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Sequence Requirements for Stable Binding and Function of Rep68 on the Adeno-Associated Virus Type 2 Inverted Terminal Repeats

JOHN A. CHIORINI,[1]* STEPHEN M. WIENER,[1] ROLAND A. OWENS,[2] SIRKKA R. M. KYÖSTIÖ,[2] ROBERT M. KOTIN,[1,3] AND BRIAN SAFER[1]

Molecular Hematology Branch, National Heart, Lung, and Blood Institute,[1] and Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases,[2] Bethesda, and Genetic Therapy, Inc., Gaithersburg,[3] Maryland

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Replication of the palindromic inverted terminal repeats (ITRs) of adeno-associated virus type 2 requires several functions of the viral nonstructural Rep proteins. These include binding to the ITR, nicking of the double-stranded replication intermediate at the terminal resolution site (trs), and then strand displacement and synthesis from the nick. This report demonstrates the ability of both recombinant fusion maltose-binding protein (MBP)–Rep68Δ produced in *Escherichia coli* and wild-type (wt) Rep68 to bind to a linear truncated form of the ITR, Δ57 ITR, with similar affinity as to the wt hairpin ITR. A dissociation constant for MBP-Rep68Δ of approximately 8×10^{-10} M was determined for the wt ITR and Δ57 ITR probes. Truncation of Δ57 ITR to generate Δ28 ITR, which retains the GCTC repeat motif but not the trs, bound at least 10 times less efficiently than Δ57 ITR. Extension of Δ28 ITR with nonspecific sequence restored the ability of MBP-Rep68Δ to bind to Δ28 ITR. Thus, high-affinity binding would appear to require stabilization by flanking sequence as well as the intact GCTC repeat motif. Cleavage of the Δ57 ITR probe with *Dde*I, which truncates the flanking sequence and was previously shown to inhibit binding by Rep68, also inhibited the binding of MBP-Rep68Δ. The requirements for stable binding were further defined with a series of oligonucleotide probes which spanned the region protected by MBP-Rep78 in DNase I footprinting. The binding activity of either MBP-Rep68Δ or wt Rep68 to hairpin ITR or Δ57 ITR was indistinguishable. However, the binding activity of MBP-Rep68Δ to DNA does not appear to correlate with trs endonuclease activity. The nicking and covalent linkage of MBP-Rep68Δ to the nonhairpin Δ57 ITR was approximately 100-fold less efficient than its linkage to a hairpin-containing ITR. Therefore, although the hairpin portion of the ITR does not appear to play a role in recognition and stabilization of MBP-Rep68Δ binding, its presence does affect the trs cleavage activity of the protein.

Adeno-associated virus type 2 (AAV2) is a human parvovirus which requires the presence of a helper virus to replicate efficiently. This single-stranded DNA virus contains two open reading frames (ORFs). The left ORF encodes the nonstructural Rep proteins, which are involved in regulation of replication and transcription in addition to the production of single-stranded progeny genomes (6–8, 14, 32, 40, 42, 45–47). Furthermore, two of the Rep proteins have been associated with the preferential integration of AAV genomes into a region of the q arm of human chromosome 19 (21–23, 41, 48). The right ORF encodes the capsid proteins, VP1, VP2, and VP3. These two ORFs are flanked by 145-nucleotide inverted terminal repeats (ITRs) which can form hairpin structures that serve as primers during replication (44). The two larger Rep proteins transcribed from the p5 promoter, Rep68 and Rep78, are referred to by their apparent masses as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. These proteins have been shown to bind to ITRs (1, 8, 16–18, 35–37, 44) and stimulate replication both in vivo (25, 26, 45) and in vitro (8, 15, 33). Furthermore, Rep68 and Rep78 have been shown to nick the duplex ITR in a site- and strand-specific manner. This cleavage, which occurs at the terminal resolution site (trs), permits the replication of the hairpin structure in a process referred to as terminal resolution (42).

In addition to their role in replication, Rep68 and Rep78 have been shown in vivo to regulate the transcription of the AAV p5, p19, and p40 promoters (2, 24, 27, 30, 46). The p5-initiated Rep proteins can also inhibit the transcription of heterologous promoters as well as cellular transformation by papillomavirus or by adenovirus E1a plus an activated *ras* oncogene (11–13, 20, 25, 26). Rep68 and Rep78 were recently shown to bind to a region on human chromosome 19 that had been identified as a preferred site for AAV DNA integration (21–23, 48). Furthermore, Rep68 was shown to mediate an interaction between the human integration locus and the ITR of AAV, suggesting a role for this protein in the targeted integration of AAV (48).

Previous literature has suggested that in order for Rep68 to bind to the ITR, it had to be in a hairpin form (1, 16). Recent work supports this idea by demonstrating that an ITR cleaved with *Dde*I, which removes the cross arms of the ITR hairpin and seven bases of the stem, would not bind Rep68 (43). Therefore, the recent observations that Rep68 and Rep78 will bind to a linear fragment from human chromosome 19 (48) and a recombinant Rep68 can bind to a full-length ITR stem (8) (see Fig. 1B) appear to be contradictory to the requirement for a hairpin structure for binding. However, other studies by Berns and colleagues have shown the importance of the

* Corresponding author. Mailing address: NIH/NHLBI/MHB, Bldg. 10/7D18, 10 Center Dr., MSC 1654, Bethesda, MD 20892-1654. Phone: (301) 496-1594. Fax: (301) 496-9985.

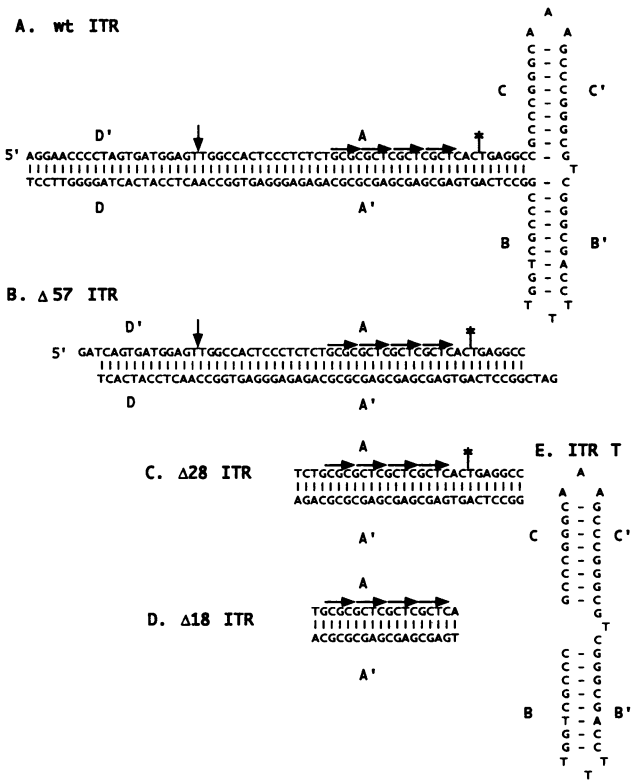

FIG. 1. Sequences of probes. wt ITR (A) was made as previously described (1). Δ57 ITR (B) is an annealed oligonucleotide pair of 57 bases in length corresponding to all of the A/A′ and part of the D/D′ region of wt ITR with a GATC 5′ overhang. Also shown are three further truncations of the ITR, Δ28 ITR (C), Δ18 ITR (D), and ITR T (E). Sequences are aligned to the GCTC imperfect repeats (horizontal arrows). The trs is indicated with a vertical arrow. The *Dde*I cleavage site in the stem of the wt ITR is indicated by an asterisk.

hairpin structure for origin function in vivo (4, 5, 29, 39). Mutations which maintained the structure were shown to retain origin activity.

Characterization of the mechanism of action of Rep68 and Rep78 has been limited by the inability to produce these proteins in large quantities. We have recently succeeded in expressing Rep68Δ and Rep78 as fusion proteins with the maltose-binding protein (MBP) in *Escherichia coli* and have demonstrated that the recombinant MBP-Rep68Δ can perform all of the in vitro activities described for the wild-type (wt) Rep protein (8). In this study, the DNA sequence and structural requirements for MBP-Rep68Δ binding are characterized. This report demonstrates that MBP-Rep68Δ is able to bind to a linear truncated form of the ITR, Δ57 ITR, with similar affinity as to wt hairpin ITR. Furthermore, binding to a linear ITR is a function not only of MBP-Rep68Δ but of wt Rep68 as well. Similar specific DNA binding activities were obtained for MBP-Rep68Δ and Rep68. However, while the hairpin is not required for Rep binding to the ITR, it does appear to have a stimulatory effect on trs endonuclease activity by Rep proteins in vitro.

MATERIALS AND METHODS

Probes. DNA probes are summarized in Fig. 1, 6, and 7A. Oligonucleotide probes were synthesized with a model 392 DNA/RNA synthesizer (Applied Biosystems). The oligonucleotides used to produce the Δ57 ITR were purified on denaturing polyacrylamide gels. All other oligonucleotides were purified by NAP 25 gel filtration chromatography (Pharmacia).

5′-^{32}P-labeled wt ITR probe was produced by *Xba*I digestion of pSub201 (38). Following calf intestinal alkaline phosphatase treatment, the 5′ terminus was labeled with [γ-^{32}P]ATP (3,000 Ci/mmol) by polynucleotide kinase, and the labeled fragment was released by *Pvu*II digestion. The linear probe was then converted to the wt ITR conformation by boiling, rapid chilling, and filling in the 5′ overhang with Klenow fragment and cold deoxynucleoside triphosphates. The unlabeled complementary strand released by *Xba-Pvu*II digestion cannot be extended by this treatment. Therefore, the wt 5′-labeled ITR can be separated from the shorter complementary strand by electrophoresis on a 6% Tris-borate-EDTA acrylamide gel, excised from the gel and eluted into 1× Tris-EDTA buffer.

3′-labeled wt ITR was prepared by boiling and quickly cooling an unlabeled *Xba*I-*Pvu*II fragment and then filling in the 5′ overhang with Klenow fragment and [α-^{32}P]dCTP. To obtain 3′-labeled ITR probe of the same specific activity as Δ57 ITR, unlabeled dCTP was added to the labeling cocktail to compensate for the greater number of cytosine residues in the 3′-labeled probe.

The Δ ITR probes were made by 5′ end labeling either oligonucleotide and annealing with the unlabeled complementary oligonucleotide. Δ57 ITR was 3′ end labeled by filling in the 5′ overhang created by the *Bam*HI restriction site included in the oligonucleotides. *Dde*I truncation of Δ57 ITR was generated by cleavage of 5′-end-labeled Δ57 ITR probe with *Dde*I restriction enzyme and shown to be greater than 90% cleaved by analysis on a denaturing acrylamide gel.

Preparation of wt Rep68 from transfected 293 cells. The preparation of wt Rep68 has been described previously (37). Briefly, a plasmid expressing Rep68 from the human immunodeficiency virus long terminal repeat (pSK9) was transfected into human 293 cells, and the cells were harvested 48 h later; 600 mM NaCl nuclear extracts were prepared as described previously (37). As negative controls, similar nuclear extracts were made from 293 cells transfected with a plasmid encoding a nonfunctional truncation mutant form of Rep68 (Rep*am*238) (37) or pBR322.

MBP-Rep68Δ and MBP-Rep78. The construction, expression, and purification of these proteins have been described previously (8). Briefly, PCR was used to amplify the Rep68 ORF from AAV nucleotides 327 to 1882. This fragment was then cloned into plasmid pPR997 (New England Biolabs). The full-length Rep78 gene (nucleotides 327 to 2186) was cloned into the same vector to produce MBP-Rep78. The fusion proteins were purified by amylose affinity chromatography. The eluted MBP-Rep68Δ and MBP-Rep78 protein fractions were found to consist of 90% full-length molecules and contain a minor fraction of prematurely truncated MBP-Rep68Δ and MBP-Rep78, as determined by Coomassie blue staining. The prematurely truncated fragments were shown to lack significant DNA binding activity by their separation from the full-length molecules, using ITR-derived DNA affinity column chromatography (data not shown).

Immunoblotting. Western immunoblot analyses of wt Rep68 and MBP-Rep68Δ were performed as described elsewhere (24). Briefly, serial dilutions of Rep68-containing 293 cell nuclear extracts or amylose column-purified MBP-Rep68Δ samples were electrophoresed on a 12.5% polyacrylamide gel containing 0.1% SDS. The proteins were electroblotted (Multiphor II Nova Blot electrophoretic transfer apparatus; Pharmacia LKB Biotechnology, Piscataway, N.J.) onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.).

The primary antibody was a rabbit antibody (anti-Rep78.93) raised against a truncated Rep78 expressed in *E. coli* (47). The secondary antibody was biotinylated goat anti-rabbit immunoglobulin G. The blots were then treated with streptavidin-alkaline phosphatase and incubated with a chemiluminescent alkaline phosphatase substrate (PhotoBlot chemiluminescent system for protein detection; Life Technologies Inc., Gaithersburg, Md.) and exposed to X-ray film. Protein concentrations in the original samples were determined by using the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.) according to the manufacturer's instructions. The amount of Rep68 protein contained in each sample was measured by densitometry of the full-length Rep protein band.

Electrophoretic mobility shift assays (EMSAs). DNA-protein complexes were detected by their reduced mobility on nondenaturing polyacrylamide gels. The assays were performed as previously described (9, 10), with the modifications described by Jacob et al. (19). Briefly, radiolabeled probes were incubated with protein fractions at 30°C for 15 min in 25 μl. The reaction mixture contained 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 4% glycerol, and 0.5 μg of poly(dI-dC) when indicated.

Specific activity determination. The specific activities of MBP-Rep68Δ and wt Rep68 produced from 293 cells transfected with pSK9 were determined as follows. The amount of shift complex formed in Fig. 2A for MBP-Rep68Δ and wt Rep68 was determined by PhosphorImager (Molecular Dynamics) analysis using a 5′-end-labeled probe. The shift bands produced by MBP-Rep68Δ per 100 ng of protein were fourfold greater than the complexes formed by 170 ng of wt Rep68-containing cell extract. The ratio of DNA to protein was shown in other experiments to be within the linear range, and this difference in binding activity was determined at several DNA/protein ratios (Fig. 4C). Thus, MBP-Rep68Δ had approximately seven times as much gel shifting activity per microgram of total protein as the 293 nuclear extract did. However, quantitation of the full-length Rep68 proteins contained in the two extracts by densitometry of a Western blot (Fig. 3, lanes 3 to 5 and 7 to 9) indicated that MBP-RepΔ contained 8 to 16 times more Rep68 protein molecules per microgram of total protein. Therefore, the specific DNA binding activities of the Rep68 proteins in the preparations of MBP-Rep68Δ and pSK9 extracts were within twofold of each other.

Dissociation constant determination. The K_d was determined by measuring the amount of DNA-MBP-Rep68Δ complex formation as a function of increasing DNA concentration, using a PhosphorImager. One hundred nanograms of amylose-purified MBP-Rep68Δ was incubated in an EMSA with increasing amounts of 5′-end-labeled wt ITR or Δ57 ITR (60 to 400 fmol). The specific activity of each probe was determined by separating the labeled probe from free ^{32}P-nucleoside triphosphate by gel filtration followed by quantitation via liquid scintillation counting. wt ITR and Δ57 ITR probes were found to have the same specific activity of 2×10^5 cpm/pmol. For the purpose of conversion from PhosphorImager units (PIUs) to counts per minute, standards were included on each gel. The conversion factors are 46 PIU/cpm of probe for Δ57 ITR and 38 PIU/cpm of probe for wt ITR. The difference in PIU for the two probes is the result of different exposure times on the PhosphorImager for the experiments. Eadie-Hofstee analysis of the data (bound versus bound/free) resulted in a straight line (slope = -1.8×10^5 PIU for Δ57 ITR and -1.87×10^5 PIU for wt ITR) and thus a single value for the slope, $-K_d$, where K_d is the dissociation constant (Fig. 4). As a confirmation of the value obtained by this method, a second determination was made by the method outlined by Meisternst et al. (31). The two methods generated K_d values in the same range.

DNase I footprinting. The substrates used for DNase I protection experiments consisted of the synthetic oligonucleotide Δ57 ITR, which contains the AD′ or A′D sequence of the ITR shown in Fig. 1B. The oligonucleotides were individually 5′ end labeled and annealed as described above. The conditions for DNase I footprinting were as described previously (16), with the following modifications. About 5×10^4 cpm of probe was used with 0.5 μg of poly(dI-dC) and 2 μg of protein. The digestion time was decreased to 30 s. The reactions were terminated in 2 volumes of stop buffer (10 mM Tris-Cl [pH 7.9], 10 mM NaCl, 0.5% SDS, 0.2 mg of yeast tRNA per ml, 2 mg of proteinase K per ml). The samples were extracted by phenol-chloroform and ethanol precipitated. The products were then resuspended in 80% formamide loading buffer, denatured by heating to 95°C for 5 min, and then fractionated on a 6% polyacrylamide–8 M urea sequencing gel.

Covalent linkage trs endonuclease assay. Activity was assayed by performing the trs endonuclease assay with 3′-labeled probes as previously described (8, 17, 18), with the following modifications. After incubation of 100 ng of MBP-Rep68Δ with either wt ITR or Δ57 ITR (50 or 500 fmol, respectively), the reactions were terminated by the addition of 1 volume of 2× sample buffer (120 mM Tris-HCl [pH 6.8], 10% SDS, 2% dithiothreitol, 40% sucrose), and the samples were heated to 95°C for 5 min. The 3′-radiolabeled probe covalently bound to MBP-Rep68Δ was separated from the free probe by electrophoresis on an SDS–10% polyacrylamide gel.

RESULTS

MBP-Rep68Δ and wt Rep68 bind to Δ57 ITR. Previous work has shown that the recombinant MBP-Rep68Δ is able to bind to a linear form of the AAV ITR (8). Δ57 ITR is the entire AAV ITR A/A′ duplex region plus 10 bp of sequence downstream of the trs (D/D′) (Fig. 1B). When MBP-Rep68Δ and 293 cell nuclear extracts containing wt Rep68 were incubated with the Δ57 ITR probe, a cluster of three reduced mobility bands was detected (Fig. 2A, lanes 2 and 3, respectively). In contrast, these bands are not obtained with a non-DNA-binding truncation mutant of Rep68, Rep*am*238 (lane 4), or in the presence of a 10-fold excess of unlabeled competitor (lanes 5 and 6). The difference in mobility of the MBP-Rep68Δ and the wt Rep68-DNA complexes is the result of a difference in the molecular masses of the two proteins (106 and 68 kDa, respectively). Some additional bands that are not produced with MBP-Rep68Δ are present when the Δ57 ITR is incubated with 293 cell nuclear extract containing wt Rep68. However, these bands are produced when the Rep*am*238 mutant is used and are thought to be the result of cellular proteins present in the 293 nuclear extract.

Therefore, the ability of either MBP-Rep68Δ or wt Rep68 to bind to the ITR does not require the presence of the hairpin cross arm (B/B′ C/C′). Furthermore, binding to a Δ57 ITR is a function not only of MBP-Rep68Δ but of wt Rep68 as well, and no binding is detected with the MBP moiety fused to LacZ (data not shown).

The ability of Rep68 to bind to a linear form of the ITR, Δ57 ITR, directly contradicts several previous observations (1, 16, 43). To resolve these discrepancies, we truncated the Δ57 ITR at the *Dde*I site, which was previously shown to inhibit binding to the wt ITR (43). Truncation at this position significantly reduced binding of MBP-Rep68Δ compared with the full-length Δ57 ITR probe (Fig. 2B, lanes 1 and 2). Competition experiments indicated that the *Dde*I-truncated fragment bound

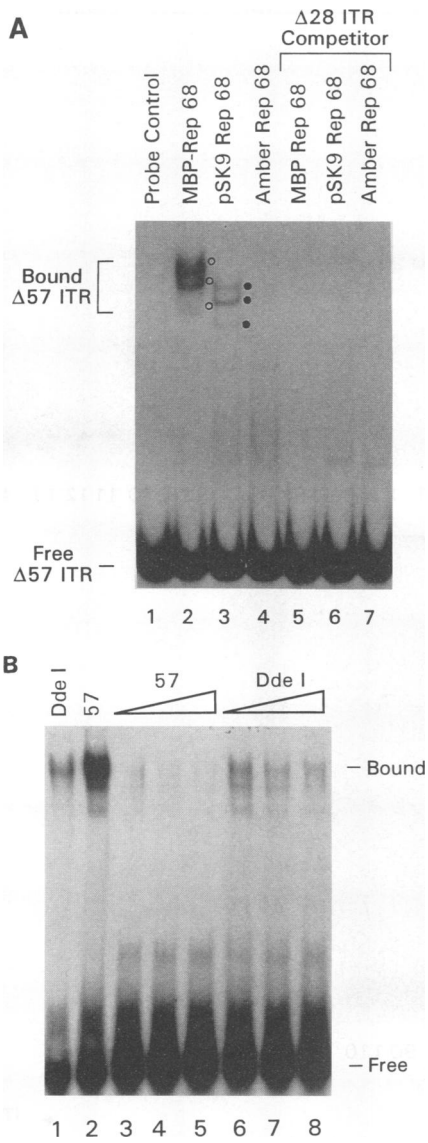

FIG. 2. Both wt Rep68 and recombinant MBP-Rep68Δ bind to Δ57 ITR. (A) Approximately 10,000 cpm of 5′-end-labeled Δ57 ITR probe was incubated with either 100 ng of MBP-Rep68Δ or 170 ng of wt Rep68 nuclear extract protein or Rep*am*238 nuclear extract protein. The positions of the free probe and bound protein-DNA complexes are indicated at the left. Rep68-DNA complexes were detected only with MBP-Rep68Δ and wt Rep68 produced from plasmid pSK9 (pSK9 Rep 68; lanes 2 and 3, respectively). No specific binding was detected by the negative controls (probe only [lane 1], MBP-LacZ [data not shown], and a truncated amber mutation, Rep*am*238 [lane 4]). The MBP-Rep68Δ and wt Rep68 shift bands could be competed for by the addition of a 10-fold excess of unlabeled Δ28 ITR competitor (lanes 5 and 6). (B) 5′-end-labeled Δ57 ITR probe was cut with *Dde*I and shown to be greater than 90% digested by separation on a denaturing gel. One hundred nanograms of MBP-Rep68Δ was incubated with approximately 0.1 ng of either *Dde*I (lane 1) or Δ57 ITR probe (lane 2) under standard EMSA conditions. The reaction mixtures were incubated at room temperature, and the gel was run at room temperature as reported previously (43). For competition, a 5-, 10-, or 15-fold excess of unlabeled duplex oligonucleotides was added to the reaction mixture with labeled Δ57 ITR probe (lanes 3 to 8).

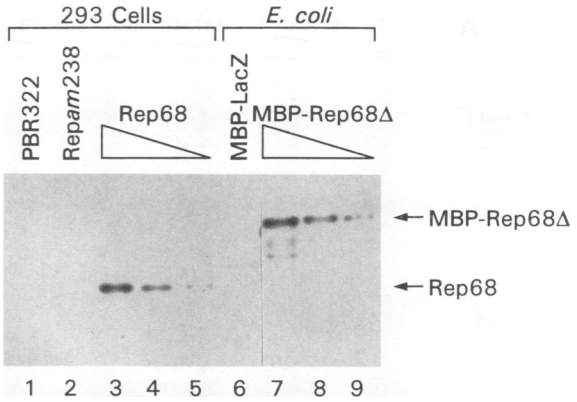

FIG. 3. Western blot comparison of wt Rep68- and MBP-Rep68Δ-containing extracts. The amounts of Rep68 protein contained in pSK9-transfected 293 cell nuclear extract and amylose column-purified MBP-Rep68Δ were compared by immunoblotting using anti-Rep78.93 antibody (47). Three negative controls were included: 4 μg of nuclear extract protein from 293 cells transfected with pBR322 (lane 1) or pHIVRep*am* (Rep*am*238; lane 2) and 4 μg of amylose column-purified MBP-LacZ produced in *E. coli* (lane 6). Lanes 3 to 5 contained 4, 2, and 1 μg, respectively, of pSK9 Rep68 nuclear extract. Lanes 7 to 9 contained 250, 125, and 62 ng, respectively, of amylose column-purified MBP-Rep68Δ. The positions of pSK9-produced wt Rep68 (Rep68) and MBP-Rep68Δ are indicated at the right.

MBP-Rep68Δ at least 10 times less efficiently than the full-length Δ57 ITR (lanes 3 to 8). This result is in agreement with the other report of Rep68 binding activity (43). However, binding is observed on the full-length Δ57 ITR (lane 2), which indicates the importance of this region in the formation of a stable complex and not necessarily the requirement of a hairpin conformation for stable binding as previously suggested.

Determination of the DNA binding activities of MBP-Rep68Δ and wt Rep68. To compare the DNA binding activities of MBP-Rep68Δ and wt Rep68, the ITR binding activity was normalized to the amount of Rep68 protein as determined by Western analysis. MBP-Rep68Δ bound approximately seven times more DNA per microgram of total protein than wt Rep68 (Fig. 2A, lanes 2 and 3) when values were corrected for the amount of protein used in each lane. The DNA/protein ratio was shown in other experiments to be within the linear range of detection. This difference in binding activity was determined at several DNA/protein ratios and is shown again in Fig. 4A and B. Western blot analysis with anti-Rep antisera showed that MBP-Rep68Δ contained approximately 8 to 16 times more full-length Rep68 than 293 cell extracts did (Fig. 3, lanes 3 to 5 and 7 to 9). Therefore, the specific activity of the Rep68 proteins in the preparations of MBP-Rep68Δ and pSK9 extracts are within twofold of each other, indicating that MBP-Rep68Δ and wt Rep68 have a similar abilities to bind the AAV ITR.

Since comparable binding activities to Δ57 ITR were obtained for wt Rep68 and MBP-Rep68Δ, the relative affinities of these protein preparations for hairpin ITR, Δ57 ITR, or a further truncation of Δ57 ITR, Δ28 ITR (Fig. 1), were compared. The binding affinities of wt Rep68 and MBP-Rep68Δ were very similar for the wt ITR and Δ57 ITR probes (Fig. 4A and B, lanes 1 to 5 and 6 to 10, respectively) but were significantly reduced upon further truncation of the Δ57 ITR probe to Δ28 ITR (lanes 11 to 15).

The data presented in Fig. 4A and B were obtained at a

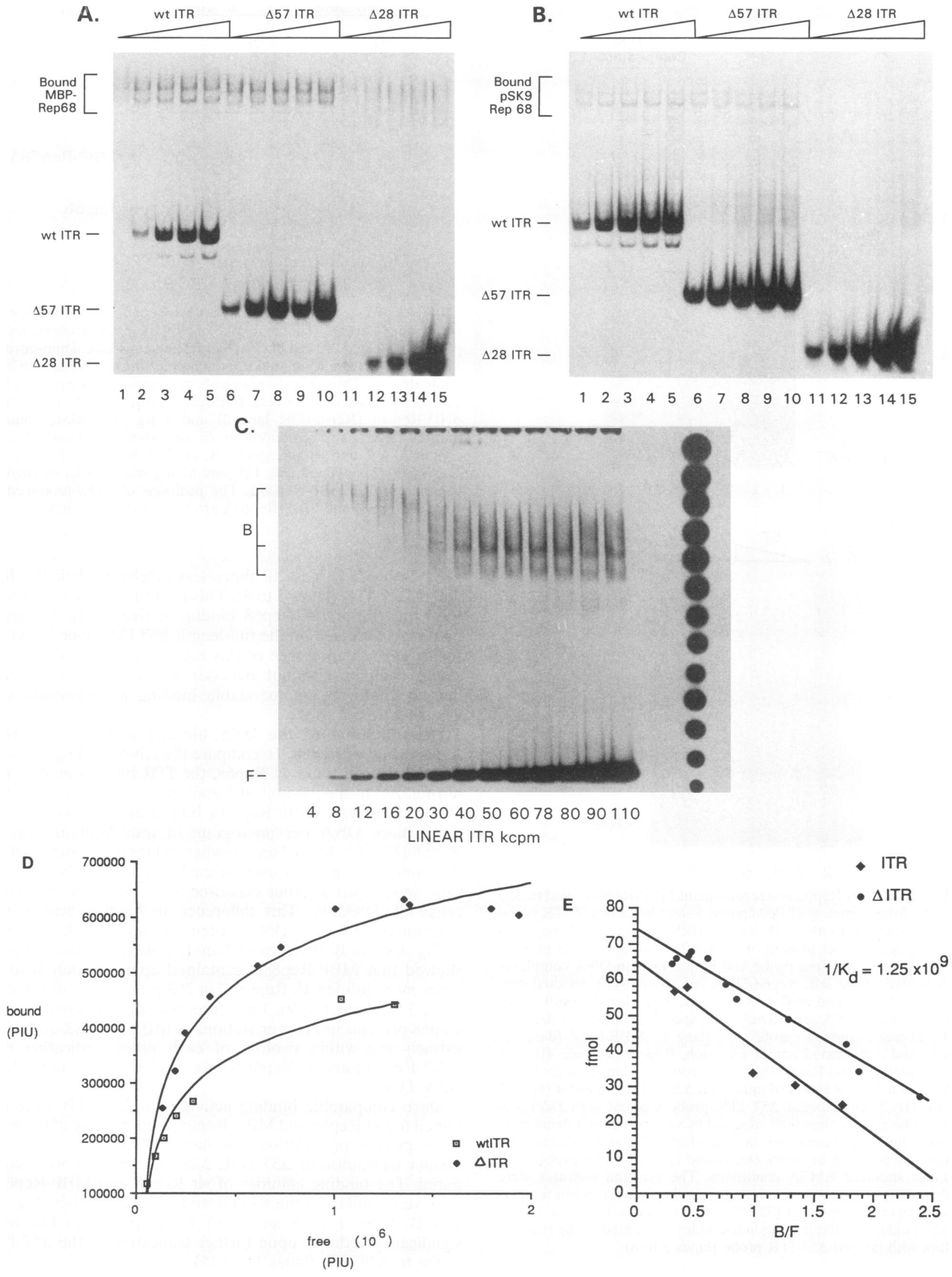
A.
wt ITR
Δ57 ITR
Δ28 ITR
Bound MBP-Rep68
wt ITR
Δ57 ITR
Δ28 ITR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B.
wt ITR
Δ57 ITR
Δ28 ITR
Bound pSK9 Rep 68
wt ITR
Δ57 ITR
Δ28 ITR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C.
B
F
4 8 12 16 20 30 40 50 60 78 80 90 110
LINEAR ITR kcpm
D
bound (PIU)
700000
600000
500000
400000
300000
200000
100000
0
1
2
free (10^6) (PIU)
wtITR
ΔITR
E
ITR
ΔITR
1/K_d = 1.25 x10^9
fmol
80
70
60
50
40
30
20
10
0
0.5
1
1.5
2
2.5
B/F

FIG. 4. wt Rep68 and MBP-Rep68Δ bind to wt ITR and ΔITR with similar affinities. The relative binding affinities of recombinant MBP-Rep68Δ (A) and wt Rep68 (B) were compared for three probes, wt ITR, Δ57 ITR, and Δ28 ITR. Increasing amounts of probe (10, 20, 30, 40, and 50 fmol) were added to a constant amount of protein (100 ng). Lanes 1 to 5 in both panels contain wt ITR as the probe; lanes 6 to 10 contain Δ57 ITR; lanes 11 to 15 contain Δ28 ITR. (C) To determine the K_d for the different protein-DNA complexes, the amount of bound and free probe was measured over a range of amounts from 60 to 400 fmol with a constant amount of protein. Bound (B) and free (F) probe bands were quantitated with a PhosphorImager (Molecular Dynamics), and the data were used to determine the dissociation constants for each of the probes with MBP-Rep68Δ. The dots at the right were used to develop a standard curve for converting from PIU to counts per minute. Graphs with plots of bound versus free probes (Δ57 ITR or wt ITR) presented in PIU (D) and bound versus bound/free (B/F) (Eadie-Hofstee plot; E) are shown.

relatively low Rep/probe ratio; hence, the amount of complex that could be formed was limited despite the addition of increasing amounts of probe (e.g., lanes 6 to 10). While these results demonstrate the similar affinities of MBP-Rep68Δ and wt Rep68 for both the wt ITR and Δ57 ITR probes, the data do not allow for more precise K_d determinations. The binding experiments were therefore repeated over a higher Rep/probe ratio. Figure 4C shows that as a fixed amount of Rep is titrated with increasing amounts of Δ57 ITR probe, the amount of complex formed increases. Similar results were obtained with both wt Rep and MBP-Rep68Δ.

The affinities of wt Rep68 and MBP-Rep68Δ for wt ITR and Δ57 ITR were determined by measuring the amount of bound and free probe over a range of protein/DNA ratios (see Materials and Methods), and a curve was fitted to these values (Fig. 4D). K_d values for MBP-Rep68Δ of approximately 8×10^{-10} and 9×10^{-10} M for Δ57 ITR and wt ITR, respectively, were determined by using an Eadie-Hofstee plot (Fig. 4E). The difference in the *y* intercepts (Fig. 4D) may result from small variations in the amount of protein added to the reaction mixtures. This is in agreement with the shift in the curve in Fig. 4D.

Binding affinity of MBP-Rep68Δ for a $(GCTC)_4$ imperfect repeat motif is increased by nonspecific flanking sequence. MBP-Rep68Δ and wt Rep68 bind to Δ57 ITR with equivalent affinities. Further truncation of Δ57 ITR to Δ28 ITR or cleavage with *Dde*I resulted in a significant decrease in binding affinity (Fig. 4A and B, lanes 11 to 15; Fig. 2B, lane 1). To determine the basis for the reduced binding affinity, DNase I protection analysis was performed with MBP-Rep78 and Δ57 ITR (Fig. 5). On the trs-containing strand of Δ57 ITR, protection extends over a 24-nucleotide sequence that is centered over an imperfect $(GCTC)_4$ repeat (lanes 5 to 7). Truncation with *Dde*I cleaves within this protected region [5′ (C/T)GAG] and further supports the importance of this region in complex formation. Protection of the non-trs-containing strand covers the complement of the GCTC repeat motif, but a total of 32 nucleotides are protected and the repeats are found at the 5′ end of the protected region (lanes 13 to 15). No protection was found on the trs region of either strand. To determine the minimal DNA sequence that would support the stable binding of MBP-Rep68Δ, several duplex oligonucleotide probes spanning the GCTC repeat region were tested for binding activity and for the ability to compete with Δ57 ITR for

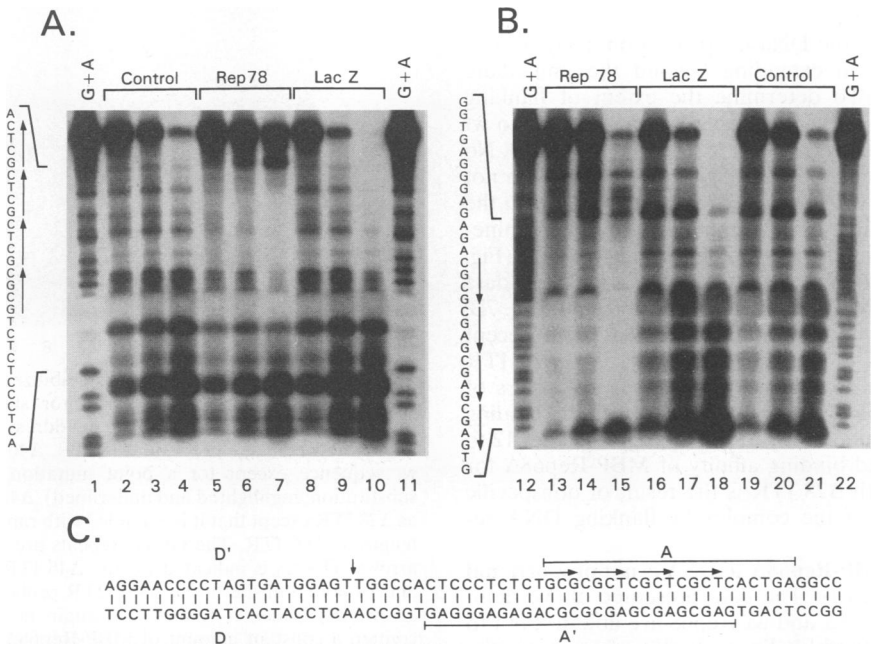

FIG. 5. DNase I footprinting of Δ57 ITR with MBP-Rep78 protects a GCTC repeat in the A/A′ region of the ITR. The region of contact between Rep protein and DNA was identified by using a DNase I protection assay. The coding (trs-containing) (A) or noncoding (B) strand of the 5′-end-labeled Δ57 ITR was incubated with 2 μg of MBP-Rep78 followed by increasing amounts of DNase I (lanes 5 to 7 and 13 to 15). As a control, Δ57 ITR was incubated with DNase I only (lanes 2 to 4 and 19 to 21) or with MBP-LacZ (lanes 8 to 10 and 16 to 18). Lanes 1, 11, 12, and 22 are G+A ladders. (C) Schematic representation of the protected regions on the coding and noncoding strands, with horizontal lines over the protected regions on the coding and noncoding strands. The GCTC repeat motif is indicated by four horizontal arrows. The trs is indicated by a vertical arrow.

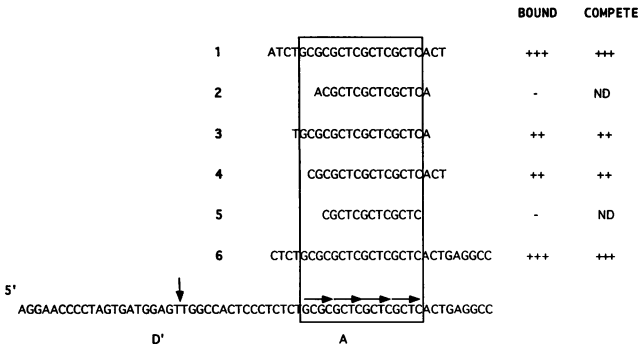

FIG. 6. Minimum binding site determination for MBP-Rep68Δ. To determine the minimum DNA sequence required for binding, several probes which spanned the protected area found by DNase I footprinting were synthesized. Only the trs-containing strands are shown. The GCTC imperfect repeat region in each probe is outlined by a box. The trs is indicated by a vertical arrow.

binding (Fig. 6). Only the trs-containing strand sequences are shown. All of the oligonucleotide probes tested had significantly weaker binding activity compared with Δ57 ITR. Oligonucleotide probes that contained both the repeats and flanking sequences were able to bind MBP-Rep68Δ as well as compete for MBP-Rep68Δ. Binding activity was proportional to the length of flanking sequence contained in the probe: the fewer the number of flanking nucleotides, the weaker the binding activity. Oligonucleotides 1 and 6, which contained at least 3 bp of flanking sequence on both sides of the imperfect $(GCTC)_4$ repeat, had the strongest binding activity. As the number of flanking nucleotides was reduced, activity was decreased. Truncation of the repeat motif (Fig. 6, 2, and 5) resulted in a total loss of binding activity.

These results support the DNase I protection assay results, which showed protection extending beyond the immediate GCTC repeat region. To determine the extent of flanking sequence needed to obtain comparable binding activity to wt ITR, Δ57 ITR was further truncated to Δ46 ITR (Fig. 7A). No loss of binding activity was observed with this probe (data not shown). To investigate the possibility that interaction with the trs was required for stable MBP-Rep68Δ binding, a thymine-to-guanine transversion was made at the trs of Δ46M ITR (Fig. 7A). Although trs endonuclease activity was abolished (data not shown), binding of MBP-Rep68Δ was not affected (Fig. 7B, lanes 7 to 10). This finding is in agreement with recent published observations (43). Binding activity of a Δ28 ITR probe extended toward the trs with additional base pairs of random sequence (Δ46R ITR) (Fig. 7A) restored the binding activity to the level obtained with Δ57 ITR (Fig. 7B, lanes 12 to 15). Thus, the increased binding affinity of MBP-Rep68Δ for Δ57 ITR compared with Δ28 ITR is the result of nonspecific sequence stabilization of the complex by flanking DNA sequences.

Binding affinity of MBP-Rep68Δ to DNA is not proportional to trs endonuclease activity. The data presented above demonstrate that MBP-Rep68Δ and wt Rep68 are able to bind to GCTC imperfect repeats within linear duplex substrates in the A/A′ region of the ITR with an affinity similar to that of the hairpin ITR. Since the hairpin structure is required for DNA replication (4, 5, 28, 29, 39), the effect of the hairpin sequence on trs endonuclease activity was examined. The amount of 5′ covalently attached MBP-Rep68Δ-DNA that results from the ATP, Mg^{2+}-dependent endonuclease activity was measured by

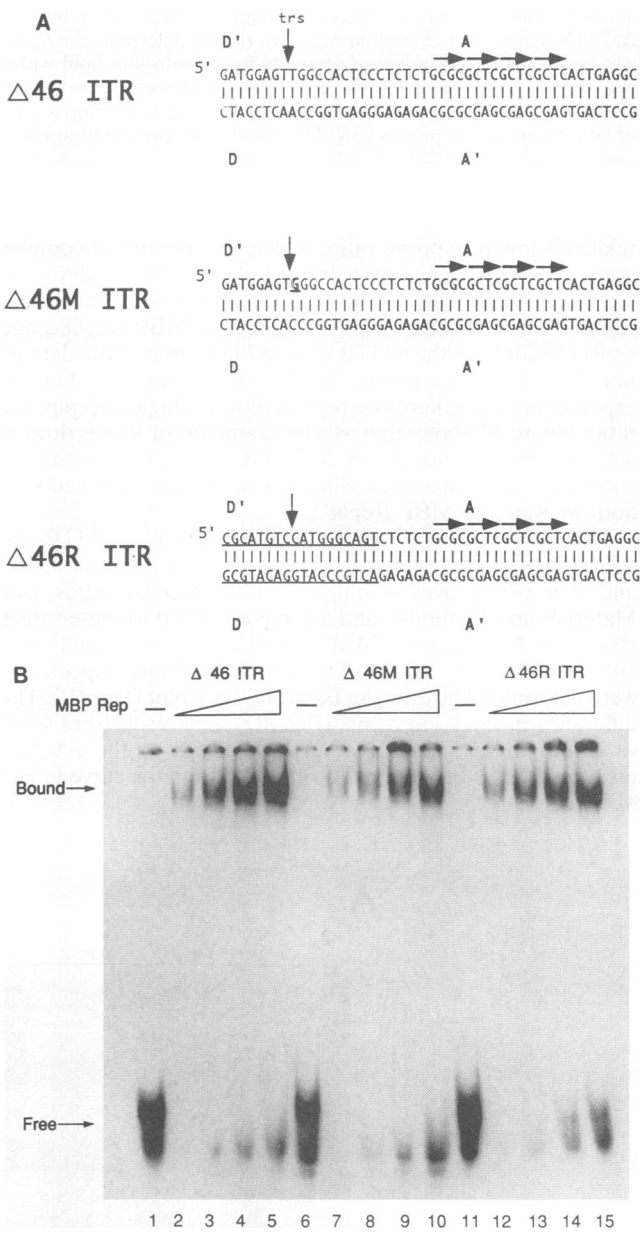

FIG. 7. MBP-Rep68 binding is stabilized by nonspecific flanking sequence, and trs is not required for stable binding. (A) Three oligonucleotide probes were made to address the differences in binding affinity between Δ57 ITR and Δ28 ITR. Δ46M ITR is identical to the wt sequence except for a point mutation at the trs site (T-to-G substitution; highlighted and underlined). Δ46R ITR is the same probe as Δ28 ITR except that it is extended with random DNA to be the same length as Δ46 ITR. The GCTC repeats are indicated with horizontal arrows. The trs is indicated for the Δ46 ITR and Δ46M ITR probes, and its potential site in the Δ46R ITR probe is indicated by a vertical arrow. (B) Lanes 1, 6, and 11 contain no protein. All other lanes contain a constant amount of MBP-Rep68Δ and increasing amounts (10, 20, 30, and 40 fmol) of Δ46 ITR (lanes 2 to 5), Δ46M ITR (lanes 7 to 10), and Δ46R ITR (lanes 12 to 15) probes. All three probes bound MBP-Rep68Δ with the same affinity, indicating that the increase in binding affinity is the result of increased probe length and not specific protein-DNA interactions.

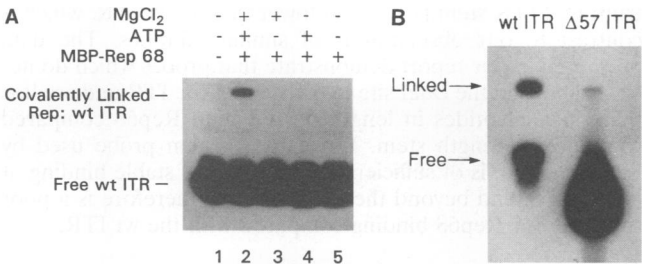

FIG. 8. Covalent linkage of MBP-Rep68Δ to wt ITR and Δ57 ITR. The standard trs endonuclease assays were performed as described by Im and Muzyczka (17), with the following modifications. After the incubation of 100 ng of protein with 50 fmol of 3′-labeled wt ITR in trs buffer, SDS sample buffer was added, the sample was heated, and the probe covalently bound to MPB-Rep68Δ was separated from the free probe by electrophoresis on an SDS–10% polyacrylamide gel. (A) To confirm the specificity of the trs endonuclease assay, the dependence of wt ITR retarded mobility on ATP, $MgCl_2$, and MPB-Rep68Δ was examined. Formation of the ITR-MPB-Rep68Δ retarded complex is dependent on ATP, $MgCl_2$, and MBP-Rep68Δ. (B) By comparing the amounts of free probe versus that covalently linked to the MBP-Rep protein, the efficiency with which the two probes were cleaved was determined. The probes used in this experiment were 3′ labeled to the same specific activity of 2×10^5 cpm/pmol. Lane 1 contains wt ITR as a substrate for MBP-Rep68Δ, and lane 2 contains Δ57 ITR. The covalently linked DNA-MBP-Rep68Δ complex (linked) and free DNA probe (free) are indicated by arrows at the left. Since the covalent linkage occurred at a greatly reduced efficiency on the Δ57 ITR probe compared with the wt ITR probe, 500 fmol of Δ57 ITR probe was used in this experiment, compared with 50 fmol of the wt ITR probe.

the formation of a lower-mobility band on an SDS-polyacrylamide gel upon incubation of MBP-Rep68Δ with 3′-radiolabeled ITR in a trs endonuclease assay (Fig. 8A). The substrate was 3′ end labeled so that the label would be present on the fragment which was covalently linked to MBP-Rep68Δ. The reduced mobility of this complex compared with the unbound fragment would allow for determination of the efficiency of linkage.

The results of this experiment (Fig. 8B) indicate that hairpin ITR is cleaved at least 100 times more efficiently than Δ57 ITR. Because of the inefficiency of linkage to the Δ57 ITR probe, 10 times the molar amount of probe was added to the reaction mixture compared with the amount of wt ITR probe. Thus, while the hairpin portion of the ITR does not play a role in binding of Rep to the ITR, its presence does stimulate trs endonuclease activity.

Previously, DNase I protection by Rep68 has been reported in the B/B′ palindromic region of the ITR (16). It is possible that interaction with this portion of the ITR could act to stimulate the covalent linkage of Rep68 to the DNA substrate. However, addition in *trans* of increasing amounts of a synthetic hairpin cross arm (Fig. 1E), which was shown to be folded into the hairpin structure by digestion with *Sma*I, had no effect on the amount of covalently bound MBP-Rep68Δ detected. Furthermore, addition of the hairpin cross arm to an EMSA had no effect on binding and itself could not bind Rep68 (data not shown).

DISCUSSION

Rep68 is a critical protein in the life cycle of AAV. Previous work has shown that a recombinant version of this protein produced in *E. coli* can catalyze all of the known in vitro activities associated with wt Rep68 (8). This report demonstrates that an additional function of wt Rep68 is its ability to bind to a linear form of the ITR, Δ57 ITR, with the same affinity as to wt hairpin ITR. MBP-Rep68Δ and wt Rep68 also appear to have similar specific DNA binding activities. A dissociation constant of approximately 8×10^{-10} M was determined for the wt ITR and Δ57 ITR probes with MBP-Rep68Δ. However, a truncated version of Δ57 ITR, Δ28 ITR, bound 10 times less efficiently than the full-length fragment. Truncation of Δ57 ITR with *Dde*I also inhibited binding, which is in agreement with previously published data (43). Footprinting analysis indicated that the *Dde*I truncation cuts within the protected region. This observation may explain why truncation with *Dde*I resulted in an inhibition of binding activity and indicates the importance of this region in the formation of a stable complex. Extension of Δ28 ITR with random sequence restored the ability of MBP-Rep68Δ to bind to Δ28 ITR. Thus, the increase in binding would appear to be the result of nonspecific sequence stabilization of MBP-Rep68Δ binding. It is not clear whether extension of the *Dde*I-truncated Δ57 ITR with random sequence would have the same stabilizing effect or if that sequence is important for stable binding. Mutation of the trs had no effect on binding, which is in agreement with other published data (43).

To further define the MBP-Rep68Δ binding site, we constructed a series of oligonucleotide probes which spanned the region protected by MBP-Rep78 in a DNase I footprinting experiment. Two 18-bp probes which contained the imperfect $(GCTC)_4$ repeat motif were found to be sufficient for the binding. While these data show that Rep68 is able to bind to nonhairpin derivatives of the ITR and identify some of the features required for binding of Rep68 to DNA, this ability of Rep68 to bind to linear DNA does not appear to correlate with trs endonuclease activity. The covalent linkage of Rep68 to the non-hairpin-containing ITR, Δ57 ITR, is approximately 100-fold less efficient than linkage to a hairpin-containing ITR. This effect of the hairpin portion of the ITR was observed only when the hairpin was part of the probe, not when it was supplied in *trans*. This difference was confirmed by the standard trs endonuclease assay (8). Thus, while the hairpin portion of the ITR does not appear to play a role in the stabilization of Rep68 binding, its presence is required for efficient trs endonuclease activity. The features of the hairpin structure that enhance cleavage activity are not clear. The presence of the hairpin structure may force the DNA into a conformation that is more favorable for cleavage. However, previous work showed that digestion of the hairpin structure with the restriction enzyme *Sma*I, which removed most of the C/C′ duplex, had little or no effect on trs endonuclease activity by Rep68 (43). Since trs endonuclease activity is thought to be central for the replication of AAV DNA, these data could indicate the importance of structure in this region for efficient trs endonuclease activity.

The data presented in this report indicate that a hairpin conformation is not required for binding of wt Rep68 or MBP-Rep68Δ to the ITR. Footprinting experiments indicate that the *Dde*I site is protected, and therefore truncation at the *Dde*I site would remove a portion of the binding site and inhibit binding. Studies in the other reports of Rep68 not binding to linear DNA used linear wt ITR as either a probe or a competitor. This fragment is a more complex substrate, and lack of binding is not as easily interpretable. The linear ITR contains two binding sites for Rep68 in close proximity and may not bind Rep68 because of interference between these sites. An alternate hypothesis is that the palindromes which are normally in a fully hairpin conformation in the wt ITR are now

in a linear conformation. This may create a partial hairpin structure that is inhibitory for Rep68 binding.

Our observation that Rep can bind to linear DNA clarifies some of the effects that p5-initiated Rep proteins have on the expression of heterologous promoters and the apparent cytostatic effects of overexpression of the Rep proteins. These effects may be due to direct Rep-DNA interaction within gene regulatory elements. It has been recently reported that a sequence with homology to the GCTC repeat motif is present in the long terminal repeat promoter of human immunodeficiency virus type 1 and may be involved in the inhibition of this virus by the Rep proteins (34). The binding of Rep68 and Rep78 to cellular promoters could have a negative effect on gene expression such as occurs with the p5 and p19 promoter of AAV2 (2, 3, 24, 27). Binding of p5 Rep proteins could also have a positive effect and stimulate the expression of silenced genes in the cell or overexpression of genes involved in down regulation of the cell. Such stimulatory effects of Rep have been reported for the p40 promoter of AAV2 (30, 46).

The results of methylation interference indicated that the guanine residues in the GCTC repeats of the AAV integration locus, AAVS1, are involved in binding (48). However, what is not known is the importance of the individual bases within the repeats or the contribution of the flanking sequence in forming a stable Rep-DNA complex. This information is necessary for defining a consensus sequence for Rep68 and identifying genes that could be regulated by Rep.

The observation that binding of MBP-Rep68Δ is distinct from trs endonuclease activity is important when one considers the life cycle of the virus. During a lytic infection, trs endonuclease activity has to be efficient to complete the replication of the large number of viral particles forming in the cell. The inefficient trs endonuclease activity on nonhairpin or defective ITRs maybe a way of ensuring that only full-length genomes are replicated. Nicking of the DNA during the process of rescue may need to occur only once and therefore can afford to be inefficient. Furthermore, cleavage of the viral and cellular promoters which are Rep responsive is not necessary for its transcriptional regulatory effects.

The ability of Rep68 to stably bind to a GCTC repeat sequence present in both the AAV ITR and the integration locus without the necessity of an adjacent palindromic hairpin structure allows for speculation that such binding may play an essential role in the process of targeted AAV integration (8, 48). The presence of stable Rep binding sites at both loci suggest that Rep-Rep interaction may align the preintegration site locus with the AAV ITR. It is unclear if other GCTC repeat sequences identified at other loci are capable of participating as alternate AAV integration sites. The data presented in this report showing that the hairpin is not required for binding of Rep68 and the identification of the binding site should facilitate the understanding of the positive and negative effects of p5-promoted Rep proteins on the expression of heterologous promoters.

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ADDENDUM

A recent publication has confirmed our earlier observation that Rep68 is able to bind to a linear truncated form of the ITR. McCarty et al. (30a) report that binding to a linear truncated ITR stem is 125-fold lower than to wt ITR, which is contrast to our observations of similar affinities. The data presented in our report demonstrate that probes which do not extend beyond the *Dde*I site in the stem of the ITR and are less than 46 nucleotides in length do not bind Rep68 compared with the full-length stem. While the A-stem probe used by McCarty et al. is of sufficient length to allow stable binding, it does not extend beyond the *Dde*I site and therefore is a poor substrate for Rep68 binding compared with the wt ITR.

REFERENCES

1. **Ashktorab, H., and A. Srivastava.** 1989. Identification of nuclear proteins that specifically interact with adeno-associated virus type 2 inverted terminal repeat hairpin DNA. J. Virol. **63**:3034–3039.
2. **Beaton, A., P. Palumbo, and K. I. Berns.** 1989. Expression from the adeno-associated virus p5 and p19 promoters is negatively regulated in *trans* by the *rep* protein. J. Virol. **63**:4450–4454.
3. **Berns, K. I., R. M. Kotin, and M. A. Labow.** 1988. Regulation of adeno-associated virus DNA replication. Biochim. Biophys. Acta **951**:425–429.
4. **Bohenzky, R. A., and K. I. Berns.** 1989. Interactions between the termini of adeno-associated virus DNA. J. Mol. Biol. **206**:91–100.
5. **Bohenzky, R. A., R. B. LeFebvre, and K. I. Berns.** 1988. Sequence and symmetry requirements within the internal palindromic sequences of the adeno-associated virus terminal repeat. Virology **166**:316–327.
6. **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.
7. **Chejanovsky, N., and B. J. Carter.** 1989. Replication of a human parvovirus nonsense mutant in mammalian cells containing an inducible amber suppressor. Virology **171**:239–247.
8. **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.
9. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9**:6505–6525.
10. **Garner, M. M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: applications to components of the Escherichia coli lactose operon regulatory system. Nucleic Acids Res. **9**:3047–3060.
11. **Hermonat, P. L.** 1989. The adeno-associated virus Rep78 gene inhibits cellular transformation induced by bovine papillomavirus. Virology **172**:253–261.
12. **Hermonat, P. L.** 1991. Inhibition of H-ras expression by the adeno-associated virus Rep78 transformation suppressor gene product. Cancer Res. **51**:3373–3377.
13. **Hermonat, P. L.** 1992. Inhibition of bovine papillomavirus plasmid DNA replication by adeno-associated virus. Virology **189**:329–333.
14. **Hermonat, P. L., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka.** 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol. **51**:329–339.
15. **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.
16. **Im, D. S., and N. Muzyczka.** 1989. Factors that bind to adeno-associated virus terminal repeats. J. Virol. **63**:3095–3104.
17. **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.
18. **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.
19. **Jacob, W. F., T. A. Silverman, R. B. Cohen, and B. Safer.** 1989. Identification and characterization of a novel transcription factor participating in the expression of eukaryotic initiation factor 2 a. J. Biol. Chem. **264**:20372–20384.
20. **Khleif, S. N., T. Myers, B. J. Carter, and J. P. Trempe.** 1991.

Inhibition of cellular transformation by the adeno-associated virus rep gene. Virology **181**:738–741.

21. **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.
22. **Kotin, R. M., J. C. Menninger, D. C. Ward, and K. I. Berns.** 1991. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. Genomics **10**:831–834.
23. **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.
24. **Kyöstiö, 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 p5 and p19 mRNA levels. J. Virol. **68**:2947–2957.
25. **Labow, M. A., and K. I. Berns.** 1988. The adeno-associated virus *rep* gene inhibits replication of an adeno-associated virus/simian virus 40 hybrid genome in cos-7 cells. J. Virol. **62**:1705–1712.
26. **Labow, M. A., L. H. Graf, Jr., and K. I. Berns.** 1987. Adeno-associated virus gene expression inhibits cellular transformation by heterologous genes. Mol. Cell. Biol. **7**:1320–1325.
27. **Labow, M. A., P. L. Hermonat, and K. I. Berns.** 1986. Positive and negative autoregulation of the adeno-associated virus type 2 genome. J. Virol. **60**:251–258.
28. **LeFebvre, R. B., and K. I. Berns.** 1984. Unique events in parvovirus replication. Microbiol. Sci. **1**:163–167.
29. **LeFebvre, R. B., S. Riva, and K. I. Berns.** 1984. Conformation takes precedence over sequence in adeno-associated virus DNA replication. Mol. Cell. Biol. **4**:1416–1419.
30. **McCarty, D. M., M. Christensen, and N. Muzyczka.** 1991. Sequences required for the coordinate induction of adeno-associated virus p19 and p40 promoters by the Rep protein. J. Virol. **65**:2936–2945.

30a. **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.

31. **Meisterernst, M., I. Gander, L. Rogge, and E. L. Winnacker.** 1988. A quantitative analysis of nuclear factor I/DNA interactions. Nucleic Acids Res. **16**:4419–4435.
32. **Mendelson, E., J. P. Trempe, and B. J. Carter.** 1986. Identification of the *trans*-acting Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. J. Virol. **60**:823–832.
33. **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.
34. **Oelze, I., K. Rittner, and G. Sczakiel.** 1994. Adeno-associated virus type 2 *rep* gene-mediated inhibition of basal expression of human immunodeficiency virus type 1 involves its negative regulatory functions. J. Virol. **68**:1229–1233.
35. **Owens, R. A., and B. J. Carter.** 1992. In vitro resolution of adeno-associated virus DNA hairpin termini by wild-type Rep protein is inhibited by a dominant-negative mutant of *rep*. J. Virol. **66**:1236–1240.
36. **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.
37. **Owens, R. A., M. D. Weitzman, S. R. M. Kyöstiö, 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.
38. **Samulski, R. J., L. S. Chang, and T. Shenk.** 1987. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised in vitro and its use to study viral replication. J. Virol. **61**:3096–3101.
39. **Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka.** 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell **33**:135–143.
40. **Senapathy, P., J. D. Tratschin, and B. J. Carter.** 1984. Replication of adeno-associated virus DNA. Complementation of naturally occurring rep- mutants by a wild-type genome or an ori- mutant and correction of terminal palindrome deletions. J. Mol. Biol. **179**:1–20.
41. **Shelling, A. N., and M. G. Smith.** 1994. Targeted integration of transfected and infected adeno-associated virus vectors containing the neomycin resistance gene. Gene Ther. **2**:1–5.
42. **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.
43. **Snyder, R. O., D.-S. Im, T. Ni, X. Xiao, R. J. Samulski, and N. Muzyczka.** 1993. Features of the adeno-associated virus origin involved in substrate recognition by the viral Rep protein. J. Virol. **67**:6096–6104.
44. **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.
45. **Tratschin, J. D., I. L. Miller, and B. J. Carter.** 1984. Genetic analysis of adeno-associated virus: properties of deletion mutants constructed in vitro and evidence for an adeno-associated virus replication function. J. Virol. **51**:611–619.
46. **Trempe, J. P., and B. J. Carter.** 1988. Regulation of adeno-associated virus gene expression in 293 cells: control of mRNA abundance and translation. J. Virol. **62**:68–74.
47. **Trempe, J. P., E. Mendelson, and B. J. Carter.** 1987. Characterization of adeno-associated virus rep proteins in human cells by antibodies raised against rep expressed in Escherichia coli. Virology **161**:18–28.
48. **Weitzman, M. D., S. R. M. Kyöstiö, 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.