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Cell Lines Inducibly Expressing the Adeno-Associated Virus (AAV) *rep* Gene: Requirements for Productive Replication of *rep*-Negative AAV Mutants

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The adeno-associated virus (AAV) *rep* gene codes for a family of nonstructural proteins which are required for AAV gene regulation and DNA replication. In addition, *rep* has been implicated in a variety of activities outside the AAV life cycle which have been difficult to study, since attempts to achieve separate and constitutive expression of *rep* in stable cell lines have failed so far. Here we report the generation of two cell lines which inducibly express Rep78 under the control of the glucocorticoid-responsive mouse mammary tumor virus promoter. In addition, one of the cell lines constitutively expresses relatively high levels of Rep52. Both cell lines showed similar plating efficiencies with and without induction of Rep78 expression, which rules out cytotoxic effects of Rep78. The cell lines efficiently support DNA replication of a *rep*-negative AAV genome and initiate the formation of AAV particles. However, despite the correct sizes and stoichiometry of the three capsid proteins, the AAV particles were noninfectious. This was found to be due to a defect in the accumulation of single-stranded AAV DNA. Transient transfection of single expression constructs for constitutive, high-level expression of individual Rep proteins (either Rep78, Rep68, Rep52, or Rep40) complemented this defect. Infectious *rep*-negative AAV progeny was produced at varying efficiencies depending on the *rep* expression construct used. These data show that functional expression of full-length Rep in recombinant cell lines is possible and that the state of Rep expression is critical for the infectivity of AAV progeny produced.

Adeno-associated viruses (AAVs) belong to the parvovirus family, a group of small single-stranded (ss) DNA viruses with unique replication properties. Whereas autonomous parvoviruses replicate independently in proliferating cells, the AAVs rely on helper viruses, either adenoviruses or herpesviruses, for efficient replication (for a review see reference 2). In the absence of a helper virus AAV integrates into the host cell genome with high efficiency and a preference for a specific site on chromosome 19 (23, 38, 40) or 17 (45).

Three viral functions on the 4.65-kb, linear genome of human AAV type 2 (AAV-2) have been mapped. The terminal 145-bp inverted repeats serve as origins of replication and as *cis* signals for integration and for packaging. The *cap* gene codes for the three structural proteins, and the *rep* gene codes for a family of multifunctional regulatory proteins. The mRNAs for Rep78 and its C-terminally spliced version Rep68 start at the p5 promoter. Two N-terminally truncated versions of Rep78 and Rep68, namely Rep52 and Rep40, respectively, are expressed under the control of the p19 promoter. Rep proteins are needed for AAV DNA replication: Rep78, Rep68, or both directly bind to the AAV origins of replication, thereby acting as ATP-dependent, sequence-specific endonucleases with helicase activity which unwind the AAV *ori* during AAV DNA replication (19, 32, 41). Genetic evidence suggests that Rep78 and/or Rep68 is required for the generation of double-stranded DNA replication intermediates, whereas Rep52 and/or Rep40 is required for the accumulation of ss AAV DNA, which is indicative of virus packaging (6). *rep* is also required for AAV gene regulation. Depending on the presence or absence of helper adenovirus functions, *rep* either activates or represses AAV promoters in *trans* (1, 44).

Autonomous parvoviruses and the AAVs have been shown to suppress tumor development in animals, and cell toxicity of the *rep*-homologous NS1 protein of autonomous parvoviruses (4) appears to provide a clue to the underlying mechanisms (35). AAV-mediated oncosuppression is less well understood. AAV has been shown to suppress oncogene-mediated cell transformation (15, 16, 21, 49) and to suppress inducible DNA amplification (13), both of which are mediated by the AAV *rep* gene. In addition, a variety of direct and indirect experimental approaches support the notion that *rep* exerts antiproliferative activity: *rep* consistently interfered with successful establishment of stable cell lines which were meant to express *rep* from a variety of heterologous promoters (25, 30). Also, AAV vectors which retain *rep* in its natural context showed reduced transduction frequencies compared with AAV vectors with deleted *rep* gene sequences (29, 39). These data appear surprising in light of the fact that AAV induces latency with high frequency in many cells of different origins. To study *rep* in more detail, we have set out to establish cell lines which express *rep* from an inducible promoter. The glucocorticoid-responsive mouse mammary tumor virus long terminal repeat (MMTV LTR) was chosen because of its low basal level of expression, which appeared to be a prerequisite for successful establishment of cell lines carrying functional *rep*. Two cell lines which express functional Rep78 upon induction of the MMTV LTR were obtained and characterized.

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MATERIALS AND METHODS

Recombinant plasmid DNAs. For inducible expression of the AAV-2 Rep proteins an expression construct based on the MMTV LTR was generated as follows. The central *Hae*III subfragment of the MMTV LTR (9) was subcloned in pBluescript (Stratagene) together with a chicken β-globin polyadenylation signal. On the same construct a tk*neo* cassette was inserted, yielding plasmid pMt*neo*. The *rep* open reading frame including the sequence for the C terminus of the spliced Rep proteins (nucleotides 264 to 2260, according to the numbering in reference 42) was inserted into pMt*neo* to yield pMt*rep*. Cloned AAV-2 genomes pTAV2 (wild type [wt]) and pTAV2-3 (*rep* negative) have been described before (13).

Expression constructs for *rep*40 (pCM*rep*40), *rep*52 (pCM*rep*52), *rep*68 (pCM*rep*68), and *rep*78 (pCM*rep*78) are based on pKEX, which carries the human cytomegalovirus immediate-early promoter (34). Separate expression of individual Rep proteins was achieved (i) by mutating the internal translational start AUG (position 993) for the synthesis of *rep*52 and *rep*40 proteins in plasmids pCM*rep*78 and pCM*rep*68, (ii) by mutating the splice donor site (G1907A) in the case of pCM*rep*52 and pCM*rep*78, or (iii) by deleting the intron (positions 1907 to 2227) in pCM*rep*68 and pCM*rep*40 by site-directed mutagenesis (see Fig. 6A). pCM*rep*78 and pCM*rep*68 carry two additional mutations which were necessary for the construction of the wild-type *rep* expression plasmid (pM1), from which these constructs were derived. pM1 was shown to be fully functional with respect to complementation of AAV DNA replication and to inhibition of DNA amplification (22).

Anti-Rep antibody. The monoclonal antibody 294-4 was generated by immunizing mice with a bacterially expressed, gel-purified Rep78 protein that is N-terminally truncated by 171 amino acids. Ascites fluid at a dilution of 1:400 was used for Western blotting (immunoblotting), in which all four Rep proteins are recognized.

Cell culture and virus stocks. HeLa cells and HeLa-derived cell clones were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's minimal essential medium supplemented with 5% fetal calf serum and penicillin-streptomycin. Unless otherwise stated, fetal calf serum was depleted of steroid hormones by activated charcoal in order to keep the basal expression level of the MMTV LTR as low as possible. Adenovirus type 2 (Ad2) stocks were prepared as described previously (47). For the preparation of AAV-2 stocks, freeze-thaw supernatants of AAV-2- and Ad2-infected cells were cleared by centrifugation and then treated for 30 min at 56°C to inactivate helper adenovirus.

Generation of stably transfected cell lines. DNA transfections by the calcium phosphate coprecipitation protocol were performed essentially as described before, but the dimethyl sulfoxide shock was omitted (14). Briefly, on the day before transfection, 2×10^6 HeLa cells were seeded on a 10-cm-diameter dish. pMt*rep* (20 μg) was linearized with *Ssp*I and transfected either directly or after concatemerization with T4 DNA ligase. On the day after transfection, cells were replated at a density of 10^5 cells per 10-cm-diameter dish. Twenty-four hours later, G418 selection was started at a concentration of 1,200 μg of G418 per ml. *rep*-expressing cell clones were subcloned twice by limiting dilution.

Plating efficiencies. Plating efficiencies were determined by plating 200 cells on each 10-cm-diameter dish in medium containing 10% untreated fetal calf serum. Twelve hours later the medium was changed, and 10^{-7} M dexamethasone (Sigma; tissue culture grade, tested for long-term stability and lack of toxicity by the supplier) was added where indicated below.

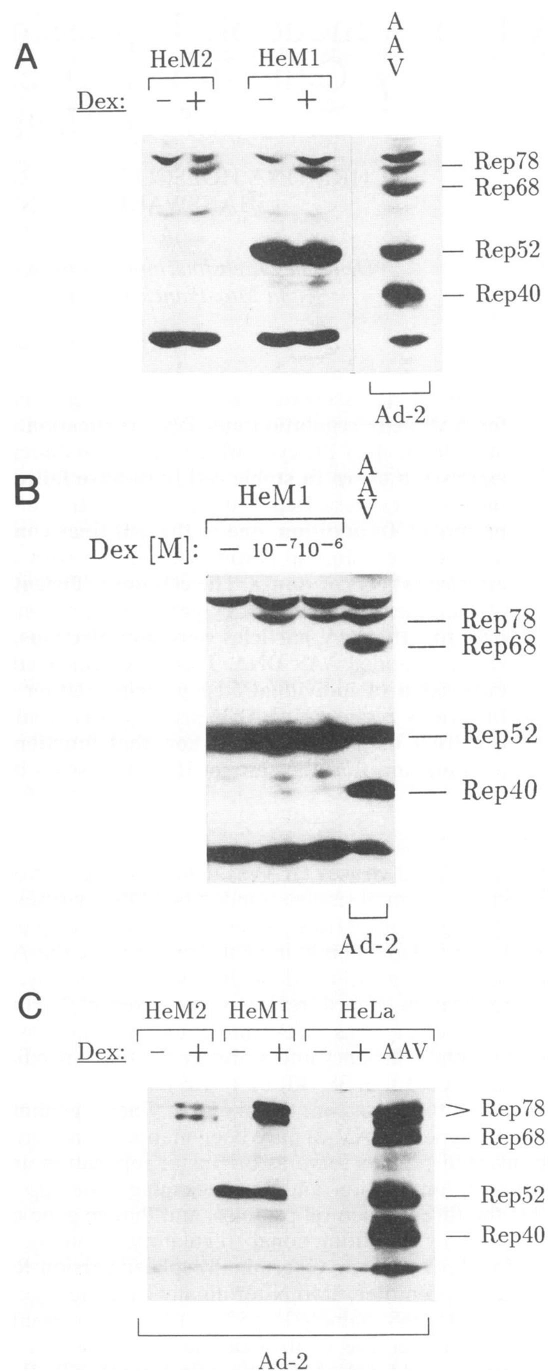


FIG. 1. Rep protein expression in stable cell lines derived from HeLa cells. HeM1 or HeM2 cells (6×10^5) were induced with dexamethasone (Dex) where indicated (+; 10^{-6} M). In the experiment for panel C cells were infected with Ad2 (MOI of 10) before induction. Whole-cell extracts were prepared 24 (A) 32 (B) and 40 h (C) later, and equivalents of 3×10^5 cells were analyzed on Western blots by using the monoclonal anti-Rep antibody 294-4. Extracts from AAV-2- and Ad2-infected HeLa cells were used as controls. The positions of the different Rep proteins are indicated. In adenovirus-infected cells Rep78 forms a double band as indicated.

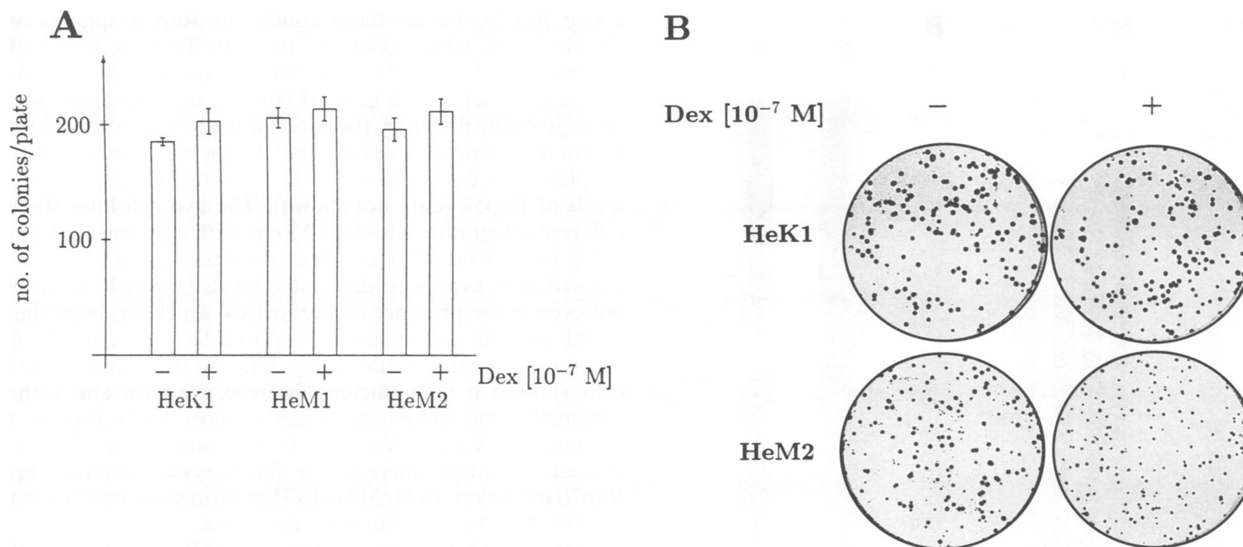


FIG. 2. Plating efficiencies of the *rep*-expressing cell lines HeM1 and HeM2. Two hundred cells were plated in triplicate, and 12 h later they were induced with 10^{-7} M dexamethasone (Dex) where indicated. Cells were stained at 13 days after induction, and colonies were counted. (A) Values indicate absolute plating efficiencies (means ± standard deviations [3]). (B) Representative dishes of the control cell line HeK1 and the *rep*-expressing cell line HeM2 are shown.

After 13 days the plates were fixed and stained with crystal violet. Colonies consisting of more than 100 cells were counted.

Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (26). Proteins were transferred electrophoretically to nitrocellulose membranes by using a semidry blotting system (transfer buffer: 20% methanol, 25 mM Tris-HCl [pH 8.0], 192 mM glycine). Western blots were reacted with the mouse monoclonal anti-Rep antibody 294-4 or with rabbit anti-VP3 antiserum as described previously (37). Detection was performed either with an alkaline phosphatase-conjugated secondary antibody according to published protocols (10) or with a peroxidase-conjugated secondary antibody detected by enhanced chemiluminescence (ECL; Amersham), as described by the supplier.

Transient transfections. On the day before transfection, 10^6 cells were plated onto 10-cm-diameter dishes. Cells were transfected by a modified calcium phosphate transfection protocol as described by Chen and Okayama (8). After overnight incubation at 35°C in 3% CO_2, the cells were washed twice with serum-free medium and then infected with Ad2. After removal of the virus inoculum, cells were incubated with growth medium with 10^{-6} M dexamethasone where indicated below. At different times postinfection (p.i.) cells were harvested and analyzed for AAV DNA replication, AAV capsid formation, accumulation of ss DNA, and production of infectious *rep*-negative AAV.

Analysis of AAV DNA replication and ss DNA accumulation. The analysis of AAV DNA replication has been described before (46). Accumulation of ss DNA was analyzed after extraction of low-molecular-weight DNA by a modified Hirt extraction protocol (33). One-third of the extracts were run on 0.7% agarose gels. Southern blotting and hybridization were performed as described previously (14).

Analysis of AAV particle formation. Heat-inactivated freeze-thaw supernatants were subjected to centrifugation at 264,000 × *g* at 4°C for 90 min. For Western blot analysis, the pellet was processed as described above. For electron microscopic examinations, the pellet was resuspended in 300 μl of a buffer containing 1% deoxycholate, 0.1% SDS, 10 mM Tris-HCl (pH 8.0), and 0.2 mM EDTA. This suspension was layered onto a double sucrose cushion of 50 and 30% sucrose (wt/vol) in 1× TE and centrifuged at 117,000 × *g* at 20°C for 2 h. The sediment was resuspended in 200 μl of 1× TE and pelleted at 117,000 × *g* at 4°C for 2 h. The pellet was resuspended in 1× TE, stained with 2% uranyl acetate, and examined with a Zeiss EM 10 electron microscope.

Hybridization with labelled oligonucleotides. To discriminate between replication of wild-type AAV and replication of *rep*-negative AAV (pTAV2-3), the following two oligonucleotides specific for the region surrounding the *Bam*HI site at position 1045 (mutated in pTAV2-3) were used: the wild-type oligonucleotide (positions 1039 to 1058) 5′ GTCCTCCTG GATCCACTGCT 3′ (antisense), and the pTAV2-3 oligonucleotide (positions 1040 to 1059), 5′ GCAGTGGATCGATC CAGGAG 3′ (sense). Oligonucleotides were end labelled with ^{32}P and hybridized at 58°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt reagent–0.1 mg of yeast tRNA per ml–1% SDS. Filters were washed at 63°C in 6× SSC.

RESULTS

Rep protein expression in two HeLa-derived cell lines. Since constitutive expression of the AAV-2 *rep* gene had repeatedly been shown to interfere with establishment of stable cell lines (21, 25, 30), the inducible MMTV LTR was used to direct *rep* gene expression. After transfection of pMt*rep* (for details see Materials and Methods) and selection with G418, resistant cell clones were analyzed on Western blots and were screened for the cell clones with the highest levels of Rep78 expression after dexamethosone induction. Of 29 G418-resistant cell clones, two showed inducible expression of Rep78 (Fig. 1). Four cell clones showed constitutive expression of Rep52 with no additional Rep78 expression upon induction of the MMTV pro-

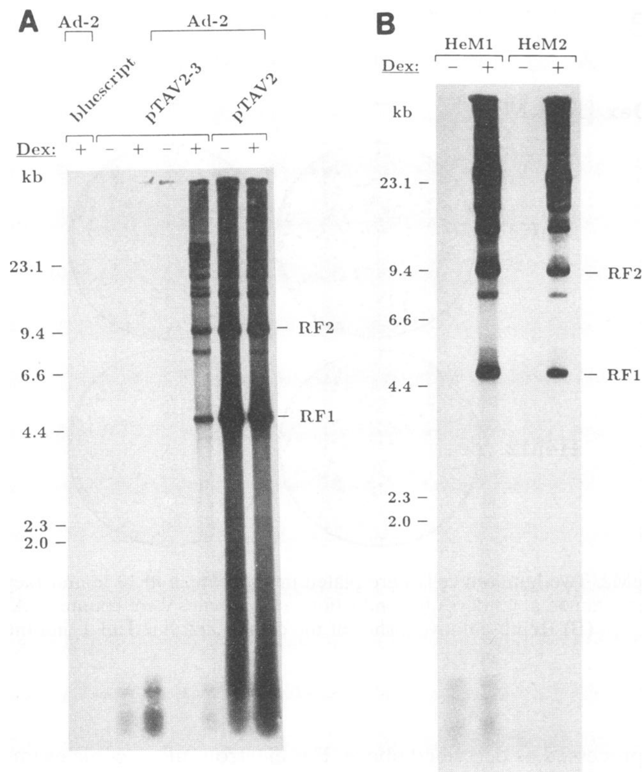


FIG. 3. DNA replication of a *rep*-negative AAV genome. (A) HeM1 cells were transfected with 4 μg of either pBluescript, pTAV2-3 (AAV *rep* negative), or pTAV2 (AAV wt), and then infected with Ad2 (MOI of 10) as indicated. (B) HeM1 or HeM2 cells were transfected with 4 μg of pTAV2-3 and infected with Ad2 (MOI of 20). Rep expression was induced where indicated (+; 10^{-6} M dexamethasone [dex]). Total cellular DNA was extracted at 50 (A) or 32 h p.i. (B). After digestion with *Xba*I and *Dpn*I, samples of 3 μg each were separated on agarose gels and then hybridized with ^{32}P-labelled AAV DNA by Southern blotting. The positions of monomeric replicative intermediates (RF1) and dimeric replicative intermediates (RF2) are marked. DNA migrating below 2.0 kb represents input DNA which was digested by *Dpn*I. In the replication-positive lanes, the band below RF2 represents residual *Dpn*I-resistant input DNA.

moter. We do not know how many additional clones might show levels of Rep undetectable by Western blotting. An additional screen of some cell clones on Northern (RNA) blots had shown a higher percentage of *rep*-expressing clones. These were found to be negative upon Western blot analysis (data not shown). The two cell lines with inducible *rep* expression were obtained from independent transfections, HeM1 from transfection of the linearized construct and HeM2 from transfection of concatemerized DNA (Fig. 1A). Both cell lines express Rep78 after induction with 10^{-6} or 10^{-7} M dexamethasone (Fig. 1B), and this expression can be clearly seen on Western blots by comparison with cell extracts from an AAV-adenovirus coinfection (Fig. 1A and B). The bands visible above Rep78 and below Rep40 in all the lanes (Fig 1A and B) represent nonspecific cross-reactions of the antibody, since these bands also light up in the absence of dexamethasone (Fig. 1A and B) and in the case of nontransfected HeLa cells (data not shown). Adenovirus infection enhances the expression level of Rep78, probably because of efficient accumulation of AAV mRNA (20). Therefore, a much shorter exposure time was needed, which explains the disappearance of the cross-reacting band above Rep78. Under these conditions Rep78 appears as a double band, which confirms previous findings from other laboratories (18, 19, 33). In HeM1, but not in HeM2, Rep52 is expressed at a rather high level (Fig. 1), and this expression is most probably driven by the constitutive p19 promoter embedded in the *rep* open reading frame. Upon infection with herpes simplex virus type 1 also, HeM2 constitutively expresses low levels of Rep52 (data not shown). The two cell lines showed different integration sites for pM*trep*, with only one copy of *rep* being present in either cell line (data not shown). Neither of the cell lines expressed detectable levels of Rep40 or Rep68, not even in the presence of adenovirus, which has been shown to enhance the accumulation of spliced Rep proteins (20, 49). After two rounds of subcloning, vitually 100% of the HeM1 cells showed a faint nuclear fluorescence with and without dexamethasone induction, which is most likely due to the presence of Rep52. With this background it was difficult to evaluate a minor increase in fluorescence intensity upon Rep78 induction. In HeM2 cells Rep expression was below the level of detection by immunofluoresence.

Lack of cell killing upon expression of Rep. To test whether induction of Rep78 expression would lead to cell toxicity, plating efficiencies and cell proliferation of HeM1 and HeM2 with and without dexamethasone induction (10^{-7}M) were measured. Induction of Rep78 had no effect on the plating efficiency of either HeM1 or HeM2. In addition, plating efficiencies were indistinguishable from that of the control cell line HeK1, which had been established by transfection of pM*tneo* (Fig. 2). This result was confirmed in a separate experiment with parental HeLa cells (data not shown). The only difference seen upon *rep* induction was the formation of smaller colonies by HeM2 (Fig. 2B) and HeM1 cells (data not shown) compared with those formed by HeK1 cells. Additional experiments with several independent *rep*-negative control clones revealed a reduction of cell proliferation after dexamethasone treatment in the case of some of them, which is indicative of a nonspecific dexamethasone-induced effect on cell proliferation. Since we obtained only two independent *rep*-expressing cell lines, it is difficult to evaluate whether the smaller colony sizes of these cells have any significance.

Efficient DNA replication of a *rep*-negative AAV mutant genome in the *rep*-expressing cell lines. To test whether Rep expressed in HeM1 and HeM2 cells is functional, the replication of a *rep*-negative AAV mutant (pTAV2-3) in these cells was tested. In pTAV2-3 a frameshift mutation at position 1045 inactivates all four Rep proteins (13). Cells were transfected with pTAV2-3 and were infected with Ad2 and then induced with 10^{-6} M dexamethasone (Fig. 3B). Thirty-two hours later cells were harvested for extraction of total cellular DNA. Samples were digested with *Xba*I and *Dpn*I and analyzed on Southern blots hybridized to ^{32}P-labelled AAV DNA. *Xba*I is a noncut enzyme for AAV-2 which leaves the typical replication intermediates (RF1 and RF2) of AAV intact. *Dpn*I digests transfected input DNA of prokaryotic origin, whereas DNA replicated in eukaryotes is resistant to *Dpn*I. In adenovirus-infected cell lines pTAV2-3 is complemented for DNA replication after induction of *rep*78 by dexamethasone (Fig. 3A), with similar efficiencies in either cell line (Fig. 3B). Replication is almost as efficient as replication of pTAV2 (wt) transfected in parallel. As expected, no replication of the *rep*-negative mutant can be detected in the absence of dexamethasone or in the absence of Ad2 infection.

AAV cap gene expression and particle formation. Apart from its role in AAV DNA replication *rep* is also required for the production of AAV particles. To test this function, HeM1 and HeM2 cells were transfected with pTAV2-3 (*rep* negative)

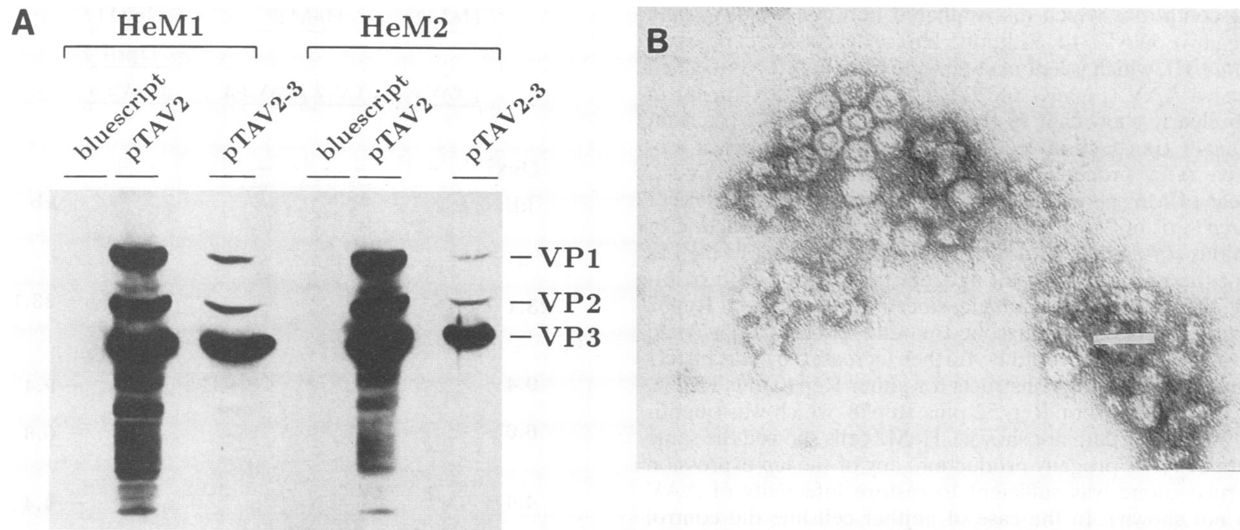


FIG. 4. Expression of capsid proteins and formation of AAV particles. (A) HeM1 and HeM2 cells were transfected with 4 μg of pTAV2 (wt), pTAV2-3 (*rep* negative), or pBluescript, infected with Ad2 (MOI of 10) and induced with dexamethasone (10^{-6} M). Cells were harvested at 52 h p.i. by three freeze-thaw cycles and subsequent water bath sonication. Cleared lysates were subjected to centrifugation at 264,000 × *g*. The resulting pellet was analyzed on Western blots by using a polyclonal anti-VP3 antiserum (37). The positions of the three VP proteins are marked, and they are as follows: VP1, 90 kDa; VP2, 72 kDa; VP3, 60 kDa. The expected stoichiometry of the three VPs, namely 1:1:10, is visible for pTAV2-3. With a short exposure of the blot this is also evident for pTAV2 (data not shown). (B) HeM1 cells were transfected with 18 μg of pTAV2-3, infected with Ad2 (MOI of 10), and induced with dexamethasone (10^{-6} M). After 52 h, cells were harvested for electron microscopic analysis (see Materials and Methods). Viral particles were negatively stained with 2% uranyl acetate and examined by electron microscopy. Bar, 40 nm.

and pTAV2 (wt), respectively, and infected with Ad2. Freeze-thaw supernatants were subjected to ultracentrifugation, which led to sedimentation of AAV particles. Pelleted material was analyzed by Western blotting. In both cell lines, capsid proteins could be detected after transfection with either pTAV2-3 or pTAV2, whereas no capsid proteins were detected in the negative control, cells transfected with pBluescript (Fig. 4A). The stoichiometry of the three capsid proteins (VP1:VP2:VP3, 1:1:10) was as expected for intact AAV particles (3, 37). These could be visualized with the electron microscope in the case of both HeM1 (Fig. 4B) and HeM2 (data not shown) cells. To our surprise, several attempts to demonstrate infectivity of particle preparations derived from pTAV2-3 failed (see below and Fig. 6C, lanes 1 and 6), whereas preparations derived from cells transfected with pTAV2 (wt) yielded infectious AAV. Therefore, in spite of efficient AAV DNA replication and production of AAV particles, some reaction necessary for the production of infectious virus particles could not be complemented by the *rep*-expressing cell lines.

Analysis of AAV ss DNA accumulation. The lack of infectivity of the AAV particles could be due to a defect in either formation or packaging of AAV ss DNA. The protocol used for the analysis of AAV DNA replication is not suitable for the detection of ss DNA. Therefore, we could not exclude the possibility that in spite of efficient synthesis of double-stranded replication intermediates (RF1 and RF2), no single strands were accumulated. To clarify this point, Hirt extracts were analyzed under conditions which minimize reassociation of complementary ss DNAs (5, 33). HeM1 cells were transfected with pTAV2 (wt) or pTAV2-3 (*rep* negative) (Fig. 5). With pTAV2, ss DNA is clearly detectable (migrating at around 2.3 kb) and the amount is comparable to the amount of RF1 DNA. In contrast, virtually no accumulation of ss DNA could be detected in cells transfected with pTAV2-3 in spite of efficient synthesis of RF DNA (Fig. 5). Similar results were obtained with HeM2 cells (data not shown). For the accumulation of ss DNA, capsid proteins are essential (17, 43), presumedly for the packaging of ss DNA derived from strand displacement synthesis (31). Since AAV particle production in the *rep* cell lines is efficient, other explanations are required for the apparent lack of single strand accumulation. An AAV mutant with a defect in *rep*52 and *rep*40 expression has been shown to be defective in the accumulation of AAV ss DNA (6). We therefore assumed that the reason for the inability to accumulate AAV ss DNA might be the lack of spliced Rep proteins, especially Rep40.

HeM1 and HeM2 can be complemented for the production of infectious AAV *rep*-negative virus by transient expression of various Rep proteins. To test the assumption that the *rep* expression pattern of the cell lines is responsible for the defect in ss DNA accumulation, thus leading to noninfectious AAV progeny, we analyzed whether the defect would be restored by transient transfection of different Rep expression constructs, each expressing only one Rep protein. HeM1 and HeM2 cells were cotransfected with pTAV2-3 and different constructs for constitutive expression of individual Rep proteins (Fig. 6A). Separate expression of either Rep78, Rep68, Rep52, or Rep40 on Western blots of transfected and Ad2-infected HeLa cell extracts was produced (Fig. 6B). Transfected HeM1 and HeM2 cells were infected with Ad2 and treated with 10^{-6} M dexamethasone. To demonstrate production of infectious progeny virus, cell-free supernatants were prepared at 31 h p.i. and then used to infect HeM1 cells to initiate a new round of pTAV2-3 DNA replication. Hirt extracts were prepared 29 h later and analyzed on Southern blots (Fig. 6C and D). Precautions were taken to minimize the chance that wt AAV would amplify because of recombination of the cotransfected constructs. First, each round of infection was cut short. Second, Hirt extracts were hybridized to an oligonucleotide specific for pTAV2-3 and then to an oligonucleotide specific for wt AAV

under conditions which discriminated between wt AAV and *rep*-negative AAV. In addition, Hirt extracts were digested with *Bam*HI, which is a noncut enzyme for pTAV2-3 but which digests wt AAV (Fig. 6C and D). The results which appear in Fig. 6 clearly show that in the case of HeM1 cells transient pCM*rep*40 transfection led to production of infectious *rep*-negative AAV progeny derived from cotransfected pTAV2-3, whereas pCM*rep*68 was less active. Also, transfection of either pCM*rep*78 or pCM*rep*52 into HeM1 cells led to production of infectious *rep*-negative AAV at an efficiency at least as high as that of the production resulting from transfection with pCM-*rep*40. This result was surprising, since both Rep78 and Rep52 are expressed in dexamethasone-treated HeM1 cells. The yield of *rep*-negative AAV could be further increased by cotransfection of two expression constructs for either Rep40 plus Rep68, Rep52 plus Rep68, or Rep 52 plus Rep78, which was quantified by titration (data not shown). HeM2 cells showed the same pattern of AAV progeny production: any of the *rep* expression constructs alone was sufficient to restore infectivity of AAV (data not shown). In the case of neither cell line did control transfections of pTAV2-3 with the addition of the empty expression vector pKEX lead to detectable production of infectious *rep*-negative AAV progeny (Fig. 6C, lanes 1 and 6).

DISCUSSION

The aim of this study was to establish cell lines with inducible *rep* expression to study *rep* functions separate from the AAV life cycle. In view of a variety of reports that constitutive *rep* expression had repeatedly interfered with successful establishment of *rep*-expressing cell lines (21, 25, 30), the use of an inducible promoter with a low basal level of expression appeared to be an absolute prerequisite for successful establishment of stable cell lines. The steroid-inducible MMTV LTR meets these requirements. Two HeLa-derived cell lines with inducible Rep78 expression were isolated. To our surprise, induction of Rep78 by dexamethasone did not lead to either a reduction in plating efficiency or a clearly measurable decrease in the proliferation rate. This is at variance with results obtained with the Rep-homologous NS1 protein of autonomous parvoviruses, which exhibits a striking cytotoxic effect when expressed under MMTV promoter control in stable cell lines (50). Recently, a 293-derived cell line with inducible *rep* expression under the control of the mouse metallothionein promoter, in the case of which an antiproliferative effect of *rep* was clearly visible, was described (48). HeLa and 293 cells may react differently upon induction of *rep* expression. However, the presence of three copies of *rep* in the 293-derived cell line may have led to levels of *rep* expression higher than those the two HeLa-derived cell lines with only one copy of *rep*. These data taken together suggest that low-level expression of *rep* does not significantly interfere with cell proliferation, whereas high levels of Rep appear to do so. A low level of *rep* gene expression is found upon AAV infection of cells at a low multiplicity of infection (MOI) in the absence of a helper virus when *rep* negatively regulates its own promoter (1, 24). The high frequency of AAV integration would be difficult to understand if *rep* were toxic under these conditions, especially in view of the fact that some *rep* expression appears to be required for site specificity of integration (reviewed in reference 37). However, proliferation rates of latently infected HeLa cell clones appear to be reduced in comparison to those of AAV-negative cell clones isolated in parallel (45). It remains to be seen whether this finding is due to the chromosomal integration site or to AAV-specific functions.

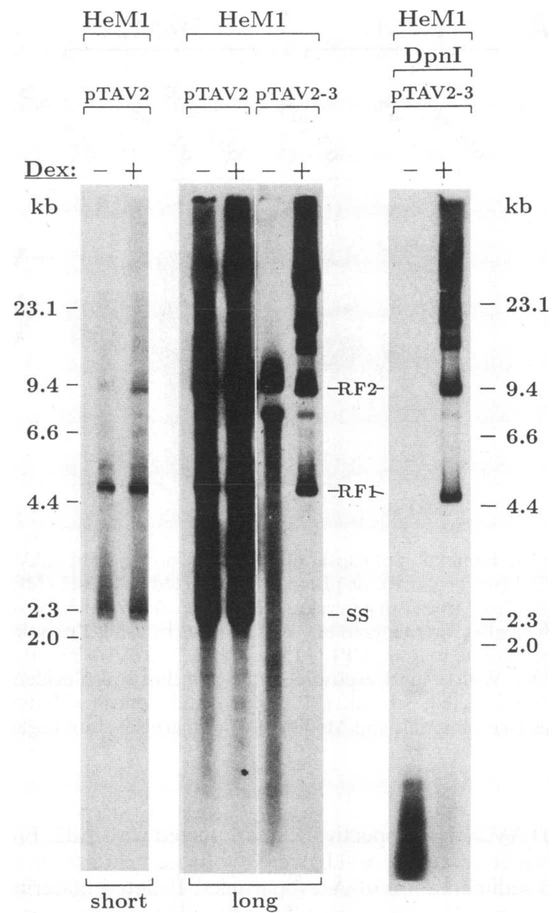
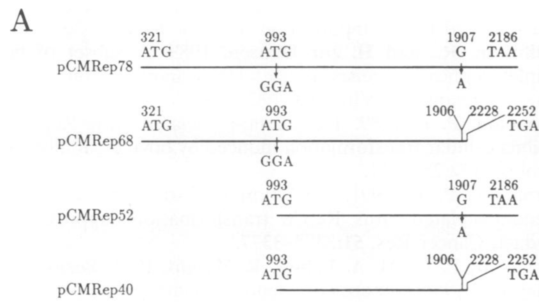
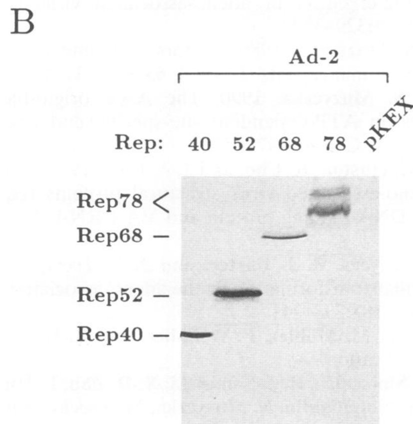
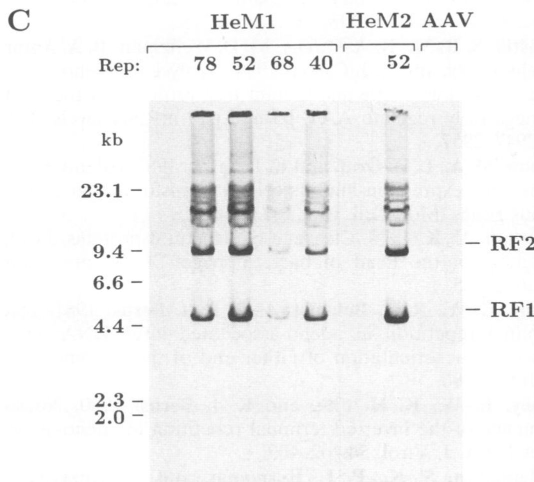


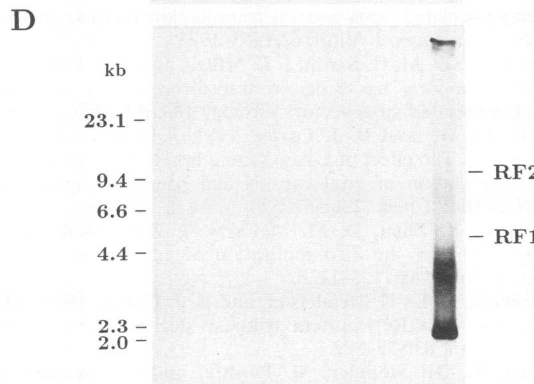
FIG. 5. Analysis of ss DNA accumulation in HeM1 cells after transfection with pTAV2-3. HeM1 cells were transfected with 4 μg of pTAV2 or pTAV2-3. Twenty-six hours after infection with Ad2 (MOI of 20) and induction with dexamethasone (Dex) (10^{-6} M), low-molecular-weight DNA was extracted by a modified Hirt extraction protocol. One-third of the total extract was analyzed on Southern blots hybridized to ^{32}P-labelled AAV DNA. The autoradiographs were exposed for 10 min (short) and 1 h (long). The positions of the different replicative intermediates (RF1 and RF2) and of ss (ss) are shown. For better distinction of the different replicative intermediates, a short exposure of the blot with extracts from pTAV2 (wt) transfection is shown. The DNA migrating at approximately 7.6 and 9.4 kb in pTAV2-3 transfected and uninduced cells (lane 5) could clearly be identified as unreplicated input DNA by its disappearance after digestion with *Dpn*I (lane 7). In contrast, after dexamethasone induction of HeM1 cells, *Dpn*I-resistant RF molecules are formed, but virtually no ss DNA is formed (lanes 6 and 8).

The low levels of Rep78 in the two cell lines described in this report can be significantly increased upon adenovirus infection. Under these conditions Rep78 is fully competent to replicate a *rep*-negative AAV genome to a degree almost as high as that at which it can replicate wt AAV. Rep52 is expressed, at least in HeM1 cells; however, neither cell line expresses Rep68 or Rep 40, both of which are derived from spliced mRNAs. This is surprising, since the entire coding sequences for all four Rep proteins, including the presumed splicing signals, are present. The defect in *rep* splicing might be due to deletion of AAV sequences downstream of *rep* which are present on the authentic AAV p5 promoter-driven mRNA. Several constructs expressing *rep* under the control of different









heterologous promoters and several different heterologous polyadenylation signals have the common characteristic that Rep proteins derived from spliced mRNAs are not properly expressed upon transient transfection, irrespective of an additional adenovirus infection (36). This consideration is in line with data obtained with minute virus of mice, in the case of which splicing of the large intron in the NS-coding region appears to be dependent on pre-mRNA sequences within the downstream small intron (50).

Rep78 expressed in Ad2-infected HeM1 and HeM2 cells induced efficient AAV DNA replication when a *rep*-negative AAV mutant genome was transfected. AAV particles of the correct size were assembled; however, they were not infectious. This defect was found to be due to an inefficient accumulation of AAV ss DNA, which was packaged into virions. The ss AAV genome is replicated by a self-priming mechanism which leads to a double-stranded replication intermediate (RF1) (11, 12, 27, 28, 32). After resolution of the terminal hairpin structure, strand displacement synthesis is initiated by using the terminal hairpin as a primer. Upon completion of strand displacement synthesis, a new AAV double strand (RF1) is formed and, in addition, a single strand is released. This displaced ss DNA is either packaged into preformed AAV capsids (31) or used as a template for a new round of DNA replication. Initially, the obvious explanation appeared to be that the lack of Rep proteins derived from spliced mRNAs might be the missing link which would be in line with genetic evidence for a role of Rep52 and/or Rep40 in the accumulation of ss DNA (6). Indeed, we found that transient expression of additional Rep proteins led to production of infectious *rep*-negative progeny.

FIG. 6. Production of infectious *rep*-negative AAV derived from pTAV2-3 after cotransfection of various Rep expression constructs. (A) Plasmid constructs for the expression of individual Rep proteins. The Rep coding sequences are transcribed from the human cytomegalovirus immediate-early promoter of pKEX. Expression of single Rep proteins is shown diagrammatically and was achieved as outlined in Materials and Methods. ATG, initiation codon; TGA and TAA, termination codons (the position of the last nucleotide of the codon is indicated). Nucleotide positions of AAV-2 are according to the numbering of Srivastava et al. (42). (B) Western blot analysis of Rep expression constructs. HeLa cells (2×10^6) were transfected with 2 μg of the indicated Rep expression construct and subsequently infected with Ad2 (MOI of 20). pKEX is the empty expression vector. Twenty-eight hours later, whole-cell extracts were prepared and analyzed by Western blotting using the anti-Rep antibody 294-4 and detection by alkaline phosphatase. Positions of authentic Rep proteins used as markers are indicated. (C and D) Detection of infectious *rep*-negative virus. HeM1 or HeM2 cells, respectively, were transfected either with 1 μg pTAV2-3 alone (lanes 1 and 6) or with 1 μg pTAV2-3 and 2 μg of various Rep expression constructs as indicated. By adding adequate amounts of the empty expression vector pKEX, the total amount of human cytomegalovirus immediate-early promoter-containing plasmids was kept constant at 6 μg in all transfections. Thirty-one hours after infection with Ad2 (MOI of 20) and dexamethasone induction (10^{-6} M) cells were lysed in a hypotonic buffer (10 mM Tris-HCl, pH 7.5) by three freeze-thaw cycles and subsequent treatment in a water bath sonicator. Growth medium was added, and the cellular debris was removed by centrifugation. Supernatants equivalent to 9×10^5 transfected cells were treated at 56°C for 30 min and subsequently used to infect 2×10^6 HeM1 cells. As a control (lane AAV), HeM1 cells were infected with wt AAV-2 (MOI of 1). For the entire experiment, 5% untreated fetal calf serum was used. Twenty-nine hours after adenovirus infection and dexamethasone induction, Hirt extracts were prepared and digested with *Bam*HI. The Southern blot was first hybridized with a radiolabelled oligonucleotide specific for *rep*-negative pTAV2-3 (C) and then rehybridized with an oligonucleotide specifically recognizing wt AAV DNA (D).

To our surprise, each of the four Reps could complement the defect in production of infectious particles independently. It is difficult to understand why additional Rep78 or Rep52 completely restored infectious virus production in HeM1 cells, since both proteins were already present. The most likely explanation appears to be that assembly of infectious AAV requires higher levels of Rep than those expressed in the cell line and that the four Rep proteins seem to be functionally redundant in this respect. In our analysis we could not document a specific function for either Rep68 or Rep40 during the AAV life cycle which would not be complemented at least partially by either Rep78 or Rep52, respectively. In addition, time-regulated *rep* expression (33) does not appear to be critical for production of infectious AAV. Apparently, the extent of AAV progeny production only depended on the overall expression levels of Rep irrespective of the promoter used to drive *rep* expression. These considerations imply that it should be possible to generate *rep*-expressing helper cell lines for the propagation of AAV vectors for gene therapy. These cell lines should also be useful for production of additional *rep*-negative AAV mutants (7) with targeted *rep* mutations to study *rep*-associated functions in animals or in situations in which DNA transfection would obscure the relevant effects.

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