A genetic screen identifies a cellular regulator of adeno-associated virus

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Adeno-associated virus type 2 (AAV2) is a human parvovirus that has attracted attention as a vector for gene transfer. Replication and site-specific integration of the wild-type virus requires binding of the AAV2 Rep proteins to a cis-regulatory element named the Rep recognition sequence (RRS). RRS motifs are found within the cellular *AAVS1* integration locus, the viral p5 promoter, and the inverted terminal repeats (ITRs). Here we report the design of a genetic screen based on the yeast one-hybrid assay to identify cellular RRS-binding proteins. We show that the human zinc finger 5 protein (ZF5) binds specifically to RRS motifs *in vitro* and *in vivo*. ZF5 is a highly conserved and ubiquitously expressed transcription factor that contains five C-terminal zinc fingers and an N-terminal POZ domain. Ectopic expression of ZF5 leads to an ITR-dependent repression of the autologous p5 promoter and reduces both AAV2 replication and the production of recombinant AAV2. By using deletion and substitution mutants we show that two different domains of ZF5 contribute to AAV2 repression. Negative regulation of the p5 promoter requires the POZ domain, whereas viral replication is inhibited by the zinc finger domain, likely by competing with Rep for binding to the ITR. Identification and characterization of proteins that bind the ITR, the only viral genetic element retained in AAV2 vectors, will lead to new insights into the unique life cycle of AAV2 and will suggest improvements important for its application as a gene therapy vector.

Adeno-associated virus type 2 (AAV2) is a nonpathogenic human parvovirus that relies on a helper virus such as adenovirus for efficient replication (1). In the absence of helper functions, the AAV2 genome is integrated into the host chromosome to establish a latent infection (2, 3). In 68–94% of reported cases, integration is targeted to a specific locus on chromosome 19 termed *AAVS1* (4–7). The AAV2 genome is rescued from the proviral state by subsequent infection of the cell with a helper virus (2).

The large nonstructural proteins of AAV2, Rep78 and Rep68, are required for replication (8–10), targeted integration (11–13), and rescue from the latent state (14) by binding to a specific cis-acting sequence named the Rep recognition sequence (RRS). The RRS (also referred to as the RBE) consists of an imperfect GCTC repeating motif, which is found within the preintegration locus *AAVS1*, the inverted terminal repeats (ITRs), and the viral p5 promoter (11, 15, 16). The Rep78/68 proteins regulate their own expression by binding the RRS in the p5 promoter (16–18). They are involved also in regulation of the AAV2 p19 and p40 promoters (19, 20), repression of heterologous promoters, and inhibition of cell growth, transformation, and replication of other viruses (21–28).

Many RRS-like motifs have been identified in promoter-proximal regions throughout the human genome in a computer-assisted homology search (29). This finding suggests that cellular genes could be regulated by Rep or cellular RRS-binding proteins. Because AAV2 depends on cellular pathways to complete its life cycle, it is likely that cellular proteins regulate the virus by binding to RRS motifs. To identify such factors we designed a genetic screen based on the yeast one-hybrid system. We screened a human cDNA library and report the isolation of the zinc finger 5 protein (ZF5) as a factor interacting with the RRS. ZF5 is a ubiquitously expressed protein identified originally by its ability to bind and repress the murine c-*myc* promoter (30). The human ZF5 homologue is 99.3% identical to the murine protein (31) and contains five C-terminal zinc fingers and an N-terminal POZ domain. The POZ domain is a conserved protein–protein interface that recruits cofactors to modulate transcription (32, 33). ZF5 mediates both transcriptional activation and repression of cellular and viral promoters (30, 33, 34). We show that ectopic expression of ZF5 leads to an ITR-dependent repression of the AAV2 p5 promoter. Expression of ZF5 also reduces AAV2 replication and production of recombinant AAV2 (rAAV2), suggesting that endogenous ZF5 is a negative regulator of the AAV2 life cycle. Identification of additional ITR-binding proteins with our genetic screen and elucidation of ZF5 functions will lead to a better understanding of the unique life cycle of AAV2 including targeted integration, viral latency, and its applications for gene therapy.

Materials and Methods

Yeast One-Hybrid Screen. All yeast manipulations were performed as described in the manufacturer's user manuals (CLONTECH). The one-hybrid screen used yeast strains containing integrated marker genes under the control of a minimal yeast promoter and upstream RRS elements. A set of antiparallel oligonucleotides containing two copies of the RRS were cloned into the polylinker of plasmid pHISi-1 (CLONTECH). The resulting plasmid was linearized with *Nco*I and integrated into the mutant *his* locus of strain YM.RRS3.LacZ (35) to generate YM.RRS2.HIS/RRS3.LacZ. For the one-hybrid screen, YM.RRS2.HIS/RRS3.LacZ was transformed with 60 μg of a HeLa cell cDNA library in vector pGAD-GH (CLONTECH), allowing expression of the cDNA as a chimeric protein fused to the GAL4 activation domain. A total of 3×10^6 transformants were screened by selection on synthetic dropout (SD) medium minus uracil, leucine, and histidine (SD/−Ura,−Leu,−His) plates supplemented with 15 mM 3-amino-1,2,4-triazole (Sigma) to suppress leaky *HIS3* expression. After 7 days, large colonies were picked and patched on SD/−Ura,−Leu plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma). Plates were incubated for 3 days at 30°C and assessed for blue colonies. Expression plasmids were isolated from positive yeast clones, amplified in *Escherichia coli* (DH5α), and transformed into control strains YM.RRS3.LacZ (35) and YM.RRS0.LacZ, which were generated by integrating the empty pLacZi plasmid (CLONTECH) into strain YM4271 (CLONTECH). Transformants were patched onto X-gal plates, and hybrid proteins that induced blue staining in YM.RRS3.LacZ but not in YM.RRS0.LacZ were analyzed further. The library

Abbreviations: AAV2, adeno-associated virus type 2; RRS, Rep recognition sequence; ITR, inverted terminal repeat; ZF5, zinc finger 5 protein; rAAV2, recombinant AAV2; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ZF5∂3 and ZF5∂4, zinc fingers 3 and 4, respectively; EMSA, electrophoretic mobility-shift assay; LMW, low molecular weight; GFP, green fluorescent protein.

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inserts were subcloned in-frame with an upstream epitope tag (6× Myc) in expression vector pCS3+MT (courtesy of T. Hunter) by digestion with *Eco*RI and *Xho*I. Plasmid pCS3+MT contains the bacteriophage SP6 and cytomegalovirus (CMV) IE94 promoters, allowing expression *in vitro* and *in vivo*.

Plasmids. Sequence analysis revealed that clone A25 encodes a C-terminal fragment of transcription factor ZF5 (residues 308–449). The full-length ZF5 cDNA was amplified from a HeLa cDNA library by PCR and subcloned into pCS3+MT. Site-directed mutagenesis (QuikChange, Stratagene) at codon positions 334/335 (AGCTGT–ACTAGT) and 362/363 (GCGTGC–GCTAGC) led to cysteine-to-serine replacements in zinc fingers 3 (ZF5∂3) and 4 (ZF5∂4). Subcloning into vector pRK5 (35) generated plasmids pRK5.ZF5C, pRK5.ZF5, pRK5.ZF5∂3, and pRK5.ZF5∂4. Reporter plasmid pGL2.p5.Luc contains nucleotides 190–320 of the AAV2 genome cloned into pGL2-Basic (Promega). Plasmids pcDNA.Rep78, pcDNA.RepTZAD, pcDNA.RepTZ, pGL3.ITR/p5.Luc, pGL3.ITR/M1.Luc, pNTC244, and pAAV.GFP have been described (35–37).

***In Vitro* Translation and Electrophoretic Mobility-Shift Assays (EMSAs).** Library clones in vector pCS3+MT were *in vitro* translated in the absence or presence of Tran-^{35}S label (ICN) by using the SP6 TNT coupled reticulocyte lysate system (Promega). The ITR probe was prepared and the EMSA was performed as described (11, 35). The 32-bp-long double-stranded RRS oligonucleotide probe contains the RRS motif of the ITR. The core sequences for the wild-type and mutant probes are 5′-CT*GCGC*(*GCTC*)$_3$AC and 5′-CT-*CCGC*(*CCTC*)$_3$AC, respectively (RRS motifs are in italics). For supershift analysis, 1 μl of anti-Myc antibody (1:5 dilution, Invitrogen) was included, and in competition experiments a 1-, 5-, or 25-fold molar excess of unlabeled oligonucleotide substrate was added to the binding reaction.

Reporter Assays and Immunoblotting. 293T and HeLa cells were obtained from the American Type Culture Collection and grown in DMEM supplemented with 10% FCS (DMEM-10). Subconfluent monolayers in 35-mm wells were transfected in duplicate by calcium phosphate precipitation. Total DNA concentrations were maintained at 4 μg per 35-mm well, and the amount of cytomegalovirus promoter-containing plasmids was kept constant by adding empty vector DNA. To normalize for transfection efficiency, a plasmid expressing β-galactosidase (pCMVβ, CLONTECH) was included. Cells were harvested in reporter lysis buffer (Promega) for 26 (293T) or 28 h (HeLa) after transfection. Luciferase and β-galactosidase activities were measured in a luminometer (Bioscan, Washington, DC) by using BrightGlo (Promega) or GalctoLight (Tropix, Bedford, MA) substrate. Statistical significance was determined by *t* tests. For immunoblot analysis, 293T cells were harvested 30 h posttransfection and resuspended in lysis buffer (35). Equal amounts of proteins were separated by SDS/PAGE and transferred to Hybond-ECL membrane (Amersham Pharmacia). Immunoblotting was performed as described (35) with antibodies specific to Rep (MAb259.5, American Research Products, Belmont, MA) or Ad5-DNA-binding protein (MAb36–8, courtesy of A. J. Levine, Rockefeller University, New York).

AAV2 Replication and Vector Production Assays. 293T cells in 60-mm wells were transfected with 0.5 μg of pNTC244, pRK5.ZF5 as indicated and empty vector DNA to 8 μg. After 18 h, the cells were superinfected with Ad5 (100 particles per cell) and harvested 22 h later. The samples were either analyzed by immunoblotting or used to isolate low molecular weight (LMW) DNA using the QIAamp DNA mini kit (Qiagen, Chatsworth, CA). LMW DNA (2.5 μg) was digested with *Dpn*I and *Bgl*II, separated on a 1% agarose gel, blotted onto Hybond-N membrane (Amersham Pharmacia), and hybridized with an AAV2-specific DNA probe that was ^{32}P-labeled with RediPrime II (Amersham Pharmacia). Replication of wild-type AAV2 and rAAV2 was assessed by real-time PCR. 293T cells in 35-mm wells were transfected in duplicate with 1.5 μg of pXX6 and 1 μg of pRK5.ZF5 (or a ZF5 mutant) and either cotransfected with 10 ng of pAAV.GFP and 0.5 μg of pcDNA.Rep78 for rAAV or superinfected with wild-type AAV2 (300 particles per cell) 16 h later. For all transfections carrier DNA was added to a total of 4 μg, and cells were harvested 40 h posttransfection. For rAAV2, 1 ng of *Dpn*I-digested LMW DNA was used as a template with the green fluorescent protein (GFP)-specific primers 5′-ATGGCCGACAAGCAGAAGAA and 5′-GCTGCCGTC-CTCGATGTT that flank a *Dpn*I site. For wild-type AAV2, 10 ng of LMW DNA was used with the Rep-specific primers 5′-AGGACCAGGCCTCATACATCTC and 5′-TGTCCAAG-GCAGCCTTGATT. Amplicons were detected by using SYBR green reporter dye in an ABI Prism 7700 sequence detection system (Perkin–Elmer/Applied Biosystems).

For rAAV2 production assays, 293T cells in 35-mm wells were transfected in duplicate with 10 ng of pAAV.GFP, 1.5 μg of pXX6, 0.5 μg of pXX2, ZF5 expression plasmids as indicated, and empty vector DNA to 4 μg. Cells were harvested at the indicated times, resuspended in 100 μl of PBS, and subjected to three cycles of freeze/thaw. Clarified supernatants containing rAAV2 were used to infect 10^5 293T cells together with Ad5 (250 particles per cell) in 24-well plates. After 24 h the titer (transducing units) was determined by flow cytometry of GFP-positive cells in the presence of propidium iodide (10 μg/ml) to exclude dead cells.

Results

A Genetic Screen to Identify Cellular Proteins That Bind to the RRS *in Vivo*. We designed a genetic screen based on the yeast one-hybrid assay (38) to identify cellular proteins that bind the RRS *in vivo*. We recently generated chimeric Rep proteins fused to a transcriptional activation domain (35) that activate expression of an integrated *LacZ* reporter gene through binding to RRS motifs upstream of a minimal promoter in *Saccharomyces cerevisiae* strain YM.RRS3.LacZ (Fig. 1*A*). To screen for cellular RRS-binding proteins, an RRS-dependent *HIS3* expression cassette was integrated into the mutant *his* locus to generate reporter strain YM.RRS2.HIS/RRS3.LacZ. The strain was validated by using hybrid proteins RepTZ and RepTZAD (Fig. 1*B*). Both proteins contain the major DNA-binding motif of Rep fused to an oligomerization domain required for binding (35). RepTZAD contains an additional transcriptional activation domain. All transformants grew on nonselective plates (YPDA). Hybrid proteins that bind the RRS and activate the *HIS3* cassette allowed growth in the absence of histidine and activated the *LacZ* gene to give rise to blue colonies on X-gal plates (Fig. 1*C*).

The reporter strain was transformed with a human cDNA library fused to a transcriptional activation domain. A total of 3×10^6 transformants were screened by selection on plates lacking histidine. Activation of *LacZ* was tested on X-gal plates, and library plasmids were rescued from the 100 most positive clones. Strain YM.RRS3.LacZ contains three RRS elements upstream of a minimal promoter driving β-galactosidase expression (35) and was used to reconfirm positive interactions independent of *HIS3* growth selection. Strain YM.RRS0.LacZ does not contain an RRS motif and was used to exclude false positives.

The cDNAs isolated from the screen were subcloned into expression vector pCS3+MT. Proteins were translated *in vitro*, analyzed by SDS/PAGE and tested for their ability to bind a ^{32}P-labeled ITR probe by EMSA. Fig. 1*D* shows an example of 4 of 55 proteins analyzed. Clone A25 interacted with the ITR specifically and contained an ORF corresponding to residues 308–449 of the human ZF5. Three more clones interacted with

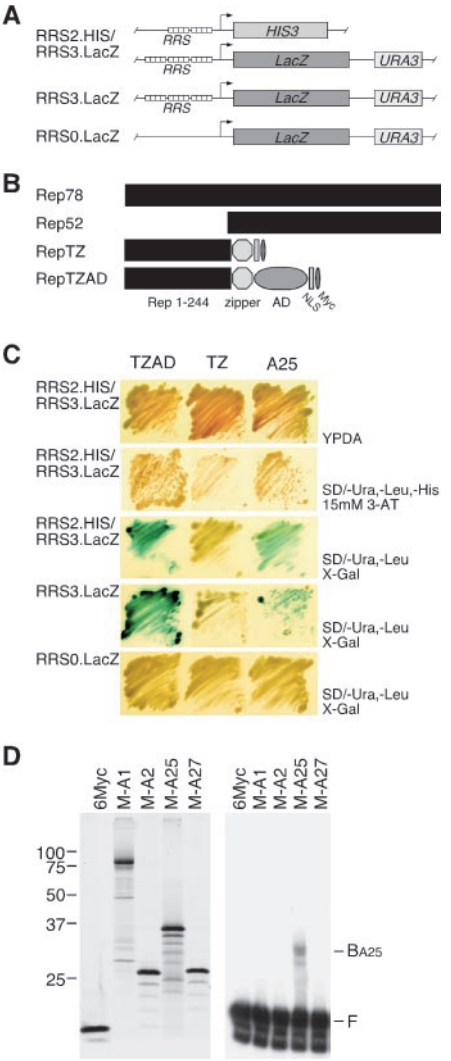

Fig. 1. A genetic screen identifies cellular proteins that bind the RRS motif. (*A*) Schematic of yeast strains. Reporter strain YM.RRS2.HIS/RRS3.LacZ contains integrated *HIS3* and *LacZ* reporter cassettes driven from minimal yeast promoters with two or three upstream tandem copies of the RRS. Control strains YM.RRS3.LacZ and YM.RRS0.LacZ contain a *LacZ* cassette with three upstream tandem copies or no RRS. (*B*) Schematic of wild-type and chimeric Rep proteins. Protein RepTZ comprises residues 1–244 of Rep, a modified leucine zipper (RepTZ), a nuclear localization signal (NLS), and a Myc epitope tag. RepTZAD additionally contains the transcriptional activation domain (AD) of VP16. (*C*) Yeast *in vivo* plate assays demonstrate RRS binding. Strains expressing RepTZAD or RepTZ served as positive and negative controls, and clone A25 was isolated in the one-hybrid screen. Transformed yeast cells were grown on nonselective medium (YPDA) and on selection medium [SD/−Ura,−Leu,−His, 15 mM 3-amino-1,2,4-triazole (3-AT)]. Interaction was confirmed on plates supplemented with X-gal to detect β-galactosidase activity. Specificity of the DNA-binding activity was confirmed in strains YM.RRS3.LacZ and YM.RRS0.LacZ. (*D*) EMSA identifies ITR-binding proteins. Positive clones were translated *in vitro* in the presence of [^{35}S]methionine and separated on a 12% SDS-polyacrylamide gel (*Left*). Size markers are indicated on the left. *In vitro* synthesized proteins were analyzed for binding to a ^{32}P-labeled ITR probe by EMSA (*Right*). The positions of free (F) and bound (B_{A25}) DNA substrate are indicated.

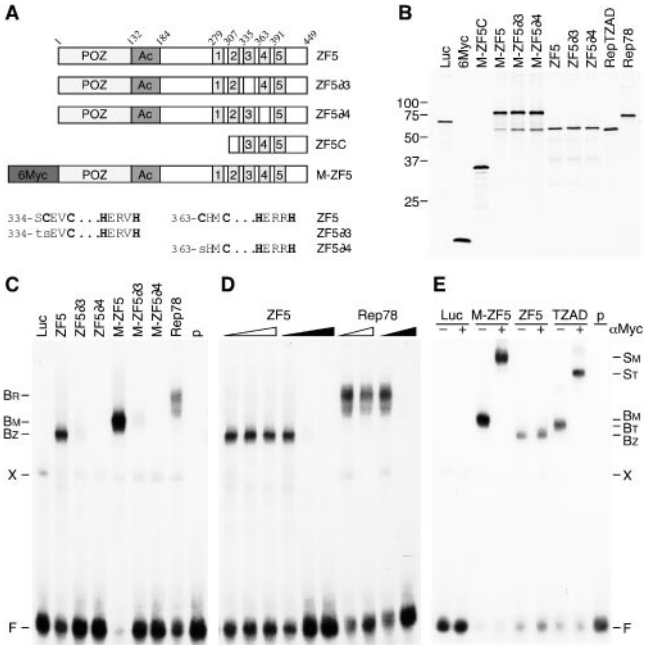

Fig. 2. ZF5 binding to the RRS *in vitro* is sequence-specific. (*A*) Schematic of wild-type and mutant ZF5 proteins. ZF5 consists of a POZ domain, a stretch of acidic residues (Ac), and five C_2H_2-type zinc fingers. Mutations in ZF5∂3 and ZF5∂4 are shown below. The C-terminal fragment of ZF5 isolated in the screen (ZF5C) contains residues 308–449. Proteins were tagged with an N-terminal Myc epitope (6Myc). (*B*) *In vitro* translation of ZF5 proteins. Proteins were synthesized in the presence of [^{35}S]methionine and separated on a 12% SDS polyacrylamide gel. Size markers are indicated on the left. (*C*) Mutations in zinc fingers 3 and 4 disrupt binding of ZF5 to the RRS. *In vitro* translated proteins were incubated with a ^{32}P-labeled RRS oligonucleotide probe, and DNA binding was analyzed by EMSA. (*D*) ZF5 binds specifically to the RRS motif. Increasing molar ratios (1, 5, 25× for ZF5, and 1, 25× for Rep78) of unlabeled DNA fragments containing the RRS (black triangles) or a mutant RRS (open triangles) were added as competitors. (*E*) The tagged ZF5-DNA complex can be supershifted by a Myc-specific antibody. The presence (+) or absence (−) of antibody is indicated above. Rep78, RepTZAD, and luciferase (Luc) were included as controls. The positions of free (F), bound (B), and supershifted (S) DNA substrate are indicated. Shifted complexes are shown for ZF5 (B_Z), M-ZF5 (B_M and S_M), Rep78 (B_R), and RepTZAD (B_T and S_T). X, indicates a nonspecific band; p, lanes that contain probe alone.

the ITR and corresponded to the same uncharacterized gene (data not shown).

Human ZF5 Binds to RRS Motifs *in Vitro*. A full-length cDNA of ZF5 was prepared by PCR, and zinc finger mutants were generated by site-directed mutagenesis (Fig. 2*A*). Because zinc finger 3 or 4 was suggested to be critical for DNA binding (39), the first cysteine of the respective zinc fingers was changed to a serine (C335S and C363S), giving rise to ZF5∂3 and ZF5∂4. Proteins were translated in the presence of [^{35}S]methionine, and expression was confirmed by SDS/PAGE (Fig. 2*B*).

Binding of proteins was assessed *in vitro* by EMSA using a ^{32}P-labeled oligonucleotide probe containing a single RRS motif (35). Whereas wild-type ZF5 shifted a substantial amount of the RRS probe (B_Z), ZF∂3 bound weakly, and no shift was detected for ZF5∂4 (Fig. 2*C*). These results were reproduced with epitope-tagged proteins. The specificity of DNA binding was shown by competition with increasing amounts of unlabeled probe containing either a wild-type or mutant RRS motif. Binding of ZF5C (not shown), ZF5, and Rep78 to the RRS probe was blocked by excess amounts of wild-type RRS but not by mutant competitor (Fig. 2*D*). Epitope-tagged protein·DNA complexes were supershifted by an antibody (Fig. 2*E*). The *in vitro* analysis demonstrates that ZF5 binds to the RRS in a sequence-specific manner and that zinc finger 4 is crucial for DNA binding.

ZF5 Represses the AAV2 p5 Promoter in an ITR-Dependent Manner. The large Rep proteins repress the AAV2 p5 promoter by binding to RRS elements within the ITR and p5 (17, 40). To

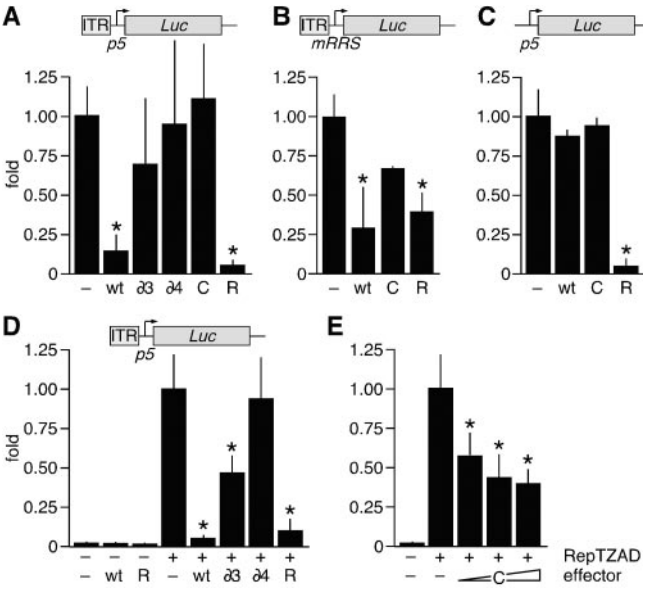

Fig. 3. ZF5 is a repressor of the AAV2 p5 promoter. (*A–C*) ZF5 represses the AAV2 p5 promoter through binding to the ITR. 293T cells were transfected with reporter plasmids pGL3.ITR/p5.Luc (*A*), pGL3.ITR/M1.Luc (*B*), or pGL2.p5.Luc (*C*) as shown schematically above. Cells were cotransfected with expression plasmids for ZF5, ZF5∂3, ZF5∂4, ZF5C, or Rep78. (*D* and *E*) ZF5 competes with Rep for binding to the ITR. HeLa cells were transfected with reporter plasmid pGL3.ITR/p5.Luc, plasmid pcDNA.RepTZAD where indicated (+) and either pRK5.ZF5, pRK5.ZF5∂3, pRK5.ZF5∂4, and pcDNA.Rep78 (*D*) or increasing amounts (0.5, 1, and 2 μg) of pRK5.ZF5C (*E*). Luciferase activity was normalized for transfection efficiency and is shown relative to transfection with empty vector (−). The graphs reflect average value and standard deviation of at least two experiments performed in duplicate. *, statistical significance ($P < 0.01$); R, Rep78; wt, ZF5; ∂3, ZF5∂3; ∂4, ZF5∂4; C, ZF5C.

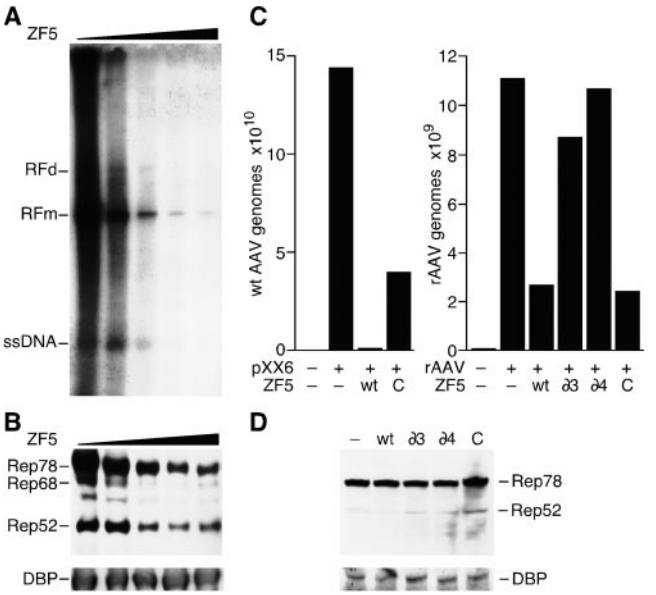

Fig. 4. ZF5 inhibits AAV2 replication. (*A* and *B*) ZF5 inhibits replication of wild-type AAV2. 293T cells in 60-mm wells were transfected with increasing amounts of pRK5.ZF5 (0, 1, 2, 4, and 6 μg) and 0.5 μg of an infectious AAV2 clone (pNTC244) and subsequently infected with Ad5. Cell pellets were processed to analyze either AAV2 replication (*A*) or protein expression (*B*). Southern blot analysis of LMW DNA was performed with a ^{32}P-labeled AAV2-specific probe. The positions of single-stranded AAV2 genome (ssDNA) and monomeric (RFm) and dimeric (RFd) replicative forms are indicated. Protein expression was assessed by immunoblotting for Rep or Ad5-DNA-binding protein (DBP). (*C* and *D*) ZF5 inhibits AAV2 replication. 293T cells were transfected with pXX6,ZF5 expression vectors as indicated and either superinfected with AAV2 (*C*, *Left*) or cotransfected with pAAV.GFP and pcDNA.Rep78 (*C*, *Right*). After 40 h the cells were harvested, and the viral replication was quantified by real-time PCR with Rep or GFP-specific primers. The columns reflect the average value of a representative experiment performed in duplicate. Protein expression was analyzed by immunoblotting for Rep or Ad5-DBP (*D*). wt, ZF5; ∂3, ZF5∂3; ∂4, ZF5∂4; C, ZF5C.

analyze whether ZF5 modulates transcription from the p5 promoter, cells were transfected with an ITR/p5 reporter plasmid (35) and ZF5 expression plasmids (Fig. 3*A*). Promoter activity of the ITR/p5 construct in 293T cells is high because of transactivation by the adenoviral E1A proteins (41). Ectopic expression of wild-type ZF5 reduced reporter gene expression significantly (7-fold), whereas the DNA-binding mutants ZF5∂3 and ZF5∂4 had little effect. The C-terminal ZF5 fragment ZF5C lacks the POZ domain and did not repress p5 transcription. To differentiate between ITR or p5-mediated repression, we used an ITR/p5 construct harboring a mutation in the p5-RRS (Fig. 3*B*) or a reporter with only the p5 promoter (Fig. 3*C*). Overexpression of ZF5 repressed the mutant ITR/p5 reporter but not the p5 alone, indicating that the p5-RRS is not involved in ZF5-mediated repression. Rep78 repressed all reporter constructs, and ZF5C had no significant repressive activity. Equal expression levels of Myc-tagged effector proteins was confirmed by immunoblotting (data not shown). These results indicate that ZF5 represses the AAV2 p5 promoter in an ITR-dependent manner that requires DNA binding and the POZ domain.

ZF5 Competes with Rep for Binding to the ITR. Next we analyzed competition between ZF5 and Rep78 for binding to the ITR in a reporter assay. Because both Rep78 and ZF5 repress the ITR/p5 promoter, we used the chimeric transactivator RepTZAD, which has a similar affinity for RRS motifs as the full-length protein (35). Transfection of the ITR/p5 reporter plasmid with a RepTZAD expression plasmid resulted in an ≈40-fold activation of the promoter in HeLa cells. Coexpression of ZF5 reduced RepTZAD-mediated activation significantly (19-fold), whereas ZF5∂3 and ZF5∂4 had less effect (Fig. 3*D*). Increasing amounts of a ZF5C also reduced activation by RepTZAD significantly and in a dose-dependent manner (Fig. 3*E*). Because ZF5C lacks the POZ domain required for transcriptional repression, a reduced activation by RepTZAD suggests direct competition for binding to the RRS. These results imply that Rep78 and ZF5 compete for binding to the ITR *in vivo*.

ZF5 Inhibits DNA Replication of AAV2. To ascertain a role for ZF5 in the AAV2 life cycle, we analyzed its effect on viral replication. A plasmid containing the AAV2 genome was transfected with increasing amounts of pRK5.ZF5, and cells were infected with Ad5 to initiate AAV2 replication. LMW DNA was extracted from cells and analyzed for AAV2 replication by Southern blot (Fig. 4*A*). ZF5 decreased AAV2 replication in a dose-dependent manner. We also observed decreased levels of the viral Rep proteins (Fig. 4*B*), consistent with ZF5-mediated repression of the p5 promoter. Helper functions provided by Ad5 were not affected by exogenous ZF5, as shown by immunoblot analysis for the adenoviral single-stranded DNA-binding protein (Fig. 4*B*). This suggests that ZF5-mediated repression of DNA replication is specific to AAV2.

These results were confirmed by using wild-type AAV2-infected cells that had been transfected with plasmids encoding Ad5 helper functions and ZF5. Quantification by real-time PCR indicated that expression of ZF5 reduced viral replication more than 400-fold, whereas ZF5C expression led to a 4-fold decrease

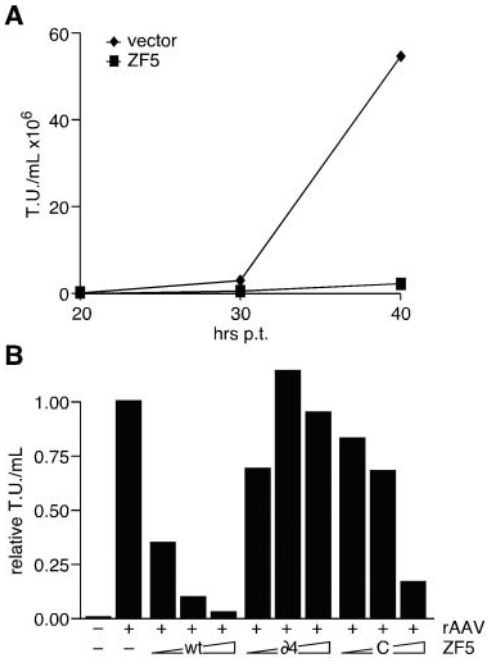

Fig. 5. ZF5 inhibits AAV2 vector production. (*A*) 293T cells in 35-mm wells were transfected with pAAV.GFP, pXX2, pXX6, and empty vector or pRK5.ZF5. Virus was harvested at 20, 30, and 40 h posttransfection, and vector titers were determined. (*B*) Cells were transfected as described for *A* with increasing amounts of pRK5.ZF5, pRK5.ZF5∂4, or pRK5.ZF5C (0.1, 0.3, and 1.0 μg). Viral vectors were harvested after 40 h and quantified. The titer is shown relative to transfection with empty vector. The graphs reflect the average value of a representative experiment performed in duplicate. −, empty vector; wt, ZF5; ∂4, ZF5∂4; C, ZF5C; T.U., transducing units.

(Fig. 4*C*, *Left*). Replication of AAV2 strictly depended on the presence of Ad5 helper functions. Because ZF5 represses the p5 promoter, inhibition of AAV2 replication could be solely due to reduced Rep levels. To assess a direct effect of ZF5 on viral replication, Rep78 was supplied *in trans*, and replication of a recombinant AAV2 genome (rAAV2.GFP) was quantified. Ectopic expression of ZF5 reduced replication of rAAV2.GFP by a factor of 4, whereas the DNA binding-deficient mutants ZF5∂3 and ZF5∂4 had little effect (Fig. 4*C*, *Right*). Conversely, expression of ZF5C decreased DNA replication to the same extent as full-length ZF5. Replication of rAAV2.GFP strictly depended on the presence of Rep and the ITRs (data not shown). A control immunoblot revealed that Rep expression and Ad5 helper functions were not affected by ZF5 expression (Fig. 4*D*). These results show that ZF5 has two effects on AAV2 replication. First it reduces Rep levels by repressing the p5 promoter, a function that requires DNA binding and the POZ domain. Second, ZF5 inhibits AAV2 replication in a POZ-independent fashion, likely by competing with Rep for binding to the viral ITR.

ZF5 Reduces AAV2 Vector Production. The effect of ZF5 on AAV2 vector production was analyzed in 293T cells. Cells were harvested at different times after transfection, and vector titers were determined. Ectopic expression of ZF5 expression reduced rAAV2 production significantly (Fig. 5*A*). Control experiments indicated that ZF5 inhibited neither the transduction by rAAV2 nor the replication of a recombinant Ad5 vector, confirming that Ad5 helper functions were not affected (data not shown). Production of rAAV2 was assessed also in the presence of increasing amounts of ZF5, ZF5∂4, and ZF5C (Fig. 5*B*). Both ZF5 and ZF5C reduced production of rAAV2.GFP in a dose-dependent manner (36- and 6-fold, respectively, at highest effector concentration), but the DNA-binding mutant ZF5∂4 did not. These results indicate that ectopic expression of ZF5 negatively regulates the AAV2 life cycle.

Discussion

In this paper, we report a genetic screen to identify cellular proteins that bind the RRS. We isolated the human ZF5 protein and show that it binds to RRS motifs *in vitro* and *in vivo*. Ectopic expression of ZF5 leads to repression of the viral p5 promoter and inhibition of AAV2 replication.

Activation of the AAV2 p5 promoter, which drives expression of the large Rep78/68 proteins, is a crucial step in the AAV2 life cycle. In the absence of helper functions, the p5 promoter is repressed by cellular YY1 (17, 18, 42) and the Rep proteins (40, 43). YY1-mediated repression is relieved after adenovirus infection (42). We have shown that ZF5 is another cellular protein that represses the p5 promoter. Unlike YY1, ZF5 regulates p5 transcription by binding the viral ITR. As observed by others (33), transcriptional repression depends on the POZ domain, a protein motif demonstrated to recruit corepressor proteins (32). ZF5 binding to the ITR also interferes with the binding of Rep proteins. Thus, ZF5 regulates the AAV2 life cycle on at least two levels: viral transcription and DNA replication. Although transcriptional repression requires the POZ domain, AAV2 replication is reduced by expressing the DNA-binding domain alone. The presence of the POZ domain further reduces vector titers, suggesting that it might recruit additional inhibitory factors. Whether ZF5 interferes with AAV2 packaging remains to be examined.

RRS-like sequences have been found in promoter-proximal regions of the human genome (29), suggesting that cellular genes may be regulated by Rep or RRS-binding proteins. Interestingly both Rep and ZF5 repress the c-*myc* promoter (30, 44). Rep may regulate other genes by binding to RRS motifs, possibly contributing to the inhibitory effects of Rep on cell growth and transformation.

It is interesting to speculate that endogenous ZF5 is a factor involved in repressing autonomous AAV2 replication and maintaining viral latency. Rescue of AAV2 infectivity from plasmid DNA has been viewed as a model system for reactivation of integrated virus (1). Ectopic expression of ZF5 prevented formation of progeny virus from plasmid DNA even in the presence of helper functions. Thus it is conceivable that in the absence of helper virus, endogenous ZF5 maintains AAV2 in the latent state by binding to the ITR. Because Rep expression has detrimental effects on the cell (22, 45, 46), it might be advantageous for the virus to use a cellular protein to maintain latency. It remains to be determined how ZF5-mediated repression is relieved after infection with a helper virus. The relief of ZF5's endogenous repression of AAV2 replication by dominant ZF5 mutants or antisense techniques also might present ways to enhance production of rAAV2 vectors for gene therapy.

The only viral genetic element retained in rAAV2 vectors is the ITR. This structure is required for replication, packaging, and integration of the virus and thus it is important to understand its interaction with cellular factors. Binding of cellular proteins to the ITR has been suggested to modulate transduction efficiency and expression from rAAV2 vectors (47–49). Our yeast system provides a powerful tool to identify cellular proteins that bind the ITR *in vivo* and regulate the AAV2 life cycle. In view of the increasing popularity of rAAV2 as a vehicle for gene transfer, it is crucial to discern interactions of the AAV2 genome with cellular pathways. This knowledge will lead to improvements in the efficacy of both production and transduction of rAAV2 vectors.

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