We have recently published the crystal structure of the adeno-associated virus type 2 superfamily 3 (SF3) helicase Rep40. Although based on its biochemical properties it is unlikely that Rep40 plays a central role as a replicative helicase the involvement of this motor protein in DNA packaging has recently been demonstrated. Here we focused our attention on residues that fall within and adjacent to the B’ motif of SF3 helicases that directly interact with single-stranded DNA during translocation of the motor protein. In vitro, alanine substitution at positions Lys-404 or Lys-406 abrogated the ability of the protein to interact with single-stranded DNA as demonstrated by electrophoretic mobility shift assay and fluorescence anisotropy, and accordingly these mutants could not unwind a partially duplex DNA substrate. Despite this loss of helicase activity, basal ATPase activity in these mutants remained intact. However, unlike the wild-type protein, K404A and K406A ATPase activity was not stimulated by DNA. As predicted, disruption of motor activity through interference with DNA binding resulted in an inability of Rep40 to package adeno-associated virus DNA in a tissue culture-based assay. Taken together, we characterized, for the first time in an SF3 helicase family member, residues that are directly involved in single-stranded DNA binding and that are critical for the Rep motor activity. Based on our findings we propose B’ as the signature motif of SF3 helicases that is responsible for the complex interactions required for the coupling of DNA binding and ATP hydrolysis.

**AAV**, best known for its potential as a gene therapy vector, is a human parvovirus that was first thought of as defective due to its inability to independently support its own replication. However, this categorization has been abandoned since it has become clear that the AAV lifecycle instead reflects a highly regulated system, consisting of both a productive replicative phase and latent infection (for a review, see Ref. 1). Productive replication occurs in the presence of helper virus functions, which can be provided by either adeno- (2, 3) or herpesviruses (4, 5). In the absence of helper virus coinfection, i.e. in healthy cells, AAV establishes a persistent infection. AAV accomplishes this by site-specifically integrating its genome into a defined locus on chromosome 19 (6). The ability of AAV to mediate site-specific integration is a feature that is unique among eukaryotic nuclear viruses and is perhaps the most intriguing aspect of the viral life style.

The AAV genome is a linear DNA molecule of ~4.7 kb and is flanked on either side by structural elements known as the inverted terminal repeats (7, 8). The inverted terminal repeats contain motifs that serve as the viral origin of replication, namely the Rep binding site and terminal resolution site (9). The right half of the genome encodes the cap open reading frame, generating the three viral capsid proteins (Vp1, -2, and -3). The left open reading frame, rep, encodes the four nonstructural proteins, Rep78, -68, -52, and -40, each designated according to their apparent molecular weight on high percent SDS-polyacrylamide gels (7, 10). The inverted terminal repeats, together with the virally encoded Rep proteins, play an essential role in virtually every aspect of the viral lifecycle.

The AAV Rep proteins possess biochemical activities consistent with their role in origin-dependent viral DNA replication, site-specific integration, transcriptional regulation, and virion assembly. These activities are separated into three domains, all of which are present in the largest of the Rep proteins, Rep78. The Rep amino terminus possesses specific DNA binding and endonuclease activity (11–13), while the central motor domain bears motifs necessary for ATPase and helicase activity as well as the nuclear localization sequence (14–16). The carboxyl-terminal zinc finger domain has been implicated in interaction with several cellular factors, although the biological significance of most of these interactions is not yet understood. The remaining Rep isoforms, Rep68, -52, and -40, are a combination of these functional domains arising from alternative splicing schemes and differential promoter usage within the rep open reading frame. Notably all four isoforms possess the motor domain at minimum represented by Rep40. This region is the most highly conserved among the parvoviral nonstructural proteins.

Most mutational studies have focused on the conserved helicase domain and have shown that disruptions in ATPase and/or helicase function disrupt several Rep activities whose active sites have been mapped to other domains. These activi-
ties include origin-specific DNA binding, endonuclease activity, and the ability to transactivate viral promoters (17, 18). The high level of interdependence between biochemical activities, and therefore biophysical domains, together with the redundancy of domain usage between the four Rep isoforms complicates the interpretation of these studies and the development of a consistent molecular model for the role of Rep in the AAV lifecycle. In efforts to address this difficulty, several laboratories have focused their attention on the study of the biophysical characterization of the AAV Rep proteins. These efforts have resulted in the crystal structure for the AAV5-derived Rep endonuclease domain (19, 20) as well as the crystal structure of AAV2 Rep40 (16).

The AAV2 Rep40 helicase is a member of the SF3 helicase family, which includes helicases encoded by small DNA and RNA viruses for which SV40 large T antigen (Tag) serves as the archetype (15). SF3 helicases are characterized by four highly conserved regions within a short stretch of ~100 amino acids: motifs A, B (also known as Walker motifs A and B), and C essentially make up the core of the helicase active site consisting of an NTP binding site, divalent metal cation coordination site, and sensor-1 site, respectively (21). Although the conservation of the fourth motif, B', among SF3 helicases has been recognized the role of these residues in DNA unwinding and ATP hydrolysis has not yet been defined on the molecular level (22). We have proposed previously that the fourth motif, B', is involved in single-stranded DNA binding necessary for helicase activity. Significantly Rep40 is structurally related to the AAA+ family of motor proteins (ATPases associated with diverse cellular activities) (16). Characteristic of the AAA+ architecture as well as that of other oligomeric NTPases is the presence of an "arginine finger" that translates ATP hydrolysis into intermolecular conformational changes in the context of a functional oligomeric complex (21, 23). Mutation of the conserved arginine finger in Rep abrogates helicase and ATPase function, consistent with a hexameric model for Rep40 (see Fig. 1 in this article, Ref. 16, and data not shown). This model is based on superimposition of AAV Rep40 with the recently published SV40 Tag hexamer structure (Fig. 1) (24).

Based on the structural information provided by our crystallographic studies, we have continued to build on our understanding of AAV Rep motor domain function through the characterization of mutants predicted to play a critical role in Rep helicase activity. In particular, we focused our study on two potential residues, lysines 404 and 406, that fall within and adjacent to the conserved B' motif, which we had hypothesized to play a role in single-stranded DNA interactions. In further support of the hypothesis that Rep40 motor activity plays a role in viral DNA packaging (25, 26), we showed that these Rep40 mutants were deficient in the ability to efficiently package AAV DNA into preformed virions. The position of Lys-404 and Lys-406 and their potential for single-stranded binding is illustrated by the models for both the Rep40 monomer and the putative hexamer that are shown in Fig. 1. In the latter model these residues are predicted to line the pore of the hexamer. However, the potential function of these residues is also consistent with a dimeric form of Rep40. Both Rep40K404A and Rep40K406A mutations led to a loss of single-stranded binding activity, helicase activity, and DNA-stimulatable ATPase activity as well as DNA packaging activity. Based on our findings we propose motif B' as the signature motif of SF3 helicases that is responsible for the complex interactions required for the coupling of DNA binding and ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Cloning of Mutant Rep Expression Constructs**—All mutant Rep proteins were generated using pHisRep40/15b, which contains the AAV2 Rep40 gene in a pET15b vector (Novagen). Rep40 variants pHisRep40K340H and pHisRep40R444A were generated by subcloning the region bearing the indicated mutation from already existing plasmids into pHisRep40/15b. Mutants pHisRep40K404A and pHisRep40K406A were generated using the QuikChange site-directed XL mutagenesis kit (Stratagene). For transfection experiments, Rep40 and its variants were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen) in which the neomycin resistance gene was removed. In all cases, regions generated by PCR were confirmed by sequencing.

**Transfection of 293T Cells and Preparation of Viral Supernatants**—Transfections were carried out in 10-cm plates (1.5 × 106 cells) using a total of 12 μg of DNA/plate by calcium phosphate precipitation. 16 h post-transfection, the cells were infected by adeno virus type 5 at a multiplicity of infection of 2 and further incubated for 48 h. Cells were then harvested, washed in phosphate-buffered saline, and lysed by three rounds of freeze-thawing (~80 °C and 37 °C). Cellular debris were pelleted by centrifugation, and the virus-containing supernatants were reserved for further analyses.

**Determination of Infectious Viral Titers**—Infectious titers were determined by replication center assay as described previously (25).

**Western Blot Analyses**—Rep and Vp expression was confirmed using monoclonal antibodies 303.9 and B1 specific for the Rep and Vp proteins, respectively, as described previously (28).

**Preparation of Capsids and Viral DNA for Quantification**—Viral supernatants were processed as described in Ref. 25. Total capsid quantification was determined by A20 capsid enzyme-linked immunosorbent assay as described previously (29). Encapsidated DNA was quantified by dot blot analysis as described previously (25).

**Coimmunoprecipitations**—Re(move-capsid complex formation was examined by coimmunoprecipitation as described previously (25, 26). Briefly 293T cells cultured in 10-cm plates were transfected by calcium phosphate coprecipitation using 8 μg of Rep40-expressing plasmid (pBlueScript as a control) and 4 μg of pCMV-Vp (encodes the AAV Vp proteins under the control of the CMV promoter). The cells were harvested 3 days post-transfection, pelleted, and resuspended in 1 ml of radioimmuneprecipitation assay buffer. Cell debris were pelleted by centrifugation, and the supernatant was reserved for coimmunoprecipitation. Immunoprecipitations were performed in 60 μl of protein A-Sepharose beads (swelled in 10% NET-N buffer) incubated with an anti-Rep polyclonal rabbit antiserum (αRep90). After washing with 100% NET-N buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40), 100–200 μl of cell lysate was incubated with the beads for 1 h at 4 °C. The beads were then washed, resuspended in SDS-PAGE sample buffer, and subjected to gel electrophoresis prior to Western blot analysis with a monoclonal Vp antibody (B1). Control lysates to verify Rep and Vp detection were prepared from lysates derived from HeLaRC cells (Rep/Cap stable cell line) that were superinfected with adeno virus.

**Recombinant Protein Isolation**—Protein isolation was performed on a Ni2+–nitrilotriacetic acid column chromatography from 1-liter cultures of BL21(DE3)pLysS cells prepared and induced according to the manufacturer's instructions (pET vectors, Novagen). Proteins were eluted in 300 mM imidazole and digested with thrombin to remove the His6 tag at 4 °C overnight while dialyzing against 1× thrombin buffer (25 mM Tris-HCl, pH 8, 200 mM NaCl, 10% glycerol, and 1 mM dithiothreitol). Thrombin cleavage was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. Following thrombin cleavage, the protein was dialyzed into MonoS buffer A (25 mM HEPES, pH 6.0, 50 mM NaCl), applied to a 5-ml S/F Sepharose column (Amersham Biosciences), and eluted via continuous gradient formed by Buffer B (25 mM HEPES, pH 6.0, 1 mM NaCl). His6 tag fragments were removed by reapplying the sample to a HiTrap column after which residual nickel was eluted by the addition of 1 mM EDTA. The protein was then applied to a HiLoad 16/60 Superdex 200 gel filtration column equilibrated in 20 mM HEPES, pH 7.5, 200 mM NaCl, and 1 mM TCEP. Prior to storage at −80 °C, the protein was dialyzed into a buffer containing 25 mM Tris-Cl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 20% glycerol. Protein concentration was estimated by Coomassie Blue staining and SDS-glycyrhydrinate gel electrophoresis using wild-type Rep40, quantified by Bio-Rad Protein Assay reagent, as a reference standard. In general, 1-liter bacterial cultures yielded between 2 and 9 mg of recombinant protein. Purity of isolated proteins was assessed by Coomassie Blue staining following SDS-PAGE and determined to be ≥98% for all proteins used in these studies. Proteins used in equilibrium binding experiments were further purified by passage through a Superdex 200 10/30GL...
column equilibrated in 25 mM Tris-Cl, pH 8.0, 200 mM NaCl, 1 mM TCEP, 0.2% CHAPS, and 5% glycerol. Following this, the proteins were concentrated using centrifugal filter devices (Millipore). The protein concentration was determined by absorbance at 280 nm using an absorbance coefficient of 56,240 and 104,831 M$^{-1}$ cm$^{-1}$ for Rep40 and Rep68 proteins, respectively, which were calculated based on amino acid composition.

Electrophoretic Mobility Shift Assays—The ability to bind nonspecifically to single-stranded DNA was assessed by gel assay using a single-stranded oligonucleotide substrate (5'-CACTCGGGTCCTTGT-CAGGTTCCTGTTACCTGCTTGT-3') containing a 5' biotin label (GeneLink). Assays were performed as follows. In a total reaction volume of 15 μl, the indicated amount of protein was incubated with 20 fmol of biotin-labeled DNA in a buffer consisting of: 0.5x TBE, pH 8.3, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 3 μl of loading buffer (40% sucrose, 0.1% xylene cyanol, and 0.1% bromphenol blue in 0.5x TBE). Reactions were incubated for 45 min at room temperature, loaded onto a 5% native polyacrylamide gel in 0.5x TBE, and run at 18 V/cm. The DNA substrate was then transferred to a nylon membrane (MagnaGraph, Osmonics Inc.) and UV cross-linked. Visualization of protein-DNA complexes was performed using the LightShift chemiluminescence EMSA kit (Pierce) according to the manufacturer’s protocol.

The same single-stranded oligonucleotide substrate, 5'-CACTCGGGTCCTTGT-CAGGTTCCTGTTACCTGCTTGT-3', was kinase-labeled with [γ-32P]ATP using a MEGALABEL kit (Takara Bio Inc.) and then isolated on an 8% polyacrylamide, 0.5x TBE gel. Following gel isolation, overnight elution into Tris-EDTA, and ethanol precipitation, the oligo concentration was determined from cpm measured by liquid scintillation.

The radiolabeled EMSAs were performed at 4 °C as follows. In a total reaction volume of 15 μl, the indicated amount of protein was incubated with 50 fmol of radiolabeled DNA in a buffer consisting of 0.5x TBE, pH 8.3, 1 mM dithiothreitol, 0.05% Nonidet P-40, 5 mM MgCl₂, and 3 μl of loading buffer (40% sucrose in 0.5x TBE). Reactions were incubated for 45 min at room temperature, loaded onto a 5% native polyacrylamide gel in 0.5x TBE, and run at 18 V/cm.

Standard DNA Helicase Assay—The ability of Rep40 to unwind DNA was assessed using an M13-based partially duplex substrate as described previously (16). Except where indicated, helicase reactions contained 14 pmol of Rep40, 0.2 mM ATP, 10 mM MgCl₂, and 50 pmol of M13 DNA substrate in a total reaction volume of 15 μl. Reactions were incubated at 37 °C for 45 min.

Colorimetric ATPase Assay—ATP hydrolysis was measured as a function of the liberation of inorganic phosphate. Assays were performed in 96-well plates using 0.5 mM ATP, 0.2% CHAPS, and 5% glycerol. Following the addition of protein, the reaction volume of 15 μl, the indicated amount of protein was incubated and substrate (final concentration, 5 nM) were incubated at 20 °C for 15 min. After a 2 min incubation, 25 μl of 34% citric acid was added to quench ATP formation in the reaction, and color development was allowed to proceed for 25 min at room temperature. Absorbance at 660 nm was measured, and the amount of inorganic phosphate released was quantified by comparison to a standard KH₂PO₄ curve (phosphate standard solution, Sigma). Error bars were calculated based on three independent assays.

Fluorescence Anisotropy Assay—Fluorescence anisotropy experiments were performed on a Beecon 2000 fluorescence polarization system (Panvera). The DNA substrate used was a single-stranded oligonucleotide 5'-go(dT)₃₈ modified by a 5' biotin label (GeneLink). The DNA substrate used was a single-stranded oligonucleotide substrate (5'-CACTCGGGTCCTTGT-CAGGTTCCTGTTACCTGCTTGT-3'). The DNA substrate in a total reaction volume of 15 μl. Reactions were incubated at 37 °C for 45 min under helicase assay buffer conditions (15 mM HEPES, pH 7.5, 0.05% Nonidet P-40, and 5 mM dithiothreitol, 0.05% Nonidet P-40, and 3 μl of loading buffer (40% sucrose in 0.5x TBE). Reactions were incubated for 45 min at room temperature, loaded onto a 5% native polyacrylamide gel in 0.5x TBE, and run at 18 V/cm.

Identification of Potential ssDNA Binding Mutants within the Motor Domain of AAV2 Rep40—Fig. 1A highlights the positions of Lys-404 and Lys-406 within the monomer structure of Rep40 that we have determined recently. In addition, based on superposition onto the hexamer structure of SV40 T-antigen solved by Li et al. (24) we generated a hexameric model for Rep40 shown in Fig. 1B. An ~18-A pore through which single-stranded DNA can pass during the process of DNA unwinding is formed in this model (16). Protruding into the central pore are a series of loops, analogous to those found in other hexameric helicases, that we hypothesized to play a role in single-stranded DNA interactions (23, 34, 35). In Rep40, the loop is part of a β-hairpin that projects from the core of the protein and is located between motifs B and C. Residues that reside in the first half of this hairpin (strand βa) form part of the B’ motif. Residues 404 and 406 (designated according to their position within the Rep68 sequence), which fall within and just outside the B’ motif, respectively, are located at the tip of the β-hairpin and may be involved in DNA interactions.

Rep40 Residues Lys-404 and Lys-406 Are Required for Single-stranded DNA Interactions—To assess the potential role of Lys-404 and Lys-406 in single-stranded DNA interactions in
the context of Rep40 motor activity, alanine mutants were generated and isolated as recombinant proteins in bacterial cultures. The Rep40 variants were then tested for their ability to interact with ssDNA as measured by EMSA. The ability of both Rep40K404A and Rep40K406A to bind to the single-stranded DNA probe was severely diminished compared with the wild-type counterpart (Fig. 2A). While the ability for DNA binding by K404A was lost (Fig. 2A, top panel), K406A retained weak ssDNA binding activity although only at the highest concentrations tested (Fig. 2A, bottom panel). To exclude the possibility of a Rep interaction with biotin, we performed EM-SAs using a radiolabeled substrate. The results of these assays were in agreement with those shown in Fig. 2A (data not shown). To further examine the influence of these residues on ssDNA interaction, we carried out equilibrium binding assays using fluorescence anisotropy. The resulting binding isotherms are shown in Fig. 2B. The dissociation constant ($K_d$) determined for Rep40 is 2.7 µM. In the case of mutants Rep40K404A and Rep40K406A, the isotherms showed a drastic reduction in their ability to bind DNA with an estimated $K_d$ of $>100$ µM. Unfortunately we were unable to obtain saturation due to the tendency of these mutants to precipitate at high concentration. Overall these results are consistent with the hypothesis that these residues play a central role in ssDNA binding. Interestingly, under the reaction conditions used, DNA binding did not require the presence of an NTP or analog as has been demonstrated for other hexameric helicases (for a review, see Ref. 36).

Biochemical Characterization of Wild-type AAV2 Rep40 Helicase Activity—To assess the motor activity of the K404A and K406A variants, standard assays were developed using the wild-type Rep40 protein. Helicase assays were performed using an M13mp18-based substrate to which a short oligo (20-mer) was annealed. As shown in Fig. 3A (left panel), wild-type Rep40 isolated from bacterial cell cultures was active in our assay at as little as 0.7 µM (10.7 pmol). To confirm that the observed helicase activity was attributable to Rep40 and not due to a co-purifying contaminant, we generated the NTP-binding (Walker A motif) mutant Rep40K340H as well as the arginine finger mutant Rep40R444A (16). As expected, these two mutants were negative for helicase activity (Fig. 3A, right panel) (1). Colorimetric ATPase assays were also performed and showed that wild-type Rep40 ATPase activity was greatly stimulated by DNA (Fig. 3B), consistent with previous findings (37). Notably both sonicated salmon sperm DNA (with an abundance of single-stranded overhangs) and single-stranded circular M13 DNA stimulated Rep40 ATPase activity (data not shown).

In general, the results of our biochemical characterization of Rep40 motor activities largely correlated with those recently published by Collaco et al. (37) with respect to NaCl sensitivity, MgCl$_2$ concentration, NTP requirements, and DNA-stimulable ATPase activity. However, our assessments differed on two significant points. While Collaco et al. (37) observed 100% helicase activity in the presence of Mn$^{2+}$, we found that Rep40 helicase activity was not efficiently supported by Mn$^{2+}$ in agreement with what has been shown previously for Rep68 (Fig. 3C) (38–40). Second we observed maximal helicase activity at an ATP concentration of only 200 µM. This is ~5- and 12.5-fold less than previously described for both Rep40 and Rep52, respectively (Fig. 3D) (32, 37). It should be noted that the amino-terminal His$_6$ tag used for isolation in our studies was removed to prevent artificial oligomerization and/or divergent metal-dependent artifacts, while the recently characterized Rep40 retained a carboxyl-terminal His$_6$ tag (37), and the biochemical characterization of Rep52 was performed using a recombinant protein that contained an amino-terminal maltose binding domain (32).

Both Lys-404 and Lys-406 Are Required for Single-stranded DNA Interactions in the Context of Rep40 Helicase and ATPase Activities—To examine the role of residues Lys-404 and Lys-
in Rep40-mediated DNA unwinding, the mutants were tested by the standard helicase assay established above. Alanine substitution at either position 404 or 406 completely abrogated helicase activity (Fig. 4A). A general characteristic of helicases is a DNA-stimulatable ATPase activity (for a review, see Ref. 36). We therefore tested the ability of K404A and K406A to hydrolyze ATP in the presence and the absence of DNA. Both mutants displayed basal levels of ATPase activity comparable to the wild-type enzyme (Fig. 4B), thereby eliminating the possibility that the loss of helicase activity was not due to misfolding of the protein as a result of the mutation. However, the ATPase activity of K404A or K406A was not stimulated by the addition of either sonicated salmon sperm DNA or single-stranded circular M13 DNA (not shown) to the reaction. This was in direct contrast to the wild-type enzyme, which displayed a nearly 8-fold enhancement of ATPase activity in the presence of DNA (Figs. 3B and 4B and Ref. 37). Again these results are in accordance with a DNA binding defect as predicted by our model.

We also examined the ATPase and helicase activities of K404A and K406A mutations in the larger Rep40 counterpart, Rep68, which differs from Rep40 by the addition of the amino-terminal origin interaction domain. The ATPase activity of Rep68 bearing either the K404A or K406A mutation was not affected (data not shown). However, while mutation of K404A still resulted in a helicase-negative phenotype, we were surprised to find that mutation of Lys-406 in the context of Rep68 had no effect on helicase activity (Fig. 4C).

We therefore tested the possibility that Rep40K406A possessed helicase activity, albeit at high molar concentrations, since this variant possesses the wild-type Lys-404 and thus the potential to interact with DNA. The observed loss of activity...
was not due to a need for a higher concentration of protein, i.e.
to compensate for a decreased affinity for the DNA substrate,
as titration of the protein to amounts as great as 224 pmol did
d not recover any activity (Fig. 4C, right panel). Thus, despite the
ability to interact weakly with ssDNA the K406A mutant pos-

ses an intrinsic defect in its ability to unwind DNA, possibly
indicating differences between Rep68 and Rep40 in the molec-
ular mechanism of DNA unwinding.

**Effect of Lys-404 and Lys-406 Mutation on Rep68 Binding to ssDNA**—To further understand the effect of Lys-404 and Lys-
406 mutations on Rep68, we carried out equilibrium binding assays using fluorescence anisotropy as described for the Rep40 protein. Binding constants were calculated for Rep68 wild type and variants. As shown in Fig. 4D, the binding constant of Rep68 is 15 nM. Mutation of Lys-404 or Lys-406 did not diminish Rep68 affinity for single-stranded DNA. The affinity of Rep68 for ssDNA was ~200-fold greater than that of Rep40, indicating that the origin binding domain of Rep68 dictates the binding for ssDNA under our experimental conditions.

Rep40 Residues Lys-404 and Lys-406 Are Necessary for Efficient AAV DNA Packaging into Capsids—Despite its biochemical properties, it is unlikely that Rep40 plays a central role as a replicative helicase. The involvement of this motor protein in DNA packaging has been demonstrated recently (25, 28). The predominant model of this process involves a direct interaction between the small Rep proteins and the assembled AAV capsid. After formation of the pre-encapsidation complex, AAV genomes are thought to be “pumped” into the capsid in a 3’ to 5’ direction by virtue of small Rep helicase activity (25, 26).

To determine whether Lys-404 and Lys-406 played a role consistent with the biological function of Rep40 in the AAV lifecycle these mutated residues were tested in the context of the previously reported packaging assay (25). Briefly, plasmid pTAV2.1, containing a mutation of the start methionine of the Rep40/K2 in an otherwise wild-type AAV genome (represented by pTAV2.0), was cotransfected into 293T cells with a plasmid expressing Rep40 or a variant, thereby complementing in trans the lack of small Rep expression in pTAV2.1. Appropriate Rep and Vp expression, relevant to the plasmids used in each transfection, were confirmed by Western blot analyses (Fig. 5A).

In the absence of small Rep protein expression from pTAV2.1, packaging efficiency was markedly reduced compared with the corresponding wild-type setting, pTAV2.0 (Fig. 5B, top panel, compare pTAV2.1 with wild-type AAV represented by pTAV2.0). However, wild-type packaging efficiencies were restored when pTAV2.1 was cotransfected with either a wild-type Rep40-expressing plasmid or plasmids encoding wild-type Rep40. B, top panel, packaging efficiency expressed as the ratio between total capsids (empty and full, quantified by enzyme-linked immunosorbent assay) and the amount of packaged DNA (as determined by dot blot using a Rep-specific probe). The results presented are derived from three independent transfections. B, bottom panel, virus infectivity expressed as the ratio between total capsids and infectious units (determined by infectious center assay). C, top and center panels, Western blot analyses performed on cell extracts used for coimmunoprecipitation assays using an anti-Rep antibody (top) and anti-capsid antibody (center). 10 μg of total protein was loaded onto each lane. wt, wild type. C, bottom panel, the capacity of the Rep40 variants to interact with capsid proteins was demonstrated by coimmunoprecipitation assays. Complexes were immunoprecipitated from cell extracts using an anti-Rep antiserum and analyzed by Western using a monoclonal anti-Vp antibody. The positions of the Rep (R) and Vp (VP) proteins are indicated as is the position of the immunoglobulin heavy chain (IgG) present in the Rep antiserum used for immunoprecipitation. Whole precipitate was loaded on each lane.

FIG. 5. Lys-404 and Lys-406 are necessary for efficient packaging of AAV DNA into capsids. A, Western blot of a representative transfection using an anti-Rep antibody (top) and an anti-Vp antibody (bottom). 10 μg of total protein was loaded onto each lane. wtR40, wild-type Rep40. B, top panel, packaging efficiency expressed as the ratio between total capsids (empty and full, quantified by enzyme-linked immunosorbent assay) and the amount of packaged DNA (as determined by dot blot using a Rep-specific probe). The results presented are derived from three independent transfections. B, bottom panel, virus infectivity expressed as the ratio between total capsids and infectious units (determined by infectious center assay). C, top and center panels, Western blot analyses performed on cell extracts used for coimmunoprecipitation assays using an anti-Rep antibody (top) and anti-capsid antibody (center). 10 μg of total protein was loaded onto each lane. wt, wild type. C, bottom panel, the capacity of the Rep40 variants to interact with capsid proteins was demonstrated by coimmunoprecipitation assays. Complexes were immunoprecipitated from cell extracts using an anti-Rep antiserum and analyzed by Western using a monoclonal anti-Vp antibody. The positions of the Rep (R) and Vp (VP) proteins are indicated as is the position of the immunoglobulin heavy chain (IgG) present in the Rep antiserum used for immunoprecipitation. Whole precipitate was loaded on each lane.

To be sure that the differences that we observed in both packaging and infectivity using the Rep40 variants was not due to an inherent defect in the ability to form pre-encapsidation complexes as a consequence of the mutations, coimmunoprecipitation experiments were performed. Using a Rep-specific antiserum, capsid proteins were effectively immunoprecipitated in the presence of each Rep40 variant tested in amounts comparable to the wild-type protein (Fig. 2C, bottom panel). Thus, the packaging defects observed were due to an in-
ability of the Rep40 variants to interact with the AAV capsid but rather the result of a step presumably occurring after complex formation. Significantly Rep40G490Δ was also able to efficiently form complexes with AAV virions, demonstrating that the carboxyl-terminal portion that is absent from this Rep40 variant (~90 amino acids) is not necessary for capsid interactions in contrast to a previous suggestion (41). Taken together, the effect of Lys-404 and Lys-406 mutations on the ability of Rep40 to function in the context of the AAV DNA-packaging system suggested an overall biochemical defect related to its motor activity.

**DISCUSSION**

A structural model for the active form of the Rep40 helicase has been proposed based on the information provided by the crystal structure (16). Although the oligomeric state(s) of Rep40 in vivo has not yet been elucidated, the presence of a functional arginine finger strongly argues for an active state that is larger than a monomer (16). A hexameric model of Rep proteins is consistent with the recently published structure of the SV40 TAg catalytic domain as well as those proposed for several other hexameric helicases (24, 36). Based on the position of positively charged residues found on β-hairpin loops extending into the central pore of the putative hexamer and the function of such residues in other hexameric NTPases, we hypothesized that lysine residues Lys-404 and Lys-406 may play a role in the ability of Rep proteins to interact with single-stranded DNA (1).

Based on the results presented here, we have shown that Lys-404 and Lys-406 are critical residues involved in Rep40-DNA interactions. These findings are supported by EMSAs and fluorescence anisotropy assays. In addition, data from ATPase and helicase assays showed that residue Lys-404 plays a critical role in the mechanism of ATP-dependent DNA unwinding. The role of Lys-406 was obscured in the context of Rep68 as the presence of the additional DNA binding activity of the origin binding domain dominates, and both mutants exhibited wild-type binding (Fig. 4D). Surprisingly, when helicase activity of the Rep40 and Rep68 variants was tested, different effects were observed between Rep40 and Rep68 bearing the Lys-406 mutation. To our knowledge, this is the first time the same mutation studied in two Rep isoforms has displayed a different phenotype. A simple explanation may be that while Lys-404 plays a critical during DNA unwinding, Lys-406 imparts only additional binding affinity thus playing a secondary role during this reaction. This is implied by the complete conservation of Lys-404 throughout the SF3 helicase family. In contrast, the conservation of Lys-406 is less stringent (16). In the case of Rep40 where most of the DNA affinity was limited to the aforementioned lysine residues located in β-hairpin 1, a mutation of either Lys-404 or Lys-406 would severely impair the formation of the initial protein-DNA complex that precedes the unwinding step, and thus both mutants are helicase-negative. However, this is not the case for Rep68, which bound ssDNA with ~200-fold stronger affinity than Rep40 due to the additional DNA binding domain. The Rep68K406A mutant thus is not the case for Rep68, which bound ssDNA with ~200-fold stronger affinity than Rep40 due to the additional DNA binding domain. The Rep68K406A mutant thus is helicase-positive due to the presence of both this domain and the critical residue Lys-404. A detailed kinetic and biochemical analysis is underway to determine the nature of these differences.

Thus, ssDNA binding through residue Lys-404 on the “DNA sensor loop” induces conformational changes that couple ATP hydrolysis to translocation of the active helicase complex along the DNA substrate. The basis of this mechanism has been laid out in the seminal report describing the RecA protein (42). In RecA similar loops containing lysine residues have the potential to interact with the phosphate groups of DNA. The binding induces conformational changes directly into the catalytic residues located in the Walker B motif and motif C. In Rep proteins motif B’ (containing and adjacent to Lys-404 and Lys-406, respectively) is located similarly between these ATPase active site motifs (highlighted in Fig. 6). Thus we suggest that motif B’, which is only present in SF3 helicases, plays multiple roles during the helicase reaction. Some residues are directly involved in DNA binding (Lys-404 and Lys-406); others are involved in the interaction with ATP in the context of an oligomeric nucleotide binding pocket (42). Another set of residues within this motif then couple DNA binding to ATP catalysis. A precedence for the coupling of ssDNA binding and ATP hydrolysis in the context of a hexamer ring is provided by the proposed mechanism for DNA translocation by the T7gp4 ring helicase (35). Single-stranded DNA binding loops within the T7gp4 helicase are asymmetrically positioned within the central pore. Upon nucleotide binding and hydrolysis, rotation of the subunits around the pore results in the translocation of the DNA through the ring.

Drawing from precedence provided by studies of SV40 TAg and other members of both the SF3 helicase and AAA+ superfamilies, it is likely that the active form of Rep78/68 helicase is a hexamer. Paradoxically there is little biophysical evidence suggesting that Rep40 could multimerize. Analytical ultracentrifugation experiments indicated that Rep40 is mostly a monomer even at high concentrations (data not shown). This is supported by data that suggests that the Rep52 isoform is monomeric as illustrated by gel filtration studies (32). In support of the oligomeric model for Rep40 motor function, we demonstrated that mutation of the putative arginine finger, Arg-444, completely abrogated the motor functions of Rep40 (Fig. 3A) as previously demonstrated for Rep68 (16). This is significant as arginine fingers (in other NTPases proteins shown to be functionally relevant only in the context of oligomers) serve to convert ATP hydrolysis into intersubunit conformational changes within a functional motor complex (21, 23). Additionally we observed at least three different protein-DNA complex species by EMSA (Fig. 2), suggesting that different multimeric combinations of both protein and DNA are possible in the context of DNA binding. Our in vitro studies do not preclude the possibility that the DNA binding function of residues Lys-404 and Lys-406 could occur in the context of a smaller oligomeric form of Rep40, for instance a dimer. Additionally the differential K406A phenotype between Rep68 and Rep40 with respect to helicase activity implies that an alternative conformation of the motor domain exists between the Rep isoforms, possibly differing in the number of active subunits. Ongoing studies in our laboratory are focused on addressing the oligomerization state of Rep40 and Rep68 in vitro and in vivo.

Previous studies have targeted Lys-404 and Lys-406 for mutation. In all cases, a replication-associated defect, consistent with a loss in helicase activity, was observed (17, 18, 27). These observations are consistent with the data provided here as we showed that both Lys-404 and Lys-406 are necessary for efficient ssDNA binding in the context of small Rep helicase activity and its associated motor functions, namely DNA-stimulated ATPase activity. In the context of AAV packaging, Rep40 Lys-404 mutants have been examined and displayed defects in capsid packaging (25, 26), observations that were confirmed by this study. Again the defect in capsid packaging is consistent with a ssDNA binding defect and supports the existing model that the small Rep proteins act to “pump” AAV genomes into the preformed capsid by virtue of their helicase activity (25, 26). Notably Rep40 basal (non-DNA-stimulated) ATPase activity (Fig. 4) was not sufficient for its function in packaging,
further supporting the role of Rep in helicase-mediated translocation of AAV genomes into capsids. Interestingly the K406A mutant, despite a total loss of helicase activity, was able to produce a number of infectious virions compared with the K404A mutant. It is important to note that the role of the large Rep proteins in packaging, expressed in all the packaging assays presented here, has not yet been clearly defined. It has been proposed that they act to traffic newly replicated genomes (possibly still covalently linked to the Rep protein via the endonuclease reaction) to the site of virion assembly and packaging by virtue of their interaction with the capsids themselves (and therefore possibly the small Rep proteins) (25, 26). While we were able to rule out a defect in the ability of the Rep40 mutants to interact with capsids as a possible source of the observed differences in virion production (Fig. 5), we did not examine the possibility that Rep40 interacts with the larger Rep proteins. The molecular mechanisms underlying AAV genome packaging, particularly with respect to the formation of the pre-encapsidation complex, therefore must be addressed.

The elucidation of the crystal structure of the Rep motor domain has made possible extensive structure-function studies that will allow for the elucidation of the molecular mechanisms underlying Rep motor activity as well as those of SF3 helicases in general. Taken together, we propose the B/H11032 signature motif of SF3 helicases that is responsible for the complex interactions required for the coupling of DNA binding and ATP hydrolysis. Based on the structural alignment of SF3 helicases (Fig. 6) we propose that the motif B/H11032 could be extended to include the residues that are involved in the formation of the second strand of the β-hairpin including lysine 406 (involved in DNA binding) as well as Gln-410 and Ile-411 that are conserved in all SF3 family members.

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