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The Rep78 Gene Product of Adeno-Associated Virus (AAV) Self-Associates To Form a Hexameric Complex in the Presence of AAV *ori* Sequences

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The Rep78 and Rep68 proteins of adeno-associated virus (AAV) are replication initiator proteins that bind the viral replicative-form origin of replication, nick the origin in a site- and strand-specific fashion, and mediate vectorial unwinding of the DNA duplex via an ATP-dependent helicase activity, thus initiating a strand displacement mechanism of viral DNA replication. Genetic and biochemical studies have identified Rep mutants that demonstrate a *trans*-dominant negative phenotype in vitro and in vivo, suggesting the possibility that multimerization of Rep is essential for certain replicative functions. In this study, we have investigated the ability of the largest of the Rep proteins, Rep78, to self-associate in vitro and in vivo. Self-association of Rep78 in vivo was demonstrated through the use of a mammalian two-hybrid system. Rep-Rep protein interaction was confirmed in vitro through coimmunoprecipitation experiments with a bacterially expressed maltose-binding protein–Rep78 fusion protein in combination with [^{35}S]methionine-labeled Rep78 synthesized in a coupled in vitro transcription-translation system. Mapping studies with N- and C-terminal truncation mutant forms of Rep indicate that amino acid sequences required for maximal self-association occur between residues 164 and 484. Site-directed mutagenesis identified two essential motifs within this 321-amino-acid region: (i) a putative α-helix bearing a 3,4-hydrophobic heptad repeat reminiscent of those found in coiled-coil domains and (ii) a previously recognized nucleoside triphosphate-binding motif. Deletion of either of these regions from the full-length polypeptide resulted in severe impairment of Rep-Rep interaction. In addition, gel filtration chromatography and protein cross-linking experiments indicated that Rep78 forms a hexameric complex in the presence of AAV *ori* sequences.

The parvoviruses are small DNA viruses that infect a wide variety of metazoan species, ranging from insects to humans (3, 17). Adeno-associated virus type 2 (AAV-2), a parvovirus of humans, is distinct from the replication-competent (or autonomous) parvoviruses in that it requires "helper" functions supplied by a coinfecting virus (typically, adeno- or herpesvirus) for efficient, productive replication (4, 5, 67). In the absence of helper virus, AAV-2 establishes a latent infection via integration into a preferred site on the long arm of chromosome 19 (6, 14, 22, 40, 41, 49, 69, 84). It has been estimated that greater than 80% of the adult U.S. population is seropositive for AAV infection; however, in contrast to human parvovirus B19, AAV has not been associated with the etiology of any human disease (4, 28, 70). Lack of pathogenicity, a preference for site-specific integration into a benign genomic locus, and the ability to infect a wide variety of both nondividing and dividing cell types (including human CD34$^+$ hematopoietic progenitor cells) have made AAV an attractive vector for human gene therapy applications (12, 21, 23, 37, 39, 56, 57, 64, 79).

The AAV-2 genome is a single-stranded, linear DNA molecule of approximately 4.7 kb (4, 74). An inverted terminal repeat of 145 nucleotides occurs at each end of the genome. The palindromic inverted terminal repeat forms a double-stranded hairpin structure that serves as the viral origin of replication (5, 30, 80). The AAV genome contains two major open reading frames (ORFs), ORF1 and ORF2 (74). ORF1, termed the *rep* gene, encodes a family of four antigenically related nonstructural proteins (known as Rep78, Rep68, Rep52, and Rep40) via a combination of differential splicing and alternative promoter utilization (48, 52, 65). ORF2, the *cap* gene, encodes the viral capsid proteins (VP1, -2, and -3) (2, 68). Genetic studies indicate that the *rep* gene is essential for viral DNA replication (10). In vitro reconstitution experiments have shown that either of the two largest *rep* gene products, Rep78 or Rep68, can mediate viral DNA replication (59, 75, 80, 81). Rep52 and Rep40 are dispensable for replication both in vitro and in vivo (9, 29, 59). Rep78 and Rep68 possess a number of enzymatic activities associated with DNA replication: these include a site- and strand-specific endonuclease activity which results in covalent attachment of Rep to the 5′ end of the cleaved DNA (34, 72, 73), an ATPase activity (85), and an ATP-dependent helicase activity (34, 85). In addition, Rep78 and Rep68 are sequence-specific DNA-binding proteins that recognize cognate binding sites within the AAV origin of replication (11, 13, 15, 33, 54, 55, 62). Thus, similarly to the bacteriophage ϕX174 gene A protein (27, 76), Rep78 and Rep68 serve as replication initiator proteins that bind the viral replicative-form origin of replication and nick the DNA duplex in a site- and strand-specific fashion, thus providing a free 3′-hydroxyl group with which to prime viral DNA synthesis. The observation of a *trans*-dominant negative phenotype for certain *rep* mutations (10, 11, 43, 62, 85) suggests that the Rep proteins interact to form multimers that are critical to Rep function.

In this study, we have investigated the ability of the largest of the Rep proteins, Rep78, to self-associate both in vivo and in vitro. Rep-Rep protein interaction could be demonstrated in vivo by using a mammalian two-hybrid system. In addition, immunoprecipitation experiments with a bacterially expressed maltose-binding protein (MBP)–Rep78 fusion protein in com-

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bination with various in vitro-translated, radiolabeled Rep polypeptides mapped amino acid residues required for Rep self-association to a 321-amino-acid (aa) region containing a nucleoside triphosphate (NTP)-binding motif and a short, coiled-coil-like domain that are essential for maximal levels of Rep-Rep interaction. Moreover, gel filtration chromatography and protein cross-linking experiments indicate that Rep78 multimerizes in the presence of AAV *ori* sequences to form a hexameric complex.

MATERIALS AND METHODS

Cell culture. CV-1-5GT cells (kind gift of Walter Schaffner, University of Zurich, Zurich, Switzerland) are a CV-1-derived monkey kidney cell line stably transfected with a plasmid containing the simian virus 40 (SV40) large T antigen gene under the control of a GAL4-inducible promoter (26). CV-1-5GT cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g of glucose per liter, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum.

Plasmids. Plasmid pT7/hPCNA (gift of Bruce Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) contains a human proliferating cell nuclear antigen (PCNA) cDNA under the transcriptional control of a bacteriophage T7 promoter. Plasmids pBXG1, pBXGVP, and pECE72 have been described previously (71). Plasmid pBXG1 encodes the 147-aa GAL4 DNA-binding domain (DBD) under the transcriptional control of the SV40 early promoter and enhancer. Plasmid pBXGVP encodes the GAL4 DBD fused to the 78-aa acidic transcriptional activation domain (AD; aa 413 to 490) of the herpes simplex virus tegument protein VP16. Plasmid pECE72, a pECE-derived expression vector (19), contains the SV40 early promoter and enhancer, as well as SV40-derived polyadenylation signals, in a pSP72 background (Promega Corp., Madison, Wis.). To generate pSR207, a 1.9-kb Rep78 expression cassette containing a methionine-initiated ORF encoding Rep78 aa 3 to 621 flanked by *Bgl*II and *Xba*I sites (10a) was ligated to a 2.7-kb *Bgl*II-*Xba*I fragment containing pUC19 sequences derived from pCAT-Control (Promega). Plasmid pSR208 was generated by cloning the 1.9-kb *Bgl*II-*Xba*I fragment of pSR207 into the *Bam*HI-*Xba*I sites of pBXG1, thus creating sequences encoding a GAL4 DBD-Rep78 fusion protein under the transcriptional control of the SV40 early promoter and enhancer. To generate plasmid pGAL4-Rep, the SV40 promoter-enhancer and the partial vector backbone of pSR208 were excised as a 1.2-kb *Sca*I-*Bgl*II fragment and replaced with the 1.8-kb *Sca*I-*Bgl*II fragment of pHSV-106 (GIBCO BRL Life Technologies, Inc., Gaithersburg, Md.), thus placing the GAL4-Rep78 transcription unit of pSR208 under the transcriptional control of the pHSV-106-derived herpes simplex virus type 1 thymidine kinase (*tk*) promoter. Plasmid pRep was constructed by ligating the 1.9-kb *Bgl*II-*Xba*I fragment of pSR207 to the 3.0-kb *Bgl*II-*Xba*I fragment of pECE72. Plasmid pVP16-AD contains a 224-bp *Eco*RI-*Rsa*I fragment (derived from pBXGVP) encoding VP16 aa 413 to 487 cloned in frame with sequences encoding an amino-terminal peptide bearing the SV40 nuclear localization signal (provided by a 74-bp *Hin*dIII-*Kpn*I fragment derived from pGAD424 [Clontech Laboratories, Inc., Palo Alto, Calif.]) in a pECE72 background. To generate plasmid pVP16-Rep, the 1.9-kb *Bgl*II-*Xba*I fragment of pSR207 was cloned in frame with the VP16 AD of *Bam*HI-*Xba*I-digested pVP16-AD.

In vitro expression plasmids expressing carboxyl-terminal truncations of Rep78 were constructed in pGEM-3Z (Promega). Rep polypeptides expressed from these constructs utilize vector-encoded stop codons and, therefore, contain from 1 to 25 vector-encoded C-terminal aa residues, depending on the construct. Plasmid pRep(1-243) was constructed as follows. Nucleotides 319 to 1056 of AAV were PCR amplified from the cloned AAV genome within plasmid pAV2 (47) by using the forward and reverse primers 5′-GGA**AGATCT**ACCATGCCG GGGTTTTACGAG-3′ and 5′-CCTCCT**GGATCC**ACTGCTTCTCCGAGG-3′, respectively. The forward primer encodes a flanking *Bgl*II restriction site, while the reverse primer contains an AAV-encoded *Bam*HI site (both in boldface). The *Bgl*II-*Bam*HI-digested PCR product was cloned into the unique *Bam*HI site of pGEM-3Z (Promega), thus placing sequences encoding Rep aa 1 to 243 under the control of the pGEM-3Z-encoded bacteriophage T7 promoter. The orientation and integrity of the clone were confirmed by DNA sequencing. To construct plasmid pRep(1-621), the 0.9-kb *Nde*I-*Bam*HI fragment of pRep(1-243) was ligated to the 3.7-kb *Nde*I-*Bam*HI fragment of pSR342. Plasmid pSR342 contains the 1.9-kb *Bgl*II-*Xba*I fragment of pSR207 in a pGEM-3Z background. Plasmid pRep(1-548) was derived from pRep(1-621) by deletion of a 0.2-kb *Pst*I fragment encoding aa 549 to 621 of Rep78. To generate plasmid pRep(1-484), the 1.5-kb *Eco*RI fragment of pRep(1-621) was cloned into the unique *Eco*RI site of pGEM-3Z. To construct pRep(1-371), pRep(1-621) was digested with *Sal*I and religated. Plasmid pRep(1-322) was constructed by cloning the 1.8-kb *Xmn*I fragment of pRep(1-621) into the *Xmn*I and *Hinc*II sites of pGEM-3Z. Plasmids expressing amino-terminal truncations of Rep78 were constructed in the pET-21(+) series of vectors (Novagen Inc., Madison, Wis.). Rep polypeptides expressed from these constructs bear a vector-encoded 11-aa leader peptide derived from the phage T7 major capsid protein (gene 10) plus an additional 2 to 13 polylinker-encoded residues. Plasmid pRep(164-621) was constructed by cloning the 1.4-kb *Sst*I-*Ava*I fragment of pSR207 into the *Sst*I and *Ava*I sites of pET-21a(+). Plasmid pRep(243-621) contains the 1.1-kb *Bam*HI-*Xho*I fragment of pSR207 cloned into the *Bam*HI and *Xho*I sites of pET-21c(+). To construct pRep(370-621), the 0.8-kb *Sal*I fragment of pSR207 was cloned into the *Sal*I site of pET-21b(+) and checked for orientation. To generate pRep(390-621), the 0.7-kb *Eae*I-*Xho*I fragment of pSR342 was cloned into the *Not*I and *Xho*I sites of pET-21b(+). Plasmid pRep(522-621) was produced by cloning the 0.3-kb *Hin*dIII-*Xho*I fragment of pSR207 between the *Hin*dIII and *Xho*I sites of pET-21a(+). Site-directed mutants are described below.

DNA transfection and mammalian two-hybrid assay. CV-1-5GT cells were seeded at a density of 5×10^5 cells per 60-mm-diameter tissue culture dish and allowed to attach overnight at 37°C in a humidified 5% CO_2 atmosphere. The next day, the cells were transfected with 1.4 pmol of a "bait" plasmid (vector 1; either the GAL4 DBD or GAL4-REP) and 0.4 pmol of an "effector" plasmid (vector 2; either the VP16 AD, REP, or VP16-REP) per dish by using LipofectAMINE Reagent (GIBCO BRL). Briefly, the cell monolayers were rinsed twice with serum-free DMEM and then covered with 3 ml of serum-free DMEM per 60-mm dish. LipofectAMINE Reagent was diluted in sterile water to a concentration of 1.2 mg/ml and then mixed in a 1:1 (vol/vol) ratio with the appropriate diluted transfecting plasmid DNA for a total volume of 100 μl of transfecting solution per 60-mm-diameter dish. After a 15-min incubation at room temperature, the DNA-lipid complexes were added to the appropriate dish (containing 3 ml of serum-free DMEM) and incubated for 5 h at 37°C in a CO_2 incubator. The medium was then replaced with DMEM containing 10% fetal bovine serum, and the dishes were incubated for an additional 60 to 62 h.

The cell monolayers were harvested by an adaption of the alkaline-sodium dodecyl sulfate (SDS) lysis method of Birnboim and Doly (7). Alkaline-SDS buffers were obtained from QIAGEN Inc. (Chatsworth, Calif.). Cell lysis buffer was prepared by mixing 5 ml of buffer P1 (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 100 μg of RNase A per ml) with 10 ml of buffer P2 (200 mM NaOH, 1% SDS). The cell monolayers were rinsed twice with ice-cold phosphate-buffered saline (PBS), and 300 μl of cell lysis buffer was added to each plate. The plates were incubated at room temperature for 2 to 3 min, after which time 150 μl of buffer P3 (3.0 M potassium acetate, pH 5.5) was added. The contents of each plate were transferred to separate microcentrifuge tubes and centrifuged for 5 min. The respective supernatants were transferred to new tubes, extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and then precipitated with an equal volume of isopropanol at −20°C for 30 min. Following centrifugation, the DNA pellets were rinsed with 70% ethanol, dried under vacuum, and dissolved in 30 μl of sterile water per sample. One-third of each sample was digested overnight with 50 U of *Dpn*I (New England Biolabs, Beverly, Mass.), extracted with phenol-chloroform-isoamyl alcohol, and then precipitated with ethanol. The *Dpn*I-resistant DNA was used to transform *Escherichia coli* MAX Efficiency DH5α competent cells (GIBCO BRL) in accordance with the supplier's instructions. The transformed cells were spread onto 15-cm-diameter Luria-Bertani agar plates containing 50 μg of ampicillin per ml. Antibiotic-resistant colonies were counted after overnight incubation at 37°C.

In vitro translation. In vitro-translated Rep polypeptides were synthesized from plasmid DNA constructs by using the TNT T7 coupled reticulocyte lysate system (Promega Corp.) as recommended by the manufacturer. Protein synthesis and radiolabeling were performed with 1 μg of plasmid DNA for 1 h at 30°C in the presence of 40 μCi of L-[^{35}S]methionine (>1,000 Ci/mmol; Amersham Life Science Inc., Arlington Heights, Ill.).

Expression and purification of recombinant MBP-Rep78. MBP-Rep78 was expressed in *E. coli* DH5α and purified by amylose affinity chromatography as described previously (11, 13).

In vitro binding assay. Prior to the in vitro binding assays, recombinant protein G-agarose beads (GIBCO BRL) were washed and resuspended in binding buffer (5.5 mM Na_2HPO_4, 1.2 mM KH_2PO_4 [pH 7.4], 154 mM NaCl, 0.5% Nonidet P-40, 1 mg of delipidized bovine serum albumin per ml) to form a 50% slurry. Ten microliters of each in vitro translation reaction mixture was diluted 25-fold with binding buffer and incubated on ice with 20 μl of the protein G-agarose bead slurry for 5 min. Following brief centrifugation, the cleared lysate was transferred to a new tube and incubated with either 1 μg of purified MBP-Rep78 or 1 μg of MBP-LacZα for 30 min at 4°C (all subsequent incubations were also performed at 4°C). Two microliters of rabbit polyclonal anti-MBP serum (New England Biolabs) was then added. Following a 30-min incubation, 40 μl of the protein G-agarose bead slurry was added, and the binding reaction mixture was incubated for an additional 30 min. The agarose beads were washed three times in binding buffer, and then the bound polypeptides were eluted from the beads by heating to 90°C for 10 min in the presence of 1× sample buffer (2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.003% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8). Eluted proteins were loaded directly onto SDS-polyacrylamide gels and electrophoresed by using the Laemmli buffer system (44). Following electrophoresis, the gels were fixed, soaked in autoradiography enhancer (Enlightning; New England Nuclear Corp., Boston, Mass.), dried, and visualized by fluorography.

Site-directed mutagenesis. Plasmid-based, oligonucleotide-directed mutagenesis was performed by using the MORF Site-Specific Plasmid DNA Mutagenesis Kit (5 Prime→3 Prime, Inc., Boulder, Colo.) and the protocol supplied by the manufacturer. Mutagenic oligonucleotides were synthesized with a 392 DNA/RNA Synthesizer (Applied Biosystems Inc., Foster City, Calif.) and purified by using an EasyPrep Oligo Prep Kit (Pharmacia, Uppsala, Sweden). The muta-

genic oligonucleotides and the resulting Rep78 mutant proteins, respectively, were as follows: 5′-CCCAGCCTGAGGGCCAGTGGGCGTGG-3′ and L165G, 5′-GCGCCTGTACGAATCTCACGGA-3′ and L180T, 5′-CAACTACCGGGCATACCAACATCG-3′ and K340H, 5′-TGGTGGATGAG^GTGGCGCAGC-3′ and d151-188, and 5′-CCATCTGGCTGTTT^GCCCACACTGTG-3′ and d334-347. Mismatched bases are underlined. In the deletion mutant oligonucleotides, the junctions of the flanking nucleotide sequences are indicated by carets. All mutations were in a pREP(1-621)-derived background and were confirmed by DNA sequencing.

Gel filtration chromatography. Gel filtration chromatography was used to estimate the molecular mass and Stokes radius of multimeric Rep78. Chromatography was performed at 4°C by using a Pharmacia fast protein liquid chromatography system equipped with a prepacked Pharmacia HR 10/30 Superose 6 column (protein separation range, 5×10^3 to 5×10^6 Da). The Superose 6 column was equilibrated with 100 mM ammonium bicarbonate buffer (pH 8.0) and calibrated with the following protein standards: thyroglobulin (molecular mass 669 kDa; Stokes radius [R_s], 85 Å), ferritin (mass, 440 kDa; R_s, 61 Å), catalase (mass, 232 kDa; R_s, 52 Å), bovine serum albumin (mass, 67 kDa; R_s, 35.5 Å), and carbonic anhydrase (mass, 29 kDa; R_s, 25 Å). Native hemoglobin within the applied in vitro translation system reticulocyte lysate served as an internal standard (mass, 65 kDa; R_s, 32 Å). By using the mathematical relationship described by Finger and Richardson (20), the Stokes radius of multimeric Rep78 was estimated from a plot of the elution volume as a function of the Stokes radii of the reference proteins described above.

[^{35}S]methionine-labeled Rep78 was synthesized in a coupled in vitro transcription-translation system (see above) for 1 h at 30°C in a total reaction volume of 50 μl. The translation reaction mixture was split into two 25-μl samples, each sample was brought to 50 μl with an equal volume of 1× PBS, and ATP was added to a 5 mM final concentration. To investigate the effect of AAV *ori* sequences on the assembly state of Rep, a 63-bp DNA oligonucleotide *ori* probe containing sequences spanning AAV nucleotides 4538 to 4584 (flanked by *Eco*RI and *Pst*I sites) was included in a subset of samples at a 2-ng/μl final concentration. The samples were incubated at 4°C for 30 min. Each sample was diluted fivefold with cold PBS and passed through a Gelman GHP Acrodisc 13 filter (0.45-μm pore size). A 200-μl volume of each sample was injected onto the Superose 6 column and developed at a flow rate of 0.4 ml/min. One-milliliter fractions were collected, and aliquots of each fraction were counted in a liquid scintillation counter (LS 6500; Beckman Instruments, Inc., Fullerton, Calif.) or concentrated by lyophilization and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Protein-protein cross-linking. Prior to protein cross-linking, a 20 mM stock of the sulfhydryl-specific, homobifunctional cross-linking reagent 1,6-bismaleimidohexane (BMH; Pierce, Rockford, Ill.) was prepared in dimethyl sulfoxide. Radiolabeled Rep78 was synthesized in vitro by using a T7 polymerase-dependent coupled transcription-translation system containing 40 μCi of [^{35}S]methionine (>1,000 Ci/mmol) per 50-μl reaction mixture. Translation was carried out at 30°C for 1 h, after which time ATP was added to a 5 mM final concentration. The translation reaction mixture was then divided in half. One half received a 63-bp AAV *ori* probe (2-ng/μl final concentration), while the other did not. Both samples were incubated at 4°C for 30 min. Following incubation, 2-μl aliquots of each sample were added to 18 μl of 0.1 M sodium phosphate buffer (pH 6.4). BMH was added in the amounts indicated in Fig. 9, and the reaction mixture was incubated at room temperature for 1 min. The cross-linking reaction was quenched by addition of at least a 25-fold molar excess of dithiothreitol. The samples received an equal volume of 2× gel loading buffer (0.2 M sodium phosphate [pH 6.7], 20% glycerol, 4% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue) and were heated to 90°C for 10 min. SDS-PAGE was performed on a 4 to 10% polyacrylamide gradient gel by using the phosphate-SDS buffer system of Weber and Osborn (82). Broad-range protein standards (29 to 200 kDa) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cross-linked phosphorylase *b* molecular mass markers (Sigma) were used to provide molecular mass standards in excess of 200 kDa. Protein standards were visualized by staining with Coomassie brilliant blue R-250. Cross-linked Rep78 was visualized by autoradiography of the dried gel.

RESULTS

Self-association of Rep78 in vivo. To investigate the ability of Rep78 to self-associate in vivo, a mammalian two-hybrid system was employed (Fig. 1). In this system, an adaption of the selection system of Gstaiger and Schaffner (26), two recombinant plasmids (vectors 1 and 2) are cotransfected into a cell line (CV-1-5GT) containing integrated copies of the SV40 large T antigen gene under the control of a GAL4-inducible promoter. Vector 1 encodes a "target protein" fused to the DNA-binding domain (DBD) of the yeast transcriptional activator GAL4. Vector 2 encodes a potential "target-binding protein" fused to the acidic transcriptional activation domain (AD) of the herpes simplex virus VP16 gene product. Protein-protein interaction between the target protein and the target-binding protein results in induction of T antigen expression and subsequent T antigen-mediated replication of vector 2, which bears an SV40 origin of replication. DNA replication within eukaryotic cells transforms bacterially derived plasmid DNA from a form that is susceptible to digestion with the restriction endonuclease *Dpn*I to a digestion-resistent form due to loss of methylated adenine residues within the *Dpn*I recognition sequence. In the absence of protein-protein interaction, T antigen is not induced, leaving the transfecting plasmid DNA sensitive to *Dpn*I digestion. Two days posttransfection, low-molecular-weight DNA is isolated from the cells, digested with *Dpn*I, and used to transform *E. coli* to ampicillin resistance. The resultant ampicillin-resistant colonies provide a "reporter signal" that is indicative of a two-hybrid interaction.

As shown in Fig. 2, cotransfection of GAL4-REP (a target protein expression vector encoding aa 3 to 621 of Rep78 fused to the 147-aa DBD of GAL4) with a negative control vector encoding the VP16 AD resulted in no detectable plasmid replication. Similarly, when GAL4-REP was cotransfected with a Rep78 expression vector (Rep) that lacked a heterologous activation domain, no significant plasmid replication was detected. However, cotransfection of GAL4-REP with a VP16-REP fusion construct resulted in greater than 2,200 CFU/pmol of the VP16-REP effector plasmid. Cotransfection of VP16-REP with a plasmid encoding the GAL4 DBD alone resulted in a very low level of plasmid replication (approximately 30 CFU/pmol of effector plasmid). Taken together, these results indicate that specific Rep-Rep protein interactions occur in vivo.

Self-association of Rep78 in vitro. To confirm the results obtained with the mammalian two-hybrid system, the ability of Rep78 to self-associate in vitro was investigated by using a coimmunoprecipitation assay (Fig. 3A). Rep78 was expressed in bacteria as an MBP fusion protein and purified by amylose affinity chromatography. MBP-Rep78 has been extensively characterized and is capable of rescuing the AAV genome in an in vitro replication assay (15, 75). Purified MBP-Rep78 was incubated with [^{35}S]methionine-labeled Rep78 synthesized in a coupled in vitro transcription-translation system and immunoprecipitated by using MBP-specific polyclonal rabbit antiserum in combination with recombinant protein G-agarose beads. As a control for specificity, the ability of Rep78 to coprecipitate the human DNA polymerase processivity factor PCNA was also examined. Figure 3B (lanes 2 to 4) shows a sample of the input polypeptides synthesized by using the coupled in vitro transcription-translation system. In the absence of programming plasmid DNA, no detectable radiolabeled polypeptides were observed (lane 2). In contrast to its sequence-predicted molecular mass of 70.6 kDa, Rep78 synthesized in mammalian cells migrates on SDS-PAGE with an apparent molecular mass of approximately 78 kDa (65). In vitro-translated Rep78, however, demonstrated an apparent molecular mass that is in close agreement with that predicted by its amino acid sequence (lane 3). Consistent with the in vivo data, radiolabeled Rep78 readily coprecipitated with bacterially expressed MBP-Rep78 (lane 6). Rep78 failed to coprecipitate with MBP-LacZα (lane 5), indicating that the observed Rep-Rep interaction was mediated by the Rep moiety of MBP-Rep78. To confirm that coimmunoprecipitation of in vitro-translated Rep78 with MBP-Rep78 was not attributable to nonspecific protein interactions or physical entrapment of Rep78 within an immune complex, MBP-Rep78 was incubated with in vitro-translated PCNA as a negative control. As shown in lane 7, PCNA failed to coprecipitate with MBP-Rep78. No detectable radiolabeled polypeptides were observed upon immunoprecipitation of

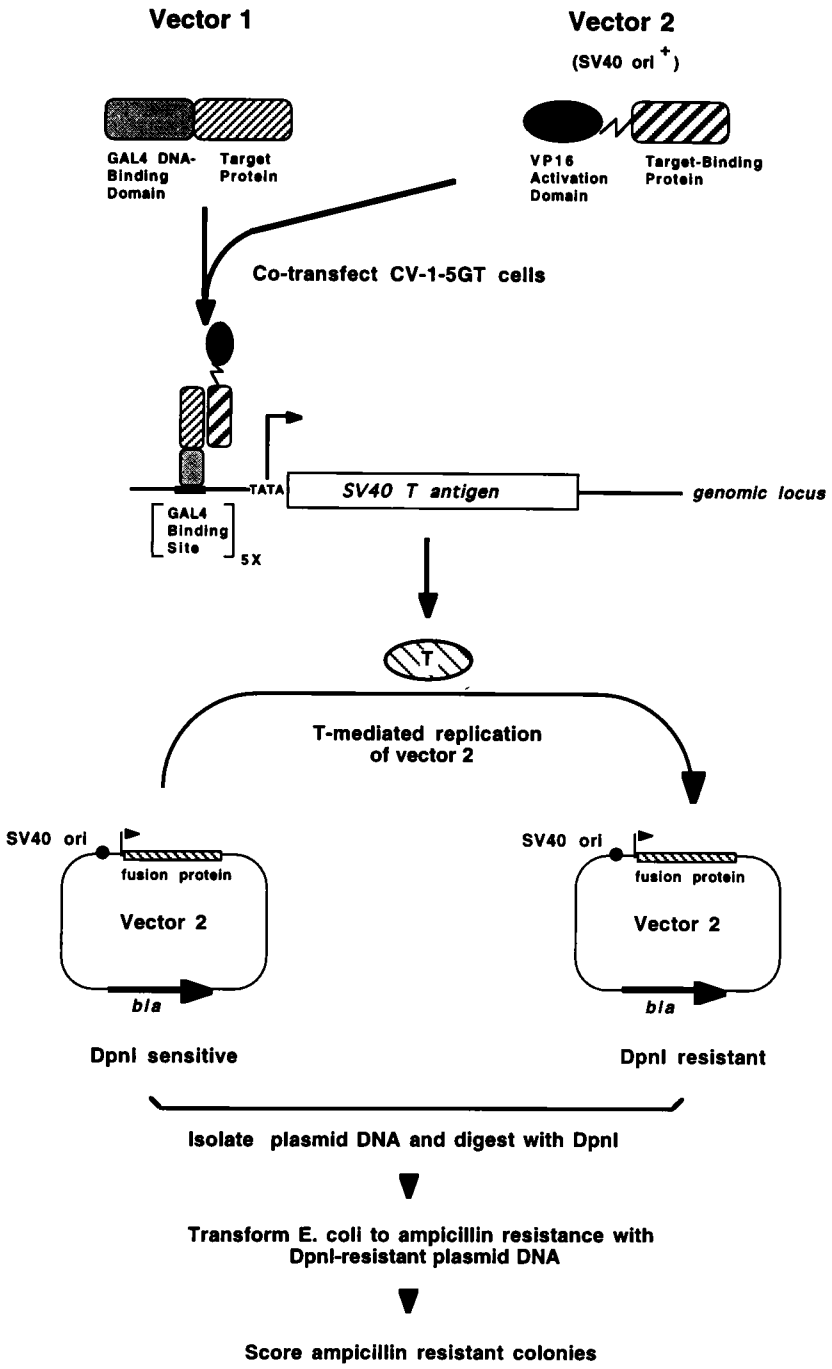

FIG. 1. Schematic representation of the mammalian two-hybrid system. Sequences encoding the bait (or target) protein are cloned into vector 1 in frame with sequences encoding the 147-aa DBD of the yeast transcription factor GAL4. Sequences encoding a potential target-binding (or effector) protein are cloned into vector 2 in frame with sequences encoding the acidic transcriptional AD of the herpes simplex virus type 1 virion protein VP16. Vectors 1 and 2 are cotransfected into CV-1-5GT cells, a monkey kidney cell line containing integrated copies of the SV40 large tumor antigen (T antigen) gene under the transcriptional control of a promoter containing five GAL4 binding sites (26). Protein-protein interaction between the bait and effector fusion proteins induces expression of T antigen. In the absence of protein-protein interaction, the T antigen locus is silent. Induction of large T antigen results in T antigen-dependent replication of vector 2 (which contains an SV40 origin of replication). Replication within eukaryotic cells converts bacterially derived vector 2 DNA from a *Dpn*I-sensitive to a *Dpn*I-resistant form. Low-molecular-weight DNA is isolated approximately 60 h posttransfection, digested with *Dpn*I, and used to transform *E. coli*. The occurrence of a two-hybrid interaction is assessed by scoring antibiotic-resistant colonies.

MBP-Rep78 from a negative control lysate that was not programmed with template DNA (lane 8).

Mapping of amino acid sequences required for Rep-Rep interaction. To map amino acid residues required for Rep-Rep protein interaction, constructs encoding either N-terminal or C-terminal truncations of Rep78 were expressed in vitro and tested for the ability to bind full-length MBP-Rep78. Figure 4 shows results obtained with the N-terminal truncation mutants. A sample of the input in vitro-translated polypeptides (IVT) is shown on the left (lanes 2 to 7), and immunoprecipitation

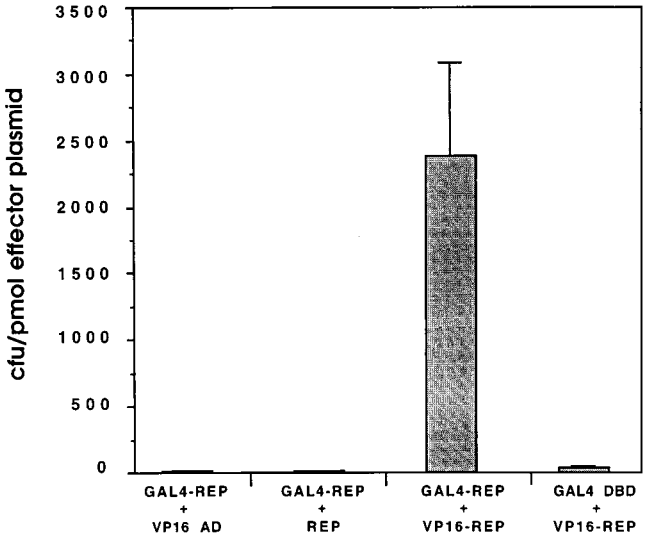

FIG. 2. Occurrence of Rep-Rep protein interactions in vivo. CV-1-5GT cells were seeded into 60-mm tissue culture dishes and cotransfected with 1.4 pmol of a plasmid expressing the indicated bait protein (either a GAL4 DBD-Rep78 fusion protein [GAL4-REP] or the GAL4 DBD alone [GAL4 DBD]) and 0.4 pmol of a plasmid expressing the indicated effector protein (either the VP16 AD, Rep78 aa 3 to 621 [REP], or a VP16 AD-Rep78 fusion protein [VP16-REP]). Transfected plasmid DNA was isolated approximately 60 h posttransfection as described in Materials and Methods, digested with restriction endonuclease *Dpn*I, and used to transform *E. coli* DH5α. Results are expressed as CFU per picomole of effector plasmid. Each transfection was performed in duplicate.

results (IP) are shown on the right (lanes 8 to 14). In agreement with the data described above, full-length Rep78 coprecipitated with MBP-Rep78 (lane 9) but not with MBP-LacZα (lane 8). Polypeptides lacking the first 163 aa residues of Rep78 also coprecipitated with MBP-Rep78 (lane 10), albeit in a lesser amount than full-length polypeptides. Deletion of aa residues 1 to 242, however, resulted in a dramatic loss of Rep-Rep interaction (lane 11). This result was also true of more extensive N-terminal truncations (lanes 12 to 14). These findings suggest that an amino acid domain critical for Rep-Rep interaction occurs, in whole or in part, between residues 164 and 243. To more clearly define the amino acid sequences required for Rep-Rep interaction, a series of plasmids encoding C-terminal truncation mutant forms of Rep78 was used to program the coupled in vitro transcription-translation system, and the resulting polypeptides (Fig. 5, left) were tested for the ability to bind full-length MBP-Rep78 (Fig. 5, right). In vitro-translated Rep polypeptides bearing aa residues 1 to 548 or 1 to 484 readily coprecipitated with MBP-Rep78 (lanes 10 and 11). Deletion of residues 372 to 621, however, resulted in a substantial decrease in binding of the corresponding Rep truncation product to MBP-Rep78 (lane 12). Truncation of Rep to residue 322 or 243 resulted in a further decrease of Rep-Rep interaction (lanes 13 and 14). Taken together, results obtained with the N-terminal and C-terminal truncation products indicate that sequence elements mapping between positions 164 and 484 of Rep are required for maximal binding in a coprecipitation assay.

Site-directed mutagenesis of specific amino acid sequences involved in Rep self-association. Examination of the Rep polypeptide sequence within the 321-aa region between aa 164 and 484 revealed two salient features: (i) a predicted alpha-helical domain occurring between aa 165 and 192 that bears a 3,4-hydrophobic heptad repeat reminiscent of those found in coiled-coil domains and (ii) a previously recognized NTP-bind-

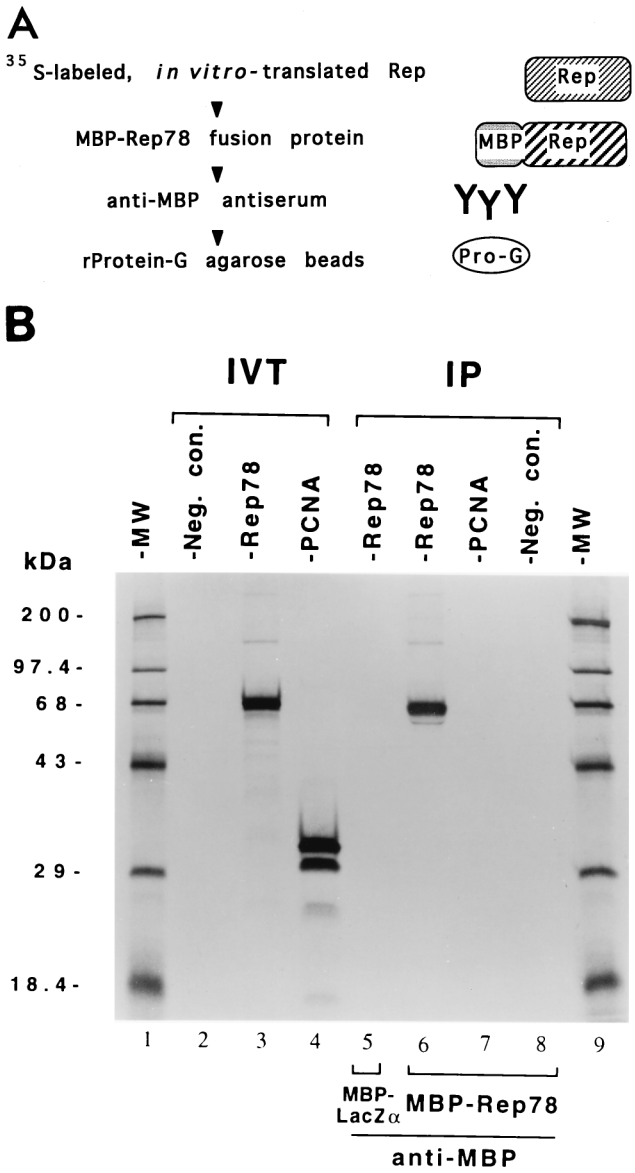

FIG. 3. Interaction of in vitro-translated Rep78 with a bacterial MBP-Rep78 fusion protein in a coimmunoprecipitation assay. (A) Schematic representation of the coimmunoprecipitation assay. Plasmids encoding wild-type Rep78 or mutant versions of Rep78 under the control of a bacteriophage T7 promoter were translated in vitro in the presence of [^{35}S]methionine by using a T7 polymerase-dependent coupled transcription-translation system. In vitro translation products were incubated with 1 μg of an MBP-Rep78 fusion protein expressed in bacteria (or, in some cases, 1 μg of an MBP-LacZα fusion product as a negative control). Protein complexes were immunoprecipitated by using MBP-specific polyclonal antiserum in combination with recombinant protein G-agarose beads, washed repeatedly, and resolved by SDS-PAGE. (B) Coprecipitation of ^{35}S-labeled Rep78 with bacterially expressed MBP-Rep78. Lanes 2 to 4 show 1-μl samples of the indicated in vitro translation product (IVT) separated on an SDS–12% polyacrylamide gel. Neg. con. represents a negative control lysate that received no programming plasmid DNA. For the immunoprecipitation assay (IP), 10 μl of in vitro-translated, ^{35}S-labeled Rep78 was incubated with 1 μg of MBP-Rep78 (lane 6) or, as a negative control, 1 μg of MBP-LacZα (lane 5), immunoprecipitated, and resolved by SDS–12% PAGE. As a control for binding specificity, the ability of MBP-Rep78 to coprecipitate ^{35}S-labeled, in vitro-translated human PCNA was also tested (lane 7). No radiolabeled polypeptides were immunoprecipitated from the negative control lysate (lane 8). MW, ^{14}C-labeled molecular weight markers (lanes 1 and 9).

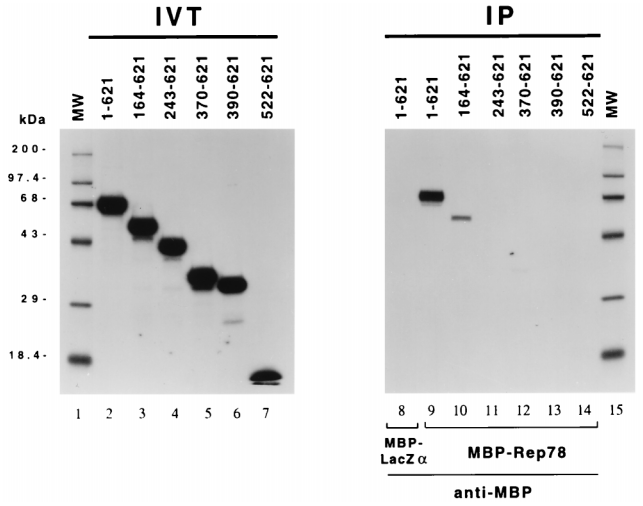

FIG. 4. Binding of bacterially expressed MBP-Rep78 to mutant forms of Rep78 with N-terminal truncations. A series of plasmids expressing N-terminally truncated forms of Rep78 was expressed in vitro in the presence of [^{35}S]methionine. The resulting polypeptides were tested for the ability to bind an MBP-Rep78 fusion protein in a coimmunoprecipitation assay. In vitro-translated proteins were resolved by SDS–12% PAGE. One-microliter samples of the indicated in vitro translation reaction (IVT) are shown in lanes 2 to 7. Ten-microliter samples of the indicated in vitro translation reaction mixtures were diluted in binding buffer, incubated with 1 μg of MBP-Rep78 (lanes 9 to 14) or 1 μg of MBP-LacZα (lane 8), and then immunoprecipitated as described in Materials and Methods. Radiolabeled proteins recovered in the immunoprecipitation assay (IP) are shown in lanes 8 to 14.

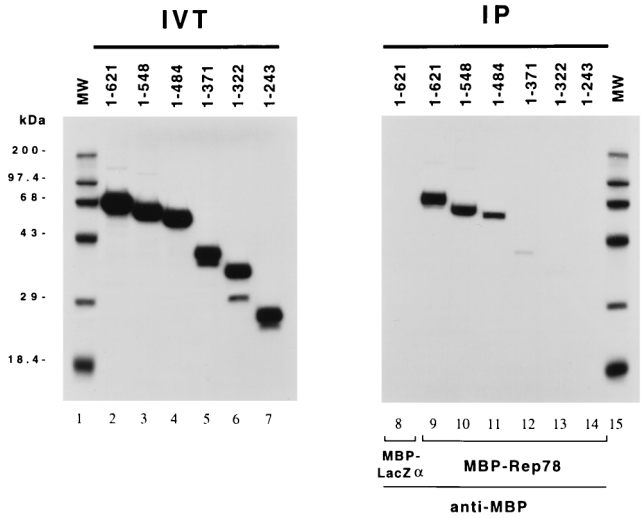

FIG. 5. Binding of bacterially expressed MBP-Rep78 to mutant forms of Rep78 with C-terminal truncations. A series of plasmids encoding C-terminally truncated mutant forms of Rep78 was expressed in vitro by using a T7 polymerase-dependent coupled transcription-translation system in the presence of [^{35}S]methionine. The resulting radiolabeled polypeptides were tested for the ability to coprecipitate with MBP-Rep78. The Rep78 amino acid residues expressed by each construct are indicated above the appropriate lane. One-microliter samples (0.1× input) of the indicated in vitro translation reaction (IVT) are shown in lanes 2 to 7. The results of the immunoprecipitation assay (IP) are shown in lanes 8 to 14. Radiolabeled proteins were resolved by SDS–12% PAGE. Lanes MW contained molecular weight markers.

ing motif occurring between aa 334 and 421 (1, 24, 25). Protein-protein interactions mediated by coiled-coil domains have been described for numerous proteins, including members of the bZIP family of eukaryotic transcription factors (e.g., Fos, Jun, and GCN4) (45, 51, 77). Moreover, binding of NTPs or nonhydrolyzable NTP analogs has been reported to enhance the oligomerization of numerous helicases of both prokaryotes and eukaryotes, including DNA helicase II of *E. coli*, the DNA replicative helicase (gp41) of bacteriophage T4, and SV40 large T antigen (8, 18, 66).

Based on the above observations, we sought to determine the contributions of the putative coiled-coil domain and NTP-binding motif to Rep oligomerization via the use of site-directed mutagenesis and coimmunoprecipitation. Figure 6 shows a series of mutant Rep proteins constructed for that purpose. Mutant L165G contains a substitution of glycine for leucine at position 165. Position 165 is predicted to occur on the protein-protein contact surface of the heptad repeat alpha helix. Mutant L180T contains a substitution of threonine for leucine at position 180. Position 180 is predicted to occur adjacent to the hydrophobic interface of the interacting coiled-coil domains. The NTP-binding motif is composed of two consensus elements, GxxxxGK(T/S) (the A site; x represents any amino acid) and uuuu(D/E)(D/E) (the B site; u represents any hydrophobic amino acid) (24, 78). An invariant asparagine residue (C site) has also been described (25). The A-, B-, and C-site elements of the conserved NTP-binding domain of Rep78 are centered at aa residues 339, 379, and 421, respectively (25). In mutant K340H, a conserved lysine residue within the A site of the consensus NTP-binding motif is replaced with histidine. The positively charged side chain of the A-site lysine residue is believed to interact with the α-phosphate of a bound NTP molecule (36). Rep polypeptides bearing a K340H mutation lack many of Rep's functional activities and exhibit a *trans*-dominant negative phenotype in vitro and in vivo (10, 11, 42, 43, 61, 85). We also used oligonucleotide-directed mutagenesis to introduce small, defined deletions within the Rep polypeptide. Mutant d151-188 bears a deletion of the 3,4-hydrophobic heptad repeat, while mutant d334-347 bears a deletion of the A site of the NTP-binding motif. In addition, a double mutant (d151-188/334-347) lacking both the heptad repeat and the NTP-binding motif A site was constructed. Figure 7 shows the in vitro translation products encoded by the various mutant constructs (lanes 2 to 8). As before, full-length Rep78 coprecipitated with MBP-Rep78 (lane 10) but not MBP-LacZα (lane 9). Deletion of the heptad repeat severely impaired the ability of Rep78 to self-associate (lane 11). Similarly, deletion of the NTP-binding motif A site greatly reduced Rep-Rep interaction (compare lanes 10 and 12). Strikingly, no detectable interaction was observed between the double deletion mutant (d151-188/334-347) and MBP-Rep78 (lane 13). Consistent with its identification as a *trans*-dominant negative mutant, K340H bound MBP-Rep78 at levels greater than or equal to that of wild-type Rep78 (lane 14). It is postulated that the differences in Rep-Rep interaction observed with A-site mutants K340H and d334-347 are due to differences in the abilities of these mutant polypeptides to bind ATP. L165G, which bears an amino acid substitution on the predicted contact surface of the heptad repeat alpha helix, bound MBP-Rep78 poorly (lane 16). L180T, which bears an amino acid substitution that is predicted to occur on the periphery of the 3,4-hydrophobic heptad repeat alpha helix, bound Rep78 at levels similar to those of wild-type Rep78 (lane 15).

Hexameric complex formation by Rep78 in the presence of AAV *ori* sequences. To examine the multimerization state of Rep78 in solution and to determine the effect of site-specific DNA binding upon Rep-Rep protein interaction, [^{35}S]methionine-labeled Rep78 was incubated in the presence or absence of a double-stranded 63-bp AAV origin probe (ORI; AAV nucleotides 4538 to 4584, flanked by *Eco*RI and *Pst*I recogni-

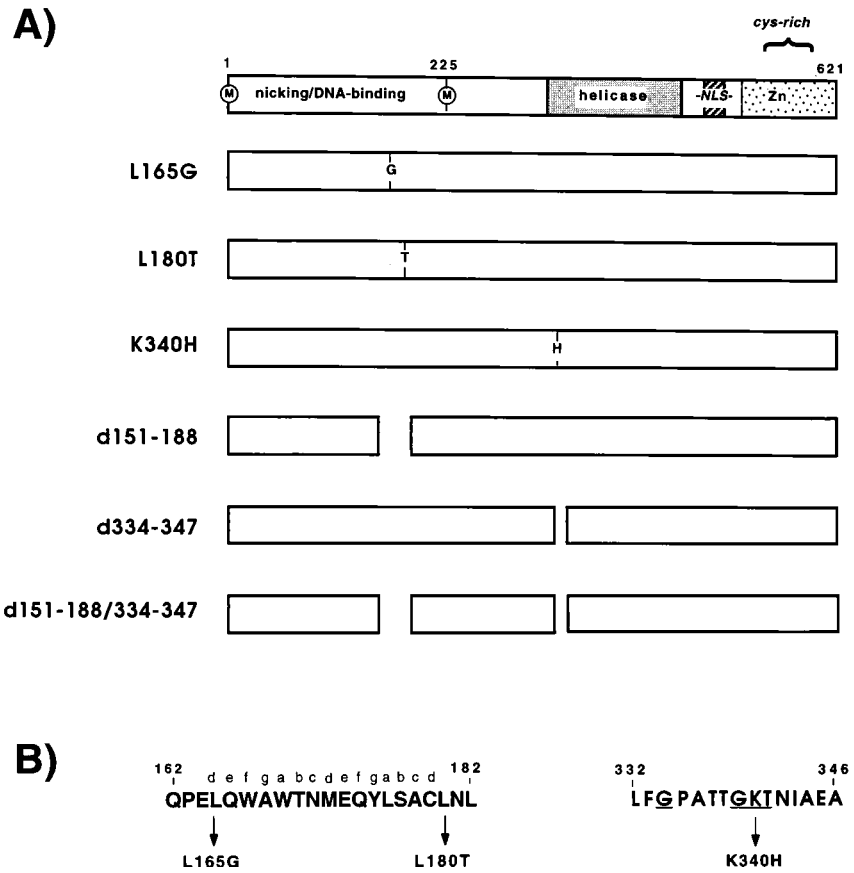

FIG. 6. Diagrammatic representation of Rep78 with site-directed mutations. (A) A schematic representation of the 621-aa Rep78 protein is shown at the top. The amino-terminal methionine residues of Rep78/68 and Rep52/40 are indicated by the letter M at positions 1 and 225, respectively. The amino-terminal portion of Rep78/68 contains a sequence-specific DBD (62, 83), as well as amino acid motifs associated with nicking and covalent attachment to replicative-form DNA that are conserved among RCR initiator proteins (32). The putative helicase domain of Rep is indicated by the shaded rectangle. This region contains motifs conserved among certain NTP-binding proteins and helicases (24, 25). The relative location of the nuclear localization signal (NLS) (38, 86) is indicated by the striped box. A cysteine-rich 91-aa region common to Rep78 and Rep52 is indicated by the stippled rectangle. This region contains a putative zinc-binding motif (58) and has been shown to bind zinc in vitro (31). The relative locations of oligonucleotide-mediated, site-directed mutations of Rep78 are indicated below. For each deletion construct, the extent of the deletion (inclusive) is indicated by the nomenclature and represented as a gap in the schematic Rep diagram. (B) At the left are aa residues 162 to 182 of Rep78, which contain a 3,4-hydrophobic heptad repeat. The heptad repeat unit is indicated by lowercase letters (a to g). Residues at the a and d positions of coiled-coil-type heptad repeats participate in a hydrophobic interface between interacting α-helices (51, 77). At the right are aa residues 332 to 346 of Rep78, which contain the A-site consensus motif (underlined) of the conserved NTP-binding domain (25).

tion sites) and subjected to gel filtration chromatography on a Superose 6 column. In the absence of ORI, Rep78 eluted in two major peaks (Fig. 8, open circles). The earliest peak represents material eluting within the void volume of the gel filtration column, while the second peak corresponds to monomeric Rep78. Incubation of Rep78 with poly(dI-dC) also yielded void and monomer peaks (data not shown). Examination of the void peak by SDS-PAGE under reducing and nonreducing conditions revealed it to contain high-molecular-weight multimers of Rep78 that were covalently linked via disulfide bonds (data not shown). Incubation of Rep78 with the AAV ORI probe (closed circles) resulted in a change in the elution profile. A novel peak with an elution volume similar to that of the ferritin molecular mass standard (440 kDa) was observed. Repeated determination of the elution volume of the novel Rep complex provided an estimated molecular mass of 442 ± 31 kDa ($n = 3$). By using the method of Finger and Richardson (20), we estimated the Stokes radius of the complex to be 63 ± 4 Å. Based upon the sequence-predicted molecular mass of a Rep78 monomer (70.6 kDa), the experimentally determined value of 442 ± 31 kDa assigned to the ORI-induced Rep complex is most consistent with a hexameric form of Rep78. To further examine the oligomeric state of Rep78, the sulfhydryl-specific cross-linking reagent BMH was used to irreversibly cross-link the Rep78 multimer in either the presence or the absence of ORI (Fig. 9). The cross-linked products were separated under reducing-denaturing conditions on a 4 to 10% polyacrylamide gradient gel by using the sodium phosphate-SDS buffer system of Weber and Osborn (82). Non-cross-linked Rep78 is shown in lane 1. In the absence of ORI DNA (lanes 2 to 4), addition of BMH in increasing amounts resulted in the appearance of six major protein species (Fig. 9, bands a through f) separated by an average molecular mass of 73 kDa and, thus, consistent with multimeric forms of Rep78 ranging from monomer to hexamer. Although bands c and d were somewhat heterogeneous, their overall migration with respect to the molecular mass standards was consistent with tri- and tetrameric forms of Rep78, respectively. Incubation of Rep78 with the AAV ORI probe prior to cross-linking greatly enhanced the formation of the tetra-, penta-, and hexameric forms of the Rep78 oligomer (Fig. 9, compare lanes 2 to 4 with lanes 5 to 7). The difference between the profiles observed in the chromatography and cross-linking experiments may be due, in part, to the efficiency of the cross-linking reaction. Indeed, increasing the

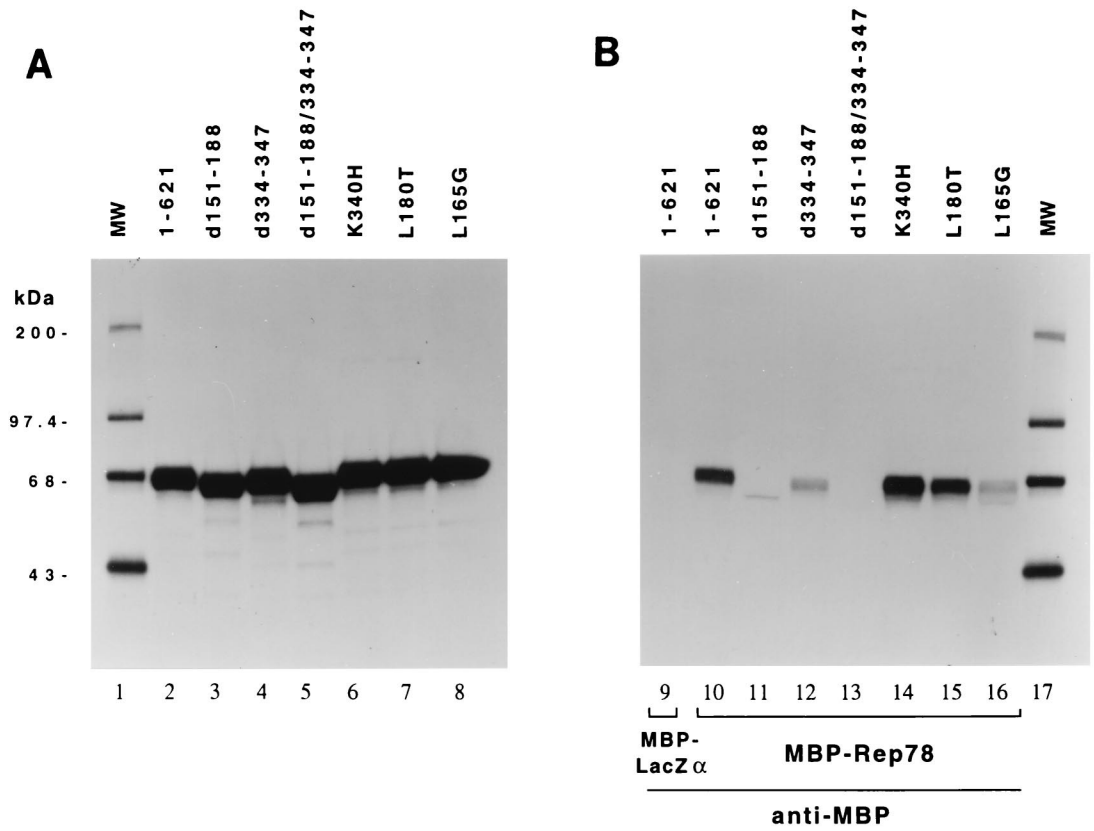

FIG. 7. Interaction of bacterially expressed MBP-Rep78 with mutant Rep78 polypeptides expressed in vitro. For lanes 1 to 8, plasmids encoding either wild-type Rep78 or mutant forms of Rep78 were expressed in vitro in the presence [^{35}S]methionine, and 1-μl samples of the resulting translation products (lanes 2 to 8) were analyzed by SDS–8% PAGE and fluorography. For lanes 9 to 17, 10 μl of each translation reaction mixture was tested for the ability to bind MBP-Rep78 (lanes 10 to 16) as described in Materials and Methods. The ability of full-length Rep78 to bind MBP-LacZα was tested as a negative control (lane 9). MW, ^{14}C-labeled molecular weight markers (lanes 1 and 17).

concentration of BMH within the cross-linking reaction mixture enhanced the observation of higher-order Rep oligomers (Fig. 9, compare lanes 5 and 7).

DISCUSSION

In this study, we have investigated the ability of the AAV Rep78 gene product, a viral DNA replication initiator protein and replicative helicase, to self-associate in vivo and in vitro. Gel filtration chromatography and protein-protein cross-linking experiments indicated that multimerization of Rep78 in the presence of AAV *ori* sequences results in the formation of hexameric oligomers. This finding identifies Rep78 as a member of a growing list of hexameric helicase proteins which includes SV40 large T antigen (53, 66), the DnaB and Rho proteins of *E. coli* (20, 50), the gp41 helicase of bacteriophage T4 (18), and the gp4A and -B helicase-primase proteins of bacteriophage T7 (63). In the absence of AAV *ori* sequences, in vitro-translated Rep78 was seen to exist in two predominant forms, (i) a high-molecular-weight form (designated HMW_{Void}) that elutes within the void volume of a Superose 6 column and (ii) a monomeric form. A relatively small amount of stable Rep78 dimer was also observed in cross-linking experiments in the absence of *ori* DNA. Gel filtration chromatography of cellular extracts derived from NIH 3T3 cells transfected with a Rep78 expression construct showed an abundance of the HMW_{Void} form of Rep78, indicating that this species of Rep is not an artifact of in vitro translation (unpublished observations). Addition of AAV *ori* sequences to a binding reaction mixture containing in vitro-translated Rep78 resulted in a dramatic rearrangement of the assembly state of Rep to a hexameric form. Unexpectedly, formation of Rep78 hexamers resulted in depletion of the HMW_{Void} fraction of Rep78, with only a relatively small reduction in the monomeric fraction (Fig. 8). In contrast, repeated cross-linking experiments with Rep78 in the presence of AAV *ori* DNA indicated that hexamer assembly was accompanied by a significant depletion of monomeric Rep78. These observations suggest that an equilibrium exists between the HMW_{Void}, monomeric, and hexameric forms of Rep78 such that, in the absence of a cross-linking reagent, any loss of Rep78 from the monomeric pool to the hexamer assembly pathway is replenished from the HMW_{Void} fraction. In the presence of a cross-linker, however, the HMW_{Void} and hexameric forms of Rep78 are immobilized and, therefore, unable to replenish the monomeric pool, resulting in the net depletion of monomer observed in the cross-linking experiments.

Speculation concerning the biological significance of an equilibrium between the HMW_{Void} and monomeric forms of Rep78 is supported by analogous observations described for the helix-loop-helix DNA-binding protein MyoD. Under experimental conditions, MyoD is observed to form high-molecular-weight aggregates (or "micelles") that are in cooperative, reversible equilibrium with MyoD monomers (46). MyoD micelle formation initiates when the dissociated form of the protein reaches a "critical micelle concentration." Once formed,

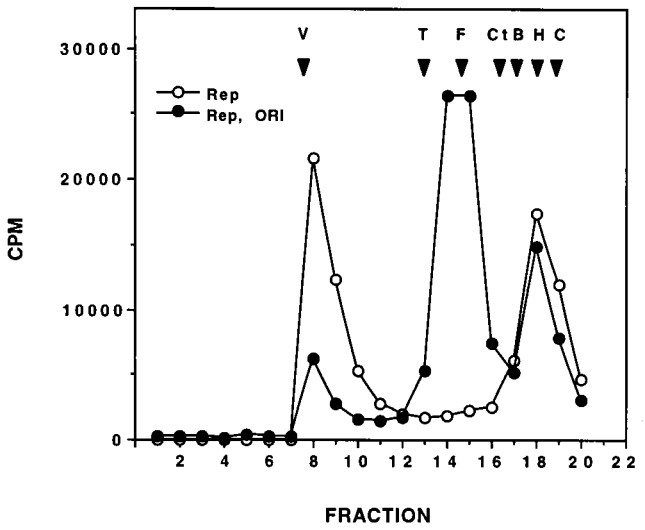

FIG. 8. Gel filtration chromatography of Rep78. In vitro-translated, ^{35}S-labeled Rep78 was incubated in the presence of 5 mM ATP (open circles) or 5 mM ATP and 2 ng of a 63-bp AAV *ori* probe per μl (closed circles) at 4°C for 30 min and then analyzed by gel filtration chromatography with a Superose 6 column. Counts per minute per 50 μl were determined for each fraction by liquid scintillation counting. The elution profile of protein standards is indicated at the top (V, excluded volume of the column; T, thyroglobulin; F, ferritin; Ct, catalase; B, bovine serum albumin; H, hemoglobin; C, carbonic anhydrase).

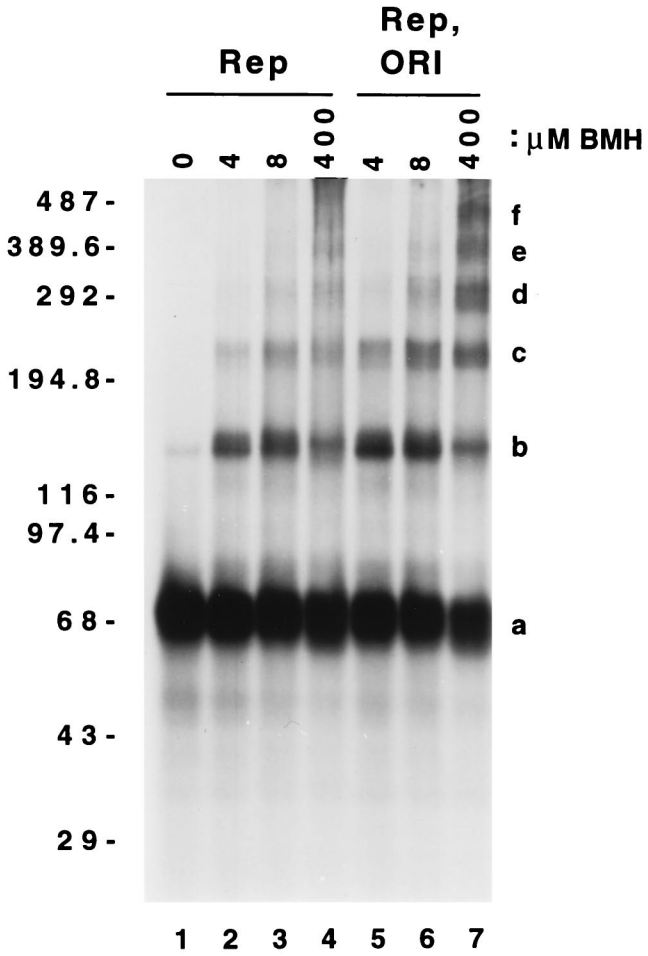

FIG. 9. Rep78 oligomers detected by protein-protein cross-linking and SDS-PAGE. ^{35}S-labeled, in vitro-translated Rep78 was incubated in the presence (Rep, ORI) or absence (Rep) of a 63-bp AAV *ori* probe under the conditions used to prepare Rep78 for gel filtration chromatography (see Materials and Methods). Following incubation, a 2-μl aliquot of each sample was diluted 10-fold in 0.1 M sodium phosphate buffer (pH 6.4) and the sulfhydryl-specific protein cross-linking reagent BMH (20 mM stock) was added to the concentration indicated at the top of each lane. Cross-linking was carried out at room temperature for 1 min, after which time the reactions were quenched with excess dithiothreitol and placed on ice. Samples were reduced and denatured by heating to 90°C for 10 min in the presence of 2% SDS and 2.5% β-mercaptoethanol. Proteins were separated on a 4 to 10% gradient gel by using the denaturing phosphate-SDS gel system of Weber and Osborn (82), stained with Coomassie blue (to visualize the molecular weight markers), dried, and exposed to film. Protein molecular mass standards in excess of 200 kDa were provided by commercially prepared, cross-linked phosphorylase *b* molecular mass markers (Sigma). Bands a to f correspond in molecular mass to monomeric through hexameric forms of Rep78, respectively.

MyoD micelles can influence the intermolecular interactions of the dissociated form of the protein via a binding competition mechanism. As a general example, if the critical micelle concentration of a hypothetical DNA-binding protein is less than the dissociation constant (K_d) of that protein's nonspecific interaction with DNA yet greater than the K_d of the protein for its cognate DNA-binding site, a "buffer" is formed in which the DNA-binding protein shuttles between its micelle and its cognate binding site over a wide range of protein concentrations (essentially avoiding low-affinity protein-DNA interactions). The same relationship can apply to protein-protein interactions. In Rep78, this mechanism may help to limit nonspecific chromosomal nicking during the establishment of latent infection and may contribute to the Rep-dependent, site-specific integration phenomenon described for AAV. For a thorough mathematical consideration of the biological consequences of micelle formation, see reference 46.

In addition to an examination of the oligomeric state of Rep78, mapping experiments identified two independent domains associated with multimerization of the protein: (i) an evolutionarily conserved NTP-binding domain and (ii) an α-helical region of approximately 30 aa bearing a short coiled-coil motif. In view of the well-characterized combinatorial interactions of certain members of the DNA-binding class of eukaryotic coiled-coil proteins (e.g., bZIP proteins) (45, 77), the existence of a coiled-coil domain within Rep78 suggests the possibility of combinatorial interaction with heterologous cellular or viral proteins (presumably helicases) bearing a compatible hydrophobic interaction domain. In addition to the putative coiled-coil domain described here, two conserved amino acid motifs common to initiator proteins involved in rolling-circle replication (RCR) have been identified within the amino terminus of Rep78 (32). These amino acid motifs are associated with DNA cleavage at the RCR origin of replication and covalent attachment of the protein to the 5′ end of the cleaved DNA. The first is a two-His motif (HuHuuu, where u is any hydrophobic residue) located between Rep78 aa 90 and 95. This motif is believed to coordinate a divalent cation that serves as a cofactor in the nicking activity of RCR initiator proteins (32, 60). Im and Muzyczka (35) have demonstrated that the endonuclease activity of Rep68 requires the presence of magnesium (manganese, zinc, and calcium cations failed to serve as cofactors). Moreover, Nuesch et al. (60) used site-directed mutagenesis to demonstrate that the HuHuuu motif of the homologous NS1 protein of minute virus of mice (a murine parvovirus) was critical for nicking activity. The second RCR-associated motif ($Yx_{3-4}K$) contains a tyrosine residue that forms a covalent bond with DNA during the strand cleavage reaction. This motif spans residues 156 to 160 of Rep78. Snyder et al. (72) have demonstrated that Rep covalently at-

taches to the 5′ end of cleaved DNA via a phosphotyrosine linkage; however, the specific Rep tyrosine residue involved was not identified. The HuHuuu and $Yx_{3-4}K$ motifs occur in proximity to the putative coiled-coil domain of Rep78. Thus, in addition to mediating Rep-Rep interaction, the coiled-coil domain may serve to juxtapose the DNA cleavage-covalent attachment domains of individual Rep molecules within oligomeric Rep complexes.

Upon noting the unusual requirement of an ATP cofactor for efficient site-specific cleavage of substrate DNA by Rep68, Im and Muzyczka (34) suggested that ATP may be needed for the assembly of an active endonuclease complex consisting of more than one molecule of Rep. In support of the hypothesis, we have shown that disruption of the NTP-binding domain of Rep78 by truncation or by a specific 14-aa deletion of the conserved A-site motif impairs the ability of Rep78 to multimerize. Moreover, Christensen et al. (16) have reported that binding of the homologous NS1 protein of minute virus of mice to the viral P38 promoter is ATP dependent; however, dependence upon ATP could be overcome by antibody-mediated cross-linking of NS1, leading those investigators to suggest that ATP binding induces NS1 to oligomerize. Furthermore, NTPs and/or nonhydrolyzable NTP analogs have been shown to increase and/or stabilize the oligomerization of certain prokaryotic helicases, including helicase II of *E. coli* (8) and the gp41 and gp4 helicases of bacteriophages T4 and T7 (18, 63), respectively. Hexamerization of SV40 T antigen is also enhanced by NTPs and nonhydrolyzable NTP analogs (53, 66). Similarly, protein-protein cross-linking experiments indicated that oligomerization of Rep78 was enhanced in the presence of the nonhydrolyzable ATP analog ATPγS (data not shown). This result indicates that hexameric assembly of Rep78 is not dependent upon ATP hydrolysis. One possibility is that the binding of ATP induces a conformational change within monomeric Rep78 that facilitates multimerization. Binding to ATPγS, for example, has been reported to result in a conformation-induced resistance of helicase II of *E. coli* to digestion with chymotrypsin (8). The possibility of an NTP-induced conformational change in Rep78 is currently under investigation in our laboratory.

In conclusion, the finding that Rep78 forms multimeric complexes in vitro and in vivo adds a new dimension to the functional complexity of this pleiotropic effector molecule. The possibility of combinatorial interaction with viral and/or cellular proteins and the in vivo significance of the apparent equilibrium among the hexameric, monomeric, and HMW_{Void} forms of Rep78 are of particular interest. In addition, the multimeric structure of Rep78 and the apparent equilibrium among its oligomeric forms may have implications for targeted integration of AAV vectors.

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REFERENCES

1. **Astell, C. R., C. D. Mol, and W. F. Anderson.** 1987. Structural and functional homology of parvovirus and papovavirus polypeptides. J. Gen. Virol. **68:**885–893.
2. **Becerra, S. P., F. Koczot, P. Fabisch, and J. A. Rose.** 1988. Synthesis of adeno-associated virus structural proteins requires both alternative mRNA splicing and alternative initiation from a single transcript. J. Virol. **62:**2745–2754.
3. **Berns, K. I.** 1990. Parvovirus replication. Microbiol. Rev. **54:**316–329.
4. **Berns, K. I., and R. A. Bohenzky.** 1987. Adeno-associated viruses: an update. Adv. Virus Res. **32:**243–306.
5. **Berns, K. I., R. M. Kotin, and M. A. Labow.** 1988. Regulation of adeno-associated virus DNA replication. Biochim. Biophys. Acta **951:**425–429.
6. **Berns, K. I., and R. M. Linden.** 1995. The cryptic life style of adeno-associated virus. Bioessays **17:**237–245.
7. **Birnboim, H., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
8. **Brosh, R. M., Jr., and S. W. Matson.** 1996. A partially functional DNA helicase II mutant defective in forming stable binary complexes with ATP and DNA. J. Biol. Chem. **271:**25360–25368.
9. **Chejanovsky, N., and B. J. Carter.** 1989. Mutagenesis of an AUG codon in the adeno-associated virus *rep* gene: effects on viral DNA replication. Virology **173:**120–128.
10. **Chejanovsky, N., and B. J. Carter.** 1990. Mutation of a consensus purine nucleotide consensus binding site in the adeno-associated virus *rep* gene generates a dominant negative phenotype for DNA replication. J. Virol. **64:**1764–1770.

10a. **Chiorini, J. A., and R. M. Kotin.** Unpublished data.

11. **Chiorini, J. A., M. D. Weitzman, R. A. Owens, E. Urcelay, B. Safer, and R. M. Kotin.** 1994. Biologically active Rep proteins of adeno-associated virus type 2 produced as fusion proteins in *Escherichia coli*. J. Virol. **68:**797–804.
12. **Chiorini, J. A., C. M. Wendtner, E. Urcelay, B. Safer, M. Hallek, and R. M. Kotin.** 1995. High-efficiency transfer of the T-cell co-stimulatory molecule B7-2 to lymphoid cells using high-titer recombinant adeno-associated virus vectors. Hum. Gene Ther. **6:**1531–1541.
13. **Chiorini, J. A., S. M. Wiener, R. A. Owens, S. R. M. Kyostio, R. M. Kotin, and B. Safer.** 1994. Sequence requirements for stable binding and function of Rep68 on the adeno-associated virus type 2 inverted terminal repeat. J. Virol. **68:**7448–7457.
14. **Chiorini, J. A., S. M. Wiener, L. Yang, R. H. Smith, B. Safer, N. P. Kilcoin, Y. Liu, E. Urcelay, and R. M. Kotin.** 1996. The roles of AAV Rep proteins in gene expression and targeted integration. Curr. Top. Microbiol. Immunol. **218:**25–34.
15. **Chiorini, J. A., L. Yang, B. Safer, and R. M. Kotin.** 1995. Determination of adeno-associated virus Rep68 and Rep78 binding sites by random sequence oligonucleotide selection. J. Virol. **69:**7334–7338.
16. **Christensen, J., S. F. Cotmore, and P. Tattersall.** 1995. Minute virus of mice transcriptional activator protein NS1 binds directly to the transactivation region of the viral P38 promoter in a strictly ATP-dependent manner. J. Virol. **69:**5422–5430.
17. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33:**91–174.
18. **Dong, F., E. P. Gogol, and P. H. von Hippel.** 1995. The phage T4-coded DNA replication helicase (gp41) forms a hexamer upon activation by nucleotide triphosphate. J. Biol. Chem. **270:**7462–7473.
19. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45:**721–732.
20. **Finger, L. R., and J. P. Richardson.** 1982. Stabilization of the hexameric form of *Escherichia coli* protein rho under ATP hydrolysis conditions. J. Mol. Biol. **156:**203–219.
21. **Flotte, T. R., S. A. Afione, R. Solow, M. L. Drumm, D. Markakis, W. B. Gugginos, P. L. Zeitlin, and B. J. Carter.** 1993. Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. J. Biol. Chem. **268:**3781–3790.
22. **Giraud, C., E. Winocour, and K. I. Berns.** 1994. Site-specific integration by adeno-associated virus is directed by a cellular DNA sequence. Proc. Natl. Acad. Sci. USA **91:**10039–10043.
23. **Goodman, S., X. Xiao, R. E. Donahue, A. Moulton, J. Miller, C. Walsh, N. S. Young, R. J. Samulski, and A. W. Nienhuis.** 1994. Recombinant adeno-associated virus-mediated gene transfer into hematopoietic progenitor cells. Blood **84:**1492–1500.
24. **Gorbalenya, A. E., and E. V. Koonin.** 1989. Viral proteins containing the purine NTP-binding sequence pattern. Nucleic Acids Res. **17:**8413–8440.
25. **Gorbalenya, A. E., E. V. Koonin, and Y. I. Wolf.** 1990. A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. FEBS Lett. **262:**145–148.
26. **Gstaiger, M., and W. Schaffner.** 1994. Strong transcriptional activators isolated from viral DNA by the 'activator trap', a novel selection system in mammalian cells. Nucleic Acids Res. **22:**4031–4038.
27. **Hanai, R., and J. C. Wang.** 1993. The mechanism of sequence-specific DNA cleavage and strand transfer by ϕX174 gene A* protein. J. Biol. Chem. **268:**23830–23836.
28. **Hoggan, M. D.** 1970. Adenovirus associated viruses. Prog. Med. Virol. **12:**211–239.
29. **Holscher, C., J. A. Kleinschmidt, and A. Burkle.** 1995. High-level expression of adeno-associated virus (AAV) Rep78 or Rep68 protein is sufficient for infectious-particle formation by a *rep*-negative AAV mutant. J. Virol. **69:**6880–6885.
30. **Hong, G., P. Ward, and K. I. Berns.** 1992. In vitro replication of adeno-associated virus DNA. Proc. Natl. Acad. Sci. USA **89:**4673–4677.
31. **Horer, M., S. Weger, K. Butz, F. Hoppe-Seyler, C. Geisen, and J. A. Kleinschmidt.** 1995. Mutational analysis of adeno-associated virus Rep protein-

mediated inhibition of heterologous and homologous promoters. J. Virol. **69:**5485–5496.
32. **Ilyina, T. V., and E. V. Koonin.** 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eukaryotes and archaebacteria. Nucleic Acids Res. **20:**3279–3285.
33. **Im, D.-S., and N. Muzyczka.** 1989. Factors that bind to adeno-associated virus terminal repeats. J. Virol. **63:**3095–3104.
34. **Im, D.-S., and N. Muzyczka.** 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell **61:**447–457.
35. **Im, D.-S., and N. Muzyczka.** 1992. Partial purification of adeno-associated virus Rep78, Rep52, and Rep40 and their biochemical characterization. J. Virol. **66:**1119–1128.
36. **Jindal, H. K., C. B. Yong, G. M. Wilson, P. Tam, and C. R. Astell.** 1994. Mutations in the NTP-binding motif of minute virus of mice (MVM) NS-1 protein uncouple ATPase and DNA helicase functions. J. Biol. Chem. **269:** 3283–3289.
37. **Kaplitt, M. G., P. Leone, R. J. Samulski, X. Xiao, D. W. Pfaff, K. L. O'Malley, and M. J. During.** 1994. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat. Genet. **8:**148–154.
38. **Kleinschmidt, J. A., M. Mohler, F. W. Weindler, and R. Heilbronn.** 1995. Sequence elements of the adeno-associated virus *rep* gene required for suppression of herpes-simplex-virus-induced DNA amplification. Virology **206:**254–262.
39. **Kotin, R. M.** 1994. Prospects for the use of adeno-associated virus as a vector for human gene therapy. Hum. Gene Ther. **5:**793–801.
40. **Kotin, R. M., R. M. Linden, and K. I. Berns.** 1992. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. EMBO J. **11:**5071–5078.
41. **Kotin, R. M., M. Siniscalco, R. J. Samulski, X. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns.** 1990. Site-specific integration by adeno-associated virus. Proc. Natl. Acad. Sci. USA **87:**2211–2215.
42. **Kyostio, S. R. M., R. A. Owens, M. D. Weitzman, B. A. Antoni, N. Chejanovsky, and B. J. Carter.** 1994. Analysis of adeno-associated virus (AAV) wild-type and mutant Rep proteins for their abilities to negatively regulate AAV p_5 and p_{19} mRNA levels. J. Virol. **68:**2947–2957.
43. **Kyostio, S. R. M., R. S. Wonderling, and R. A. Owens.** 1995. Negative regulation of the adeno-associated virus (AAV) P_5 promoter involves both the P_5 Rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. J. Virol. **69:**6787–6796.
44. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–687.
45. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a class of DNA binding proteins. Science **240:**1759–1764.
46. **Laue, T. M., M. A. Starovasnik, H. Weintraub, X.-H. Sun, L. Snider, and R. E. Klevit.** 1995. MyoD forms micelles which can dissociate to form heterodimers with E47: implications of micellization on function. Proc. Natl. Acad. Sci. USA **92:**11824–11828.
47. **Laughlin, C. A., J.-D. Tratschin, H. Coon, and B. J. Carter.** 1983. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. Gene **23:**65–73.
48. **Laughlin, C. A., H. Westphal, and B. J. Carter.** 1979. Spliced adenovirus-associated virus RNA. Proc. Natl. Acad. Sci. USA **76:**5567–5571.
49. **Leonard, C. J., and K. I. Berns.** 1994. Adeno-associated virus type 2: a latent life cycle. Prog. Nucleic Acid Res. Mol. Biol. **48:**29–52.
50. **Lohman, T. M.** 1993. Helicase-catalyzed DNA unwinding. J. Biol. Chem. **268:**2269–2272.
51. **Lupas, A.** 1996. Coiled coils: new structures and new functions. Trends Biochem. Sci. **21:**375–382.
52. **Lusby, E. W., and K. I. Berns.** 1982. Mapping of the 5′ termini of two adeno-associated virus RNAs in the left half of the genome. J. Virol. **41:**518–526.
53. **Mastrangelo, I. A., P. V. C. Hough, J. S. Wall, M. Dodson, F. B. Dean, and J. Hurwitz.** 1989. ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature (London) **338:**658–662.
54. **McCarty, D. M., D. J. Pereira, I. Zolotukhin, X. Zhou, J. H. Ryan, and N. Muzyczka.** 1994. Identification of linear DNA sequences that specifically bind the adeno-associated virus Rep protein. J. Virol. **68:**4988–4997.
55. **McCarty, D. M., J. H. Ryan, S. Zolotukhin, X. Zhou, and N. Muzyczka.** 1994. Interaction of the adeno-associated virus Rep protein with a sequence within the A palindrome of the viral terminal repeat. J. Virol. **68:**4998–5006.
56. **McKeon, C., and R. J. Samulski.** 1996. NIDDK workshop on AAV vectors: gene transfer into quiescent cells. Hum. Gene Ther. **7:**1615–1619.
57. **Miller, J. L., R. E. Donahue, S. E. Sellers, R. J. Samulski, N. Y. Young, and A. W. Nienhuis.** 1994. Recombinant adeno-associated virus (rAAV)-mediated expression of a human γ-globin gene in human progenitor-derived erythroid cells. Proc. Natl. Acad. USA **91:**10183–10187.
58. **Muramatsu, S.-I., H. Mizukami, N. S. Young, and K. E. Brown.** 1996. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology **221:**208–217.
59. **Ni, T.-H., X. Zhou, D. M. McCarty, I. Zolotukhin, and N. Muzyczka.** 1994. In vitro replication of adeno-associated virus DNA. J. Virol. **68:**1128–1138.
60. **Nuesch, J. P. F., S. F. Cotmore, and P. Tattersall.** 1995. Sequence motifs in the replicator protein of parvovirus MVM essential for nicking and covalent attachment to the viral origin: identification of the kinking tyrosine. Virology **209:**122–135.
61. **Owens, R. A., J. P. Trempe, N. Chejanovsky, and B. J. Carter.** 1991. Adeno-associated virus Rep proteins produced in insect and mammalian expression systems: wild-type and dominant-negative mutant proteins bind to the viral replication origin. Virology **184:**14–22.
62. **Owens, R. A., M. D. Weitzman, S. R. M. Kyostio, and B. J. Carter.** 1993. Identification of a DNA-binding domain in the amino terminus of adeno-associated virus Rep proteins. J. Virol. **67:**997–1005.
63. **Patel, S. S., and M. M. Hingorani.** 1993. Oligomeric structure of bacteriophage T7 primase/helicase proteins. J. Biol. Chem. **268:**10668–10675.
64. **Podsakoff, G., K. K. Wong, Jr., and S. Chatterjee.** 1994. Efficient gene transfer into nondividing cells by adeno-associated virus-based vectors. J. Virol. **68:**5656–5666.
65. **Redemann, B. E., E. Mendelson, and B. J. Carter.** 1989. Adeno-associated virus Rep protein synthesis during productive infection. J. Virol. **63:**873–882.
66. **Reynisdottir, I., H. E. Lorimer, P. N. Friedman, E. H. Wang, and C. Prives.** 1993. Phosphorylation and active ATP hydrolysis are not required for SV40 T antigen hexamer formation. J. Biol. Chem. **268:**24647–24654.
67. **Richardson, W. D., and H. Westphal.** 1983. Adenovirus early gene regulation and the adeno-associated virus helper effect. Curr. Top. Microbiol. Immunol. **109:**147–165.
68. **Ruffing, M., H. Heid, and J. A. Kleinschmidt.** 1994. Mutations in the carboxy terminus of adeno-associated virus 2 capsid proteins affect infectivity: lack of an RGD integrin-binding motif. J. Gen. Virol. **75:**3385–3392.
69. **Samulski, R. J., X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter.** 1991. Targeted integration of adeno-associated virus (AAV) into human chromosome 19. EMBO J. **10:**3941–3950.
70. **Shade, R. O., M. C. Blundell, S. F. Cotmore, P. Tattersall, and C. R. Astell.** 1986. Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J. Virol. **58:**921–936.
71. **Smith, R. H., Y. Zhao, and D. J. O'Callaghan.** 1994. The equine herpesvirus type 1 immediate-early gene product contains an acidic transcriptional activation domain. Virology **202:**760–770.
72. **Snyder, R. O., D.-S. Im, and N. Muzyczka.** 1990. Evidence for covalent attachment of the adeno-associated virus (AAV) Rep protein to the ends of the AAV genome. J. Virol. **64:**6204–6213.
73. **Snyder, R. O., R. J. Samulski, and N. Muzyczka.** 1990. In vitro resolution of covalently joined AAV chromosome ends. Cell **60:**105–113.
74. **Srivastava, A., E. W. Lusby, and K. I. Berns.** 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J. Virol. **45:**555–564.
75. **Urcelay, E., P. Ward, S. M. Wiener, B. Safer, and R. M. Kotin.** 1995. Asymmetric replication in vitro from a human sequence element is dependent on adeno-associated virus Rep protein. J. Virol. **69:**2038–2046.
76. **van Mansfeld, A. D. M., H. A. A. M. van Teeffelen, P. D. Baas, and H. S. Jansz.** 1986. Two juxtaposed tyrosyl-OH groups participate in ϕX174 gene A protein catalysed cleavage and ligation of DNA. Nucleic Acids Res. **14:**4229–4238.
77. **Vinson, C. R., T. Hai, and S. M. Boyd.** 1993. Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. Genes Dev. **7:**1047–1058.
78. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
79. **Walsh, C. E., A. W. Nienhuis, R. J. Samulski, M. G. Brown, J. L. Miller, N. S. Young, and J. M. Liu.** 1994. Phenotypic correction of Fanconi anemia in human hematopoietic cells with a recombinant adeno-associated virus vector. J. Clin. Invest. **94:**1440–1448.
80. **Ward, P., and K. I. Berns.** 1995. Minimum origin requirements for linear duplex AAV DNA replication *in vitro*. Virology **209:**692–695.
81. **Ward, P., E. Urcelay, R. Kotin, B. Safer, and K. I. Berns.** 1994. Adeno-associated virus DNA replication in vitro: activation by a maltose binding protein/Rep 68 fusion protein. J. Virol. **68:**6029–6037.
82. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244:**4406–4412.
83. **Weitzman, M. D., S. R. M. Kyostio, B. J. Carter, and R. A. Owens.** 1996. Interaction of wild-type and mutant adeno-associated virus (AAV) Rep proteins on hairpin DNA. J. Virol. **70:**2440–2448.
84. **Weitzman, M. D., S. R. M. Kyostio, R. M. Kotin, and R. A. Owens.** 1994. Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. Proc. Natl. Acad. Sci. USA **91:**5808–5812.
85. **Wonderling, R. S., S. R. M. Kyostio, and R. A. Owens.** 1995. A maltose-binding protein/adeno-associated virus Rep68 fusion protein has DNA-RNA helicase and ATPase activities. J. Virol. **69:**3542–3548.
86. **Yang, Q., A. Kadam, and J. P. Trempe.** 1992. Mutational analysis of the adeno-associated virus *rep* gene. J. Virol. **66:**6058–6069.