Upstream AP1- and CREB-Binding Sites Confer High Basal Activity on the Adeno-Associated Virus Type 5 Capsid Gene Promoter[▽]

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In contrast to the prototype adeno-associated virus type 2 (AAV2), the capsid gene P41 promoter of AAV5, within viral constructs that lack inverted terminal repeat sequences, displays a high basal level of expression in 293 cells in the absence of coinfecting adenovirus. Here we demonstrate that this was due to differences in the relative strengths of the core promoter elements and to the presence of active binding sites for the transcription factors CREB and AP1 within the upstream region of P41 that are absent from the AAV2 capsid gene promoter P40. These differences also governed the relative basal activity of the AAV capsid gene promoters within near-full-length viral genomes.

Members of the *Parvovirus* and *Dependovirus* genera use internal promoters to regulate capsid gene expression (6, 21). The activity of these promoters is typically low in all cell types examined prior to activation either by a viral protein alone (e.g., the large nonstructural viral protein NS1 for the *Parvovirus* genus), or, in the case of the adeno-associated virus type 2 (AAV2)-like viruses, by the viral Rep protein plus helper virus (2, 6, 21). For the prototype AAV2, transactivation of P40 capsid protein requires binding of the large Rep proteins to the transcription template at either the AAV2 inverted terminal repeat (ITR) or the P5 promoter (13) and has been proposed to work, at least in part, by stabilizing a loop-like structure that localizes the P5 promoter and, presumably, P5-associated transcription factors, to the P40 promoter (11, 16). Efficient activation of AAV2 P40 by AAV2 Rep requires coinfection by adenovirus (Ad), even in 293 cells, which constitutively express both the well-characterized transcription activator E1A and E1B proteins (2, 20, 25).

AAV5, the most divergent of the AAV serotypes, shares only 64% overall nucleotide identity with AAV2 (1, 3, 28). The AAV2 and AAV5 Rep proteins share 67% identity (4, 28). Both AAV2 and AAV5 Rep proteins bind the Rep-binding element (RBE) that is present in both AAV2 and AAV5 ITRs (3) and the AAV2 P5 promoter (28). In contrast to AAV2, however, the AAV5 large Rep gene promoter (P7) contains only a poor-consensus RBE (28), which suggested that activation of the AAV5 capsid gene promoter P41 may be governed differently from its AAV2 counterpart (28).

We have recently shown that within RepCap constructs lacking ITR sequences, the expression level of the AAV5 P41 capsid gene promoter in 293 cells was surprisingly high, and this higher level of activity required adenovirus E1A and/or E1B (19, 28). However, in contrast to AAV2 P40, neither adenovirus infection nor the large Rep protein was required to

achieve these higher levels of expression in these cells (19, 28). In addition, the AAV5 Rep protein was itself a poor activator of the inducible AAV2 P40 promoter (28).

In this article, we show that the higher basal activity of AAV5 P41 relative to AAV2 P40 is due to differences in the relative strengths of the core promoter sequences, but more importantly, to the presence of active binding sites for the transcription factors CREB and AP1 within the upstream region of AAV5 P41 that are absent from AAV2 P40. These differences also govern the relative basal activity of the AAV5 capsid gene promoters within near-full-length viral genomes.

MATERIALS AND METHODS

Cells and virus. HeLa and 293 cells were propagated as previously described (14). Transfections, using Lipofectamine and the Plus reagent (Invitrogen, Carlsbad, CA), were performed as previously described (20), and when Ad5 was coinfecting, this was done 5 h after transfection at a multiplicity of infection of 5.

Plasmid constructs. (i) **Luciferase reporter constructs.** All luciferase reporter constructs are based on pGL3-Basic (Promega, Madison, WI). The core promoters (CP40 and CP41) and the mutant sequences shown in Fig. 1A were inserted between XhoI and HindIII sites in the polylinker region. The constructs shown in Fig. 2A, designed to analyze upstream sequences, were based on CP40 and CP41 constructs; upstream sequences were inserted between the NheI and XhoI sites. 2L, 2S, and V2min span from nucleotides (nt) 1655, 1708, and 1744 to nt 1822, respectively. 5L, 5S, and V5min span from nt 1681, 1733, and 1770 to nt 1879, respectively. V2min AP1 is based on V2min with GAGCAT at nt 1761 to 1766 changed to ACTCAC. V2min CRE is based on V2min with extra CRE-binding sequence from AV5 CTGGGTGACGTCACCAATACTAGC added at the 3' position. V2min AP1 CRE contains both changes mentioned above. V5min AP1m is based on V5min with the sequence ACTCAC at nt 1787 to 1792 changed to GAGCAT. V5min CREm is based on V5min with the GT at nt 1865 to 1866 changed to TC. V5min AP1m CREm contains both changes.

(ii) **E1A and E1B expression constructs.** E1A, E1B, 13SE1A, and 12SE1A expression constructs were gifts from Greg Tullis (Boston University). Based on the 13SE1A plasmid, d1N has a deletion of the N-terminal 40 amino acids (aa) of E1A, and d1CR1 has deletion of E1A aa 41 to 80 generated similarly to mutants characterized in reference 27. All of the wild-type and mutant E1A expression constructs routinely generated similar levels of protein following transfection, as determined by Western blot analysis.

(iii) **RepStopCapTR and RepStopDMCapTR plasmids.** The AAV2 RepStopCapTR and AAV5 RepStopCapTR constructs are based on AAV2 RepCap (containing AAV2 nt 145 to 4492) (20) and AAV5 RepCap (containing AAV5 nt 185 to 4448) (19) constructs and contain premature termination codons introduced at nt 489 and 480, respectively, in the amino terminus of their respective Rep proteins, as previously described (28). In addition, half of the ITR

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FIG. 1. Core promoter comparison of AAV2 P40 and AAV5 P41. (A) Sequences of AAV2 P40 core promoter (CP40 nt 1823 to 1887) and AAV5 P41 core promoter (CP41 nt 1880 to 1970). The core promoters start from the TATA box of each promoter, and the luciferase genes from pGL3 are cloned downstream for the reporter assay below. TATA box and initiator sequences are indicated in boldface letters. The vertical lines in the boxes indicate the borders of TATA box and initiator elements in the hybrid promoters (AV2 CP40 V5TATA, AV2 CP40 V5Inr, AV5 CP41 V2TATA, and AV5 CP41 V2Inr) tested below. (B) Luciferase (Luc) activity of the wild-type, mutant, and hybrid promoters in 293 cells. The activity of each promoter is the average of duplicate samples from at least three experiments. All values are standardized to *Renilla* luciferase (R-Luc) activity generated by a TK promoter-driven *Renilla* luciferase expression vector used as an internal control.

sequence from AAV2 (nt 4489 to 4626) or AAV5 (nt 4476 to 4587), respectively, was added at the 3' portion of the genome to allow Rep binding and activation. The AAV2 RepStopDMCapTR and AAV5 RepStopDMCapTR plasmids contain the same promoter upstream region as the reporter constructs V2min AP1 CRE and V5min AP1mCREm, respectively, as described above.

Luciferase assays. Luciferase assays were performed according to the manufacturer's suggested protocols (Promega, Madison, WI). Briefly, 293 cells grown in 12-well plates were transfected with 0.1 μ g per well of the luciferase reporter constructs along with 0.35 μ g per well of empty pBluescript SK(+) (Stratagene, La Jolla, CA) using the Lipofectamine reagent (Invitrogen). In addition, 0.05 μ g per well of thymidine kinase (TK)-driven *Renilla* luciferase gene reporter was cotransfected as an internal control. The total amount of DNA transfected into each well was kept at 0.5 μ g. Thirty-six hours after transfection, cells were lysed and the luciferase activity was tested using the Promega dual-luciferase reporter assay system (Promega). Each experiment represents the average of duplicates from three individual experiments (error bars shown), each normalized to *Renilla* luciferase activity.

Expression of the TK-driven *Renilla* luciferase gene varied in response to E1A. Therefore, *Renilla* luciferase gene activity was not suitable as an internal control when testing E1A function in HeLa cells. Thus, these experiments were carried without an internal control but were repeated multiple times. Using the Lipofectamine reagent (Invitrogen, Carlsbad, CA), HeLa cells grown in six-well plates were transfected with 0.3 μ g per well of luciferase reporter constructs together with 0.6 μ g per well of empty pBluescript SK(+) DNA or different E1A constructs. In addition, 0.6 μ g E1B was added for the E1A-E1B cotransfection group. Otherwise, 0.6 μ g pBluescript SK(+) DNA was added to make up the amount of total DNA. Thirty-six hours after transfection, one-fifth of the cells were lysed with buffer containing 1% Triton X-100 and subsequently tested for luciferase activity as previously described (12). Four-fifths of the cells were used to assay E1A expression levels by Western blot analysis.

Nuclear extract preparation. The method for nuclear extract preparation was adapted from that described by Schreiber et al. (24). Briefly, 293 cells were collected and washed with phosphate-buffered saline (PBS). Cells were then resuspended in 2 volumes of buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, and 1.0 mM dithiothreitol [pH 7.9]), lysed with a tight Dounce homogenizer and centrifuged at low speed to collect nuclei. Pellets were resuspended in 2 volumes of buffer C (20 mM HEPES, 1.5 mM MgCl₂, 1.0 mM dithiothreitol, 0.2 mM EDTA, 0.44 M KCl, 25% glycerol), and centrifuged at 3,000 rpm for 10 min. Supernatants were then dialyzed against buffer D (20 mM HEPES [pH 7.9], 100

mM KCl, 0.2 mM EDTA, 20% glycerol), and centrifuged at 3,000 rpm for 10 min to remove particulate material.

EMSA and UV cross-linking immunoprecipitation. Oligonucleotide sequences for electrophoretic mobility shift (EMSA) probes (lowercase nucleotides indicate mutations) were as follows: AP1 sense, 5'-GTGCCGGTGACTC ACGAGTTT-3'; AP1 antisense, 5'-TTTAAACTCGTGAGTCACCGGC-3'; AP1m sense, 5'-GTGCCGGTGgaTCAAGAGTTT-3'; AP1m antisense, 5'-TT TAAACTCaTGATcCACCGGC-3'; AP1sense for cross-link, 5'-GTGCCGGBr GACBrCACGAGTTT; AP1m sense for cross-link, 5'-GTGCCGGBrGgaBrCAcT GAGTTT-3'; CRE sense, 5'-GCCCACTGGGTGACGTACCAAT-3'; CRE antisense, 5'-TAGTATTGGTGACGTACCCAGT-3'; CREm sense, 5'-GCC CACTGGGTGACtCACCAAT-3'; and CREm antisense, 5'-TAGTATTGGTG gaGTCACCCAGT-3'.

Briefly, annealed oligonucleotides were labeled with [³²P]dATP using the DNA polymerase Klenow fragment. After incubation with nuclear extract for 15 min, the binding complexes were resolved by 6% native polyacrylamide gel electrophoresis. For the supershift assay, 4 μ l anti-CREB antibody was added to the reaction mixture for an additional 30 min before being resolved by polyacrylamide gel electrophoresis.

For AP1 cross-linking-immunoprecipitation, the reaction mixture was cross-linked using a 310-nm UV transilluminator (Fisher Scientific) for 30 min. The cross-linked complexes were then immunoprecipitated using anti-c-Fos, anti-c-Jun, or anti- β -actin antibody in nondenaturing RAF solution (20 mM Tris, 139 mM NaCl, 10% glycerol, 1% NP40 [pH 8.0]). The precipitated complexes were collected for scintillation counting.

RNase protection assays. Total RNA was isolated 36 to 41 h posttransfection as previously described (15, 23). RNase protection assays were performed as previously described (15, 23), using homologous antisense probes, termed "RP," which spanned nt 1767 to 1906 for the AAV2 promoter and nt 1846 to 1985 for the P41 promoter of AAV5, respectively. The probes designated "DM" start and end at the same original nucleotide position but contain the corresponding AP1 and CRE debilitating mutation (for AAV5) or enabling mutation (for AAV2).

RESULTS

The core capsid promoter of AAV5 is stronger than that of AAV2. Previous comparison indicated that in Ad5 E1A- and E1B-expressing 293 cells, the basal activity within RepCap

FIG. 2. Upstream sequence analysis of the capsid promoters. (A) Upstream sequence alignment of AAV2 P40 promoter (USP40 nt 1655 to 1822) and AAV5 P41 promoter (USP41 nt 1681 to 1879). Identical nucleotides are shaded, and the abbreviations L, S, and min indicate the different lengths of the upstream sequences linked to the core promoters in the luciferase constructs tested below. The consensus AP1 and CRE sites are also indicated in the boxes. (B and C) AAV5 upstream sequence enhances promoter activity greater than AAV2 upstream sequence. Different lengths of AAV2 and AAV5 upstream sequences are linked to core promoter CP40 (B) or CP41 (C), and the activity of each promoter is the average of duplicate samples from at least three experiments. All values are standardized to *Renilla* luciferase (R-Luc) activity generated by a TK promoter-driven *Renilla* luciferase expression vector used as an internal control. (D and E) AP1 and CRE are the major elements enhancing AAV5 P41 promoter activity. AAV2 core promoter CP40 is linked with wild-type AAV2, AAV5 upstream sequence, or AAV2 upstream sequence mutated to contain AP1, CRE, or both sites (D). A similar set was tested with AAV5 core promoter CP41 linked with wild-type AAV2, AAV5 upstream sequence, or AAV5 upstream sequence with AP1 and with the CRE site mutated (E).

plasmids of the AAV5 P41 capsid gene promoter was significantly greater than that of the P40 capsid gene promoters of AAV serotypes 1, 2, 3, 4, and 6 in similar backgrounds (28). To systematically identify *cis*-acting sequences that might account for these different expression levels, we used luciferase reporter assays to first compare the core promoter activities of AAV2 P40 and AAV5 P41 (Fig. 1).

The major determinants of both core promoter activities (as diagrammed in Fig. 1A) were found, as expected, to be the TATA box and initiator element. Both elements were required for full promoter activity, as mutation of either element in

either of the core promoter constructs CP40 or CP41 decreased activity to about half of the wild-type level (compare AV2 CP40 to AV2 CP40 TATAm and AV2 CP40 Inrm and AV5 CP41 to AV5 CP41 TATAm and AV5 CP41 Inrm, Fig. 1B). Consistent with our previous evaluations, the AAV5 core promoter had about five times greater activity than the AAV2 core promoter (compare AV2 CP40 and AV5 CP41, Fig. 1B).

To further identify which elements within the core promoter regions might determine the difference in their activity, we exchanged their TATA boxes and initiator regions plus associated flanking regions. Replacement of the AAV2 TATA box

with the AAV5 TATA box resulted in greater than threefold higher activity compared to that of the wild-type AAV2 core promoter (Fig. 1B, compare AV2 CP40 with AV2 CP40 V5TATA), whereas replacement of the AAV2 initiator with the AAV5 initiator resulted in a chimera with only about half of the activity of the wild type (Fig. 1B, compare AV2 CP40 with AV2 CP40 V5Inr). In reciprocal experiments, replacement of the AAV5 TATA box with that of AAV2 resulted in a chimera with only half of the original activity (Fig. 1B, compare AV5 CP41 with AV5 CP41 V2TATA), while replacement of the AAV5 initiator sequence with that of AAV2 resulted in only a slight increase in activity (Fig. 1B, compare AV5 CP41 with AV5 CP41 V2Inr). These results indicated that AAV5 contains a stronger TATA box while AAV2 has a stronger initiator, yet the wild-type AAV5 core promoter had an overall stronger activity than the wild-type AAV2 core promoter. These differences, however, did not seem strong enough to explain the differences in expression observed in the parent RepCap constructs (28), and so further comparison of the upstream regions of the two promoters was performed.

AP1 and CRE elements in AAV5 P41 upstream sequence contribute to its high basal activity. We next examined the roles of the upstream regions of AAV2 and AAV5 by linking various portions of these regions to AAV2 or AAV5 core promoters driving the luciferase gene. An alignment of the upstream regions of AAV5 P41 and AAV2 P40, designed to maximize sequence identity, is shown in Fig. 2A. These regions show significant differences, and identity between the two drops dramatically downstream of nt 1744(AAV2)/1770(AAV5), the region designated as “min” in Fig. 2A.

AAV2 or AAV5 upstream elements starting at either nt 1655(AAV2)/1681(AAV5) (L), 1708(AAV2)/1733(AAV5) (S), or 1744(AAV2)/1770(AAV5) (min) stimulated the basal activity of the core promoters to various extents (Fig. 2B and C, compare CP40 and CP41 to their relevant chimeras); however, all AAV5 upstream sequences tested consistently increased both AAV5 and AAV2 core promoter activity to a greater extent than AAV2 upstream sequences (compare 2L, 2S, and V2min chimeras to 5L, 5S, and V5min chimeras for CP40 in Fig. 2B and CP41 in Fig. 2C). Thus, it was likely that the AAV5 region from nt 1770 to the TATA box contained elements absent from the analogous region in AAV2 that stimulated basal activity of these core promoters.

Inspection of the region downstream of AAV5 nt 1770 identified consensus binding sites for transcription factors AP1 and CREB (8) that are absent from AAV2. To determine if these sites influence promoter activity, we carried out mutational analysis of the AAV5min and AAV2min constructs described above. The AAV5 AP1 site was destroyed by changing it to the resident AAV2 sequence (V5minAP1mCP41), and the CRE site was destroyed by changing the GT nucleotides at nt 1865 to 1866 to TC (V5minCREmCP41), a mutation shown by others (22) to prevent CREB binding. The double-knockout mutant was also made (V5minAP1mCREmCP41). Reciprocal mutations were made in AAV2min by changing the region corresponding to the AAV5 AP1 site (nt 1761 to 1766) so as to add the AAV5 sequence (V2minAP1CP40) or by adding the AAV5 CRE site directly downstream of AAV2 nt 1822 (V2minCRECP40). The double-addition mutation was also made (V2minAP1CRECP40).

As predicted, the AAV5 promoter with either AP1 or CRE

FIG. 3. Physical interaction between cellular factors and AP1 and CRE sites. (A and B) EMSA of AP1 and CRE with 293 cell nuclear extract. Free probes (F) and specific shifted (S) bands are indicated on the left. Complexes formed with wild-type and mutant (m) probes are shown in lanes 1 and 2, respectively. Tenfold cold wild-type (lane 3) and labeled mutant (lane 4) probe competition results are also included. The bands marked with an asterisk may represent additional unidentified complexes since they are removed by competition. (C) Supershift assay for CRE probe. In lane 2, after incubation of the probe and nuclear extract, 4 μ l anti-CREB (a-CREB) antibody was added to the reaction mixture for an additional 30 min. A supershifted band is indicated by SS. (D) UV cross-link coupled immunoprecipitation to test the binding of c-Fos and c-Jun to the AP1 element. The radioactivity of the immunoprecipitated complexes was measured using scintillation counting. A wild-type AP1 probe with anti- β -actin (α - β -actin) antibody and an AP1 mutant probe (AP1m) with anti-c-Fos (α -c-Fos) or anti-c-Jun (α -c-Jun) antibodies were used as controls. The data shown are the average of duplicate samples from at least three experiments.

mutated had reduced activity compared to the P41 core promoter containing the wild-type AAV5 upstream sequence (compare V5minAP1mCP41 and V5minCREmCP41 to V5minCP41, Fig. 2E). The double-knockout mutation decreased promoter activity even further, to approximately the level generated by

FIG. 4. E1A activates AAV5 P41 promoter, but the activation is not directly mediated by AP1 or CRE sites. Shown is the luciferase activity of P41 reporter constructs with either the wild-type or AP1 and CRE mutant upstream regions, tested in HeLa cells together with cotransfection of different E1A isoforms, E1A mutants, or empty vector SK+. The activity is the average of duplicate samples from at least three experiments, as described in Materials and Methods.

AAV2min (compare V5minAP1mCREmCP41 to V2minCP41, Fig. 2E). Within the AAV2 background, creation of either an AP1 or CRE site doubled the basal promoter activity (compare V2minAP1CP40 and V2minCRECP40 to V2minCP40, Fig. 2D). When both sites were added, improvement almost to the level achieved by AAV5min was observed (compare V2minAP1CRECP40 to V5minCP40, Fig. 2D). These results suggested that the AP1 and CRE elements present in the AAV5 upstream region were at least partially responsible for the elevated basal activity of AAV5 P41 in 293 cells.

The AAV5 AP1 and CRE elements bind their cognate factors in 293 cells. EMSA was used to investigate the binding of cellular factors to the AAV5 AP1- and CRE-binding sites. Both wild-type, but not mutant, probes were able to specifically bind cellular factors present in 293 cell nuclear extracts (compare Fig. 3A and B, lanes 1 to 2). Excess cold wild-type probes effectively competed for binding (Fig. 3A and B, compare lanes 1 and 3), and excess labeled mutant probes exhibited no additional complex formation (Fig. 3A and B, lanes 4). To determine the specificity of these complexes, supershift assays were performed. AP1 sites most commonly bind to a heterodimer formed by c-Fos and c-Jun (9), while CREB is the primary factor that binds to CRE sites (5). A supershifted complex was clearly detected for the CRE probe when anti-CREB antibody was added (Fig. 3C, lane 2), suggesting that CREB indeed forms a complex with this site in the AAV5 P41 promoter. Anti-c-Fos or anti-c-Jun antibodies failed to generate detectable supershifted bands complexing with the AAV5 AP1 element (data not shown), perhaps because of instability of the complex; however, UV-cross-linking of cellular complexes with this element allowed specific immunoprecipitation (compared to anti- β -actin antibodies), by either anti-c-Fos and anti-c-Jun antibodies, of a wild-type radioactive probe to greater extents than a mutant radioactive probe (Fig. 3D), suggesting that c-Fos and c-Jun form a complex with the AAV5 AP1 site.

Activation by E1A is independent of the AAV5 AP1 and CRE sites. As previously reported (28), the basal activity of AAV5 P41 is higher in 293 cells than HeLa cells. To test whether the adenovirus E1A and/or E1B gene products endogenously produced in 293 cells were responsible for this difference, the expression of P41 from a construct containing wild-type AAV5 upstream sequences (V5min CP41) was tested in HeLa cells, following cotransfection with various combinations of the Ad5 E1 genes. E1A, or E1A together with E1B, increased promoter activity in this assay, while E1B alone did not, indicating that E1A was responsible for the activation effect (Fig. 4, left panel). The Ad E1A gene product interacts with many cellular proteins (including CBP, which is a binding partner of CREB) to alter the transcription of both viral and cellular genes, although it does not bind DNA directly (7). Surprisingly, we found that while basal levels of expression were lower, a P41 reporter construct containing mutations of the AP1 and CRE sites (V5minAP1mCREm CP41) was still activated to similar degrees by E1A (compare the first two columns of each panel in Fig. 4). These results suggested that the increase in P41 expression in response to E1A was not mediated by the AP1 or CRE sites, but rather the effect of E1A was likely targeted directly to the basic transcription machinery.

The E1A gene generates 13S and 12S isoforms by alternative splicing which differs mainly in the retention of conserved region 3 (CR3) in the 13S isoform (7). Furthermore, the CR1 and N-terminal regions of E1A, present in both isoforms, contain domains that interact with p300/CBP (27), which might be predicted to be important for CRE-mediated E1A activation if such a pathway exists. We found that the 12S E1A product, supplied from a cDNA expression vector, increased promoter activity from each of the two reporter constructs tested to levels similar to that induced by the 13S E1A product (Fig. 4), suggesting that CR3 was not essential for its transactivating activity. Similarly, deletion of the CR1 region from the 13S

FIG. 5. AP1 and CRE enhance promoter activity in the viral RepCap background. (A) Illustration of the constructs used in the assay. AAV2 RepStopCapTR contains AAV2 sequence nt 145 to 4626 and a premature stop codon at nt 489. AAV2 RepStopDMCapTR has additional AP1 and CRE sites present in the P40 region. AAV5 RepStopCapTR contains AAV5 sequence nt 185 to 4587 and a premature stop codon at nt 480. AAV5 RepStopDMCapTR has additional AP1 and CRE mutation to knock out those sites in the P41 region. The probes used in the RNase

cDNA expression vector did not abrogate its ability to activate either of the two reporter constructs. However, deletion of the N-terminal region from this protein did abolish its activating activity, although all mutant proteins were expressed at levels equivalent to those of the wild-type protein (data not shown). These results suggested that the N-terminal domain was required for E1A function, consistent with a role for p300/CBP in this process.

The presence or absence of AP1 and CRE elements helps determine expression of the capsid gene promoter within the viral RepCap background. To further examine the roles of the AP1 and CRE elements, the debilitating mutations within AAV5 P41 and the enabling mutations within AAV2 P40 described above were introduced into AAV5 and AAV2 RepCap constructs. To prevent confounding expression of potentially mutant Rep proteins from these constructs, a premature termination codon was introduced into the NH₂-terminal region of the large Rep proteins, as indicated in Fig. 5A, and activation of these constructs was then evaluated in the presence or absence of the AAV2 and AAV5 Rep proteins provided in *trans*. The AAV2 P5 promoter region provides an RBE competent for directing Rep activation; however, the AAV5 P7 region does not, and hence simple AAV5 RepCap constructs would not be expected to support Rep activation (28). The AAV5 ITR, however, does contain an RBE which effectively functions as a binding platform and supports AAV5 Rep activation of weak promoters (28). Therefore, serotype-matched, RBE-containing half-ITR sequences were added at the 3' end of the four reporter constructs to provide RBEs for effective Rep activation, yet not allow replication which would introduce another variable into the evaluation of expression levels. Expression of these constructs was then tested in 293 cells in the presence and absence of cotransfected human immunodeficiency virus promoter-driven Rep-expressing plasmids (19, 20).

Similar to previous results (19, 28), we observed a much higher basal level of wild-type AAV5 P41 than wild-type AAV2 P40 from these constructs in 293 cells (Fig. 5B, compare lane 1 to lane 9), and the basal activity of both wild-type promoters was stimulated only slightly by adenovirus (Fig. 5B, compare lanes 2 to 1 and 10 to 9). The addition of AAV5 Rep slightly increased wild-type P41 activity (about threefold) (Fig. 5B, compare lane 4 to lane 2), while added AAV2 Rep had a negligible effect on P41 (Fig. 5B, compare lane 3 to lane 2). Mutation of the AAV5 AP1 and CRE binding sites significantly decreased P41 basal activity in both the absence (to approximately 1/10 of the original level, Fig. 5B, compare lane 5 to lane 1) and presence (to approximately 1/17 of the original level, Fig. 5B, compare lane 6 to lane 2) of adenovirus. Interestingly, this weakened promoter was activated by AAV5 Rep

(more than sevenfold), but not by AAV2 Rep (Fig. 5B, compare lanes 8 and 7 to lane 6). These results demonstrate the importance of the AP1 and CRE elements in maintaining high basal promoter activity in 293 cells. In addition, these results confirm that AAV5 Rep can act as a transcriptional activator under certain circumstances (a weaker promoter and a good binding site) as previously described (28).

The addition of an AP1 binding site and a CRE binding site conferred to the AAV2 P40 promoter a much higher basal activity, in both the absence and presence of adenovirus (Fig. 5B, compare lane 13 to lane 9 and lane 14 to lane 10). Both wild-type and AP1-plus-CRE site-containing promoters were activated by AAV2, but not AAV5, Rep (Fig. 5B, compare lanes 11 and 12 to lane 10 and lanes 15 and 16 to lane 14). While the final activated levels of the AP1-plus-CRE binding site-containing P40 promoters were higher than for the wild-type P40, the activation (fold) induced by AAV2 Rep was somewhat lower for the AP1-plus-CRE site-containing P40 compared to the wild type (3.5-fold versus 5-fold, Fig. 5B, compare lanes 11 to 10 and 15 to 14). This result suggested that the addition of AP1 and CRE sites did not disrupt the elements required for Rep activation; however, activation levels were lower due to increased basal activity.

DISCUSSION

The capsid promoter of AAV5 differs from that of the AAV2-like *Dependovirus* members (AAV1, -3, -4, and -6) in that it exhibits high constitutive activity in Ad5 E1A- and E1B-expressing 293 cells, but not HeLa cells, either as part of a minimal P41 expression construct or as part of a larger, partial ITR-containing, RepCap construct. We show in this article that this is due to both a stronger core promoter and the presence in AAV5 P41 of CREB and AP1 binding sites immediately upstream of the core sequences. Increased expression from the core elements depends upon the Ad5 E1A protein, but activation due to the upstream CREB and AP1 binding sites is independent of E1A.

The AP1 and CREB-binding sites are found upstream of the capsid gene promoters of other AAV5-like *Dependovirus* members, including bovine AAV (B-AAV), and caprine AAV (Go.1-AAV) (Fig. 6). These elements are lacking from all of the AAV2-like viruses, as well as the avian AAV (A-AAV), which is the most divergent of the nonprimate AAV5-like viruses (18). It has become clear that the AAV5-like AAVs (including those nonprimate AAVs so far characterized) are quite distinct from the AAV2-like viruses, both phylogenetically and in genetic organization (17, 18). The presence of the AP1 and CREB sites, and hence differences in capsid promoter activity, is another feature that discriminates between

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protection assay are also shown. (B) RNase protection assay, performed as previously described (15, 23), using homologous RP probes to protect RNA generated in 293 cells following transfection of the constructs mentioned above. pBluescript SK+ was used as an empty vector control. AAV2 or AAV5 Rep was provided in *trans* (V2 or V5, respectively), driven by human immunodeficiency virus promoter, as previously described (19, 20). pEGFPC1 (Clontech, Mountain View, CA) was cotransfected as an internal control. Transfection of samples for lanes 2 to 4, 6 to 8, 10 to 12, and 14 to 16 was followed by adenovirus infection to allow activation, as indicated. (C) Quantification, using Fujifilm MultiGauge software, of RNase protection. A representative example is shown. Data from at least three experiments, with standard error bars, are presented as the activation (fold) of P40 (P41) promoters comparing transcription levels. The wild-type P40 (P41) level without Rep cotransfection and adenovirus infection is set as 1.

FIG. 6. Genome sequence comparison of AAV2 and AAV5-like viruses. The P40/P41 upstream sequence alignments of AAV2, AAV5, bovine AAV (BAAV), and caprine AAV (Go-AAV) are shown. The upstream promoter sequences from each virus were aligned using Vector NTI software (Invitrogen) with AAV5 as the consensus reference. Identical nucleotides in all AAVs are shown in the darkest shade of gray. Nucleotides identical to the AAV5 sequence are shown in the lighter shade of gray. The consensus AP1 and CRE sites are indicated in boxes.

these two groups and which potentially can be used to evaluate evolutionary distances. Although the animal AAVs have been shown to cocirculate with adenovirus species (17, 18), the natural host for AAV5 has not been unambiguously established. Thus, it remains possible that the response of AAV5 to herpes simplex virus may be different.

AP1 and CREB have been extensively studied as part of the cellular signal transduction pathways in many different systems (5, 9). Both sites have been found in the adenovirus E3 promoter and have been implicated in stabilization of the TATA box binding complex (22). Because of the intimacy between the AAV and Ad life cycles, it is perhaps not surprising to find that they perform at least in part a similar function during AAV5 gene regulation. As found previously for the Ad5 E3 promoter (22), the disruption of the AAV5 AP1 and CREB-binding sites did not eliminate activation by E1A, which suggested that E1A may affect the general transcription machinery directly at the core promoter and thus synergistically enhance promoter activity with transcription factors bound upstream.

Regulation of transcription from an incoming authentic infectious viral genome may ultimately show differences from the plasmid transfection results shown here. However, within AAV5 RepCap constructs bearing a single RBE-containing partial AAV5 ITR sequence, the presence of CREB- and AP1-binding sites upstream of the capsid gene promoter is the main determinant governing its high basal activity. Furthermore, AAV5 Rep only slightly increases P41 activity in this background in the presence of Ad5. When the AP1- and CREB-binding sites in this background were disrupted, the basal level of expression of P41 was severely decreased, and, interestingly, in the mutant background AAV5 Rep, but not AAV2 Rep, was able to significantly activate this promoter, although both Rep

proteins have been shown to bind to the RBE resident on the AAV5 ITR with similar affinities (3). In a reciprocal set of experiments, introduction of these transcription factor binding sites into the AAV2 background containing the AAV2 half-ITR increased the basal activity of the capsid promoter, thus decreasing the relative activation (fold) in response to AAV2 Rep. AAV5 Rep remained a very poor activator of P40 in this context. Although the AAV2 and AAV5 Rep proteins have been shown to bind both the AAV5 and AAV2 ITRs with similar affinities (3), activation by Rep could certainly be influenced by a specific configuration adopted during the binding of each Rep to its own ITR (10, 26). The two Rep proteins have conserved their activation ability; however, the magnitude of activation seems to be determined by both the relative strength of the target promoters and the nature of their cognate binding sites.

It may be that the relative expression level of the capsid gene promoter of AAV5 needs to be more significantly regulated during viral infection, and thus capsid gene expression during infection by wild-type viral genomes that contain two ITRs may be different. In fact, following transfection of the AAV5 infectious clone, the basal activity of P41 was found to be relatively lower, and greater (though still modest) levels of transactivation were observed from these full-length molecules (19). However, it should be noted that while the genomes of both minute virus of mice and AAV2 are expressed in a temporal manner (i.e., the capsid-encoding genes are upregulated by the prior expression of the nonstructural proteins), steady-state levels of promoter activity are reached quite quickly, after which there is little change in the relative strength of the viral promoters or the ratio of the abundance of the various transcripts.

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