Multiple human papillomavirus genes affect the adeno-associated virus life cycle

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Abstract

The risk of cervical cancer, one of the most prevalent cancers in the world, is determined by two viruses. Human papillomavirus (HPV) is the main risk factor for developing cervical cancer. However, although little known, it is well substantiated that the human Parvovirus adeno-associated virus type 2 (AAV), and its encoded Rep78 protein, interacts with HPV and lowers the risk of cervical cancer. HPV also contributes to AAV inhibition by serving as a helper virus for AAV and stimulating higher AAV replication levels. Here we surveyed four HPV-16 early genes, E1, E2, E6 and E7, for their ability to increase/decrease the basal level of AAV replication in stratifying squamous epithelium (the epithelial raft culture system). It was found that the HPV-16 E1, E2 and E6 genes were able to help/enhance AAV-2 replication in epithelial raft cultures. Under these conditions, with all the HPV genes being expressed from the AAV p5 promoter, E1 appeared to have the strongest enhancing effect on AAV DNA replication (Southern blot), RNA expression (RT-PCR), protein expression (Western blot) and AAV virion production (2 plate-Southern blot). Further study of E1 mutants showed that the carboxy-half of E1, the putative helicase/ATPase domain, was the main contributor of helper activity. These data are important for understanding the HPV–AAV interaction and its effect on modifying cervical cancer risk. These data also suggest the possibility that the identified HPV helper genes may be useful in the generation of recombinant (r)AAV virions for gene therapy, as rAAV is increasing in popularity for such purposes.

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Introduction

Taxonomically AAV is a Dependovirus, a genus of Parvoviridae. This categorization reflects AAV’s usual requirement for co-infection with adenovirus (Ad) (Casto et al., 1967; Mayor et al., 1967), herpes simplex virus (HSV) (Buller et al., 1981; Mayor et al., 1974) or human papillomavirus (HPV) (Ogston et al., 2000; Meyers et al., 2001) in order for AAV replication to occur. Thus, AAV receives ‘helper functions’ from the helper virus (Ad, HSV or HPV) which allows AAV to replicate and produce progeny AAV. In return, AAV usually inhibits the DNA replication and oncocgenic abilities of its helper viruses. This helper virus dependence and bi-directional interaction is nearly unique among wild-type viruses and is important for the generation of systems for generating recombinant (r)AAV for gene therapy purposes. Moreover, the relationship of AAV with HPV is particularly important in regards to human health. With two new epidemiologic studies published recently (Coker et al., 2001, Smith et al., 2001), there is now significant data which confirm earlier studies (Georg-Fries et al., 1984; Mayor et al., 1976) and firmly establish that...
AAV inhibits cervical cancer development (odds ratio of \(~0.3–0.4\). There is also a plethora of laboratory data supporting this conclusion (for example, Hermonat, 1994; Hermonat et al., 1997; Horer et al., 1995; Su and Wu, 1996).

While the adenovirus (Duan et al., 1999; Samulski and Shenk, 1988; Richardson and Westphal, 1981; West et al., 1987) and herpes virus helper genes (Mishra and Rose, 1990; Weindler and Heilbronn, 1991) have been studied, HPV helper genes are largely unstudied. Walz et al. (1997) demonstrated that AAV and HPV were preferentially found co-infesting the cervical epithelium. While not a direct demonstration of helper function, it provided coincidental and consistent data with this hypothesis first presented in 1989 (Hermonat, 1989). Ogston et al. (2000) next demonstrated that AAV replication takes place in HPV-16 E2 positive cells. Most recently, Meyers et al. (2001) demonstrated in a study in a human skin model in culture, in which both HPV and AAV are productive, that co-HPV infection increased AAV DNA replications many fold over AAV-only infection. With AAV’s ever increasing popularity as a gene therapy vector (Hermonat and Muzyczka, 2000, 2001), in this study, we use the raft culture system to demonstrate equivalent DNA loading, Fig. 4D shows the ethidium bromide stained agarose gel (corresponding to the DNA (10 μg total cellular DNA each lane) was DpnI digested, size separated and Southern blotted. Fig. 4B shows a representative Southern blot analysis, while Fig. 4C shows a quantification of five equivalent experiments upon densitometric analysis. To demonstrate equivalent DNA loading, Fig. 4D shows the ethidium bromide stained agarose gel (corresponding to the Southern blot in B) before transfer, demonstrating total DNA content. Finally, Fig. 4E lists the P values of the indicated comparisons. These data indicated that under our experimental conditions, the E1 gene stimulated the highest AAV DNA replication level, followed by E2 and E6. In contrast, E7 had no significant effect compared to the AAV/Neo control vector. We further analyzed E1 to observe if the helper effect was dosage-dependent. As shown in Fig. 5, E1 showed a dosage-dependent

Results

**E1, E2 and E6 help AAV DNA replication**

We have shown earlier that AAV-2 has the ability to autonomous replicate in differentiating keratinocytes of the organotypic epithelial raft cell culture system, which closely mimics normal stratified squamous epithelium and likely represents the natural host tissue for AAV-2 (Meyers et al., 2000, 2001). In this study, we use the raft culture system to observe potential HPV early genes (E1, E2, E6 and E7) helper functions for AAV replication. The structure of the expression plasmids is shown in Fig. 1. The plasmid AAV/Neo (no HPV gene) served as a negative control. All of the HPV genes were expressed from the same transcriptional promoter, AAV p5. There were no RNA splice donor or acceptor sites remaining in the vector sequences outside that present in the specific HPV gene. PHFK monolayers were transfected with the HPV expression plasmids for E1, E2, E6 and E7 on day 0 and then 1 day later infected with wt AAV as described in more detail in the Materials and methods section. This order of introduction was done so that the HPV product would be present at the initiation of AAV DNA replication as AAV initiates DNA replication relatively fast in stratified squamous epithelium (Meyers et al., 2000). Our plasmid transfection efficiency for the keratinocytes, determined by lipofecting a plasmid expressing green fluorescent protein (GFP), was 46% (average of four experiments) as shown in Fig. 2. The HPV-transfected/AAV-infected keratinocytes were then trypsinized and seeded onto collagen disks with embedded fibroblasts. The next day, the rafts were raised to the air to stimulate formation of a stratified squamous epithelium.

First, the ability of our vectors to express the HPV genes was analyzed. The expression plasmid of each HPV early gene (E1, E2, E6 and E7) was individually transfected into PHFK, then subsequently infected with AAV, the cells used to form skin rafts, and polyA-selected RNA was isolated on day 5. Fig. 3 (representative of three experiments) demonstrates that the transfected HPV genes were significantly expressed as analyzed by RT-PCR. The cellular housekeeping gene expression control was TFIB. Relative transcript levels could be judged by comparing the HPV and TFIB cDNA levels. As shown, the mRNA levels of all of HPV genes were comparable, with E2 mRNA levels being perhaps slightly lower. PCR-only failed to generate any products indicating that our polyA RNA samples were free of DNA.

Next, AAV replication levels were compared. The structure of the experiment is shown in Fig. 4A. As in Fig. 3, each HPV early gene (E1, E2, E6 and E7) was individually transfected into PHFK, then subsequently infected with AAV, then used to form skin rafts. DNA was harvested from total rafts on day 5 and resulting AAV DNA levels compared. The DNA (10 μg total cellular DNA each lane) was DpnI digested, size separated and Southern blotted. Fig. 4B shows a representative Southern blot analysis, while Fig. 4C shows a quantification of five equivalent experiments upon densitometric analysis. To demonstrate equivalent DNA loading, Fig. 4D shows the ethidium bromide stained agarose gel (corresponding to the Southern blot in B) before transfer, demonstrating total DNA content. Finally, Fig. 4E lists the P values of the indicated comparisons. These data indicated that under our experimental conditions, the E1 gene stimulated the highest AAV DNA replication level, followed by E2 and E6. In contrast, E7 had no significant effect compared to the AAV/Neo control vector. We further analyzed E1 to observe if the helper effect was dosage-dependent. As shown in Fig. 5, E1 showed a dosage-dependent
effect on AAV replication, with increasing amounts of AAV/E1 resulting in increasing AAV DNA levels.

**E1, E2 and E6 increase AAV mRNA expression by RT-PCR analysis**

Next, an analysis of HPV early genes effects on AAV transcriptional activity was undertaken.

The experiment was identical to those AAV DNA experiments shown in Figs. 4 and 5 with the exception that polyA-selected RNA was isolated. The mRNA samples were then analyzed by RT-PCR and PCR for AAV rep and lip-cap containing mRNA as described in the Materials and methods section. Plasmid pSM620, which contains the complete AAV genome, was a template for PCR amplification to give the correct sized product. The cellular housekeeping gene expression control was TFIIB. Again, relative transcript levels could be judged by comparing the rep and TFIIB cDNA levels. Three such RT-PCR experiments gave consistent results. Shown in Fig. 6A, E1-transfected rafts had higher AAV rep mRNA expression (a higher rep:TFIIB ratio), than those transfected with E2 or E6, while the control Neo-only and E7 transfections gave the lowest rep mRNA levels. These results are fully consistent with the hierarchy of the AAV DNA replication levels. This is noteworthy as rep is required for AAV DNA replication. Fig. 6B (three separate experiments) similarly studied cap mRNA expression. As determined in three such experiments, the hierarchy of cap mRNA also followed DNA replication levels, with E1 having the highest helping activity, E2 and E6 with intermediate helper activity and E7 with little effect or possibly a slight decrease in cap expression. The mRNA samples were also analyzed by direct PCR. As shown in Fig. 6C, PCR-only failed to generate any products indicating that our polyA RNA samples were free of DNA.

**E1, E2 and E6 increase AAV capsid protein production by Western blot analysis**

Next, the effect of HPV helper genes on AAV protein levels was carried out, again as in Figs. 4 and 5. Total cellular protein was isolated from individual rafts and analyzed for AAV rep and cap proteins. Coomassie blue stained gel was used to show that the same amount of total protein was loaded (Fig. 7C). Fig. 7A shows a Western blot using an anti-Rep monoclonal antibody. Three similar experiments gave consistent results and demonstrated that Rep78/68 protein levels were not significantly changed by any of the HPV genes. This is different than the rep mRNA levels, but this was a fully reproducible result in all three experiments.

Equivalent Western blots were probed with anti-Cap antibody as shown in Fig. 7B. Unlike Rep proteins, HPV E1-transfected rafts expressed the highest levels of VP3 capsid protein. Transfection of E2 and E6 also slightly increased VP3 protein expression. Similar to Southern blot and RT-PCR
results, E7 appeared to mildly decreased VP3 protein level. The results were also repeatable over three experiments. As single stranded DNA is believed to reflect genomic DNA encapsidation, this provides mutual confirmation and support for correlating E1 with high VP3 levels.

**E1 helps to produce more AAV infectious virions by two-plate assay**

Next, we analyzed HPV helper gene effects on the complete AAV life cycle, the production of AAV infectious units. These infectious units were measured using a two-plate assay.

**Fig. 5. E1 helps AAV DNA replication in a dose-dependent manner.** Shown is a similar experiment to those in Fig. 4, but in which increasing amounts of E1 were introduced. Note that E1 enhanced AAV replication in a dose-dependent manner.

(Meyers et al., 2000). The first plate part of the experiment was again carried out as depicted in Fig. 8A. However, instead of isolating DNA, RNA or protein, the rafts were processed to result in putative virus stocks as described in the Materials and methods section. The stocks were treated with DNase I to prevent the possibility of free DNA transfer. These putative virus stocks were then tested for the level infectious units (virions) by infecting a second plate of adenovirus-infected 293 cells. At 36 h post-infection of 293 cells by putative AAV virus from the original rafts, total DNA (10 μg) was isolated and analyzed by Southern blot for AAV DNA levels. As shown in Fig. 8B, it can be seen that the production of AAV infectious...
units generally followed effects on AAV DNA replication. E1 helped AAV virion production the most, followed by E6, then E2. Again, E7 appeared to inhibit AAV virion production (consistent among three experiments). It should be noted that infectious unit levels (Fig. 8), VP3 levels (Fig. 7) and single stranded DNA levels (Fig. 2) are all mutually supportive and provide a firm conclusion with regards to the hierarchy of level of helper function provided by E1, E2, E6 and E7.

The carboxy-half of E1 helps AAV replication

We then focused more closely on HPV E1 as it had the highest level of helper activity. The E1 protein is divided roughly into two halves, an E2-binding/ori interaction domain in the amino-half and a helicase/ATPase domain in the carboxy-half (Amin et al., 2000; White et al., 2001; Wilson et al., 2002; Woytek et al., 2001). Therefore, we divided E1 into two half genes and generated AAV vectors AAV/E1-5' or AAV/E1-3' each containing either the E2 interaction or helicase domain (Fig. 9A). Both of these truncated E1 genes were compared to full-length E1 in experiments designed essentially as in Fig. 4A to observe AAV helper function. As shown in Figs. 9B and C, AAV/E1-3' clearly had a much stronger enhancing activity than E1-5'. These data suggest that the carboxy-half of E1 contains the major AAV helper domain and suggest that the helper function may be through E1 helicase activity.

Discussion

While AAV is able to replicate without the presence a helper virus under certain conditions and in certain tissue (Meyers et
al., 2000, 2001; Wang and Srivastava, 1998; Yakobsen et al., 1987, 1989; Yalkinoglu et al., 1988), it is clear that the presence of viral helper genes greatly facilitates the production of both wild type and rAAV. This study demonstrates that multiple HPV-16 genes, including E1, E2 and E6, contribute in enhancing/helping AAV replication and progeny formation in stratified squamous epithelium. E1, E2 and E6 were all expressed from the same transcriptional promoter, and there are no significant dual splice donor–acceptor sites remaining within the single HPV genes. Thus, if all the HPV genes were expressed at approximately the same level, as suggested by Fig. 3, this indicates that E1 provided the highest amount of helper function. Moreover, it is interesting that three different HPV genes appear to enhance AAV’s full life cycle. That is, DNA, RNA and protein (cap protein anyway) levels are all up-regulated. This is somewhat different from the adenovirus helper genes which appear to help AAV in more specific steps of gene expression. The lack of helper function of E7 may reflect its ability to bind the AAV Rep78 replication protein and to inhibit some of its functions (Hermonat et al., 2000).

It should be pointed out that E1, E2 and E6 are proteins with very different biochemical activities and biological functions. E1’s primary function is being a helicase, opening up the HPV origin of replication (ori) and thus promoting the initiation of DNA synthesis (Hughes and Romanos, 1993; Wilson et al., 2002). E2 is a transcription factor and facilitates E1 binding to the HPV ori (Berg and Stenlund, 1997; Woytek et al., 2001). E6 is an oncoprotein and is important for wart formation (Munger and Howley, 2002). Thus, we find it surprising that most aspects of AAV biology are up-regulated by all three of these genes in a relatively similar manner. We likely can gain some insight regarding the mechanism of action of HPV-16 helper gene function by reviewing and comparing them to adenovirus helper gene functions. Presumably many of the Ad helper functions must be duplicated by HPV. Ad is the best studied helper for AAV, and five regions of this virus are involved in providing AAV helper function. These include E1A, E1B, E2A, E4 (ORF6) and VA (Janik et al., 1989; Jay et al., 1989; McPherson et al., 1982; Strauss et al., 1976). Yet, the only straightforward similarity is HPV E6 and adenovirus E1A, both of which are oncoproteins which bind p53 (38).

Focusing on E1, we hypothesize that E1’s helicase function may add to or augment Rep78’s own endogenous helicase function. There is also the potential that Rep78 and E1 directly interact, both being the respective replication proteins of these two virus types. Mutational analysis of E1, which we have initiated here, will ultimately provide additional information as to the mechanism of E1 helper function. It should also be noted that the E1 stimulated higher rep RNA levels (Fig. 6), yet very little change (possibly slightly lower) in Rep78 protein levels (Fig. 7). We are not aware that E1 has shown any activity in affecting translation, yet this appears superficially consistent with our data. Perhaps HPV E1’s closest match among the adenovirus helper genes may be adenovirus E2A. E1 has an ATP-requiring helicase activity, and E2A has a single stranded DNA binding activity which may give outcomes similar to a helicase. In fact, we can map the helper effect of E1 to the domain which includes the helicase function, supporting the hypothesis that E1 helicase function is the helper effect mechanism of action.

Similar analyses can and will be done on E2 and E6 to determine their mechanisms of action for helper function on AAV. It seems reasonable that E6 has an analogous helper role to adenovirus E1B, both known to bind p53. E2’s match in the adenovirus helper gene list is more difficult as E2 functions largely as a transcription factor, and while adenovirus E1A has such an activity, these two proteins are otherwise very dissimilar. These data also suggest the possibility that, upon understanding each helper gene (adenovirus, herpes virus or HPV) mechanism of action, we can combine various cocktails of helper genes to get better or more specific results in the generation of rAAV products or gene therapy. In fact, preliminary results suggest this is the case (Liu, You and Hermonat, unpublished). Information as to how HPV helps AAV is important for developing new techniques for the generating rAAV for gene therapy as well as understanding the HPV–AAV interaction and its effect on cervical cancer risk. Clearly, as demonstrated in co-infection experiments in skin, both HPV and AAV, when present at moderate levels, accentuate each other’s replication. This suggests that these two viruses will always be preferentially found together as traveling partners in the genital tract. If you have only HPV infection or low AAV infection, you are at greater risk for developing cancer (Coker et al., 2001; Smith et al., 2001). This study establishes the level of contribution of specific HPV-16 genes for their helper function for increasing AAV replication in the stratified squamous epithelium. Such knowledge is important for our understanding of AAV/HPV interaction with regards to the development of cervical cancer as well as for the potential that certain HPV genes may be useful in the generation of recombinant AAV virus for gene therapy.

Materials and methods

Construction of plasmids

We used standard recombinant DNA methodologies to generate expression plasmids for E1, E2, E6 and E7 (Liu et al., 2000, 2001, 2005; Chiriva-Internati et al., 2002, 2003; You et al., 2002). Briefly, the wild-type AAV genome pSM620 was partially digested with BsaI to delete the internal AAV sequences from map units 6–95 (nt 286–4460), and a specially designed polylinker was ligated in place, resulting in the AAV vector plasmid d66-95/PL1. Into this polylinker, the neomycin (Neo) resistance gene and one of the HPV16 E1, E2, E6 and E7 open reading frames (cloned by PCR amplification from the HPV-16 plasmid pAT-HPV-16) were sequentially ligated (see Fig. 1 for structures). AAV/Neo, with no HPV gene, was also generated. The role of AAV/Neo was to serve as an HPV-negative control vector. Two additional E1 vectors were also generated. One contained the amino-half of E1, E1-5’ (aa 1 to 365). The primer used was 5’-ATA-GATCTTCTACCATGGCTAGCTCGG-3 and 5’-ATTCGGAG-TAGTTAGGCATGTACCATC-3 which targeted
amplification of the HPV-16 sequences from nt 859 to 1959. The other was the carboxy-half, E1-3' (aa 353 to 649). The primer used was 5'-ATAATAGATCTATGAGACA CGCCA-GAATGGGATAC-3' and 5'-CCGGTCCGAGTATATGGTCAGCG-TAGGTC-3' which targeted amplification of the HPV-16 sequences from nt 1866 to 2844. In the case of E1-5', an in-frame stop codon TAG was included at the end of the open reading frame. In the case of E1-3', a new in-frame initiation methionine codon was included to allow for translation of the open reading frame. The enhanced green fluorescent Protein (GFP) cDNA was derived from plasmid pEGFP-N1 (BD Biosciences Clontech) and inserted to the downstream of p5 promoter of dl6-95 vector to generate AAV/GFP.

Analysis of transfection efficiency

Keratinocytes (Cambrex) were cultured in six-well plates with the keratinocyte medium (Cambrex). When the cell density was at 80% confluence, they were transfected with 1.0 μg of the AAV/GFP plasmid using FuGENE 6 liposomal reagent (Roche Diagnostics) according to the manufacturer’s recommendation. At 72 h of post-transfection, the keratinocytes were observed under an inverted fluorescent microscope. At the same time, the cells were also harvested and detected by FACs as the routine method.

Assaying AAV replication and HPV helper gene activity in the organotypic epithelial raft culture system

Primary human foreskin keratinocytes (PHFK) were purchased from Clonetics Inc. (San Diego, CA) and cultured in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) in 37 °C under 5% CO2. Cells were fed every other day. A representative experiment of this design is shown in Fig. 2A. On day 0, 10⁶ PHFK monolayers were transfected with 4 μg of the AAV/HPV (AAV/E1, E1-5', E1-3', E2, E6 or E7) plasmid using Fugene6 (Roche, Indianapolis, IN) according to manufacturer’s instructions. Transfection of 4 μg AAV/Neo (no HPV gene) was served as a control. The next day (day 1), these cells were infected with 10⁸ infectious units of wild-type AAV-2 virus (MOI ~100). Six hours later, the cells were trypsinized and seeded onto J2 fibroblast-containing collagen rafts as described previously (Meyers et al., 2000, 2001). Next, the rafts (later on day 1) were raised to the air interface and allowed to form a stratified squamous epithelium and were harvested on day 5. Experiments were repeated 3–8 times.

Southern blot analysis of AAV DNA replication

All rafts were harvested on day 5. After washing with PBS twice, total DNA was extracted as previously described (Meyers et al., 2000, 2001). Total cellular DNA (10 μg) was agarose gel sized separated, Southern blotted and probed with 32P-AAV capsid gene DNA. This allowed for the specific identification of the wild-type AAV genome, without the complication of also identifying the AAV/HPV helper plasmid DNA. Finally, a quantification of the Southern blot was done by densitometric analysis using the Alpha Imager 2000 (Alpha Innotech Corporation, San Leandro, CA).

RT-PCR analysis of AAV and HPV mRNA expression

Total RNA was isolated from rafts on day 5 using Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer protocol and treated with 5 U/μg of RNase-free DNase I at 37 °C for 2 h. Messenger polyA RNA then was isolated using the Oligotex mRNA Mini Kit (QIAGEN Inc. Valencia, CA) according to the supplier’s instruction. The first-strand cDNA synthesis was performed at 37 °C for 1 h in a final volume of 25 μl reaction buffer (1 μg mRNA; 50 mM Tris–HCl, pH 8.3; 7.5 mM KCl; 3 mM MgCl2; 10 mM DTT; 0.5 μg oligo(dT)15; 0.5 mM each of the four dNTPs; 30 U of RNasin and 200 U of M-MLV Reverse Transcriptase RNase HMinus (Promega Co., Madison, WI)). PCR amplification (32 cycles) of the cDNA was performed in a 100-μl reaction volume which contained 2.5 U Taq DNA polymerase; 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 2 mM MgCl2; 0.2 mM each of the four dNTPs; 1 μM of each upstream and downstream primer specific for the cDNA template and 10 μl cDNA templates.

The primer set used for AAV rep was 5'-TGAAGCGGG-GAGTTTGAACG-3' and 5'-TCCATATTAGTCCACGGCC-3', which targeted amplification of the AAV sequences from nt 291 to 322. The primer set used for AAV cap was 5'-CCCTCGAGGAGAAAAGCAG-3' and 5'-GTGGTGACAGGCA-3' which targeted amplification of the AAV sequences from nt 2417 to 3175. The TFIIB (housekeeping gene) was also analyzed in each RT-PCR mix. The products were size separated by agarose gel electrophoresis, stained with ethidium bromide and photographed.

For analysis of HPV mRNA expression keratinocytes were lipofected with 1.0 μg of AAV/E1, AAV/E2 AAV/E6 or AAV/ E7 plasmid using FuGENE 6. The cells were harvested at 72 h post-lipofection, and HPV mRNA expression was measured similar to that done for AAV mRNA analysis with the exception that different analytical primers were used, as shown below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer position</th>
<th>Primer sequences</th>
<th>Size of PCR products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Up</td>
<td>nt 859 TCTACCATGGCTGATCTCTG</td>
<td>1986</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>nt 2844 TATATTAGTCCAGGTCG</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>Up</td>
<td>nt 2742 ACAAGGAAGAAACGATGAGGAGAC</td>
<td>1136</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>nt 3877 GTTGGTGATGACATCAAG</td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>Up</td>
<td>nt 57 CGGTCTAGTATAAAACGAGC</td>
<td>523</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>nt 579 GGCTCGAGGTGATCTCC</td>
<td></td>
</tr>
<tr>
<td>E7</td>
<td>Up</td>
<td>nt 559 ATCATGCATGGAGATACACC</td>
<td>322</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>nt 880 GGCTCCAGCAGCTACGCC</td>
<td></td>
</tr>
</tbody>
</table>

The TFIIB (housekeeping gene) was also analyzed as previously described (Liu et al., 2000).

Western blot analysis of AAV protein expression

For protein isolation, the rafts were washed with PBS twice and lysed with Passive Lysis Buffer (Promega, Madison, WI).
5 µg of protein from each raft was then briefly boiled and then electrophoresed by 10% SDS-PAGE and electroblotted. The resulting nitrocellulose membrane was blocked by 10% milk TBS for 1 h. Purified mouse anti-human AAV rep or cap monoclonal antibody (mouse IgG2a, American Research Products Inc., Belmont, MA) was added (duplicate membranes) to the buffer (1:100 dilution). The membrane was incubated at 4 °C overnight and then washed with TBS three times. The membrane was then put into peroxidase-labeled secondary antibody and incubated at room temperature for 1 h. After washing the membrane three times, ECL chemiluminescence was applied.

Assaying for AAV virion production in the organotypic epithelial raft (skin) culture system

Putative virus stocks were generated by freezing day 5 rafts and grinding the rafts with mortar and pestle. The remains of the raft were placed in 1 ml of DMEM medium, vortexed for 1 min and centrifuged at 8000 g for 15 min to remove debris, and the supernatant was filtered through a 20-µm filter. Raft lysates were treated with Dnase I at 100 U/ml for 1 h to eliminate unencapsidated DNA contamination. One third of the putative virus stock was used to infect a 6-cm plate on 80% confluent monolayer 293 cells. These cells were also infected with Ad helper virus at an MOI of 5. Any AAV infectious units produced in the original raft would be eliminated by unencapsidated DNA contamination. One third of the putative virus stock was used to infect a 6-cm plate on 80% confluent monolayer 293 cells. These cells were also infected with Ad helper virus at an MOI of 5. Any AAV infectious units produced in the original raft would be amplified in the Ad-infected 293 cells. After 36 h of infection, total DNA was extracted, and 10 µg of total cellular DNA was analyzed for AAV DNA replication levels by agarose gel electrophoresis, Southern blotting, and probing with 32P-AAV cap DNA probe.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.virol.2005.08.039.

References


