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Encapsidation of Adeno-Associated Virus Type 2 Rep Proteins in Wild-Type and Recombinant Progeny Virions: Rep-Mediated Growth Inhibition of Primary Human Cells

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The adeno-associated virus type 2 (AAV) arrests the growth of primary human fibroblasts in vitro at high particle-to-cell ratios. To test the role of AAV gene expression in the observed growth inhibition, primary human cells were infected, under identical conditions, with wild-type (wt) AAV or with recombinant AAV that lacked all viral promoters and coding sequences. Significant, dose-dependent growth inhibition of primary human cells was observed with both wt and recombinant AAV at particle-to-cell ratios equal to or exceeding 10^4. In contrast, neither virus affected the growth of immortalized human cells even at a 10-fold-higher particle-to-cell ratio. AAV-induced growth arrest could be overcome by reculturing cells after treatment with trypsin. Even after reculturing, cells still harbored the proviral AAV genome. Thus, neither integration nor expression of the AAV genome appears to be required for the virus-induced growth-inhibitory effect on primary human cells. The growth-inhibitory effect of AAV was hypothesized to be mediated by virion-associated AAV Rep proteins, since these proteins have been reported to inhibit cellular DNA synthesis. Rep proteins tightly associated with wt as well as recombinant AAV could be detected on Western blots. Coinfection by adenovirus was necessary and sufficient for ample replication of recombinant AAV genomes lacking the *rep* gene. Although wt AAV-like particles arose during production of the recombinant AAV stocks, their low-titer levels were insufficient to cause the observed growth inhibition. AAV *rep* gene expression from these contaminating particles was not required for replication of the recombinant AAV genomes, which could be detected even in the absence of de novo Rep protein synthesis. Exposure of recombinant AAV to anti-AAV Rep protein antibodies did not abrogate viral infectivity. These results suggest that biologically active Rep proteins are encapsidated in mature progeny AAV particles. AAV Rep protein-mediated growth inhibition of primary human cells has implications in the use of AAV-based vectors in human gene therapy.

The adeno-associated virus type 2 (AAV) is a single-stranded DNA-containing, nonpathogenic human parvovirus that requires coinfection with a helper virus (5) or conditions of genotoxic stress (54, 55) to proliferate. Under conditions which are not conducive for replication, wild-type (wt) AAV establishes a latent infection where the viral genome integrates into host chromosomal DNA in a site-specific manner (24, 25, 41). If a latently infected cell is superinfected with a helper virus, such as adenovirus (Ad), then the AAV genome is excised (or rescued) from the chromosomal DNA, replicated, and packaged into progeny virions. The helper virus also undergoes productive infection, and eventually both viruses are liberated by cell lysis. If AAV and a helper virus coinfect a cell, then replication of both viruses ensues (28).

The AAV genome consists of two open reading frames, *rep* and *cap*, flanked by palindromic inverted terminal repeats. The *cap* gene encodes three viral structural proteins (45). The *rep* gene encodes four viral nonstructural proteins named according to their apparent molecular weights. The Rep52 and Rep40 proteins are involved in packaging single-stranded viral genomes into capsid structures (8). The Rep78 and Rep68 proteins function as positive and negative regulators of AAV gene expression (6), and it has recently been shown that Rep52 can form a complex with Rep78 or Rep68 that may be involved in attenuating Rep repression at the p5 promoter (31). Rep78 and Rep68 are required for AAV DNA replication, and they may also be required for site-specific integration of the viral DNA and rescue of the DNA from the integrated state (6). The AAV Rep proteins are involved in the terminal resolution process by which the viral genome replicates (43), and functions of Rep78 and Rep68 involved in this process include binding to the AAV terminal repeat (1, 27), a site-specific, strand-specific endonuclease activity which cleaves at the terminal resolution site, and covalent attachment to this site (20). Rep78 and Rep68 also possess an ATP-dependent DNA helicase activity (20).

Effects of AAV infection on cells can be grouped into at least three categories based on permissiveness for AAV gene expression and replication (51). Effects of AAV infection which fall into the first group (type I) are those which do not require AAV gene expression. These effects include induction of differentiation (23) and inhibition of growth and development (4, 7, 51). Type II refers to conditions under which AAV transcription occurs in the absence of detectable AAV DNA replication. An example of a type II effect is inhibition of the growth and oncogenicity of Ad-transformed hamster cells by AAV infection (30). In type III, complete AAV replication occurs due to genotoxic stress and/or helper virus coinfection. Experiments carried out under conditions that fall into the third category have shown that AAV infection inhibits devel-

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opment of tumors in rodents which can otherwise be induced by Ad, by herpesvirus, or by transplantation of cells transformed by these viruses (42). In vitro studies have documented that AAV inhibits transformation of tissue culture cells induced by viral and cellular oncogenes, and this inhibition has been mapped to the AAV *rep* gene (16, 17, 22, 30). AAV *rep* gene products have also been shown to inhibit viral and cellular DNA synthesis (15, 18, 56, 57) and a variety of enhancer/promoter elements (26, 48, 49). Thus, AAV Rep proteins are most likely involved in oncosuppression by AAV. Whether the molecular mechanisms underlying effects of AAV infection (types I to III) on different cell types under different conditions have similarities or are fundamentally distinct remains to be investigated.

We studied the effect of AAV infection on primary and immortalized human cells in the absence of a helper virus and genotoxic stress (type I conditions). Consistent with reports from others (4, 51), we observed inhibition of the growth of primary human cells at high particle-to-cell ratios. In theory, Rep could mediate growth inhibition, given its ability to inhibit transformation and DNA synthesis. Based on the observation that UV-inactivated AAV inhibits growth (51), however, *rep* gene expression does not appear to be required. To substantiate that AAV gene expression is not necessary for growth inhibition, diploid human cells were infected with wt as well as with recombinant AAV that lacked all viral promoters and coding sequences. We report here that recombinant AAV inhibits growth of primary human cells as efficiently as wt AAV. Neither recombinant nor wt AAV perturbed the growth of immortalized human cells, even though these cells could be latently infected with AAV. Furthermore, AAV-induced growth arrest could be reversed by reculturing infected cells after treatment with trypsin. Even after reculturing, cells still harbored the proviral genome. Thus, neither AAV gene expression nor integration of the viral genomes into chromosomal DNA appears to be required for AAV-mediated arrest of cell growth. Although expression of the AAV *rep* gene per se is not required for the observed growth inhibition, we provide evidence here that biologically active AAV Rep proteins are encapsidated in mature, intact wt as well as recombinant AAV particles. We suggest, therefore, that encapsidated Rep proteins mediate growth inhibition of primary human cells when the particle-to-diploid cell ratio is high.

MATERIALS AND METHODS

Viruses, cells, and plasmids. AAV and Ad type 2 (Ad2) stocks were kindly provided by Kenneth I. Berns (Cornell University Medical College, New York, N.Y.) and Kenneth H. Fife (Indiana University School of Medicine, Indianapolis, Ind.), respectively. Human bone marrow cells were obtained from healthy volunteer donors through a protocol approved by the Institutional Review Board. Primary human bone marrow stromal (BMS) cells were propagated as adherent monolayer cultures in Iscove's-modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum (FBS) and antibiotics. Human BMS cells immortalized with the simian virus 40 (SV40) large T antigen were kindly provided by David A. Williams (Indiana University School of Medicine). Primary human embryonic lung fibroblasts (WI-38 cells) were obtained from the American Type Culture Collection (Rockville, Md.). Monolayer cultures of WI-38 cells, immortalized BMS cells, the human cervical carcinoma cell line HeLa, and the Ad-transformed human embryonic kidney cell line 293 were maintained in IMDM supplemented with 10% FBS and antibiotics. AAV plasmids pSM620 and pAAV/Ad have been described elsewhere (39, 40) and were a kind gift from Richard J. Samulski (University of North Carolina, Chapel Hill, N.C.). The AAV plasmid pSM620 contains the wt AAV genome. Plasmid pAAV/Ad contains the wt AAV coding sequences flanked by Ad5 terminal sequences in place of the AAV termini; therefore, it is defective for packaging into AAV virions. Plasmid pAAV/Ad serves as a helper plasmid during production of recombinant AAV by supplying *rep* and *cap* genes in *trans*. Recombinant AAV plasmid pWP-8A, containing the gene for resistance to tetracycline and the herpesvirus thymidine kinase promoter-driven gene for resistance to neomycin (*neo*^{r}), has been described elsewhere (29). Recombinant AAV plasmid pCMVp-*lacZ*, containing the human cytomegalovirus immediate-early promoter-driven β-galactosidase (*lacZ*) gene, has also been described elsewhere (35). Recombinant AAV plasmids pWP-8A and pCMVp-*lacZ* lack all viral promoters and coding sequences, but they contain the AAV inverted terminal repeats which are required in *cis* for replication and packaging.

Preparation and purification of AAV stocks. Wild-type AAV and two AAV recombinants (vTc-Neo and vCMVp-*lacZ*) were generated from plasmids pSM620, pWP-8A, and pCMVp-*lacZ*, respectively. The virus stocks were prepared as described previously (35, 39), with the following modifications. Plasmid transfections were carried out by electroporation of 293 cells with 10 μg each of the recombinant AAV plasmid and the pAAV/Ad helper plasmid per confluent 100-mm-diameter dish of 293 cells. Transfected cells were infected with 10 PFU per cell of Ad2. Crude cell lysates were prepared 65 to 72 h postinfection by three cycles of freezing and thawing followed by heat inactivation of Ad2 at 56°C for 30 min. Clarified supernatants were digested with DNase I (100 U/ml) for 1 h at 37°C and purified by sedimentation through 40% (wt/wt) sucrose cushions at 35,000 rpm for 6 h at 20°C in a Beckman SW41 rotor. In some experiments, DNase I-digested, clarified supernatants were adjusted to a density of 1.40 g/ml by addition of CsCl and centrifuged at 35,000 rpm for 40 h at 20°C. Equilibrium density gradients were fractionated by collecting drops through a puncture in the bottom of the centrifuge tube. The densities of all fractions were determined from refractive index measurements. All fractions were dialyzed against 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) and analyzed for viral DNA by slot blot analysis as previously described (46). Viral DNA was detected in fractions which banded at densities ranging from 1.35 to 1.50 g/ml, with a peak at 1.39 g/ml. Viral titers of these fractions and virus stocks purified through a sucrose cushion ranged from 10^{11} to 10^{13} particles/ml, as determined by quantitative DNA slot blot analysis (46). In other experiments, AAV stocks were further purified by centrifuging combined fractions (1.38 to 1.45 g/ml) twice more at 35,000 rpm for 40 h at 20°C in Tris-buffered CsCl at an average density of 1.39 g/ml.

Infection of bone marrow cells and cell density assays. Equivalent numbers of primary human bone marrow low-density mononuclear cells were either mock infected or infected with increasing amounts of wt AAV (10^3 to 10^5 particles/cell) at 37°C for 1 h, washed with sterile phosphate-buffered saline (PBS), and incubated in IMDM containing 20% FBS and antibiotics at 37°C for 10 days in a 5% CO_2 incubator. After removal of the nonadherent cells by washing with PBS, the adherent cells were photographed with a Nikon inverted light microscope (100× objective). In some experiments, uninfected primary human bone marrow mononuclear cells were incubated at 37°C for 10 days to allow BMS cells to attach. Following removal of nonadherent cells by washing with PBS, the BMS monolayer was trypsinized, subcultured, and incubated at 37°C for 6 to 16 h to allow cells to reattach to the culture dish. Equivalent numbers of BMS cells were then mock infected or infected with increasing amounts of wt AAV or vTc-Neo (10^2 to 10^5 particles/cell). Cells were maintained in IMDM with 20% FBS and antibiotics at 37°C. The culture medium was replaced every fourth day. Adherent cells were photographed 10 days postinfection as described above. Sixty days postinfection, adherent cells were fixed in 10% formaldehyde (pH 7.0), and the nuclei were stained with a fluorescent dye (Hoechst 33342; Molecular Probes, Inc., Eugene, Oreg.). The numbers of viable cell nuclei/0.005-mm^2 field in 10 or more randomly selected fields were counted with a Leitz epifluorescence microscope. Statistical differences in the numbers of viable cell nuclei among the study groups were determined by using an unpaired Student *t* test.

Infection of cells and [^{3}H]thymidine incorporation assays. Primary BMS cells, immortalized BMS cells, and primary lung fibroblasts (WI-38 cells) were prelabeled with [2-^{14}C]thymidine (53 mCi/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) at 0.1 μCi/ml for 24 h prior to seeding to allow correction for any difference in the number of cells attaching or surviving during the experiment. Equivalent numbers of ^{14}C-labeled cells were infected (as described above) in triplicate with sucrose cushion-purified wt AAV or vCMVp-*lacZ*, or with various fractions of CsCl gradient-purified vCMVp-*lacZ* that spanned the equilibrium density gradient. In the case of CsCl gradient fractions of vCMVp-*lacZ* that did not contain viral DNA, as determined by slot blot analysis, BMS cells were exposed to a volume equal to the largest volume used of the DNA-containing fractions. As controls, cells were mock infected with equilibrium density gradient fractions of lysates from cells that were transfected with pAAV/Ad and infected with Ad2. Lysates from cells transfected with pAAV/Ad and infected with Ad2 were also sedimented through sucrose cushions and used as controls in experiments where cells were infected with sucrose cushion-purified virus stocks. Four days postinfection, cells were pulsed with [6-^{3}H]thymidine (27 Ci/mmol; Amersham, Arlington Heights, Ill.) at 5 μCi/ml for 24 h. Five days postinfection, the medium was removed and the cells were washed with PBS. Cells were lysed directly on the plates by addition of 0.5% sodium dodecyl sulfate (SDS) in 10 mM Tris–1 mM EDTA (pH 8). After a 10-min incubation at room temperature, an equal volume of ice-cold 10% trichloroacetic acid was added to each plate and the precipitate was collected on Millipore HA 0.45-μm-pore-size filters. The ^{3}H and ^{14}C radioactive counts were determined in a Beckman liquid scintillation counter, and cross-channel correction was performed. In some experiments, mock-infected and AAV-infected cells were passaged for 1 month before addition of [^{3}H]thymidine to equivalent numbers of cells from each group and analyzing incorporation as described above.

Rescue and replication of AAV from latently infected cells. Equivalent numbers of primary and immortalized BMS cells were either mock infected or

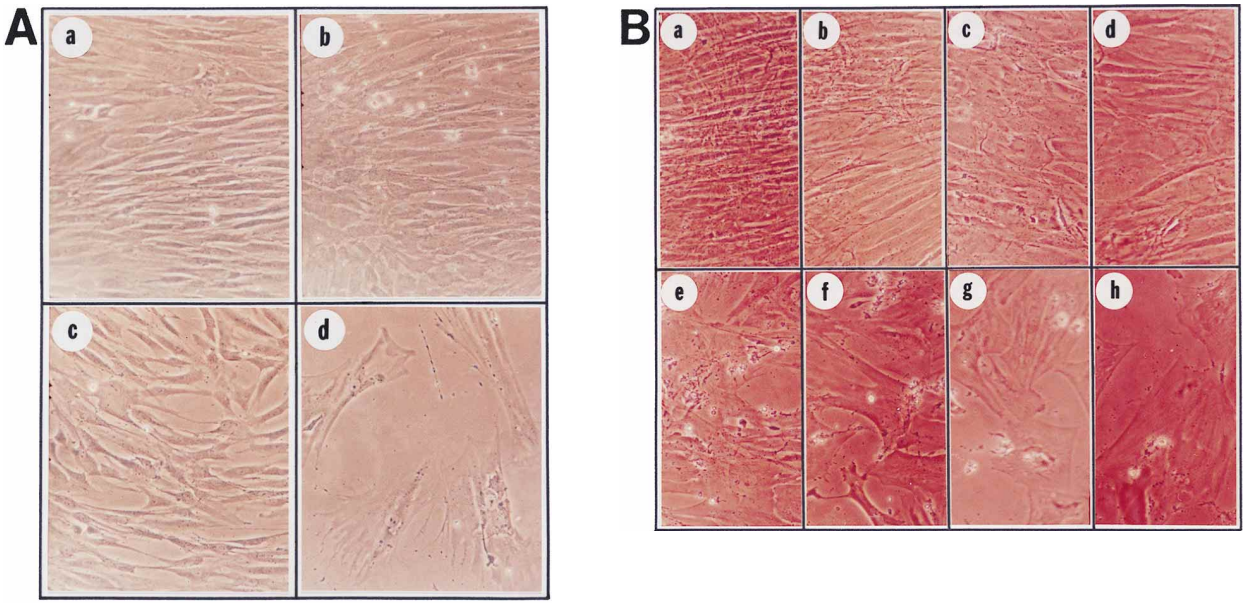

FIG. 1. Wild-type AAV infection perturbs the growth of primary human BMS cells. (A) Equivalent numbers of primary human low-density bone marrow mononuclear cells were either mock infected (a) or infected with wt AAV at 10^3 (b), 10^4 (c), or 10^5 (d) particles/cell, allowed to incubate for 10 days at 37°C, and photographed following removal of nonadherent cells. (B) Equivalent numbers of primary human BMS cells were either mock infected (a) or infected with wt AAV at 10^3 (b), 2×10^3 (c), 5×10^3 (d), 10^4 (e), 2.5×10^4 (f), 5×10^4 (g), or 10^5 (h) particles/cell, allowed to incubate for 10 days at 37°C, and photographed as described above (magnification, ×100).

infected with wt AAV at 10^4 or 10^5 particles/cell. After passaging cells for 1 month, equivalent numbers of cells from each study group were infected with 10 PFU/cell of Ad2. Cells were harvested 72 h postinfection with Ad2, and low-M_r DNA was isolated by the method described by Hirt (19). Equivalent amounts of DNA samples were electrophoresed on 1% agarose gels, transferred to nylon membranes, and analyzed on Southern blots, using ^{32}P-labeled DNA probes specific for AAV DNA sequences, as described previously (44).

Western blot analysis of AAV Rep proteins. Purified AAV stocks were denatured and electrophoresed on SDS–12.5% polyacrylamide gels. Cell lysates prepared from 293 cells following infection with Ad2 or coinfection with AAV and Ad2 were electrophoresed as negative and positive controls, respectively. Lysate from a Rep$^+$ cell line that expresses all AAV Rep proteins following Ad2 infection (34) served as an additional positive control. Proteins were transferred to polyvinylidene difluoride membranes and probed with a polyclonal anti-Rep rabbit antiserum kindly supplied by James P. Trempe (Medical College of Ohio, Toledo) as previously described (58).

Recombinant AAV DNA replication assays. Equivalent numbers of 293 cells were mock infected or infected with vCMVp-*lacZ* (10^2 to 10^4 particles/cell) in the presence or absence of Ad2 (10 PFU/cell). Cells were harvested 24, 48, and 72 h postinfection. Equivalent amounts of low-M_r DNA samples were electrophoresed on 1% agarose gels, transferred to nitrocellulose membranes, and analyzed on Southern blots, using ^{32}P-labeled probes specific for *lacZ* (3.5 kb) and AAV (4.3 kb) DNA sequences lacking the viral inverted terminal repeats, as previously described (44). All ^{32}P-labeled DNA probes were synthesized by using random oligonucleotide primers. In some experiments, equivalent numbers of HeLa cells were infected with Ad2 at 10 PFU/cell. At approximately 18 h after infection with Ad2, actinomycin D or cycloheximide (both from Sigma Chemical Co., St. Louis, Mo.) was added to the cells at a final concentration of 0 to 100 μg/ml. Eight hours later, cells were infected with CsCl density gradient-purified vTc-Neo (10^4 particles/cell) for 1 h in the continued presence of actinomycin D or cycloheximide. After addition of fresh medium and inhibitor, cells were incubated for 2 days at 37°C. Low-M_r DNA samples were then isolated and analyzed on Southern blots as described above, using random-primed, ^{32}P-labeled probes specific for the *neo* gene (1.7 kb) and AAV (4.3 kb) DNA sequences. In other experiments, vTc-Neo virions were incubated on ice for 90 min with IMDM or with various dilutions of anti-Rep rabbit serum, human serum from a commercial source, or human serum from a volunteer donor. The mixtures were then used to infect 293 cells at 5×10^3 particles/cell. Low-M_r DNA samples isolated 72 h postinfection were analyzed on Southern blots as described above.

RESULTS

Intact wt and recombinant AAV particles inhibit growth of primary human cells at high particle-to-cell ratios. Wild-type AAV has been shown to inhibit growth of primary human fibroblasts (4, 51). Since we are interested in using AAV-based vectors for transduction of the human hematopoietic system, we examined the effect of wt AAV infection on primary human bone marrow mononuclear cells. Primary human bone marrow mononuclear cells were either mock infected or infected with increasing amounts of wt AAV (10^3 to 10^5 particles/cell) and incubated at 37°C for 10 days. Whereas nonadherent cells appeared to remain unaffected as determined by trypan blue exclusion (data not shown), perturbation of the growth of BMS cells that form the adherent layer was clearly evident. These results are depicted in Fig. 1A. It can be seen that whereas mock-infected BMS cells formed a confluent monolayer, BMS cells infected with increasing amounts of AAV achieved decreasing cell densities. It was possible that AAV had only an indirect effect on BMS cells in that it interfered with attachment of BMS cells to the culture dish. To address this possibility, uninfected bone marrow mononuclear cells were incubated at 37°C for 10 days to allow BMS cell attachment to occur. The adherent BMS cells were then passaged and allowed to reattach prior to mock infection or infection of subconfluent cultures with increasing amounts of wt AAV (10^3 to 10^5 particles/cell). Again, AAV infection was observed to interfere with the growth of BMS cells in a dose-dependent manner (Fig. 1B). The morphology of infected cells was altered in that they appeared significantly larger than mock-infected cells, which is indicative of growth arrest (2, 3).

To evaluate the role of AAV gene expression in the observed perturbation of the growth of BMS cells, we compared the effects of wt and recombinant AAV infections on BMS cells by the quantitative cell density assay described in Materials and Methods. The wt AAV used in these and all subsequent experiments was generated from plasmid pSM620 as previously described (39). Both wt AAV and the recombinant AAV, vTc-Neo, used here were sedimented through sucrose cushions. The cell density data shown in Fig. 2A indicate that

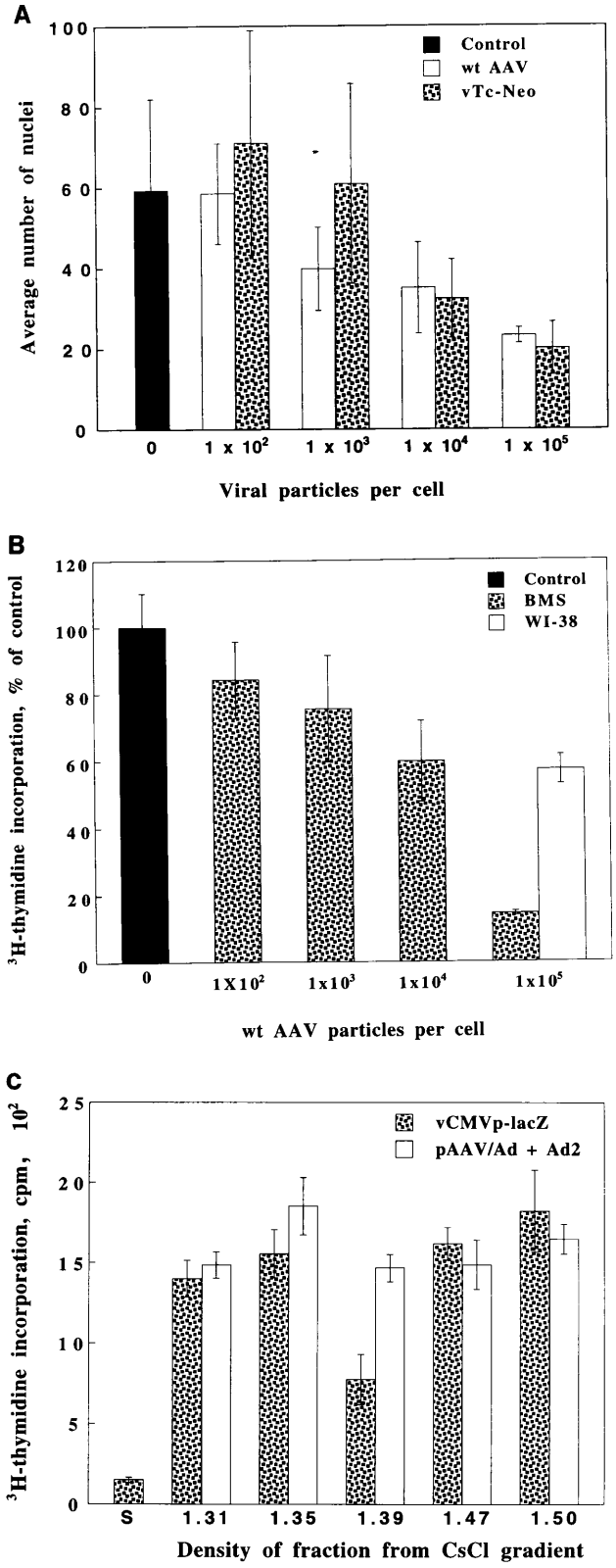

FIG. 2. Intact wt and recombinant AAV particles inhibit growth of primary human cells. (A) Equivalent numbers of BMS cells were mock infected (closed bars) or infected with wt AAV (open bars) or recombinant AAV (stippled bars) at the indicated particle-to-cell ratios. The average number of nuclei/0.005 mm^2 was determined 60 days postinfection as described in Materials and Methods. (B) Equivalent numbers of primary human BMS cells (stippled bars) or primary human lung fibroblasts (open bars) were mock infected (closed bars) or infected in triplicate with wt AAV at the indicated particle-to-cell ratios. Incorporation of [^{3}H]thymidine was measured 5 days postinfection as described in Materials and Methods. (C) Equivalent numbers of ^{14}C-labeled BMS cells were infected in triplicate with 1.6×10^5 particles of vCMVp-*lacZ* per cell (stippled bars) purified on sucrose cushions (S) or fractionated on CsCl equilibrium density gradients. As controls, cells were mock infected in triplicate with CsCl equilibrium density gradient fractions (densities of the fractions are indicated) of lysates from cells transfected with pAAV/Ad and infected with Ad2 (open bars). The [^{3}H]thymidine incorporation 5 days postinfection was determined for each study group as described in Materials and Methods.

BMS cells infected with greater than or equal to 10^4 particles/cell of either wt or recombinant AAV grew to a significantly lower cell density than mock-infected cells ($P < 0.001$). Differences in cell density induced by the wt and recombinant AAV were not significant at any virus particle-to-cell ratio ($P > 0.05$). These results indicate that the observed growth perturbation is not a consequence of AAV gene expression.

We next wished to investigate whether the decreased cell density achieved by AAV-infected BMS cells relative to control cultures was due to inhibition of growth or cell death. To this end, equivalent numbers of BMS cells, prelabeled with [^{14}C]thymidine, were either mock infected or infected with increasing amounts of sucrose cushion-purified wt AAV (10^2 to 10^5 particles/cell). Primary human lung fibroblasts (WI-38 cells), prelabeled with [^{14}C]thymidine, were also mock infected or infected with 10^5 particles of the wt AAV per cell. As controls, cells were mock infected with sucrose cushion-purified lysates from 293 cells that were transfected with pAAV/Ad and infected with Ad2. Incorporation of [^{3}H]thymidine and ^{14}C counts was determined 5 days postinfection. In agreement with the cell density data (Fig. 2A), the [^{3}H]thymidine incorporation data shown in Fig. 2B indicate that BMS cells infected with greater than or equal to 10^4 wt AAV particles/cell incorporated significantly less [^{3}H]thymidine than control cultures ($P < 0.02$). Although the lung fibroblasts were less susceptible to growth inhibition than the BMS cells (Fig. 2B), WI-38 cells infected with 10^5 particles of wt AAV per cell incorporated significantly less [^{3}H]thymidine than control WI-38 cultures ($P = 0.009$). It is noteworthy that WI-38 cells grow approximately 10 times faster than BMS cells, as estimated from the extent of [^{3}H]thymidine incorporation by equivalent numbers of cells (data not shown). There were no significant differences in ^{14}C counts between mock-infected and virus-infected cultures (data not shown). Thus, differences in [^{3}H]thymidine incorporation were not attributable to differences in numbers of cells plated or differential cell survival. These results indicate that AAV infection induces growth arrest, not death, of primary human cells.

Since the wt AAV that has previously been shown to inhibit growth of primary human cells was purified by equilibrium density gradient centrifugation in CsCl (4, 51), we wished to determine if CsCl density gradient-purified recombinant AAV could also inhibit cellular growth. We therefore fractionated cell lysates containing vCMVp-*lacZ* by one round of ultracentrifugation on a CsCl density gradient and infected BMS cells with various fractions spanning the gradient. The [^{3}H]thymidine incorporation data shown in Fig. 2C document that the only fraction of the gradient that inhibited growth was the one that banded at a density of about 1.39 g/ml. This fraction was expected to contain intact vCMVp-*lacZ* particles, based on the fact that the major infectious component of wt AAV-containing cell lysates bands at 1.41 g/ml (11). In fact, this fraction (1.39 g/ml) of the vCMVp-*lacZ* gradient contained the most viral DNA by slot blot analysis and the most capsid proteins by Western blot analysis with monoclonal anti-AAV capsid pro-

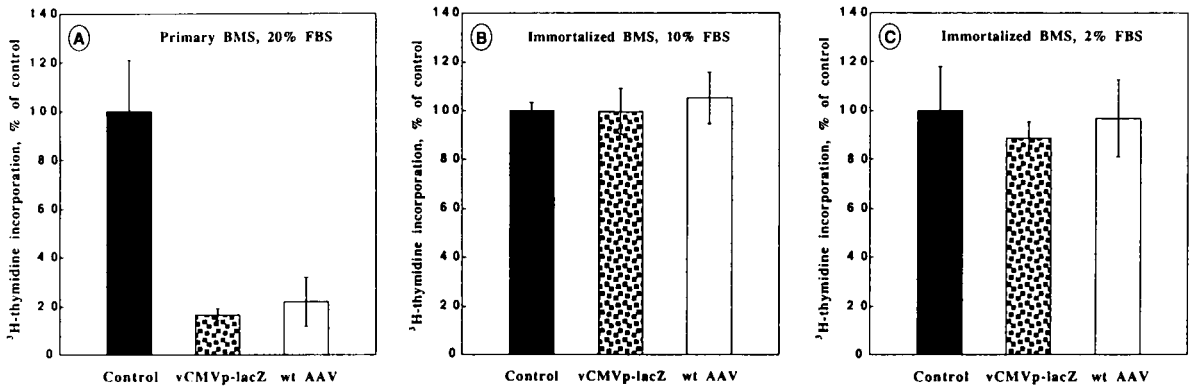

FIG. 3. Immortalized human BMS cells are resistant to AAV-mediated growth inhibition. Primary (A) and immortalized (B and C) BMS cells were either mock infected (closed bars) or infected with 10^5 particles of recombinant AAV (stippled bars) or wt AAV (open bars) per cell. Cells were maintained in the presence of the indicated concentrations of FBS, and incorporation of [^{3}H]thymidine was measured 5 days postinfection as described in Materials and Methods.

tein antibodies (data not shown). Although the growth-inhibitory effect of sucrose cushion-purified vCMVp-*lacZ* was approximately fivefold higher than that of the 1.39-g/ml fraction of the vCMVp-*lacZ* density gradient, there were no significant differences in ^{14}C counts between the study groups. Therefore, differences in [^{3}H]thymidine incorporation were not due to differences in number of cells plated.

In Fig. 2C, controls represent [^{3}H]thymidine incorporation by BMS cells that were mock infected with CsCl equilibrium density gradient fractions of lysates from cells transfected with pAAV/Ad and infected with Ad2. The pAAV/Ad helper plasmid is defective for packaging but supplies wt AAV gene products in the presence of Ad2. Thus, the control lysates contain AAV Rep and capsid proteins and lack viral particles. No portion of the fractionated control lysate inhibited [^{3}H]thymidine incorporation by BMS cells (Fig. 2C). It appears, therefore, that only intact AAV particles inhibit growth.

Immortalized cells are resistant to AAV-induced growth arrest. The growth-inhibitory effect of AAV has been shown to be cell type specific (2, 3, 51). All primary cells examined have been shown to be susceptible. Whereas many immortal lines are relatively resistant, freshly established malignant human tumor cells (melanoma and carcinoma) and a permanent line of human melanoma cells, MKr, are susceptible (2, 3). Interestingly, a different established line of human melanoma cells, HUM, was found to be resistant (51). Since an SV40-transformed line of adult human skin fibroblasts, XP20-S, has been reported to be relatively resistant to AAV-induced growth inhibition (51), we investigated whether immortalization of primary human BMS cells with SV40 large T antigen would decrease their sensitivity. Equivalent numbers of primary and immortalized BMS cells were either mock infected or infected with 10^5 particles of sucrose cushion-purified wt AAV or recombinant AAV (vCMVp-*lacZ*) per cell. Primary BMS cells were maintained in IMDM with antibiotics and 20% FBS, while immortalized BMS cells were maintained in IMDM with antibiotics and either 10 or 2% FBS. Incorporation of [^{3}H]thymidine was determined 5 days postinfection as described in Materials and Methods. It is interesting that whereas both wt and recombinant AAV inhibited growth of primary BMS cells (Fig. 3A), neither virus affected incorporation of [^{3}H]thymidine by immortalized BMS cells (Fig. 3B), even when infected cells were maintained in fivefold less serum (Fig. 3C). Similar results were obtained with HeLa cells (data not shown). It would appear, therefore, that cellular growth rate alone is insufficient to account for the differential sensitivities of primary and immortal human cells to AAV-mediated growth inhibition.

AAV-mediated growth inhibition is reversed upon subculturing. In contrast to freshly established malignant human tumor cells, fibroblasts are able to at least partially overcome wt AAV-induced growth arrest upon subculturing after trypsin treatment (2, 4). To determine if primary BMS cells can overcome the antiproliferative effect of recombinant AAV, BMS cells were mock infected or infected with 10^5 particles of sucrose cushion-purified vCMVp-*lacZ* per cell and maintained at 37°C. At 1 week postinfection, infected cells incorporated significantly less [^{3}H]thymidine than control cultures (Fig. 4). However, infected cells that were treated with trypsin and subcultured every week for a month incorporated just as much [^{3}H]thymidine as control cultures (Fig. 4). Thus, primary BMS cells overcome growth arrest with trypsinization. It is interesting that growth arrest persists for at least 2 months if the cells are not treated with trypsin and recultured (Fig. 2A).

Integration of the AAV genome is not responsible for growth arrest. Reversion of the transformed phenotype in virus-transformed cells is often due to alteration or loss of the integrated

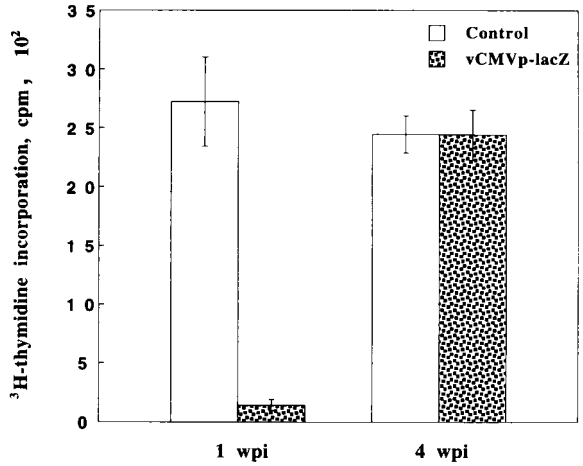

FIG. 4. Recombinant AAV-mediated growth inhibition of primary human BMS cells is reversed upon subculturing. Equivalent numbers of primary human BMS were mock infected (open bars) or infected with 10^5 particles of recombinant AAV per cell (stippled bars). Incorporation of [^{3}H]thymidine was measured 1 week postinfection (1 wpi) or following repeated subculturing for 4 weeks (4 wpi) as described in Materials and Methods.

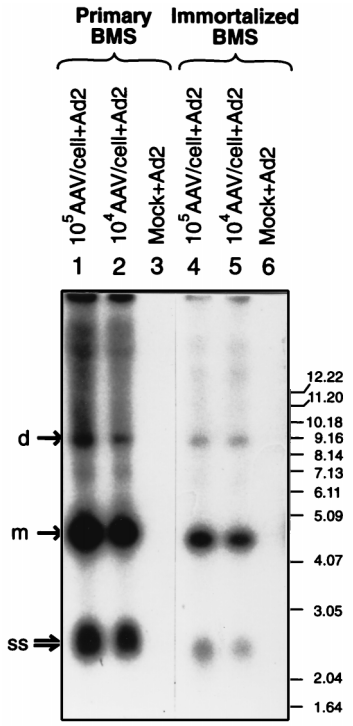

FIG. 5. Southern blot analysis for rescue and replication of the proviral wt AAV genome in latently infected primary and immortalized human BMS cells. Equivalent numbers of primary and immortalized BMS cells were mock infected or infected with wt AAV at the indicated particle-to-cell ratios, passaged for 1 month, and superinfected with Ad2. Low-M_r DNA samples were analyzed on Southern blots, using an AAV-specific DNA probe as described in Materials and Methods. Monomeric and dimeric replicative intermediates and single-stranded DNA are denoted by m, d, and ss, respectively. Sizes are indicated in kilobases.

viral DNA (14). Similarly, escape from AAV-induced growth arrest could be associated with a loss of AAV DNA. Furthermore, resistance of immortalized BMS cells to AAV-induced growth arrest could also be due to an inability of AAV to establish a latent infection in these cells. To investigate these possibilities, we attempted to rescue and replicate wt AAV DNA sequences from primary and immortalized BMS cells that had been subcultured every week for a month after infection with wt AAV. Under these conditions, AAV-induced growth arrest is reversed in primary BMS cells (Fig. 4). Upon superinfection with Ad2, AAV DNA could be rescued and replicated in both cell types (Fig. 5). That replication occurred from episomal forms of the AAV genome was unlikely since these forms were not detected on Southern blots 1 month postinfection (data not shown). These results indicate that AAV can establish and maintain a latent infection in primary BMS cells under conditions where growth inhibition is reversed. Thus, growth arrest does not appear to be a consequence of integration of the AAV genome. This finding is in accordance with our recent observation that wt and recombinant AAV genomes do not appear to have the same integration locus in human chromosomal DNA (33). While immortalized BMS cells could be latently infected with AAV, rescue and replication of the provirus was less efficient than in primary cells. Based on slot blot analysis of genomic DNA from both cell types, however, the immortalized BMS cells harbored at least as much AAV DNA as primary BMS cells (data not shown). To determine whether Ad2 replication was impaired in immortalized cells relative to primary cells, equivalent amounts of low-M_r DNA samples from the latently infected primary and immortalized BMS cells were digested with *Kpn*I and analyzed on Southern blots, using a probe specific for Ad2 DNA sequences. Ad2 replicated much less efficiently in the immortalized cells (data not shown), which probably accounts for the less efficient rescue and replication of AAV DNA in these cells. It is possible that the SV40 large T antigen interferes with Ad2 replication or that Ad2 does not infect immortalized BMS cells as efficiently as primary BMS cells. These possibilities were not examined further. The fact that AAV could establish a latent infection in immortalized BMS cells further suggests that AAV-mediated growth inhibition of primary BMS cells is not due to integration of the proviral sequences or to a difference in permissiveness for AAV infection of the two cell types.

Rep proteins associate tightly with wt and recombinant AAV. Since the AAV recombinants (vTc-Neo and vCMVp-*lacZ*) lack the *rep* gene, we reasoned that the observed growth inhibition of BMS cells by these viruses could be due to the presence of virion-associated AAV Rep proteins. Sucrose cushion-purified AAV stocks were analyzed on Western blots for the presence of AAV Rep proteins. As is evident from Fig. 6A, Rep78 and Rep68 (which appear to comigrate in this figure) and Rep52 were readily detected in sucrose cushion-purified vCMVp-*lacZ*, vTc-Neo, and wt AAV. When preparations of sucrose cushion-purified vCMVp-*lacZ* were electrophoresed longer, Rep68 in addition to Rep78 and Rep52 could be readily detected (Fig. 6B, lane 3). These data suggest that Rep proteins associate with AAV particles.

The vCMVp-*lacZ*-containing cell lysates fractionated by equilibrium density gradient centrifugation in CsCl were also analyzed for AAV Rep proteins on Western blots. As can be seen in Fig. 6B, AAV Rep proteins were detected along the

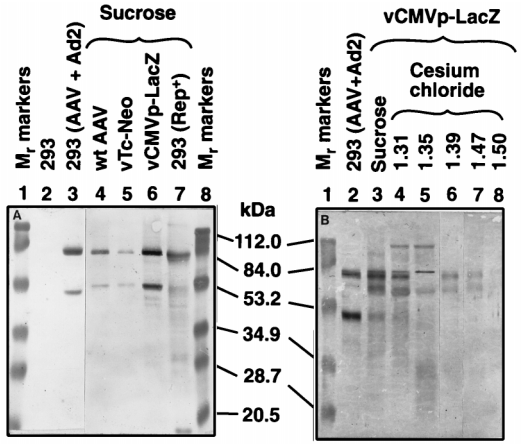

FIG. 6. Partially purified recombinant AAV virions have associated Rep proteins. (A) Approximately 10^8 particles of wt AAV (lane 4), recombinant vTc-Neo (lane 5), and recombinant vCMVp-*lacZ* (lane 6) virions purified on sucrose cushions were analyzed for the presence of AAV Rep proteins by Western blotting. Lysates from Ad2-infected (lane 2) and wt AAV- plus Ad2-coinfected (lane 3) 293 cells were included as negative and positive controls, respectively. Lysate from a cell line that expresses AAV Rep proteins following Ad2 infection also served as a positive control (lane 7). (B) Recombinant vCMVp-*lacZ* virions purified on sucrose cushions (lane 3) or fractionated on CsCl equilibrium density gradients (lanes 4 to 8) were analyzed as described above. Approximately 3×10^8 particles (2.5 μl) of sucrose cushion-purified vCMVp-*lacZ* were analyzed. An amount of each equilibrium density gradient fraction corresponding to approximately 3×10^9 particles (10 to 22 μl) of vCMVp-*lacZ*, as determined by DNA slot blot analysis, was analyzed. In the case of the fraction which banded at 1.31 g/ml (lane 4), and did not contain viral DNA, 2.5 μl was analyzed. Lysate from wt AAV-Ad2-coinfected 293 cells was included as a positive control (lane 2).

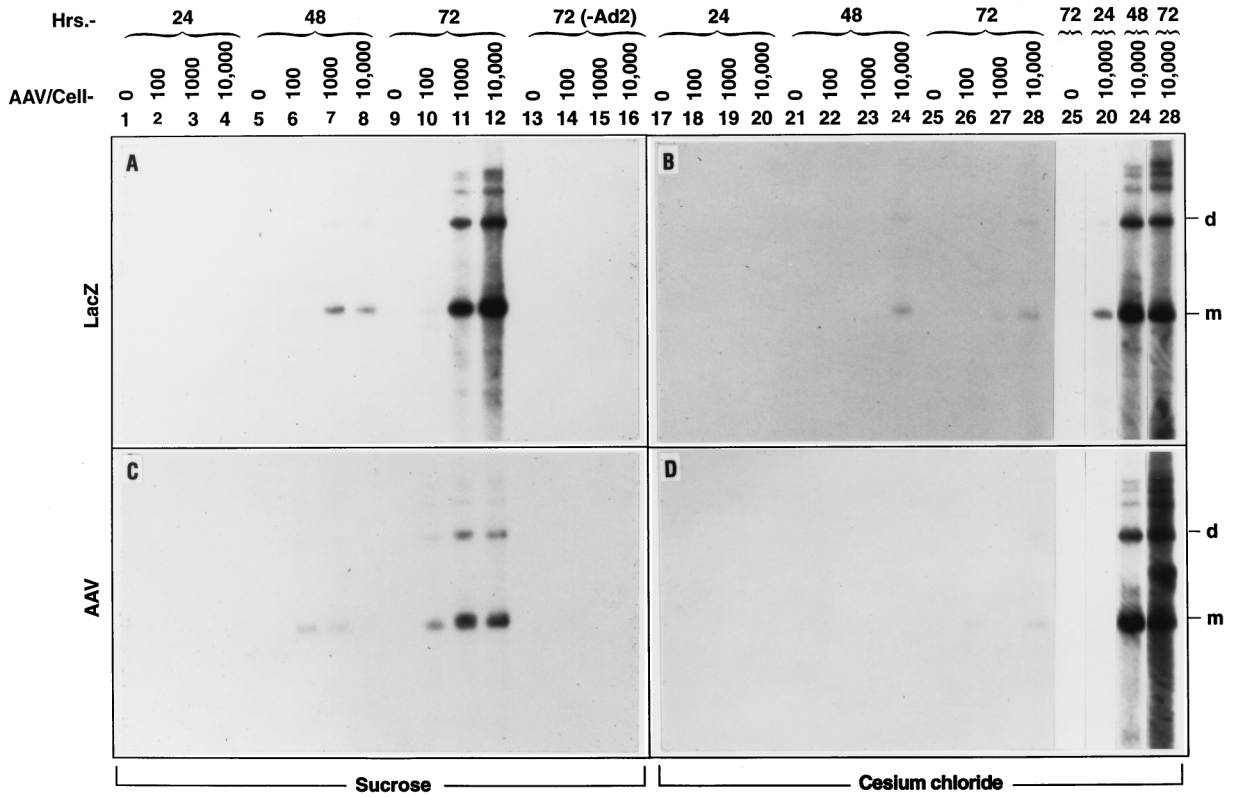

FIG. 7. Southern blot analyses for replication of recombinant AAV genomes. (A) 293 cells were mock infected (lanes 1, 5, 9, and 13) or infected with the indicated viral particle-to-cell ratio of recombinant vCMVp-*lacZ* virions purified on sucrose cushions in the presence (lanes 1 to 12) or absence (lanes 13 to 16) of Ad2. At the indicated times postinfection, low-M_r DNA samples were isolated and analyzed on Southern blots, using a *lacZ* DNA probe as described in Materials and Methods. The blot was autoradiographed for 1 h. (B) Same as panel A except that cells were coinfected with Ad2 and vCMVp-*lacZ* virions purified on CsCl density gradients. Lanes 25, 20, 24, and 28 are from the same blot autoradiographed for 24 h. (C and D) Blots shown in panels A and B were stripped of the *lacZ* probe and reprobed with the wt AAV DNA probe. The monomeric and dimeric forms of the viral replicative DNA intermediates are denoted by m and d, respectively.

entire equilibrium density gradient. The highest concentration of Rep proteins was at the top of the gradient (1.31 g/ml), where free proteins are expected to band (13). No viral DNA was detected in this portion of the gradient by slot blot or Southern blot analyses (data not shown). The free Rep proteins did not inhibit growth of BMS cells (Fig. 2C, 1.31-g/ml fraction). Fractions which banded at densities ranging from 1.35 to 1.50 g/ml contained viral DNA that was not susceptible to DNase I digestion, as determined by slot blot analysis (data not shown). Thus, Rep proteins detected in these fractions were most likely associated with virus particles. This was confirmed by analyzing CsCl equilibrium density gradient fractions of lysates from cells transfected with pAAV/Ad and infected with Ad2 on Western blots for AAV Rep proteins. Virtually all proteins in the control lysates banded at the top of the gradient, and Rep proteins could not be detected in fractions that banded at higher densities where virus particles would band (data not shown).

Sucrose cushion-purified vCMVp-*lacZ* contained significantly more Rep protein than the fraction of the vCMVp-*lacZ* density gradient which banded at 1.39 g/ml (Fig. 6B). Sucrose cushion-purified vCMVp-*lacZ* also inhibited growth to a greater extent than did this fraction (Fig. 2C). Thus, the Rep protein content appeared to correlate with the magnitude of the growth-inhibitory effect induced. When recombinant AAV was purified by three rounds of ultracentrifugation in CsCl, Rep proteins could no longer be detected by Western blot analysis, even when 10^{10} particles per lane were analyzed (data not shown). Even though these virions contained no detectable Rep proteins, they were able to inhibit growth of BMS cells as efficiently as the sucrose cushion-purified vCMVp-*lacZ* shown in Fig. 2C (data not shown). Therefore, growth inhibition is not attributable to the Rep proteins that are detectable by Western blot analysis (Fig. 6), which associate tightly with the outside surface of virions. It remained possible, however, that Rep proteins were also encapsidated in intact virions, since it would not be possible to detect only a few Rep proteins per virion by Western blot analysis. We therefore performed functional assays to determine if Rep proteins are indeed encapsidated in AAV virions.

Rep proteins are encapsidated in mature AAV particles. Previous studies have established that the Rep78 and Rep68 proteins are indispensable for AAV DNA replication in Ad-infected cells, whereas Rep52 and Rep40 are not essential (6). Although the AAV recombinants do not contain the *rep* gene, we reasoned that if Rep78 and Rep68 proteins were encapsidated in recombinant AAV virions, then the viruses should be able to undergo DNA replication in Ad2-infected cells. To test this, 293 cells were mock infected or infected with fractions spanning the vCMVp-*lacZ* equilibrium density gradient or with sucrose cushion-purified vCMVp-*lacZ*, in the presence and in the absence of coinfection with Ad2. The only fraction which yielded replicative forms of the recombinant AAV upon coinfection of 293 cells with Ad2 was that which banded at a density of 1.39 g/ml (data not shown). Thus, the only growth-inhibitory fraction (Fig. 2C, 1.39 g/ml) also appears to be the

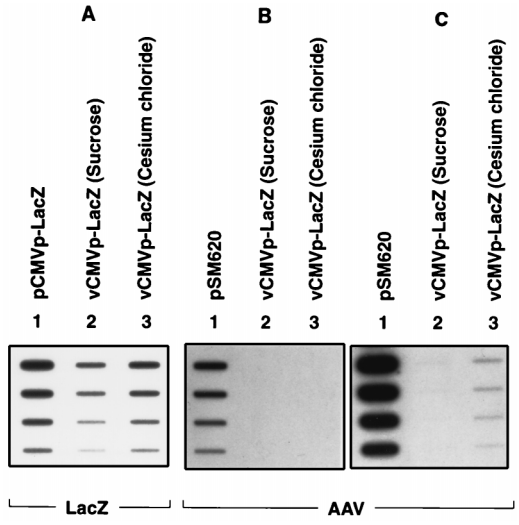

FIG. 8. DNA slot blot analysis of viral titers. (A) Twofold serial dilutions of known quantities of plasmid pCMVp-*lacZ* (lane 1) and recombinant vCMVp-*lacZ* viral stocks purified on sucrose cushions (lane 2) or on CsCl density gradients (lane 3) were filtered through a nitrocellulose membrane and probed with the *lacZ* DNA probe. The blot was autoradiographed for 5 h. (B) Equivalent amounts of the recombinant viral stocks purified by the two different methods (lanes 2 and 3) were also analyzed by using the AAV DNA probe along with twofold serial dilutions of the AAV plasmid, pSM620 (lane 1). The blot was autoradiographed for 5 h. (C) Same as B except that autoradiography was performed for 5 days.

only fraction that contains intact particles. Sucrose cushion-purified vCMVp-*lacZ* particles were able to replicate much more efficiently than CsCl density gradient-purified particles (data not shown). The ability of a virus stock to inhibit growth appears, therefore, to correlate with its infectious particle titer.

Equilibrium density gradient fractions of vCMVp-*lacZ*-containing cell lysates that banded at densities from approximately 1.40 to 1.45 g/ml were pooled and used in the replication assay shown in Fig. 7. Again, sucrose cushion-purified vCMVp-*lacZ* replicated much more efficiently than CsCl density gradient-purified particles (compare Fig. 7A and B). Twenty-four hours of autoradiographic exposure was required to observe the same intensity of the replicative forms for the CsCl gradient-purified recombinant AAV particles as was observed for the sucrose cushion-purified particles in only 1 h of exposure. To check for the presence of contaminating wt AAV in sucrose cushion- and CsCl gradient-purified viral preparations, the blots shown in Fig. 7A and B were stripped of the *lacZ* probe and hybridized with the AAV probe. As is evident from Fig. 7C and D, replicative intermediates which hybridized to the AAV probe were indeed generated in cells coinfected with Ad2 and recombinant AAV purified on a sucrose cushion or a CsCl density gradient. More of these replicative intermediates were generated from the sucrose cushion-purified recombinant AAV preparation than from the CsCl density gradient-purified recombinant AAV preparation. However, the CsCl density gradient-purified recombinant virus stock contained more wt AAV DNA than did the sucrose cushion-purified stock. This observation was substantiated experimentally by quantitative DNA slot blot analysis of approximately equivalent numbers of vCMVp-*lacZ* particles purified by the two different methods. These results are shown in Fig. 8. The *lacZ* probe (Fig. 8A) detected roughly the same number of vCMVp-*lacZ* particles in the two preparations. The AAV DNA probe failed to detect wt AAV genomes in 5 h of autoradiographic exposure (Fig. 8B). However, upon autoradiographic exposure for 5 days, wt AAV DNA could be detected in the CsCl density gradient-purified, but not in the sucrose cushion-purified, recombinant vCMVp-*lacZ* virus preparation. The most likely explanation for these seemingly contradictory results is that the replicative forms which hybridize to the AAV probe derive from wt AAV-like recombination products rather than true wt AAV. Wild-type AAV-like particles can be generated by recombination events involving the recombinant AAV plasmid and the AAV helper plasmid, pAAV/Ad, during production of recombinant AAV, and different recombination events yield wt AAV-like particles which undergo replication at various efficiencies (50).

In view of the presence of wt AAV-like particles in purified preparations of vCMVp-*lacZ*, it remained possible that the growth inhibition documented in Fig. 2A and C was due to contaminating wt AAV-like particles rather than to recombinant AAV. This possibility was ruled out by the observation that CsCl density gradient-purified wt AAV did not inhibit [^{3}H]thymidine incorporation by BMS cells exposed to 1,000 particles/cell (data not shown). The ratio of wt AAV-like particles to recombinant AAV particles was estimated to be less than 1 in 1,000 (Fig. 8).

It was also possible that replication of the recombinant AAV genome was due to wt AAV-like *rep* gene expression rather than encapsidated Rep proteins. To address this possibility, replication assays were performed in the presence of actinomycin D (RNA and DNA synthesis inhibitor) or cycloheximide (protein synthesis inhibitor) as described in Materials and Methods. CsCl gradient-purified vTc-Neo virions devoid of detectable Rep proteins (by Western blot analysis of 10^{10} particles) were used in these replication assays. As shown in Fig. 9, replication of vTc-Neo genomes could be detected even in the presence of 100 μg of cycloheximide per ml (lane 8). The lowest concentration of cycloheximide that gives maximal (>95%) inhibition of protein synthesis in HeLa cells is 25 μg/ml (21). These data are consistent with the hypothesis that Rep proteins are encapsidated in recombinant AAV virions. Replication was inhibited by actinomycin D, as expected (Fig. 9, lanes 3 to 5). Replication of wt AAV-like particles could also be detected in the absence of inhibitors and in the presence of up to 10 μg of cycloheximide per ml (Fig. 9, lane 15). It is reasonable to speculate that replication of wt AAV-like genomes was catalyzed by encapsidated Rep proteins in these virions or, alternatively, by encapsidated Rep proteins in recombinant AAV.

To determine if Rep proteins associated with mature particles are accessible to anti-Rep antibodies (Rep Ab), AAV recombinants were mock treated or preincubated with sera containing Rep Ab prior to infection of Ad2-infected 293 cells. Preincubation with anti-AAV capsid Ab (Cap Ab) was also carried out as a control. The recombinant AAV DNA replication assays were performed as described in Materials and Methods. The results are depicted in Fig. 10. It is interesting that pretreatment of recombinant AAV with Rep Ab had no significant effect on viral infectivity (Fig. 10, lane 3). Even when the Rep Ab-to-viral particle ratio was increased 10-fold, no significant decrease in viral infectivity was observed (data not shown). The slight decrease in infectivity observed in Fig. 10 (compare lanes 2 and 3) may therefore be due to nonspecific interference of the antibody with viral attachment. Pretreatment with human sera from a commercial source or from a volunteer donor, presumably containing Cap Ab, abrogated the viral DNA replication (Fig. 10, lanes 4 and 5). Similar results were obtained with monoclonal Rep and Cap Ab (data not shown). These data provide additional evidence that Rep proteins are encapsidated in AAV particles during viral assembly.

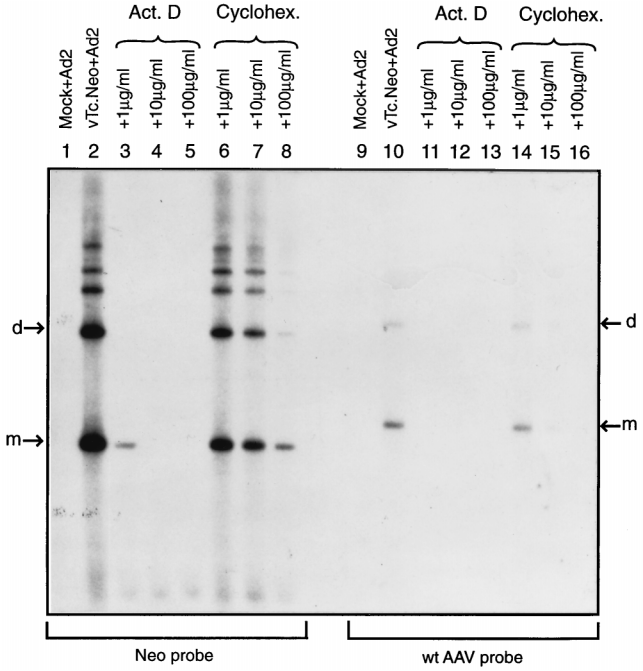

FIG. 9. Highly purified recombinant AAV virions have associated Rep proteins. HeLa cells were either not treated (lanes 2 and 10) or treated with the indicated amounts of actinomycin D (Act. D; lanes 3 to 5 and 11 to 13) or cycloheximide (Cyclohex.; lanes 6 to 8 and 14 to 16) approximately 18 h after infection with Ad2. Eight hours later, equivalent numbers of cells were mock infected or infected, in the continued presence of the inhibitors, with recombinant vTc-Neo virions purified by three rounds of centrifugation in CsCl. Two days later, low-M_r DNA samples were isolated and analyzed on Southern blots, using either *neo*-specific (lanes 1 to 8) or AAV-specific (lanes 9 to 16) DNA probes as described in Materials and Methods. The monomeric and dimeric forms of each of the viral replicative intermediates are denoted by m and d, respectively.

DISCUSSION

AAV-mediated growth inhibition of primary human fibroblasts has been speculated to result from an interaction between a cellular target and the incoming AAV virion or one of its components (51). Consistent with this hypothesis, we found that growth inhibition depends on high particle-to-cell ratios, that the effect can be reversed, and that AAV gene expression is not required. Furthermore, our results suggest that growth arrest is not attributable to AAV integration, since primary BMS cells remain latently infected under conditions where growth arrest has been reversed. It is conceivable that growth arrest is mediated by interaction of a protein component(s) of the incoming virion with a cellular protein. It could also be envisaged that upon stimulation of growth-arrested cells to reenter S phase by treatment with trypsin and subculturing, cellular targets, but not virion components, are newly synthesized. Since there would not be enough virion components to interact with the newly synthesized cellular targets, growth could resume.

We hypothesized that growth inhibition could be mediated by virion-associated AAV Rep proteins that are delivered into cells to a significant extent at high particle-to-cell ratios. The protein content of AAV particles has been analyzed on SDS-polyacrylamide gels, but Rep proteins were never found (38, 47). The most likely explanation is that the amount of Rep protein is below the detection limit by Coomassie blue staining. We found that Rep proteins do indeed associate tightly with AAV particles, but they can be removed by three rounds of ultracentrifugation in CsCl. That the Rep proteins associate tightly is evidenced by the observation that they remain associated with virions sedimented through a sucrose cushion. Even after one round of ultracentrifugation in CsCl, some virion-associated Rep proteins could be detected. These results are consistent with a report demonstrating that complexes of AAV Rep and capsid proteins exist during a productive infection of HeLa cells with AAV and Ad2 (52). Although virions purified by three rounds of ultracentrifugation in CsCl contained no detectable Rep proteins, they did inhibit growth of BMS cells. To determine if these virions could contain encapsidated Rep proteins undetectable by Western blot analysis, we performed functional assays for Rep activity. We found that recombinant AAV lacking the *rep* gene could replicate in Ad2-coinfected cells even in the absence of *rep* gene expression from contaminating wt AAV-like particles. These results provide evidence that AAV Rep proteins are encapsidated in virions in addition to being tightly associated with virions externally. Encapsidated Rep proteins could enter the nucleus, bind to Rep recognition sequences in regulatory regions of cellular genes involved in growth and differentiation (53), and alter expression of these genes such that cellular growth is arrested. Of course, cellular transcription factors are most likely required; indeed, it has recently been reported that Rep78/68 can form a complex with the cellular transcription factor SP1 (32).

Although the mechanism of association of the Rep proteins with virions during AAV assembly remains unknown, it is noteworthy that a genome-linked copy of the nonstructural (NS-1) polypeptide has been shown by Cotmore and Tattersall

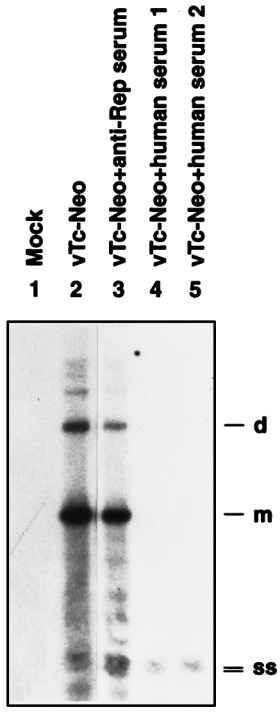

FIG. 10. Rep proteins are encapsidated in AAV virions. 293 cells were mock infected (lane 1) or infected with recombinant vTc-Neo virions following preincubation with no serum (lane 2), with anti-Rep rabbit serum (lane 3), with commercially available human serum (lane 4), or with serum from a volunteer donor (lane 5). Low-M_r DNA samples isolated 72 h postinfection were analyzed on Southern blots, using a *neo* DNA probe as described in Materials and Methods. Monomeric and dimeric replicative intermediates and single-stranded viral DNA are denoted by m, d, and ss, respectively.

(10) to be present on the outside of the infectious autonomous murine parvovirus minute virus of mice. The NS-1 protein is covalently linked to the 5′-ends of the replicating viral DNA strands (9). Similarly, a covalent linkage of the AAV Rep78 and Rep68 proteins to the replicative forms of the AAV genome has been demonstrated in in vitro studies (43). Furthermore, it has recently been reported that Rep78 is covalently attached to viral DNA in a preformed virion and that the Rep78 is probably located on the outside of the particle, based on susceptibility of the DNA linkage to nuclease digestion and accessibility of Rep78 to antibody binding and protease digestion (36, 37). However, in our studies, the association between Rep78/68 proteins and infectious virions was not sensitive to digestion with DNase I, and the virion-associated Rep proteins were not accessible to the same anti-Rep antibodies used by Prasad and Trempe (36). These apparent differences are most likely due to the fact that we analyzed mature viral particles that were assembled 65 to 72 h postinfection, in contrast to viral particles that were assembled 20 h postinfection and were probably more immature since they sedimented more slowly than mature virions on sucrose gradients (36). Another possibility is that encapsidated Rep proteins are bound to the viral terminal repeats which are common to wt and recombinant AAV (1, 27).

Previous studies with wt AAV have documented that each infectious particle contains one full-length, single-stranded AAV genome and bands at a density of approximately 1.41 g/ml in a CsCl gradient (11). Particles which band at densities less than 1.41 g/ml on CsCl gradients are defective in that they are unable to replicate. These particles contain either the left- or the right-terminal 10 to 50% of the single-stranded AAV genome (11). Particles which band at densities greater than 1.41 g/ml probably contain higher DNA-to-protein ratios than intact particles. It appears from our studies that these principles also apply to recombinant AAV. Interestingly, only intact recombinant AAV particles inhibited growth. This was discovered by fractionating recombinant AAV-containing cell lysates on a CsCl density gradient and testing individual fractions for growth-inhibitory effect. The only component which inhibited growth was that which banded at a density of approximately 1.39 g/ml and was therefore expected to contain predominantly intact particles. More direct evidence that the fraction which banded at 1.39 g/ml contained intact recombinant AAV particles was derived from the observation that this was the only fraction of the gradient that yielded replicative forms of recombinant AAV in Ad2-coinfected 293 cells. Thus, even though recombinant AAV DNA and/or AAV Rep proteins were detected in fractions of the density gradient that banded above and below 1.39 g/ml, these fractions most likely contained incomplete particles or no particles at all.

In agreement with a previous report (51), we found that cells immortalized by SV40 are resistant to AAV-induced growth inhibition. Interestingly, we found that BMS cells were more sensitive than lung fibroblasts (WI-38 cells). With each successive subculture of a primary culture, the component of the population with the ability to proliferate most rapidly will gradually predominate, and nonproliferating or slowly proliferating cells will be diluted out (12). Consistent with this observation, the fresh BMS cell cultures used in this study, which had been passaged only a few times, grew much more slowly than the WI-38 cells that had been passaged >30 times. The WI-38 cells had more chances to undergo selection and phenotypic drift (especially since they were cultured in antibiotics to cure a bacterial infection) than the BMS cell cultures, which may account for the difference in sensitivity of the two cell types to AAV-induced growth arrest. Increased cellular growth rate does not by itself account for resistance to growth arrest; rather, it appears to be a consequence of the event that makes cells resistant. Such an event could be a change in cell cycle regulation as a consequence of a cellular target protein(s) being overexpressed, overridden, bypassed, or destroyed. Differences in susceptibility to AAV-mediated growth arrest among various immortal cell types are likely due to differences in the molecular mechanisms whereby the cells were immortalized or transformed.

The presence of contaminating wt AAV-like particles even in highly purified recombinant viral preparations precludes accurate estimation of the infectious particle titers of recombinant AAV vectors. In addition, the growth-inhibitory property of Rep proteins encapsidated in recombinant AAV impinges on the use of this vector system to transduce primary cells in human gene therapy.

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