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The Adeno-Associated Virus *rep* Gene Suppresses Herpes Simplex Virus-Induced DNA Amplification

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Herpes simplex virus (HSV) induces within the host cell genome DNA amplification which can be suppressed by coinfection with adeno-associated virus (AAV). To characterize the AAV functions mediating this effect, cloned AAV type 2 wild-type or mutant genomes were transfected into simian virus 40 (SV40)-transformed hamster cells together with the six HSV replication genes (encoding UL5, UL8, major DNA-binding protein, DNA polymerase, UL42, and UL52) which together are necessary and sufficient for the induction of SV40 DNA amplification (R. Heilbronn and H. zur Hausen, J. Virol. 63:3683–3692, 1989). The AAV *rep* gene was identified as being responsible for the complete inhibition of HSV-induced SV40 DNA amplification. Likewise, *rep* inhibited origin-dependent HSV replication. *rep* neither killed the transfected host cells nor interfered with gene expression from the cotransfected amplification genes. This points to a specific interference with HSV-induced DNA amplification.

Adeno-associated viruses (AAVs) are members of the parvovirus family, a group of small single-stranded DNA viruses with unique replication properties. In contrast to the autonomous parvoviruses which can replicate independently in proliferating cells, the AAVs rely on helper viruses, either adenoviruses or herpesviruses, for efficient replication (for a review, see reference 4). However, cells treated with chemical or physical carcinogens can support low-level AAV replication in the absence of a helper virus (13, 34, 45, 46). Thus, the replication defectiveness of AAV is not absolute. The autonomous and helper-dependent parvoviruses have unique biological properties in common. Members of both groups efficiently suppress tumor growth in animals, irrespective of the mode of tumor induction. Parvoviruses inhibit spontaneous tumor formation (37) and tumors induced by various oncogenic viruses as well as by chemical carcinogens (9, 10, 19, 28, 30, 39). In addition, there is evidence from seroepidemiological studies that high antibody titers against the human AAV types 2, 3, and 5 (AAV-2, AAV-3, and AAV-5) are associated with a reduced cancer incidence (11, 27, 35). The mechanisms, however, by which parvoviruses exert their oncosuppressive effect are not yet understood. In an attempt to unravel the underlying molecular mechanisms, the effect of parvoviruses was studied in various in vitro transformation systems. Minute virus of mice, an autonomous parvovirus, suppressed transformation of mouse fibroblasts by simian virus 40 (SV40) (29). Likewise, AAV suppressed the transformation of hamster fibroblasts by different adenovirus strains (5) and the transformation of the mouse fibroblast cell line C127 by bovine papillomavirus. AAV-2 $p78^{rep}$ appeared to be responsible for this effect (16).

Three viral functions have been mapped on the 4.65-kilobase (kb) AAV-2 genome (Fig. 1). The 145-base-pair (bp) terminal repeat structures serve as origins of replication. The right-hand open reading frames (ORFs) encode the three capsid proteins (*cap*), whereas the ORFs on the left-hand side of the genome code for *rep*, a family of multifunctional nonstructural AAV proteins. The mRNAs coding for $p78^{rep}$ and the spliced $p68^{rep}$ start at the p_5 promotor, and those coding for $p52^{rep}$ and the spliced $p40^{rep}$ start at the p_{19} promotor. The *rep* proteins are required for AAV DNA replication (17, 40). $p78/68^{rep}$ is responsible for the accumulation of replicative intermediates, whereas $p52/40^{rep}$ is required for the generation of single-stranded monomer AAV and for the packaging of the virus (6). The role of $p78/68^{rep}$ in the accumulation of replicative intermediates is also reflected by their binding to the AAV origins of replication (18). The *rep* proteins are required not only for AAV DNA replication but also for AAV gene regulation. Depending on the presence or absence of helper adenovirus functions, they either activate or repress the AAV promotors in *trans* (3, 41). Although most of these experiments were performed with human cells, the different AAV mutants exhibited the same replication phenotype in rodent cells (16; R. Heilbronn, unpublished data).

We have shown previously that AAV can severely inhibit herpesvirus- or carcinogen-induced DNA amplification (2, 13, 34). DNA amplification plays a central role not only in the development of drug resistance (20, 26, 36) but also in the course of tumor development, where amplified oncogene sequences are a frequent finding (for a review, see reference 1). We therefore decided to analyze the AAV-mediated inhibition of DNA amplification at the molecular level. This was possible by cotransfecting cloned AAV mutants in combination with the recently identified set of herpes simplex virus (HSV) amplification genes (15), thus introducing the amplification-inducing and -inhibiting functions into the same fraction of cells. By the use of AAV wild-type (wt) or mutant genomes, we show here that *rep* alone is sufficient to completely suppress DNA amplification induced by the set of six HSV amplification genes.

MATERIALS AND METHODS

Recombinant plasmid DNAs. The cloned AAV-2 wt genome (pAV2) was obtained from C. Laughlin (24). AAV-2 was subcloned into the *Bam*HI site of Bluescript (Stratagene), resulting in plasmid pTAV2 (Fig. 1). *ori* mutants

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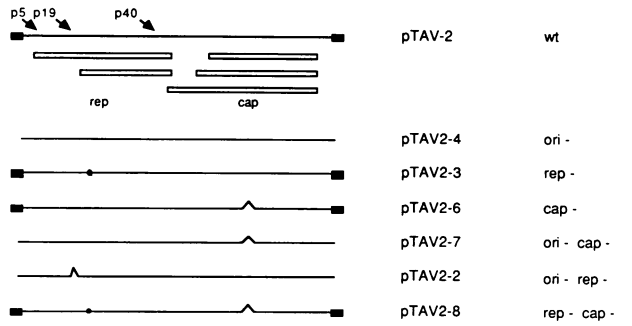

FIG. 1. Structure of AAV wt and mutant genomes. The AAV-2 genome is represented schematically with the inverted repeats serving as origins of replication at the ends of the genome (■). The ORFs encoding the nonstructural proteins (*rep*) and three capsid proteins (*cap*) are shown (□). Construction of the AAV mutant genomes is explained in the text. Frameshift deletions (/\) and insertions (●) are indicated. Mutant phenotypes are indicated to the right of the plasmid designations.

(pTAV2-2, pTAV2-4, and pTAV2-7) were generated from pTAV2 by using the *Bal*I sites at nucleotide positions 121 and 4554. *rep* mutants were generated either by introducing a 4-bp frameshift insertion at position 1045 by using the unique *Bam*HI site (pTAV2-3 and pTAV2-8) or by introducing a 150-bp deletion encompassing the p_{19} promotor region (positions 814 to 964, pTAV2-2). Both mutations inactivated the *rep* genes starting from the promoters p_5 and p_{19}. *cap* mutants (pTAV2-6, pTAV2-7, and pTAV2-8) were generated by introducing a 164-bp deletion between positions 3326 and 3490.

The series of plasmids carrying the HSV amplification genes pH6 (pol and DBP), pH7 (IE175 and IE110), pH8 (UL42 and UL52), pH9 (UL5 and UL8), and the respective ORFs expressed under the control of the human cytomegalovirus (HCMV) immediate early (IE) promoter (−598 to +52) (pCM-UL5, -UL8, -UL9, -*pol*, -DBP, -UL42, and -UL52) have been described before (15). pH10 carries the HSV ori_S (15). pCMcat (kindly provided by Hubert Stöppler) expresses the chloramphenicol acetyltransferase (CAT) gene under the control of the same HCMV IE promoter fragment (−598 to +52) as the pCM-UL series of HSV expression constructs.

Cells and viruses. The Elona11 cell line and propagation of HSV-1 strain 17 have been described elsewhere (15, 33). AAV-2 was propagated in HeLa cells with adenovirus 2 as the helper as described previously (46).

Transfection procedure. Calcium phosphate coprecipitation followed by a dimethyl sulfoxide shock was performed exactly as described before (15). Each transfection experiment was repeated at least three times with two different plasmid preparations, leading to similar results.

AAV replication assay. HeLa cells (3×10^5) were plated onto 6-cm-diameter dishes. The next day, cells were transfected with AAV-2 mutant plasmids which had been excised from the vector with *Pvu*II. After the dimethyl sulfoxide-shock, cells were infected with HSV-1 diluted in 1 ml of medium (multiplicity of infection, 10) and incubated at 37°C for 40 h. The cultures were lysed in situ by three freeze-thaw cycles. The disrupted cells were pelleted and used for the analysis of AAV DNA replication. Genomic DNA was extracted by digestion with proteinase K, repeated extractions with phenol and chloroform, digestion with RNase A, and dialysis against 1× TE (15). The supernatants were treated at 56°C for 30 min to inactivate the helper virus (HSV) and then used for the titration of infectious AAV in 1:10 dilution steps on HeLa cells grown on 96-well plates. Cells were infected with HSV-1 as the helper virus 16 h later. At 40 h after HSV infection, cells were dotted onto GeneScreen (Dupont, NEN Research Products, Boston, Mass.) and hybridized to ^{32}P-labeled AAV-2 DNA.

Analysis of genomic DNA. For the analysis of AAV DNA replication, total genomic DNA was doubly digested with *Xba*I and *Dpn*I. A 5-μg sample of each digest was run on 0.7% agarose gels, blotted onto GeneScreen Plus (Dupont, NEN Research Products), and hybridized to a ^{32}P-labeled, full-length AAV-2 probe. SV40 DNA amplification was assayed on Southern blots of *Sac*I-digested genomic DNA hybridized to ^{32}P-labeled SV40 DNA, and HSV ori_S replication was assayed with the *Dpn*I assay as described before (15).

CAT assays. Protein contents of freeze-thaw extracts were determined by using the Bradford assay (Bio-Rad Laboratories, Munich, Federal Republic of Germany). CAT enzymatic activity was assayed by using defined amounts of heat-treated (10 min at 60°C) protein extract (8). Final concentrations in the assay were 1.25 μCi of ^{14}C-chloramphenicol per ml and 1 mmol of acetyl coenzyme A per liter. Ethyl acetate-extracted reaction products were analyzed by thin-layer chromatography. Spots representing unreacted chloramphenicol and acetylated chloramphenicol were excised, and radioactivity was quantitated by liquid scintillation counting.

RESULTS

Replication phenotype of AAV mutant plasmids. Single and double mutations were generated in cloned wt AAV-2 DNA (pTAV2) according to published genetic data (Fig. 1) (17, 40). The phenotypes of this series of AAV plasmids were verified by transfecting the individual mutants into HeLa cells with HSV-1 as the helper virus. At 40 h after HSV infection, cultures were processed for the determination of AAV DNA replication and, in parallel, production of infectious viral particles (Fig. 2). With pTAV2 (wt), the typical AAV replication intermediates appeared (Fig. 2A, lane 2). Titration of freeze-thaw supernatants of transfected cells on fresh HeLa cells in the presence of HSV-1 as the helper virus clearly demonstrated the rescue of infectious viral particles from pTAV2 (Fig. 2B, lane 2). HSV-1 served as a helper virus with an efficiency equal to that of adenovirus type 2 (data not shown). The *cap* mutant pTAV2-6 gave rise to replicative intermediates (Fig. 2A, lane 5) whose slightly shorter lengths (compared with that of wt pTAV2) are due to the 150-bp deletion within the *cap* ORF. The *cap*-negative phenotype of pTAV2-6 was documented by the inability of the mutant to generate infectious viral particles (Fig. 2B, lane 5). As expected, *rep* mutants did not give rise to either replicative intermediates or infectious AAV particles (Fig. 2A and B, lanes 4 [pTAV2-3], 7 [pTAV2-2], and 8 [pTAV2-8]). Transfection of *ori* mutants with deletions of the terminal repeats but intact *rep* genes (pTAV2-4 and pTAV2-7) gave rise to a high AAV-2-specific background hybridization signal on the Southern blot without the typical replication intermediates (Fig. 2A, lanes 3 and 6). The absence of similar hybridization signals in all the lanes with *rep* mutants (Fig. 2A, lanes 4, 7, and 8) raises the possibility that *rep* gene expression per se mediates unspecific initiation of DNA synthesis on the AAV template in the absence of origin sequences. However, no infectious particles were formed.

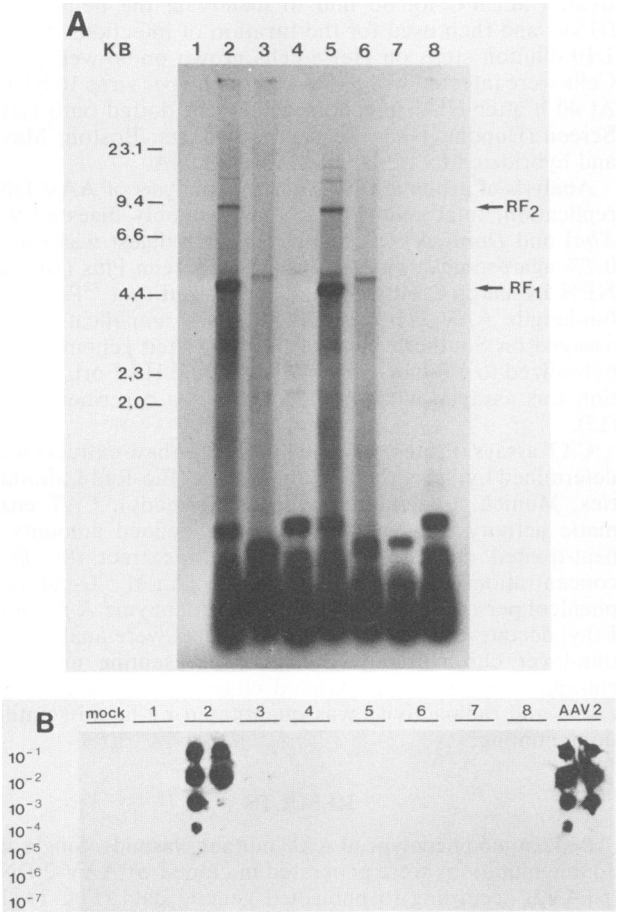

FIG. 2. Phenotypes of AAV-2 mutants with HSV as helper virus. HeLa cells were transfected with AAV-2 wt or mutant genomes and infected with HSV-1. At 40 h p.i., cultures were freeze-thawed three times and cells were pelleted. Genomic DNA was extracted from the pellet for Southern blot analysis (A). Supernatants were used for titration of infectious AAV particles (B). (A) Southern blot analysis of *Xba*I-*Dpn*I-digested genomic DNA hybridized to ^{32}P-labeled AAV DNA. *Xba*I was a noncut enzyme for AAV DNA. *Dpn*I digested the transfected procaryotic plasmid DNAs visible in the lower part of the figure, whereas AAV molecules replicated in eucaryotic cells were resistant to *Dpn*I and appear as the typical replicative intermediates in lanes 2 and 5. RF_1 (4.65 kb) represents a double-stranded monomer, and RF_2 (9.3 kb) is a double-stranded dimer. In addition, higher oligomeric forms are visible faintly. Transfected DNAs (20 μg) are as follows: lane 1, Bluescript; lane 2, pTAV2 (wt); lane 3, pTAV2-4 (*ori* negative); lane 4, pTAV2-3 (*rep* negative); lane 5, pTAV2-6 (*cap* negative); lane 6, pTAV2-7 (*ori* and *cap* negative); lane 7, pTAV2-2 (*ori* and *rep* negative); lane 8, pTAV2-8 (*rep* and *cap* negative). Because of the high level of background hybridization which was most likely due to single- and partially double-stranded replicative intermediates of all size classes, it is difficult to detect single-stranded, full-size AAV molecules. The bands around 5 kb visible in lanes 2 to 6 most likely represent a small amount of input plasmid DNA resistant to *Dpn*I. The various sizes of these bands correspond to the different sizes of the mutants. (B) Titration of infectious AAV-2 particles generated after transfection of AAV mutants and subsequent HSV-1 infection. Supernatants were treated at 56°C for 30 min to inactivate the helper virus (HSV-1) and titrated in duplicate in 1:10 dilution steps on HeLa cells grown in 96-well plates. After 16 h, cells were infected with HSV-1 as the helper virus. After 40 h, cells were dotted onto GeneScreen and hybridized to ^{32}P-labeled AAV-2 DNA. Lanes: mock, no supernatant; 1 to 8, same as in panel A; AAV2, titration of heat-inactivated (30 min, 56°C) prediluted AAV-2 stock virus used as a positive control.

The transfections described above were repeated in Elona11 cells with similar results.

Effect of AAV mutants on DNA amplification induced by the HSV amplification genes. HSV infection leads to DNA amplification of chromosomally integrated SV40 DNA sequences. This effect can be suppressed by coinfection with AAV (2, 34). Recently, we have identified the six HSV-1 genes which together are necessary and sufficient for the induction of SV40 DNA amplification. The locations of the amplification genes (UL5, UL8, DBP, *pol*, UL42, and UL52) on the HSV genome are depicted in Fig. 3. Transfection of the set of plasmids carrying the HSV amplification genes (pH6, pH8, pH9, and pH7) induced a strong amplification signal (Fig. 4A, lane 3) as described before (15). Cotransfection of pTAV2 (wt) with pH6, pH7, pH8, and pH9 suppressed the amplification effect (Fig. 4A, lane 4). Bluescript DNA or cloned AAV-2 wt DNA (pTAV2) was used as a negative control (Fig. 4A, lanes 1 and 2).

To evaluate which of the AAV-2 functions mediated the suppression of DNA amplification, individual AAV-2 mutant genomes (Fig. 1) were cotransfected. pTAV2-4 (*ori* negataive) (Fig. 4A, lane 5) as well as pTAV2-6 (*cap* negative) (Fig. 4A, lane 7) suppressed the induction of DNA amplification as well as pTAV2 (wt), whereas pTAV2-3 (*rep* negative) did not affect HSV-induced DNA amplification (Fig. 4A, lane 6). The conclusion that the *rep* gene is responsible for this effect was confirmed by the use of double mutants. pTAV2-7 (*ori* and *cap* negative) suppressed DNA amplification (Fig. 4A, lane 8), which is in line with the fact that this mutant carried an intact *rep* gene. However, double mutants with a disrupted *rep* gene, pTAV2-2 (*ori* and *rep* negative) and pTAV2-8 (*rep* and *cap* negative), were unable to suppress HSV-induced DNA amplification (Fig. 4A, lanes 9 and 10). In conclusion, the *rep* gene is responsible for the suppression of HSV-induced DNA amplification.

The AAV *rep* gene suppresses DNA amplification induced by HSV amplification genes under control of a heterologous promoter. Several targets can be envisaged for the *rep* gene-mediated inhibition of HSV-induced DNA amplification. To exclude the possibility that *rep* interferes with HSV IE gene-mediated transactivation of the early promoters which drive the HSV amplification genes, we asked whether *rep* is able to inhibit DNA amplification induced by the set of amplification genes driven by a heterologous promoter (pCM-UL5, -UL8, -DBP, -*pol*, -UL42, and -UL52; Fig. 3). A transfection experiment similar to the one described above was performed, with replacement of the authentic HSV amplification genes by the HCMV IE-driven constructs. Again, the controls, i.e., transfection of either Bluescript DNA or pTAV2, did not show any DNA amplification (Fig. 4B, lanes 1 and 2), whereas the set of HCMV IE-driven HSV amplification genes induced a strong amplification effect (Fig. 4B, lane 3) as described before (15). This effect was suppressed upon cotransfection of pTAV2 (wt), pTAV2-4 (*ori* negative), or pTAV2-6 (*cap* negative) (Fig. 4B, lanes 4, 5, and 7). pTAV2-3 (*rep* negative), however, did not have an effect on HSV-induced DNA amplification (Fig. 4B, lane 6). Again, double mutants confirmed the conclusion that *rep* mediates this inhibition. The mutant pTAV2-7 (*ori* and *cap* negative) was able to suppress DNA amplification (Fig. 4B, lane 8), whereas the two double mutants with disrupted *rep* genes, pTAV2-2 (*ori* and *rep* negative) and pTAV2-8 (*rep* and *cap* negative), did not influence DNA amplification (Fig. 4B, lanes 9 and 10). Transfection of equimolar amounts of pTAV2-7 (*ori* and *cap* negative) compared with the HCMV IE-driven HSV amplification genes (1 μg of each) still led to

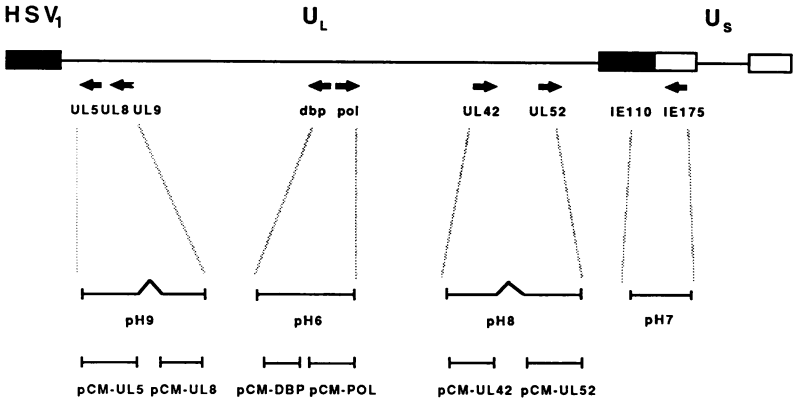

FIG. 3. Structure of HSV-1 genome and cloned amplification genes. Schematic representation of the 150-kb HSV-1 genome with the unique long (U_L) and unique short (U_S) regions and the flanking inverted repeats (■ and □). The replication- and amplification-inducing genes are indicated: UL5, -8, -9, -42, -52, *dbp* (single-stranded DNA-binding protein), and *pol* (DNA polymerase). These genes are early genes which have to be transactivated by the HSV IE genes, IE110 (ICP0), and IE175 (ICP4), for efficient transcription. The arrows indicate the direction of transcription: black arrows are genes necessary for both HSV DNA replication and SV40 DNA amplification (UL5, UL8, *dbp*, *pol*, UL42, UL52, and IE175); speckled arrows indicate genes required only for HSV DNA replication (UL9 and IE110). The set of plasmids covering the individual genes are shown. Below, the pCM series of expression constructs is represented. These constructs are named according to the ORF they express under the control of the HCMV IE promotor.

complete suppression of DNA amplification (data not shown). These experiments confirm that the *rep* gene function alone mediates complete suppression of HSV-induced DNA amplification. In addition, the HSV IE gene-mediated transactivation of early genes does not represent the target for this interference.

***rep* mediates suppression of DNA amplification by mechanisms other than nonspecific down regulation of expression of HSV amplification genes.** To monitor the transfection efficiency of the above described experiment, an HCMV IE promoter-driven CAT construct (pCMcat) was cotransfected (at a molar ratio of 1:20 compared with pTAV2-7) and CAT enzyme activity was assayed in parallel to the amplification assay in Fig. 4B. CAT enzyme activity was in the same range in all transfections (Fig. 4C), reflecting comparable transfection efficiencies. In addition, this result excludes the possibility that the suppression of DNA amplification in the range of 100-fold is due to a *rep*-mediated repression of the HCMV IE promoter, which drives the HSV amplification genes. However, some minor, two- to threefold reduction of pCMcat expression was observed whenever AAV wt or mutants with intact *rep* genes were cotransfected (Fig. 4C). To study the influence of *rep* on pCMcat expression in more detail, pCMcat was transfected into Elona11 cells together with increasing amounts of pTAV2-7 under conditions identical to the ones used for the amplification studies described above. A 2-fold inhibition of CAT expression was observed with equimolar concentrations of pTAV2-7, and up to 3.5-fold inhibition was observed with a 25-fold molar excess of pTAV2-7 versus pCMcat (Fig. 5). These results confirm the above conclusion that this mild reduction of pCMcat expression cannot explain the drastic inhibition of DNA amplification. Our results are in line with recent reports which describe a moderate (about twofold) *rep*-mediated inhibition of CAT expression from an SV40 or bovine papillomavirus promoter (3, 16). We conclude that *rep* mediates inhibition of HSV-induced SV40 DNA amplification by mechanisms other than down regulation of the expression of the HSV amplification genes.

***rep* inhibits origin-dependent HSV replication parallel to the inhibition of DNA amplification.** The six HSV amplification genes encode proteins necessary but not sufficient for HSV DNA replication. An additional gene coding for a HSV origin-binding protein (UL9) is required for origin-dependent HSV replication (15, 44). We therefore asked whether *rep* could also interfere with origin-dependent HSV replication. The seven HSV replication genes under HCMV IE promoter control were transfected into Elona11 cells together with increasing amounts of pTAV2-7 and a plasmid carrying the HSV ori_S (pH10) to test for origin-dependent HSV replication. ori_S replication was assayed with the *Dpn*I assay (Fig. 2A). ori_S replication induced by the seven HSV replication genes was suppressed by increasing amounts of pTAV2-7 (*ori* and *cap* negative) (Fig. 6), thus paralleling the suppression of SV40 DNA amplification which was measured in the same experiment (data not shown). ori_S replication appeared not to be repressed as strongly as SV40 DNA amplification, but this might be due to a higher copy number of transfected ori_S sequences compared with the single integrated copy of SV40. In summary, *rep* markedly inhibits HSV ori_S replication induced by the seven HSV replication genes in parallel to the inhibition of SV40 DNA amplification. At present, we do not know which of the different *rep* proteins is responsible for the above-described effects. We have generated a HCMV IE promoter-driven construct for the *rep*78 ORF which leads to suppression of DNA amplification and ori_S replication similar to those of pTAV2-7 (*ori* and *cap* negative) (data not shown). However, the *rep*52 ORF was embedded in the *rep*78 ORF, so an additional role of $p52^{rep}$ cannot be excluded.

DISCUSSION

AAV inhibits DNA amplification induced by HSV or carcinogens. In this report, we show that the AAV *rep* gene is responsible for the inhibition of HSV-induced DNA amplification.

SV40 DNA amplification as a model system for the amplification of authentic cellular genes. Two lines of evidence support the notion that DNA amplification plays an important role in tumor development. On the one hand, amplification of cellular or integrated viral DNA sequences can be induced in cell lines by chemical or physical carcinogens, by a variety of chemotherapeutic drugs, and also by viruses like

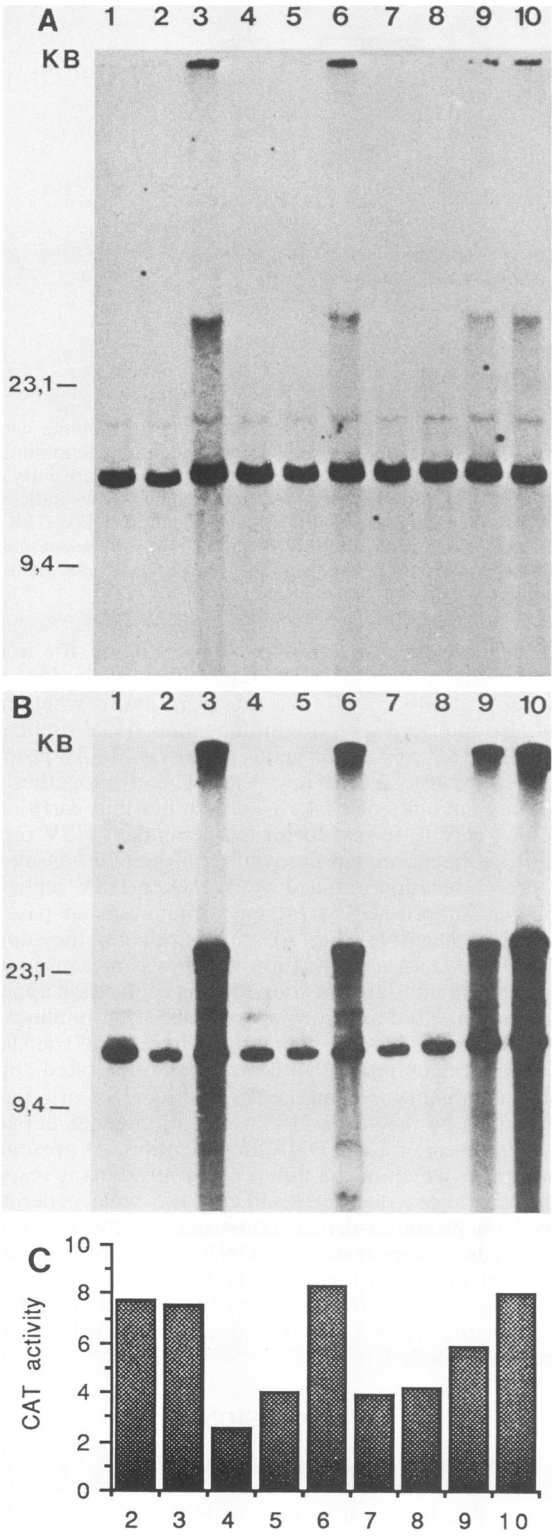

FIG. 4. Effects of wt and mutant AAV genomes on HSV-induced SV40 DNA amplification. (A) Southern blot of *Sac*I-restricted genomic DNA of Elona11 cells transfected with the set of HSV amplification-inducing genes (pH9, pH6, pH8, and pH7) and the different AAV-2 mutant plasmids. The blot was probed with a ^{32}P-labeled *Bst*XI-*Kpn*I fragment of SV40 DNA. The 15-kb (KB) band which is present in every lane corresponds to the genomic restriction fragment carrying the integration locus of SV40, whereas the bands around 40 kb represent amplified copies of this locus. Bands with intensities far below the level of 1 copy per cell always became visible in all the lanes upon long exposure of the blots and most probably represent cellular sequences cross-hybridizing with SV40 DNA. The following combinations of DNAs were transfected (see Fig. 1 and 3 for reference): lane 1, Bluescript DNA, 20 μg; lane 2, pTAV2 (wt), 20 μg; lane 3, pH9, pH6, pH8, and pH7, 4 μg each; lane 4, same as lane 3 plus pTAV2 (wt), 10 μg; lane 5, same as lane 3 plus pTAV2-4 (*ori* negative), 10 μg; lane 6, same as lane 3 plus pTAV2-3 (*rep* negative), 10 μg; lane 7, same as lane 3 plus pTAV2-6 (*cap* negative), 10 μg; lane 8, same as lane 3 plus pTAV2-7 (*ori* and *cap* negative), 10 μg; lane 9, same as lane 3 plus pTAV2-2 (*ori* and *rep* negative), 10 μg; lane 10, same as lane 3 plus pTAV2-8 (*rep* and *cap* negative), 10 μg. (B) As described for panel A, but with the HSV amplification genes under the control of a heterologous constitutive promotor, HCMV IE (pCM-UL5, -UL8, -DBP, -*pol*, -UL42, and -UL52; 1 μg each). For the CAT assay performed in parallel, 0.5 μg of pCMcat was cotransfected. Lanes correspond to those in panel A. (C) CAT assays were performed in parallel to the amplification assay in panel B with 30 μg of protein extract for 1 h. CAT enzymatic activity is represented as percent CAT conversion. Numbers on the *x* axis correspond to lanes in panel A.

HSV (13, 15, 20, 25, 26, 33, 36). On the other hand, amplified oncogene sequences are frequently detected in tumor cell lines as well as in primary tumors (1). The amplification of cellular genes after carcinogen treatment has been well documented, as in the case of inducible dihydrofolate reductase (DHFR) gene amplification, which leads to methotrexate resistance (20, 26, 36). Since inducible amplification of cellular genes is a rare event whose detection requires selection for the amplified phenotype, short-term assay systems were developed. A variety of carcinogenic agents and also viruses like HSV induce a high degree (over 100-fold) of DNA amplification of chromosomally integrated SV40 DNA sequences in SV40-transformed hamster cells within 2 to 5 days (2, 13, 15, 20, 25, 33). This made these cell lines a convenient model system for analysis of the amplification-inducing or -inhibiting potential of many different agents in a short-term assay system. Parallel analysis demonstrated that although SV40 DNA amplification occurred at a much higher rate than DHFR gene amplification, the two events exhibited the same dose responses and time courses and, in addition, occurred in the same subpopulation of cells (20, 21). This parallelism suggested that similar intracellular events lead to SV40 as well as DHFR gene amplification. Thus, SV40 DNA amplification can be considered to be a suitable model system for the molecular analysis of the mechanisms leading to DNA amplification. This is further supported by the observation that AAV appears to interfere with both SV40 DNA amplification and DHFR gene amplification: AAV severely inhibits not only carcinogen-induced SV40 DNA amplification (34) but also carcinogen-induced resistance against methotrexate, which has been associated with amplification of the DHFR gene (A. Ö. Yalkinoglu, J. R. Schlehofer, and H. zur Hausen, submitted for publication). It will be interesting to see whether the AAV *rep* gene is also responsible for these carcinogen-induced effects.

Targets for *rep* interference with DNA amplification. Three different mechanisms can be envisaged for the *rep*-mediated interference with DNA amplification: an interference of *rep* with the expression of the cotransfected HSV amplification genes, a concentration-dependent toxicity of *rep* for the host cell, and a specific interference of *rep* with DNA amplification.

Interference of *rep* with the expression of the cotransfected HSV amplification genes could be excluded. A minor

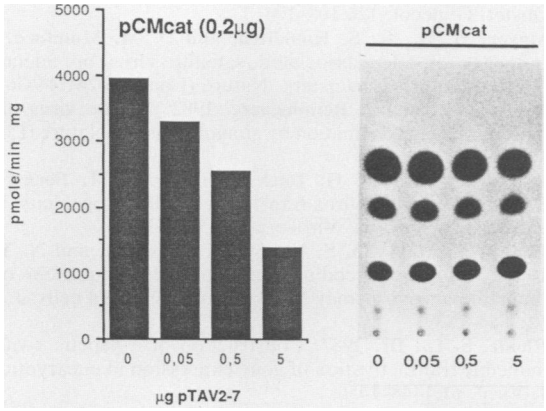

FIG. 5. Influence of AAV *rep* gene on HCMV IE promoter-driven CAT gene expression. CAT assays were performed with extracts from Elona11 cells transfected with 0.2 μg of pCMcat in the presence of increasing amounts of pTAV2-7 (*ori* and *cap* negative). CAT enzymatic activity was assayed with 10 μg of protein extract for 1 h as outlined in Materials and Methods. Autoradiograms of the thin-layer chromatographies are shown to the right. Quantitation of the enzyme activity is given to the left as picomoles of acetylchloramphenicol generated per minute per milligram of total protein.

(2- to 3-fold) effect of *rep* on gene expression does not affect this conclusion, since the repression of DNA amplification is in the range of 100-fold. Moreover, to exclude competition between the HCMV IE promoter driving the HSV amplification genes and the AAV promoters driving the *rep* genes, we cotransfected an HCMV IE promoter-driven construct for the *rep*78 ORF which suppressed HSV-induced DNA amplification in a manner similar to that of pTAV2-7 (R. Heilbronn, unpublished data). Thus, the amplification-inducing HSV genes and the amplification-inhibiting AAV *rep* genes behave similarly, irrespective of whether they are expressed by their cognate or by a heterologous promoter (HCMV). This further argues against an interference at the level of gene expression under natural coinfection conditions.

An alternative mechanism of *rep* interference with DNA amplification could be a concentration-dependent toxicity of *rep* for the host cell. Low-level *rep* expression is certainly not toxic, since AAV is known to establish latency in cell cultures with high frequency (7, 12) in the absence of helper viruses. The AAV promoters driving *rep* are active without a helper virus, though at a low level (3, 41, 42). However, it

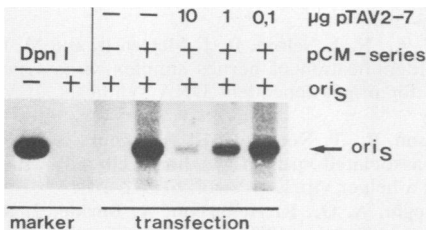

FIG. 6. *rep* inhibits ori_S replication induced by HSV replication genes. Elona11 cells were transfected with the set of seven HSV replication genes (UL5, UL8, UL9, DBP, pol, UL42, and UL52; 1 μg of each) together with pH10 (ori_S, 1 μg) and increasing amounts of the *rep*-expressing plasmid pTAV2-7 (0.1, 1, and 10 μg). A Southern blot of *Eco*RI-*Hin*dIII-*Dpn*I-digested genomic DNA was probed with a ^{32}P-labeled ori_S fragment. −, Not present; +, present.

is well established that AAV infection alone already leads to a reduced plating efficiency of the infected cells (43; R. Heilbronn and A. Bürkle, unpublished observation). Treatment of AAV-infected cells with carcinogens leads to AAV DNA replication and a concomitant drastic reduction of plating efficiency and killing of the cells, irrespective of whether infectious progeny is produced (14, 43, 45, 46). Furthermore, overexpression of NS1, the *rep* gene homolog of autonomous parvoviruses, leads to cell toxicity (31, 32). Toxicity of overexpressed *rep* has also been assumed because it proved difficult if not impossible to generate cell lines which constitutively express *rep* (23). In spite of all the aforementioned observations, toxicity of *rep* does not seem to play a role within the 48 h of the DNA amplification assay, because there is no major cell killing by *rep*. This can be concluded from the amplification experiments described in this report in which CAT expression from transfected pCMcat was not significantly lowered by the expression of *rep*, whereas DNA amplification induced by the cotransfected HSV amplification genes was completely suppressed. We can therefore exclude the possibility that *rep* leads to loss of the successfully transfected cell.

From our data, we conclude that *rep* interferes with DNA amplification by a specific mechanism. Many intracellular targets can be envisaged. However, since *rep* interferes with both SV40 DNA amplification and HSV ori_S replication, one should consider that *rep* might directly interact with the HSV replication and amplification complex. Recently, Labow and Berns reported *rep*-mediated inhibition of hybrid virus genomes carrying AAV terminal repeats attached to an SV40 replicon (22). It is difficult to compare the two systems, but it appears that *rep* inhibits replication of the AAV/SV40 hybrid virus through the AAV termini. This assumption is further supported by the recent demonstration that $p78/68^{rep}$ binds to the AAV terminal repeats (18). Whatever intracellular target *rep* might use for the inhibition of inducible DNA amplification, further detailed analysis of *rep* gene interference with DNA amplification will hopefully lead to an understanding of AAV-mediated oncosuppression as well.

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