Adeno-Associated Virus Rep78/Rep68 Promotes Localized Melting of the Rep Binding Element in the Absence of Adenosine Triphosphate

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We have applied fluorescence anisotropy and molecular beacon fluorescence methods to study the interactions between the Adeno-associated virus Rep78/Rep68 protein and the 23-bp Rep binding element (RBE). Rep78/Rep68 stably interacted with both the single- and double-stranded conformations of the RBE, but the interaction mechanisms of single- and double-stranded DNA appeared to be fundamentally different. The stoichiometry of Rep78 association with both the separate top and bottom strands of the RBE was 1:1, and the relative dissociation constant (K_D) values of these associations were calculated to be 2.3×10^{-8} and 3.2×10^{-8} M, respectively. In contrast, the stoichiometry of Rep78 association with the double-stranded RBE was 2:1, and the dissociation constant was determined to be 4.2×10^{-15} M^2. Moreover, Rep78/Rep68 interaction with the 23-bp duplex RBE appeared to cause localized melting of the double-stranded DNA substrate in the absence of adenosine triphosphate (ATP). This melting activity showed slower kinetics than binding and may contribute to the initiation of ATP-dependent Rep78 helicase activity.

Introduction

The Adeno-associated virus (AAV) is a single-stranded DNA virus flanked by repeated sequences that self-anneal to form terminal, hairpin structures. The small AAV genome contains only two open reading frames, *rep* and *cap*. The Rep proteins are a family of overlapping, alternatively spliced peptides that are transcribed from two AAV promoters, p5 and p19. The larger p5 Rep proteins, Rep78 and Rep68, possess a number of characterized biochemical activities, including single-stranded DNA binding, sequence-specific double-stranded DNA binding, adenosine triphosphatase, 3′ to 5′ DNA helicase, and sequence-specific single-stranded cleavage activities.[1, 2] During AAV replication, Rep68 and Rep78 bind to the AAV terminal repeats (TRs) and introduce a single-stranded nick into the downstream terminal resolution site (*trs*) to create a 3′-hydroxy primer used to initiate repair synthesis of the viral TRs.[3] Initial Rep interaction with the TRs during this activity is directed through a double-stranded DNA sequence termed the Rep binding element (RBE).[4–10]

The 23-bp duplex RBE was initially defined by mutational analysis and is both necessary and sufficient for stable Rep interaction with double-stranded DNA.[6, 7, 9–11] Interference assays indicate that Rep makes a number of binding contacts with both strands of the RBE, centered around the core tetranucleotide repeat, 5′-GAGC-3′.[6] However, these same assays indicate that the actual base contacts are context dependent and vary between linear and hairpin TR substrates, a concept supported by oligonucleotide selection assays and mutagenesis studies that indicate some degeneracy to Rep binding specificity.[6, 12] RBE homologues are also present at the AAV p5 promoter and the proviral integration site on human chromosome 19, and mutation of these sequences disrupts both viral transcription and proviral integration.[7, 13–15] Despite the importance of this process to the AAV life cycle, the mechanism by which Rep mediates these viral activities through RBE homologues is unknown.

It is now clear that the RBE is required for at least two reasons during *trs* cleavage. First, the RBE aligns the Rep complex along the AAV TR, thereby directing cleavage to the downstream *trs*.[4, 5, 8, 10] Second, Rep requires an RBE homologue to unwind the double-stranded *trs* and form the single-stranded nicking intermediate.[16, 17] This second RBE requirement is a curious feature of AAV biochemistry. Rep is unable to unwind blunt, double-stranded DNA substrates unless these DNAs contain an RBE. Yet, Rep does not require an RBE to unwind duplex DNAs with extensive 3′ single-stranded tails, a fact suggesting that Rep helicase activity can be initiated through a single-stranded DNA intermediate.[17–19] Apparently, the RBE facilitates formation of an appropriate initiation intermediate in the absence of a single-stranded tail. Since Rep requires adenosine triphosphate (ATP) as

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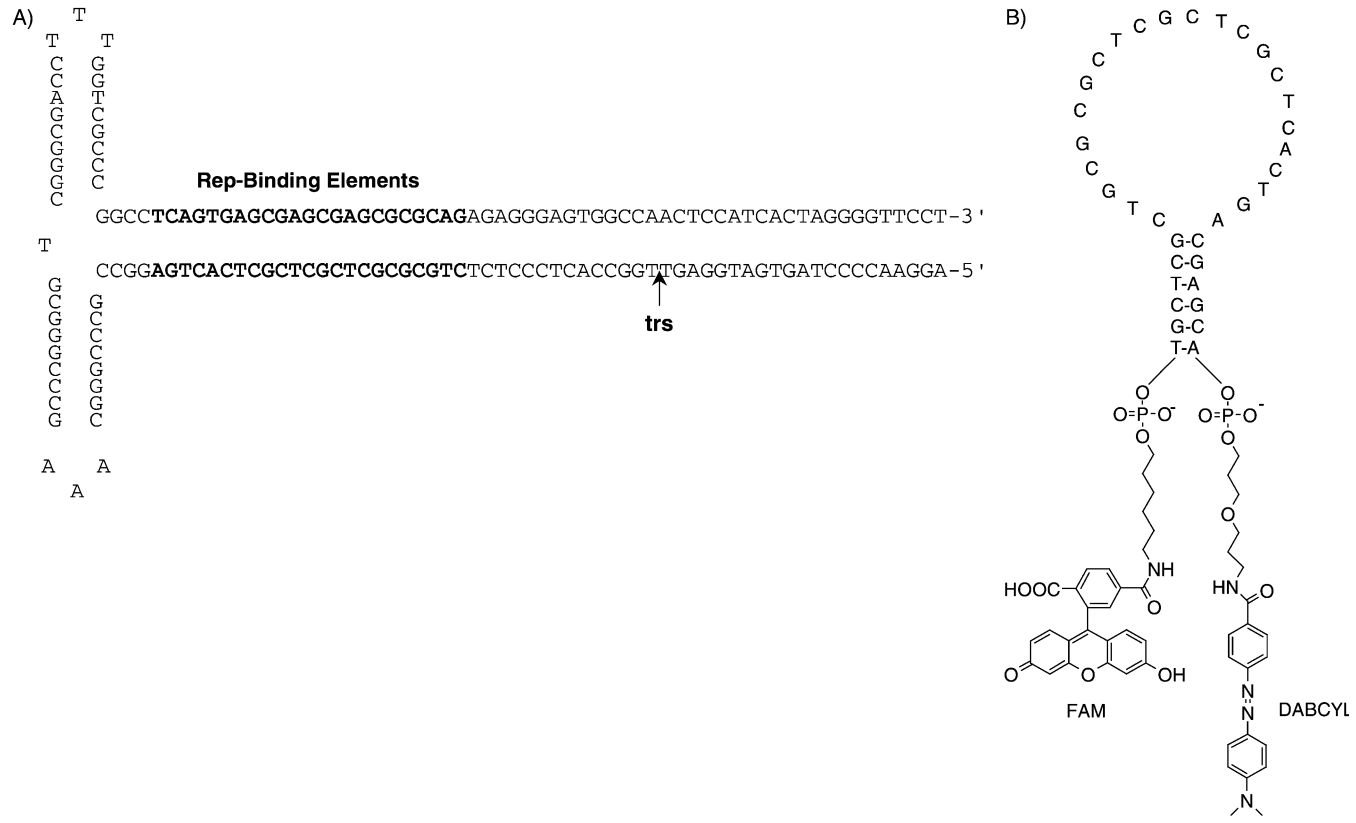

Scheme 1. *A) Schematic representation of the AAV TR showing the RBE. B) Structure of the MB1 probe.*

a cofactor during helicase activity it has been assumed that Rep is able to promote ATP-dependent DNA melting after association with the RBE.

Enzymatic activities such as DNA melting are dynamic events, yet most traditional techniques assay the accumulation of reaction products and, hence, do not give real-time data. Recently, several fluorescence methods have been developed to accurately monitor molecular interactions at equilibrium in real time. These include fluorescence anisotropy and molecular beacon (MB) fluorescence.[20–25] MBs are DNA oligonucleotides labeled with a fluorophore at one end and a fluorescent quencher at the other end. These moieties are held in close proximity by self-annealing terminal sequences, which cause the MB to adapt a stem-loop structure. This juxtaposition of fluorophore and quencher effectively quenches fluorescence by energy transfer. When a molecule binds the target sequence within the MB loop, the DNA undergoes conformational reorganization, the stem is opened, and fluorescence is restored.

To better understand the mechanism of Rep interactions with the RBE, we have synthesized fluorescent probes derived from the AAV TRs and characterized the interaction of purified Rep78/Rep68 with both the single-stranded and double-stranded conformations of the RBE (Scheme 1). Our data indicate that Rep is able to interact stably with both strands of the RBE in a sequence- and salt-dependent manner. Initial interaction with single-stranded RBE substrates appears to be mediated by a Rep monomer although higher order complexes are also observed at higher protein concentrations. In contrast, Rep78/Rep68 appears to bind the double-stranded RBE as two monomers. Furthermore, Rep interaction with the duplex RBE appears to promote localized melting of double-stranded sequences in the absence of ATP.

Results

Kinetics of Rep association with the single-stranded RBE

Rep interaction with the RBE located within the viral TRs is required for a number of viral activities, including DNA replication and transcription. However, the nature of Rep contacts with the RBE is not known. Previous in vitro studies demonstrated that Rep makes contact with both strands of the double-stranded RBE during binding to the AAV TR.[6] Given the affinity of Rep for single-stranded DNA, this observation raises the possibility that Rep may interact discretely with the individual strands that comprise the duplex RBE.[2, 26] To investigate the nature of the Rep interaction with the RBE, a molecular beacon (MB) was synthesized containing 23 nucleotides from the bottom strand of the AAV RBE flanked by a short stretch of complementary nucleotides that anneal in solution to form a stem-loop structure (Scheme 1). This MB substrate was labeled with a fluorophore (6-carboxyfluorescein, FAM) at the 5′ end and a quencher moiety (4-(4′-dimethylaminophenylazo)benzoic acid, DABCYL) at the 3′ end. In the absence of Rep, the self-annealed stem positions the two moieties in close proximity, effectively quenching FAM fluorescence by energy transfer.

The hairpin-shaped MB represented the same DNA conformation as the single-stranded RBE. After it interacted with the

single-stranded DNA binding protein (SSB), an apparent fluorescence enhancement was obtained, a result indicating that the loop section of the MB is a single-stranded DNA.

When Rep78 was incubated with the bottom-strand RBE MB, a significant increase in fluorescence intensity was observed (Figure 1 A). Thus, binding of Rep78 to the single-stranded RBE target sequence causes a structural rearrangement of the MB,

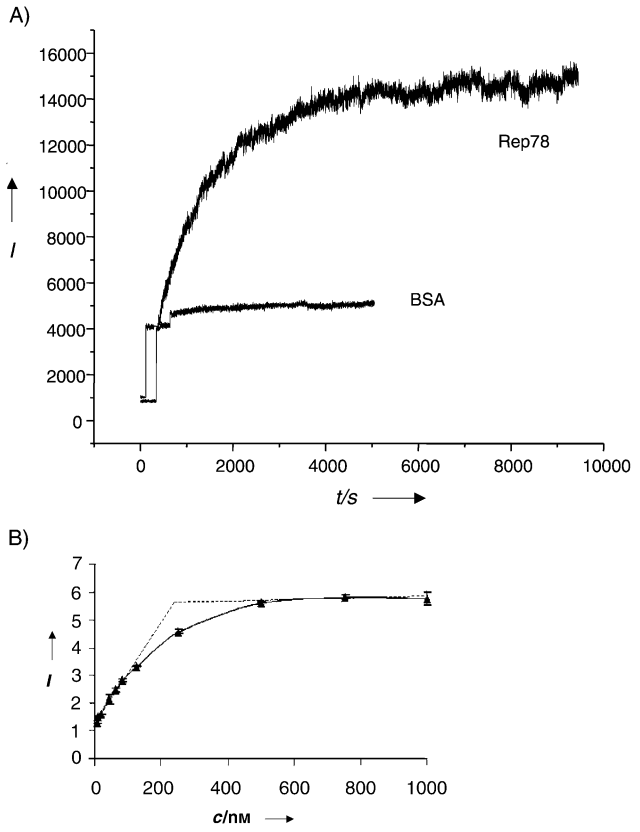

Figure 1. *Rep78 interaction with an MB derived from the RBE bottom strand. A) Fluorescence intensity time scans of the binding reaction of the MB1 with Rep78 and BSA. The concentrations of Rep78, BSA, and MB1 were 250 nM. The first segment of the data is due to the fluorescence of the buffer, the second segment is due to the fluorescence of MB1 in the reaction buffer, and the third segment shows the increase in fluorescence intensity that occurs upon addition of Rep78 protein. B) Rep78 was titrated into MB1 binding reactions and the fluorescence intensity was measured after the reactions reached equilibrium (1 h). The concentration of MB1 was 250 nM, and each titration point was repeated in 10 replicates.*

which separates the fluorophore and quencher in space and restores MB fluorescence. In contrast, incubation of this probe with bovine serum albumin (BSA), a protein that does not bind DNA, did not result in significant fluorescence enhancement. We also repeated these experiments with several preparations of bacterially expressed, homogeneously pure Rep68 and observed a similar increase in fluorescence over time (data not shown). Together these observations suggest that the observed increase in fluorescence was the result of Rep78-specific interaction with the MB.

Unlike traditional binding assays, MB probes allow real-time monitoring of protein interactions with DNA without the use of gel electrophoresis. To determine the time course of Rep78 binding to the single-stranded RBE, Rep78 and the MB probe were incubated in a binding reaction and fluorescence was monitored over time. As shown in Figure 1 A, Rep78 binding to probes appears to occur in at least two discrete steps. During the initial step the relationship between Rep78 binding and time is linear. However, once 60% of the maximal fluorescence is achieved, the slope of the time-course graph flattens out and finally reaches a plateau.

To further investigate the kinetics of Rep association with the single-stranded RBE, Rep78 was titrated into the MB binding reactions, and fluorescence was monitored after 1 hour. As expected the fluorescence intensity increased with the Rep78 concentration, to yield a curve similar in slope to the time-course graph of MB binding. A linear relationship between fluorescence intensity and Rep78 concentration was observed at lower protein concentrations (Figure 1 B). When the Rep78 concentration exceeded 500 nM, the intensity reached a plateau. By extending the initial linear and the plateau parts of the curve, an intersection point was obtained at a Rep concentration of 250 nM. Given a probe concentration of 250 nM, the stoichiometry of Rep78 association with the bottom-strand RBE probe appears to be 1:1.

Purified AAV Rep78 is a monomer in solution

To determine the solution oligomerization state of the Rep78 protein used in this study, an aliquot was resolved over a 10–40% sucrose gradient, and individual fractions were collected. These fractions were then incubated with the single-stranded MB probe derived from the bottom, T-rich strand of the RBE, and binding activity was monitored by fluorescence measurements. Protein molecular-weight standards were also loaded onto the same gradient, and these were visualized within recovered fractions on polyacrylamide gels stained with Comassie Blue. As can be seen in Figure 2, a single peak of MB binding activity was recovered between fractions containing the 66 and 150 kD molecular-weight standards, a result implying that Rep78 is a monomer in solution.

The binding constant of Rep78 association with the single-stranded RBE

Since Rep78 appears to be a monomer in solution and the stoichiometry of Rep78 association with the MB probe is 1:1, it seems reasonable to assume that one Rep molecule binds to a single bottom-strand RBE substrate when protein concentration is limiting. Thus, we propose that Rep78 association with this DNA follows first-order kinetics [Eq. (1)]:

$$\begin{array}{lllll} \mathrm{B} & + & \mathrm{P} & \rightleftarrows & \mathrm{BP} \\ [\mathrm{B}] & & [\mathrm{P}] & & 0 \\ [\mathrm{B}](1-\theta) & & [\mathrm{P}]-[\mathrm{B}]\theta & & [\mathrm{B}]\theta \end{array} \quad (1)$$

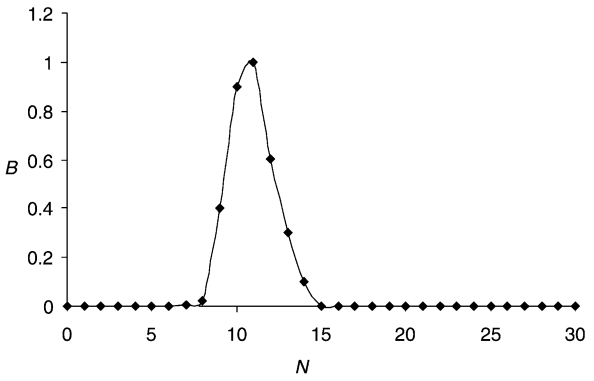

Figure 2. *Determination of Rep78 oligomerization state. Rep78 was fractionated over a sucrose gradient as described in the Experimental Section. Individual fractions were collected, aliquots were resolved on sodium dodecylsulfate (SDS) polyacrylamide gels, and molecular-weight standards were visualized by staining with Coomassie blue. Equal volumes of each fraction were then incubated with 250 nM MB1 as described in the Experimental Section. Rep78 was detected in individual fractions by monitoring the fluorescence intensity.*

At equilibrium, the binding or association constant (K_1) is given by Equation (2):

$$K_1 = \frac{[B]\theta}{[B](1-\theta)([P]-[B]\theta)} \quad (2)$$

$$F = F_0(1-\theta) + F_1\theta$$

In these equations B and P represent the MB and Rep78, θ is the fraction of MB bound to Rep78, F is the relative fluorescence intensity at a particular concentration of Rep78, F_0 is the relative fluorescence intensity caused by the MB background, and F_1 is the relative fluorescence intensity caused by the complex after the MB binds to Rep78.

Based on the above equations, the binding constant (K_1) of Rep78 association with the single-stranded RBE substrate was determined to be $3.1(\pm 0.9) \times 10^7\,\text{M}^{-1}$. The apparent dissociation constant (K_D) of this interaction was calculated to be 3.2×10^{-8} M. We have also examined the analytical sensitivity of Rep78 interactions with the MB probe. The concentration detection limit ($S/N = 3$) for Rep78 was determined to be 5.0×10^{-10} M. It is worth noting that this detection limit is achieved by a conventional spectrofluorimeter with a xenon lamp as the light source and can be further improved by using lasers for excitation and a more efficient optical detection system for emission collection. Thus molecular beacons provide a very sensitive method for the detection and characterization of protein interactions with DNA in general and specifically for Rep interactions with the AAV TR sequences.

Rep78 forms multimeric complexes with the single-stranded RBE

Fluorescence anisotropy is a simple signaling method that requires a fluorescently labeled substrate, but not a quencher. A plane of polarized light is used to excite the fluorophore, and the horizontal and vertical components of the fluorescence emission are monitored. Information about the size, shape, and flexibility of the fluorophore or the fluorophore-linked molecule can be obtained from these components. Fluorescence anisotropy techniques have been used to assay the binding of proteins to DNA, to detect conformational changes in proteins, and to study the self-association of peptides and proteins.[24, 27, 28] Our MB, containing both a fluorophore and quencher, should be ideally suited for anisotropy measurements because it is a relatively small molecule, and Rep78 binding should significantly increase the molecular weight, thereby reducing the rotational speeds of the fluorophore-labeled substrate and resulting in detectable variations in anisotropy.

To confirm our fluorescence intensity data, we titrated Rep78 into the bottom-strand MB binding reaction and monitored fluorescence anisotropy. As expected, the fluorescence anisotropy of the reaction increased with the Rep78 concentration, a result confirming an association between Rep78 and the single-stranded RBE MB substrate (Figure 3 A). A linear relationship

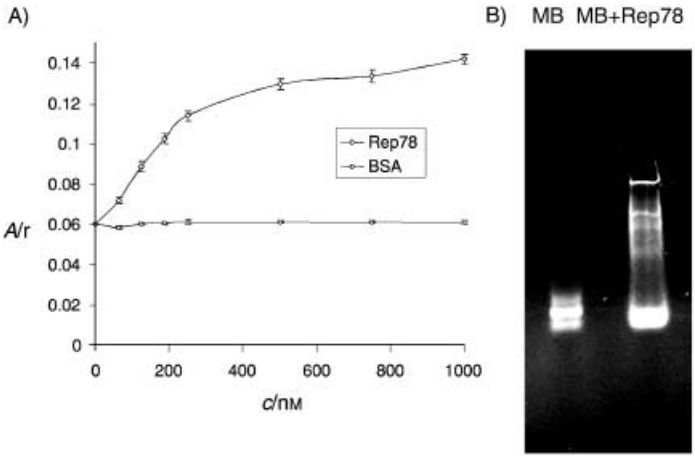

Figure 3. *Rep78 oligomerization on the RBE bottom strand. A) Rep78 was titrated in MB1 binding reactions, and Rep78 association with MB1 was monitored by fluorescence anisotropy as described in the Experimental Section. Each titration point was repeated in 10 replicates. B) A binding reaction containing 3 μM MB1 and 3 μM Rep78 was resolved on a 4–20% native polyacrylamide gel and visualized with UV/visible light.*

between anisotropy and Rep78 concentration was observed at lower protein concentrations (Figure 3 A), which was similar to that observed in the fluorescence intensity experiments. However, the anisotropy does not reach a plateau at Rep78 concentrations above 250 nM as would be expected for a simple first-order reaction. Rather, there appear to be two linear sections to the titration curve. To eliminate the possibility that high protein concentrations were causing a change in the chemical environment and increasing fluorescence anisotropy, a control experiment was performed with BSA. No increase of fluorescence anisotropy was observed no matter what concentration of BSA was used. Therefore, it appears that both segments of the titration curve are due to interactions between Rep78 and the RBE substrate.

To clarify the nature of Rep78 complexes formed on the single-stranded RBE we directly visualized the products of Rep78 binding reactions on polyacrylamide gels. Yet another advantage

of MB probes is that they can be visualized upon excitation by UV/visible light and do not require radioactive labeling. When binding reactions were repeated with equal molar amounts of protein and MB substrate and the products resolved on a native polyacrylamide gel, at least three distinct Rep78 complexes with the MB probe were observed (Figure 3B). This observation indicates that Rep78 is able to form a number of complexes with the single-stranded RBE and appears to confirm the data obtained from our anisotropy experiments. Since the kinetic data suggest that initially a single molecule of Rep78 associates with the MB substrate, we assume that the additional complexes arise from interactions between this initial, first-order complex and free Rep78 or other Rep78–DNA complexes. This seems plausible because higher order Rep complexes with single-stranded DNA have been previously observed.[26]

Specificity of the Rep78 interaction with the single-stranded RBE

The 23 nucleotides from the AAV RBE within our MB probe include the tetranucleotide GCTC repeat that has been found in all known Rep binding sites. Interference assays indicate that these nucleotides comprise the core Rep recognition element within the double-stranded RBE. Indeed, mutation of these repeated sequences dramatically inhibits Rep association with the RBE as well as viral DNA replication.[6, 7, 9, 29] To determine whether Rep interaction with the bottom strand of the RBE was also dependent on specific sequences, we performed a competitive binding assay with unlabeled oligonucleotides containing either wild-type (wt) or mutant RBE sequences (Figure 4).

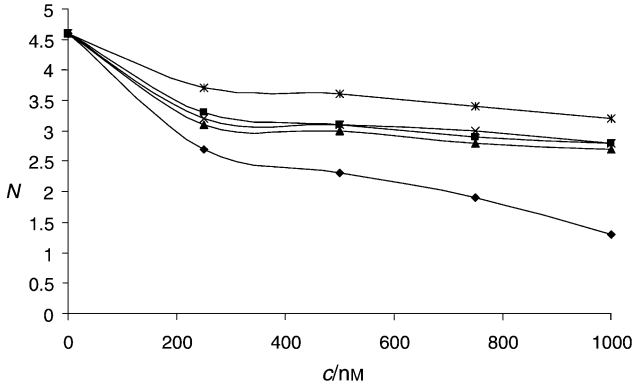

Figure 4. *Specificity of Rep78 binding to single-stranded RBE. The concentrations of MB1 and Rep78 were both 250 nM. Relative fluorescence intensity was measured after reactions reached equilibrium. Competitor sequences are:* ◆ *AGTCACTCGCTCGCTCGCGCGTC;* ■ *AGTCA**AGAT**CTCGCTCGCGCGTC;* ▲ *AGTCACTCG**AGAT**CTCGCGCGTC;* × *AGTCACTCGCTCG**AGAT**CGCGTC;* * *AGTCA**AGATAGATAGAT**CGCGTC.*

Binding assays were repeated as before with equal molar concentrations of MB and Rep78 and with multiple concentrations of each competitor.

The results from this competition assay indicate that Rep interacts with the single-stranded RBE with some sequence specificity. As expected the wt RBE competitor oligonucleotide significantly inhibited Rep association with the MB probe, especially at a higher concentration than that of the MB. Moreover, competitor DNAs containing individually or collectively mutated GCTC motifs were less inhibitory than the wt RBE oligonucleotide. These data suggest that the GCTC repeat motifs are important to Rep association with single-stranded DNA. It is worth noting that all of the mutant competitors inhibited Rep association with the MB probe to a greater extent than expected. This implies that Rep78 maintained some affinity for the mutant single-stranded DNAs. However, mutation of the GCTC motifs in previous competitor studies reduced Rep affinity for double-stranded RBE substrates by as much as tenfold. Thus, our data suggest that the three GCTC repeat motifs are involved in Rep78 association with single-stranded DNA but to a lesser extent than observed with double-stranded RBE substrates. This suggests that Rep78 interaction with single-stranded and double-stranded DNA is fundamentally different in terms of sequence specificity.

We also examined the specificity of Rep association with single-stranded DNAs by synthesizing two fluorescence anisotropy probes derived from both the top and bottom strands of the RBE (see Scheme 1). Unlike our bottom-strand MB probe, these substrates did not contain a quencher moiety and stem nucleotides. Rep78 binding activity on these DNAs was then monitored by fluorescence anisotropy, and Rep78 binding constants were calculated (Figure 5). The binding constant (K_1) of Rep78 association with the top-strand RBE substrate was determined to be $4.4(\pm 0.8) \times 10^7\,\text{M}^{-1}$, and the apparent dissociation constant (K_D) of this interaction was calculated to be 2.3×10^{-8} M. The K_D value of the Rep78 interaction with the bottom strand was again determined to be 3.2×10^{-8} M. These values are essentially the same, a fact implying that Rep78 interacts with both the top and bottom strands of the RBE with the same specificity. This is rather surprising given that these two strands contain different sequences. Indeed, sequence identity between these two strands is restricted to four or five GC repeats interspersed throughout both strands of the RBE.

Kinetics of Rep78 association with the double-stranded RBE

We next wished to determine the Rep78 affinity for the complete, double-stranded RBE. To this end, a double-stranded RBE substrate containing a 5′ fluorophore was synthesized, and Rep78 binding activity on this substrate was monitored by fluorescence anisotropy (see Scheme 1). Similarly to the anisotropy results obtained with the single-stranded RBE probes, a rather complex curve was obtained as Rep78 was titrated into double-stranded RBE binding reactions (Figure 6). This curve appears to be composed of two discrete sigmoidal sections, a result implying that Rep78 association with the double-stranded RBE is a multiorder reaction. This seems reasonable since at least six discrete Rep complexes with double-stranded RBE substrates are observed when products of binding reactions are resolved on native polyacrylamide gels.[7, 11, 30, 31]

To determine the stoichiometry of Rep78 association with the double-stranded RBE, the initial linear and the plateau sections of the titration curve were extended, and an intersection point was obtained. From this intersection point the stoichiometry of

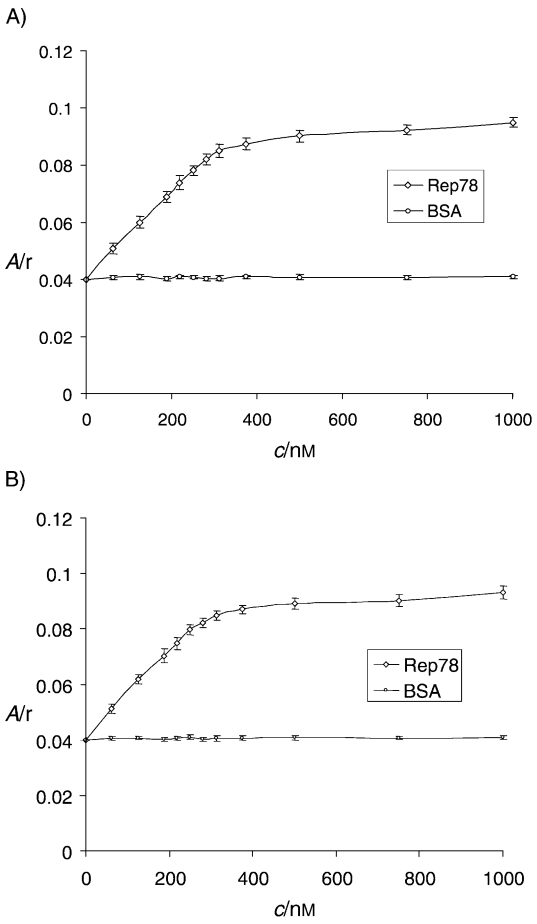

Figure 5. *Fluorescence anisotropy measurements of Rep78 association with the RBE top and bottom strands. A) Rep78 was titrated into binding reactions containing 250 nM bottom-strand RBE anisotropy probe 1. Each titration point was repeated in 10 replicates. B) Rep78 was titrated into binding reactions containing 250 nM top-strand RBE anisotropy probe 2. Each titration point was repeated in 10 replicates.*

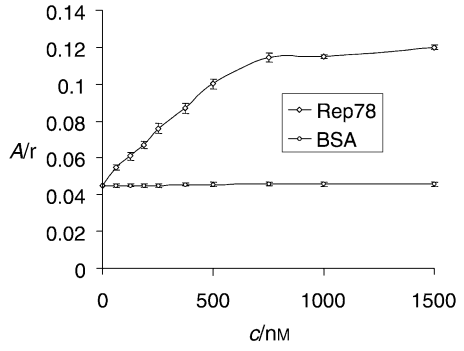

Figure 6. *Fluorescence anisotropy measurements of Rep78 association with the double-stranded RBE. Rep78 was titrated into binding reactions containing 250 nM double-stranded RBE anisotropy probe 3, and Rep78 binding was monitored by fluorescence anisotropy as described in the Experimental Section. Each titration point was repeated in 10 replicates.*

Rep78 association with the double-stranded RBE was determined to be about 2:1, which suggests that two Rep molecules bind each double-stranded RBE substrate when protein concentration is limiting. Based on this result, we assume that the binding interaction between Rep78 and double-stranded RBE is described by Equation (3):

$$\begin{array}{lll} D & +\,2P & \rightleftarrows DP_2 \\ [D] & [P] & 0 \\ [D](1-\theta) & [P]-2[D]\theta & [D]\theta \end{array} \quad (3)$$

At equilibrium, the binding or association constant (K_2) is given by Equation (4):

$$K_2 = \frac{[D]\theta}{[D](1-\theta)([P]-2[D]\theta)^2} \quad (4)$$

$$A = A_0(1-\theta) + A_1\theta$$

In these equations, D and P represent the fluorescent probe and Rep78, respectively, θ is the percentage of the probe bound to Rep78, A is the measured anisotropy at a particular concentration of Rep78, A_0 is the anisotropy caused by the fluorescent probe, and A_1 is the anisotropy caused by the complex after the probe binds to Rep78. By using the two equations above, the association constant (K_2) of Rep78 bound to double-stranded RBE was determined to be $2.4(\pm 0.8) \times 10^{14}\,M^{-2}$. The apparent dissociation constant (K_D) of this interaction was calculated to be $4.2 \times 10^{-15}\,M^2$. Unfortunately, this apparent K_D value is hard to compare with the K_D values obtained from single-stranded RBE substrates, since the two reactions appear to proceed through different reaction kinetics, second order compared to first order.

Rep mediates conformational changes within the double-stranded RBE

To further characterize the biochemical properties of Rep78 interaction with the RBE, a second, double-stranded substrate was synthesized. This probe was identical to that used in anisotropy experiments except it contained both fluorophore and quencher moieties. When Rep78 was added to binding reactions containing this MB substrate, a dramatic increase in fluorescence was observed over time (Figure 7 A). This observation implies that Rep78 binding induces conformational changes in the double-stranded probe that, in turn, physically separate the fluorophore and quencher in space, thereby restoring fluorescence. Although the exact nature of these structural alterations is not known, the dramatic increase in fluorescence suggests that the labeled ends of the RBE MB are unraveled, or melted, during interaction with Rep78. A linear relationship was observed between Rep78 concentration and fluorescence in our binding reactions, a result implying that the structural changes to the MB substrate are mediated by a monomer of Rep78.

The results from these binding experiments were rather surprising since our reactions did not contain ATP, and Rep-mediated DNA conformational changes had not been previously observed in the absence of this cofactor. Initially, we were concerned that a contaminant in our Rep78 preparation was stimulating these conformational changes to our binding

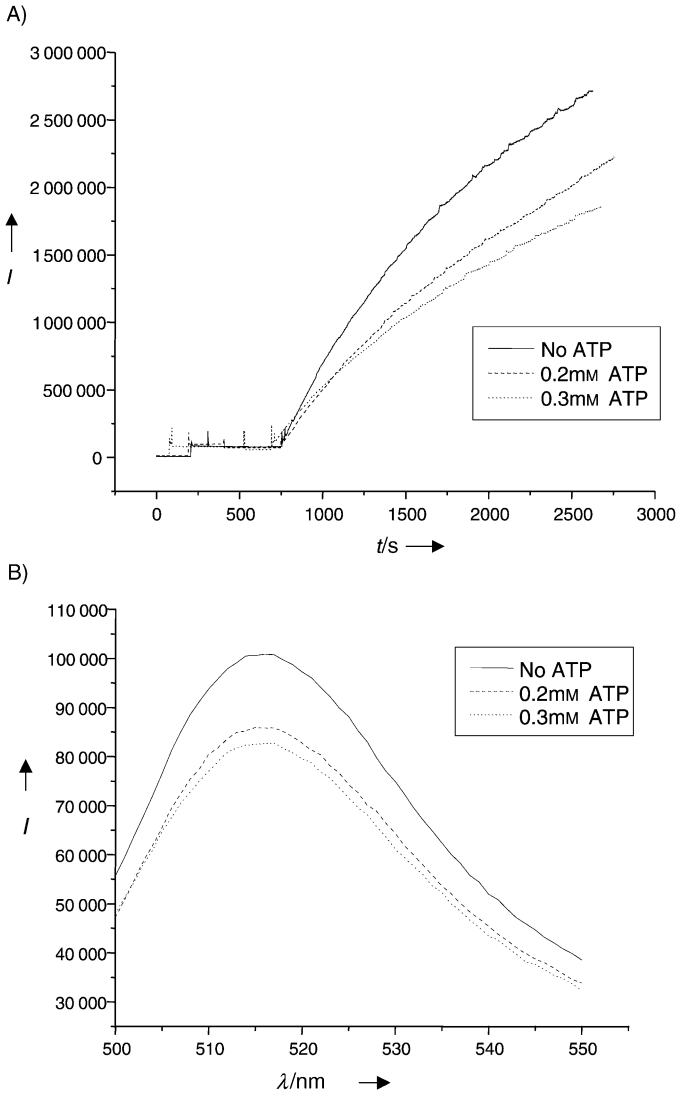

Figure 7. *Rep78 melts double-stranded RBE. A) The time course of Rep78-mediated fluorescence of the double-stranded RBE MB2 was measured as described in the Experimental Section. Binding reactions contained 250 nM MB2 and 500 nM Rep78. The effect of ATP on this activity was determined by adding this cofactor to reactions at a final concentration of 0.2 or 0.3 mM. B) The quenching effect of ATP was determined by adding 0.2 or 0.3 mM ATP to solutions containing 250 nM MB2. Fluorescence intensity was monitored 5 min after addition of ATP.*

substrate. However, this same activity has been observed with several bacterially expressed, homogeneously pure Rep68 preparations as well (data not shown). Hence, it appears that this fluorescence enhancement activity is intrinsic to the Rep78 and Rep68 enzymes and is a direct result of Rep interaction with the double-stranded RBE. Although our previous Rep helicase studies did not observe RBE melting in the absence of ATP, the RBE substrates used in these earlier studies contained 14 additional base pairs of AAV TR derived sequences.[17] Thus, the Rep-mediated conformational changes observed within our binding reactions appear to be localized within the RBE itself and are not transmitted downstream.

We next attempted to determine the contribution of ATP to the fluorescence enhancement observed in our binding reactions. We would expect ATP to increase the rate of this reaction since this cofactor appears to enhance Rep binding to the double-stranded RBE by about twofold and stimulates endogenous Rep helicase activity, which would, in turn, stimulate unwinding of the double-stranded MB sequences present and increase fluorescence.[1, 15, 17, 19] Unfortunately, ATP and other nucleotide cofactors have a general quenching effect on FAM fluorescence (Figure 7B), and even 0.2 mM ATP significantly quenched the fluorescence of our MB probe, to make comparisons between reactions done in the absence and presence of ATP difficult. A possible reason is that the probe concentration is much lower than that of ATP (1000-fold). In the solution, every fluorophore has 1000 ATP molecules around and thus the fluorescence was quenched by ATP. In any event, there was no increase in fluorescence when ATP was added to these Rep78 binding reactions, even after factoring the quenching effect of this cofactor.

Discussion

Nearly every aspect of the AAV life cycle, including viral DNA replication, transcription, and proviral integration, requires coordinated interaction between the viral Rep proteins and RBE homologues. In this paper, we have constructed several fluorescently labeled substrates derived from the RBE present within the AAV TRs. These probes allowed us to use both fluorescence anisotropy and MB fluorescence to monitor Rep78/Rep68 interaction with both single- and double-stranded conformations of the RBE in real time. Our studies with these probes indicate that Rep78/Rep68 stably associates with both conformations of the RBE in a sequence-dependent manner, although marked differences between Rep interactions with the two conformations were also observed.

By using both MB and more traditional fluorescent RBE substrates, we were able to compare the two types of assays in otherwise identical conditions. Although Rep interactions with RBE substrates immediately increased fluorescence anisotropy, an hour was required for reactions to reach equilibrium when monitored by MB fluorescence intensity. This discrepancy probably reflects the conformational changes necessary to separate the fluorophore and quencher moieties within the MB to restore fluorescence. Thus, it is likely that Rep association with the MB substrate occurred almost immediately after addition of the protein to the reactions, but the conformational changes necessary to restore fluorescence took longer to reach equilibrium.

Rep complexes formed on single-stranded RBE substrates

Our fluorescence anisotropy experiments showed no definitive plateau as Rep78 was titrated into binding reactions. This response to enzyme concentration is similar to that observed during fluorescence anisotropy studies of NtrC association with its cognate DNA binding site.[32] In these studies the lack of anisotropy plateau was attributed to the additional interaction

of free protein with protein–DNA complexes. Presumably, additional protein interactions increase the molecular weight of the initial complex and, thus, enhance the anisotropy. According to this rational, our anisotropy assays suggest that Rep78 forms first-order as well as higher order complexes with the single-stranded RBE. This conclusion is supported by our gel electrophoresis experiments that clearly show a number of Rep complexes with the bottom-strand RBE probe.

Smith and Kotin also observed higher order Rep complexes on RBE substrates that included the RBE and sequences flanking the *trs*.[26] Our data here indicate that the RBE itself is sufficient to drive formation of higher order Rep78 complexes. It is worth noting that six Rep complexes with the duplex RBE are observed on native polyacrylamide gels.[7, 11, 30, 31] These complexes seem to differ in number of Rep molecules, and the largest complex appears to contain six Rep molecules.[31] There is also evidence that individual Rep complexes with the RBE can associate with one another to form higher order complexes composed of multiple DNA substrates.[33] However, it is unclear how any of these complexes contribute to enzyme function.

Rep affinity for single- and double-stranded RBE substrates

Little is known about Rep interaction with single-stranded DNA. Rep recognizes a discrete single-stranded sequence during *trs* cleavage, but a nicking-site structure also seems to be important to this interaction.[4] During this study, we observed marked differences in Rep interaction with single- and double-stranded RBE substrates. The stoichiometry of Rep78 association with either strand of the RBE was determined to be 1:1, but the stoichiometry of Rep78 interaction with duplex RBE association was determined to be 2:1. This implies that single-stranded DNA interaction is mediated by one Rep protein, but two Rep molecules mediate duplex DNA interaction.

The specificity of Rep association with single- and double-stranded DNAs also appears to be different. When the GCTC repeats of the RBE are mutated within the bottom strand of the RBE, Rep78 binding affinity decreases, but not to the extent seen previously with duplex RBE substrates.[6, 7] Moreover, the nearly identical K_D values for Rep associations with either RBE strand indicate that the GCTC repeats themselves are not responsible for this association. The top and bottom strands are dissimilar in sequence, and homologies are limited to four or five CG dinucleotides found on each strand. This may indicate that Rep recognition of single-stranded DNA is somewhat degenerate and context dependent, similar to Rep recognition of the duplex RBE.[6, 12] However, these discrepancies may also indicate that the mechanisms of Rep interaction with single- and double-stranded DNA are not the same.

Rep catalyzes localized RBE melting

During our investigation, we observed that Rep stimulated structural alterations to the duplex RBE MB. These alterations were sufficient in magnitude to separate the fluorophore and quencher moieties in space, a fact suggesting that Rep stimulated unraveling, or melting, of duplex sequences. Since we did not observe this ATP-independent activity in previous Rep helicase studies with a 37-bp RBE substrate, the Rep-mediated melting of our 23-bp RBE MB substrate is localized and not transmitted to flanking sequences. Hence, it seems reasonable to refer to this Rep-mediated activity as a localized melting activity.

There seem to be at least two explanations for this Rep-mediated activity. First, Rep may bend the duplex RBE and cause downstream sequence distortion and strand separation, similar to the activities ascribed to the HMG family of proteins.[28, 34, 35] It is also possible that Rep association with the duplex RBE includes both double- and single-stranded binding components. After initial contact with the duplex RBE, Rep may locally displace annealed strands and initiate single-stranded contacts with the RBE, similar to the transcriptional coactivator PC4.[36] This second possibility may be supported by the relatively high Rep78 affinity for both strands of the RBE.

The observed Rep-mediated melting activity might explain a feature of Rep biochemistry. Rep requires an RBE to unwind double-stranded DNAs unless these contain an extensive 3′ single-stranded tail. This requirement suggests that Rep helicase activity is loaded onto the template DNA through a single-stranded intermediate and implies that the RBE functionally substitutes for single-stranded DNA. Our findings here indicate that Rep interaction with the duplex RBE causes the template DNA to open up, presumably allowing Rep access to single-stranded DNA. This would, in turn, allow Rep to initiate ATP-dependent translocation along the template and to unwind downstream sequences.

Experimental Section

Design of the molecular beacons, anisotropy probes, and other DNA sequences: Based on literature regarding Rep protein binding with the AAV sequence, we have designed two types of MBs. We used FAM as the fluorophore and DABCYL as the quencher. The molecular beacon I, 5′-(FAM) TGC TCG CTG CGC GCT CGC TCG CTC-ACT GA CGA GCA (DABCYL)-3′, was custom designed and synthesized by the Gemini Biotech (Alachua, FL). The molecular beacon II, 5′-(FAM) CTG CGC GCT CGC TCG CTC ACT G TTT CAGT GAG CGA GCG A-GC GCG CAG (DABCYL)-3′, was custom synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Three fluorescence anisotropy probes have been labeled with FAM at the 5′ end. The sequences are: Anisotropy probe I (designed based on bottom-stand RBE), 5′-(FAM) CTG CGC GCT CGC TCG CTC ACT GA-3′; anisotropy probe II (designed based on top-strand RBE), 5′-(FAM) TCA GTG AGC GAG CGA GCG CGC AG-3′; anisotropy probe III (designed based on double-stranded RBE), 5′-(FAM) CTG CGC-GCT CGC TCG CTC ACT G TTT CAGT GAG CGA GCG AGC GCG CAG-3′.
The anisotropy probes and other unlabeled DNAs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). All the probes and DNAs were purified with reverse-phased HPLC. The probes were dissolved in buffer (20 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), pH 7.5). Ultrapurified water from a compact ultrapure water system (EASYpure LF) was used for the preparation of all the solutions. Unless stated otherwise, chemical reagents were from Sigma (St. Louis, MO).

Preparation of the Rep78 protein: The Rep78 and Rep68 proteins were extracted from recombinant baculovirus-infected SF9 insect

cells. Rep78 was purified by chromatography on a P-11 phosphocellulose column (Whatman). The Rep78 nuclear extract in 1 M NaCl was loaded onto the column, which was previously equilibrated with buffer F (0.1 M NaCl, 50 mM Tris-HCl, 20% glycerol, 0.1 mM ethylenediaminetetraacetate (EDTA), 0.05% Nonidet P-40, 1 mM 1,4-dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride, 0.05 μg mL^{-1} leupeptin, 0.7 μg mL^{-1} pepstatin, pH 7.5). The column was washed with buffer F and eluted with a 0.1 – 0.5 M NaCl gradient in buffer F. Rep78 was then further purified by single-stranded-DNA-cellulose (ssDNA-cellulose) affinity chromatography.[2, 7] Rep78 was detected by immunoblotting, and the concentration of the Rep78 protein was determined to be 1.0 mg mL^{-1} with the Bradford reagent (Bio-rad), with gamma globulin used as the standard.

Rep68 was purified to homogeneity from baculovirus-infected SF9 cells as previously described.[7, 37] Rep68 was purified by sequential chromatography on phenyl-Sepharose, ssDNA – cellulose, and DNA affinity matrices (fraction A) or by sequential chromatography on phenyl-Sepharose, ssDNA-cellulose, and diethylaminoethyl-cellulose (fractions V and S). Both types of preparations were more than 99% pure as judged by SDS polyacrylamide gel electrophoresis and silver staining.[7, 37] The Rep68 concentration was determined to be 0.22 mg mL^{-1} by the Bradford assay, with gamma globulin used as the standard.

Fluorescence intensity and anisotropy measurements: The fluorescence intensity and anisotropy measurements were carried out on a spectrofluorometer (JOBIN YVON-SPEX Fluorolog-3) with an external circulating water bath for temperature control. All experiments were carried out at 37 °C. Anisotropy measurements are based on the principles of photoselective excitation of fluorophores by polarized light, where polarized light is used to excite a fluorophore and polarized components of the emission are detected. The degree of fluorescence anisotropy, *r*, was calculated from measurements of the emission intensity, *I*, according to Equation (5), where *G* is the instrumental correction factor, $G = I_{hv}/I_{hh}$, and the subscripts v and h refer to the vertical and horizontal orientation of the polarizer, respectively. The first and second subscripts refer to the orientation of the excitation and the emission polarizers, respectively.

$$r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh}) \quad (5)$$

The sample cuvette contained 80 μL of buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM KCl, pH 7.2). The spectral bandwidth was set to be 3 nm for intensity measurements and 5 nm for anisotropy measurements. The sample was excited at 488 nm and emissions were monitored at 520 nm. Data corrections and control experiments were carried out for all quantitative analyses.

Gel electrophoresis: Gel electrophoresis was used to confirm the binding of Rep78 protein to the MB. Binding reactions were conducted with 3 μM MB probe and 3 μM Rep78 protein in buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM KCl, pH 7.5). The samples were incubated at 37 °C for 10 min to complete the binding reaction, and were then supplemented with 50% glycerol solution (5 μL). Reactions were analyzed by electrophoresis through a 4 – 20% gradient Tris/boric acid/EDTA (TBE) polyacrylamide gel (89 mM Tris, 89 mM boric acid, 2.0 mM EDTA; Biorad, Hercules, CA). Electrophoresis was performed in 1.0 × TBE buffer supplemented with 5 mM $MgCl_2$ at room temperature for 50 min (15 V cm^{-1}). The resulting gel was excited with a UV illuminator and imaged with a Kodak DC290 digital camera (Eastman Kodak Company, Rochester, New York).

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