control, 20 μg genomic DNA from HPV-18 DNA-positive HeLa cells was used. PCR products were separated by electrophoresis through a 1% agarose gel containing ethidium bromide. The products were visualized under UV light and photographed.

Cells

Baby hamster kidney cells (BHK) and 293 cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with antibiotics (10 μg/ml penicillin and 20 μg/ml streptomycin) and 10% fetal calf serum.

Cells from mouse tongue from URR-18-*LacZ* transgenic mice were isolated and propagated as follows. Mice were sacrificed by cervical dislocation and the tongue was removed and carefully cut into small pieces with a sterile scalpel in sterile medium (4 ml DMEM, supplemented with 20% FCS and antibiotic–antimycotic solution, Gibco-BRL, Grand Island, NY) in a 10-cm culture dish. Medium containing the fragments was transferred into a 50-ml Falcon tube and 1 ml HEPES buffer (pH 7.0), 250 μl collagenase IV (stock, 60 mg/ml, Sigma, Deisenhofen, Germany), 250 μl DNase I (stock, 25 mg/ml, Boehringer, Mannheim, Germany) and 400 μl hyaluronidase (stock, 10 mg/ml, Sigma) were added. Cells of the tissue were separated with constant shaking for at least 4 h at 37°C. Finally the digested tongue cells were suspended by repeated pipetting and transferred into six-well plates and incubated with medium (as above). Medium was changed after 8 and 16 h.

Virus

AAV-2 was propagated in HeLa cells with adenovirus type 2 (ATCC, Rockville, MD) as the helper and purified as described (Yakobson *et al.,* 1987). Infectious particles were analyzed by end-point titration (Bantel-Schaal and zur Hausen, 1988) and by a capsid-specific ELISA to determine the number of physical particles (Grimm *et al.,* 1999). The "mock" inoculum was a dialyzed fraction (1.41 g/cm^3) from a CsCl gradient from HeLa cells only infected with adenovirus type 2.

Empty AAV-2 particles ("Virus-like particles")

293 cells were infected with recombinant adenovirus expressing AAV VP genes (Grimm *et al.,* 1999) and maintained in culture until complete adenovirus-induced cytopathic effect (CPE) appeared. Cells were harvested

and sedimented by centrifugation (5 min, 200 *g,* 4°C), and VLPs were purified as described by Wistuba *et al.* (1997). The number of empty particles was adjusted to the one of physical particles present in the preparation of infectious AAV, as determined by the capsid ELISA (Grimm *et al.,* 1999) and by estimating protein content in Western blot analyses with anticapsid antibodies recognizing only entire particles. This allowed similar numbers of input particles in experiments with infectious and empty particles.

Plasmids

The following plasmids were used for transfection in BHK cells. p-URR-18-*lacZ* [whole URR from HPV-18 as 1050-bp fragment cloned in front of the *E. coli* β-galactosidase gene in the plasmid pC4AUG-β-gal (Cid *et al.,* 1993)]; pURR-18-LUC (whole URR from pURR-18-*lacZ* subcloned into the firefly luciferase expression vector pBL into the *Bam*HI/*Pst*I site); pURR-16-LUC (whole URR from HPV-16 cloned into pBL into the *Bam*HI/*Pst*I site); pURR-6b-LUC (whole URR from HPV-6b cloned into the *Bam*HI/*Pst*I site of pBL); pURR-11-LUC (whole URR from HPV-11 cloned into the *Bam*HI/*Pst*I site of pBL); pTK-RL [cDNA of *Renilla luciferase* cloned behind the TK-promoter-enhancer as described by manufacturer (Promega, Madison, WI)]; pTK-LUC [positive control for the *Firefly luciferase* cloned behind the TK-promoter-enhancer (Promega)]; pAAV-Δ-VP (AAV genome without *VP* genes, expressing the nonstructural *rep* genes from their own promoters p5 and p19); pCMV-rep78 (AAV *rep 78* gene cloned behind the early CMV promoter).

With these plasmids, 80% confluent growing cells were transfected or cotransfected with different HPV-URR-LUC constructs, the internal control pTK-RL, and constructs from AAV described above. Two micrograms of DNA were transfected with LipofectAMINE (Life Technologies, Eggenstein, Germany) in 2×10^5 cells according to the manufacturer's recommendations. Control experiments using the pBluescript plasmid instead of the internal control pTK-RL excluded specific effects of the pTK-RL.

In vivo infection/treatment

Transgenic animals as well as nontransgenic littermates were infected with purified AAV-2 or VLPs by injection into the tail vein (10^{10}–10^{11} infectious units per animal). To induce strong expression of the reporter gene from the HPV promoter in the transgenics, dexamethasone was administered at the time of AAV infection by addition to the drinking water [4 μg/ml (10^{-5} M) from a 10 mg/ml stock of crystalline hormone (Sigma-Aldrich, Deisenhofen, Germany) in ethanol]. Control animals were either mock-infected or did not receive dexamethasone. Nontransgenic littermates were negative controls receiving the same treatment as the transgenics. The animals were sacrificed by cervical dislocation, 12 h after onset of treatment and infection.

Southern blot analysis

DNA was extracted from tissue samples of spleen, liver, lung, kidney, heart, gut, stomach, tongue, blood, and brain of infected or mock-infected animals (12 h p.i.) using standard procedures. Twenty micrograms of each DNA sample was digested with *Eco*RV (not cutting within the AAV DNA genome), separated on a 0.7% agarose gel, and transferred onto Nylon membranes. Blots were hybridized with genomic AAV DNA [pTAV2; (Heilbronn *et al.,* 1990)] and autoradiographed.

In vivo β-galactosidase assay

The β-galactosidase activity in the tongue tissue of the animals was determined with the ONPG (*o*-nitrophenol-β-D-galactopyranoside) assay. Tongues from sacrificed animals were cut into small pieces using a sterile scalpel. The tissue fragments were transferred into PM2 buffer [33 mM NaH_2PO_4, 66 mM Na_2HPO_4, 0.1 mM $MnCl_2$, 2 mM $MgSO_4$, 40 mM β-mercaptoethanol (pH, 7.3)] and homogenized on ice with a polytron homogenizer. Tissue homogenates were centrifuged and resuspended three times at 12,000 *g* for 15 min at 4°C. The supernatant was transferred into a microcentrifuge tube and protein concentration was determined as described (Bradford, 1976). Protein (300 μg) and ONPG (800 μg) were transferred into a final volume of 1 ml PM2 buffer at 37°C. Immediately after adding ONPG, the initial absorption (A_{420}) of each sample was read. Readings were taken every 30–60 min over a time of incubation at (37°C) of up to 10 h. The β-galactosidase activity was calculated after subtraction of the individual initial values: units = [$380 \times A_{420}$/time (in minutes) × 3,3333; 380 is a constant, so that 1 unit is equivalent to the conversion of 1 nM ONPG per minute at 37°C]. Since 300 μg protein extract was in the reaction, the final unit value for 1 mg protein was calculated by multiplying with 3.3333.

In vitro infection/treatment

Primary cells prepared from the tongue of transgenic animals, or BHK cells (80% confluent), were transfected with the various HPV plasmid constructs. They were then inoculated with AAV-2 (m.o.i. = 10^3–10^4 IU/cell), or with VLPs (adjusted for the number of particles, see above) and incubated with medium containing dexamethasone (final concentration, 4 μg/ml) at 37°C for 12–16 h prior to analyses of HPV promoter activity (see below). As controls, cells treated only with dexamethasone or untreated cells were used.

In vitro β-galactosidase assay

Explanted tongue cells (transgenic for HPV18-URR-*lacZ*) or BHK cells (transfected with the HPV-18-URR-

LacZ plasmid) were washed twice with PBS and fixed with 3% formaldehyde in PBS (pH 7.5) at 4°C for 20 min. Fixed samples were washed with PBS at RT and incubated with X-gal reaction solution [1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in 2 mM $MgCl_2$ 0.02% Nonidet P-40 (NP-40) 0.01% Na deoxycholate in 0.1 M phosphate buffer (66 mM Na_2HPO_4, 33 mM NaH_2PO_4; pH 7.3)] for 1–12 h at 37°C in a humidified atmosphere. The number of cells staining blue was determined microscopically.

Incubation of cells with extracts from tongue tissue of mice

Proteins were extracted from tongue tissue of AAV- or VLP-infected and control mice as described above. Protein extracts were treated with DNase to remove potential AAV DNA sequences. In addition, 5 μl of DNase-treated extract was inoculated in HeLa cells infected with adenovirus, demonstrating the absence of infectious virus. Similar extracts were prepared from AAV-infected BHK cells. After complete adenovirus-induced CPE, cells were transferred onto a nylon membrane and lysed as described (Walz *et al.,* 1997). The lysed cells were hybridized with radioactively labeled AAV DNA to detect replication of AAV. Virus-free protein extract (100 μg diluted in 5-ml full medium) was added to BHK cell cultures (80% confluent, treated or not with dexamethasone) that had been transfected 12–16 h before with the URR-18-*lacZ* reporter plasmid. After 16 h, *in vitro* β-galactosidase assay was performed (see above) and the number of blue cells was determined microscopically.

In vitro luciferase assay

BHK cells transfected with luciferase constructs of various HPV types (see above) were either infected with AAV (m.o.i. = 10^3–10^4 IU/cell) or transfected with AAV constructs (see above). Cultures were incubated overnight in medium containing dexamethasone or not, as described in the figures. After lysis of the cells, the firefly luciferase activity in the cell extracts was measured against the internal control of the *renilla* luciferase in a luminometer using the Dual-Luciferase Reporter 1000 Assay System from Promega (Madison, WI).

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