Adeno-Associated Virus Rep Protein-Mediated Inhibition of Transcription of the Adenovirus Major Late Promoter In Vitro

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Adeno-associated virus (AAV) is a human parvovirus that normally requires a helper virus such as adenovirus (Ad) for replication. The four AAV replication proteins (Rep78, Rep68, Rep52, and Rep40) are pleiotropic effectors of virus integration, replication, transcription, and virion assembly. These proteins exert effects on Ad gene expression and replication. In transient plasmid transfection assays, Rep proteins inhibit gene expression from a variety of transcription promoters. We have examined Rep protein-mediated inhibition of transcription of the Ad major late transcription promoter (AdMLP) in vitro. Rep78/68 are the strongest transcription suppressors and the purine nucleotide binding site in the Rep proteins, and by implication, the ATPase activity or conformational change induced by nucleotide binding is required for full repression. Rep52 has modest effects, and Rep40 exerts no significant effect on transcription. Rep78/68 and their N-terminal 225-residue domain bind to a 55-bp AdMLP DNA fragment in gel shift assays, suggesting that protein-DNA interactions are required for inhibition. This interaction was confirmed in DNase I protection assays and maps to a region extending from the TATA box to the transcription initiation site. Gel shift, DNase I, and chemical cross-linking assays with TATA box-binding protein (TBP) and Rep68 indicate that both proteins interact with each other and with the promoter at adjacent sites. The demonstration of Rep interaction with TBP and the AdMLP suggests that Rep78/68 alter the preinitiation complex of RNA polymerase II transcription. These observations provide new insight into the mechanism of Rep-mediated inhibition of gene expression.

Adeno-associated virus (AAV) is a helper-dependent human parvovirus that normally requires coinfection with adenovirus (Ad) to establish a productive infection. AAV has two translation open reading frames that encode three capsid (Cap) proteins and four replication (Rep) proteins. Rep78 and Rep68 are translated from mRNAs originating from a transcription promoter at map unit 5 (p_5). Rep52 and Rep40 are translated from mRNAs originating from a transcription promoter at map unit 19 (p_{19}). Rep68 and Rep40 differ from Rep78 and Rep52 as a result of mRNA splicing that replaces 92 amino acids from the carboxyl terminus with 9 amino acid residues. Rep78/68 are required for viral DNA replication, regulation of AAV gene expression, and site-specific integration into human chromosome 19, which occurs in the absence of helper virus infection (39). The smaller Rep proteins, Rep52/40, play roles in virus assembly (11, 32).

Upon entering the nucleus, host DNA polymerase synthesizes a complementary strand to the AAV single-stranded DNA genome. The primary role of Rep78/68 in AAV DNA replication is to resolve the covalently closed, T-shaped, inverted terminal repeat (ITR) element, resulting in complete synthesis of a replicative-form monomer viral DNA. Rep78/68 mediate viral replication by interacting with a Rep binding site (RBS) in the ITR and nicking the terminal resolution site (*trs*) (2, 29, 49). All four Rep proteins are ATPases and SF3 family helicases (22). In one model of AAV DNA replication,

Rep78/68 oligomerize on the ITR, induce localized denaturation at *trs*, cleave *trs*, and covalently link to the 5' end of the DNA (7, 8). The ITR is then unwound, and DNA synthesis is completed on the single-strand region that results from *trs* cleavage.

Rep78/68 regulate AAV gene expression. Rep78/68 repress p_5 promoter-directed transcription, and an RBS in the p_5 promoter is essential for inhibition (6, 28, 33, 44). During Ad coinfection, Rep78/68 activate p_{19} and p_{40} promoter transcription (37, 45). Transactivation of the AAV p_{40} promoter requires the RBS in the ITR or p_5 promoter, a CarG-like element in the p_{19} promoter, and a Sp1 binding site in the p_{40} promoter (45). AAV and the Rep proteins also regulate Ad gene expression during coinfection. The Rep78/68 proteins bind to the Ad E2a promoter, reducing the amount of E2a mRNA (10, 30, 40). Rep proteins interact with cellular proteins involved in transcription regulation, including Sp1 (25, 45), high-mobility-group nonhistone protein 1 (15), the transcriptional coactivator PC4 (54), TATA-binding protein (26, 51), a putative protein kinase (protein kinase X or PKX), and protein kinase A (PKA) (13, 17). The biological effect of the PKX association is inhibition of the steady-state levels of cyclic AMP-responsive-element-binding protein (CREB) and cyclin A protein. Rep expression in the absence of virus infection inhibits gene expression at the transcription and translation levels from a variety of transcription promoters (1, 28, 30, 31, 34). While the number of Rep-interacting partners is growing, the mechanism whereby the Rep proteins regulate transcription remains undefined.

We have initiated a biochemical characterization of Rep-mediated inhibition of transcription by studying Ad major late

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transcription promoter (AdMLP) transcription in vitro using purified Rep proteins. The results of our studies demonstrate that Rep68 suppresses AdMLP transcription in vitro (40). Here we show that Rep78/68 are the strongest transcription suppressors and that the purine nucleotide binding site (PNB) in the Rep proteins, and by implication, the ATPase activity or conformational change induced by nucleotide binding is required for full repression. Rep68 and its N-terminal domain bind to the AdMLP element in electrophoretic mobility gel shift assays (EMSAs). DNase I mapping localizes Rep interaction to the region extending from the TATA element to the transcription initiation site. When combined with purified TATA-binding protein (TBP), Rep68 alters TBP-AdMLP interactions. The results of these studies indicate that the larger Rep78/68 proteins alter the transcription preinitiation complex, resulting in transcription suppression.

MATERIALS AND METHODS

Purification of Rep proteins. Rep68 (10), RepNT (10), Rep52, and Rep40 (14) were expressed and purified from *Escherichia coli* as previously described.

Purification of MBPRep78 and MBPRep68. Rep78 and Rep68 were expressed individually in *E. coli* as maltose-binding protein (MBP) fusions encoded by the pRepMal78 plasmid (12). Cells were grown at 37°C in LB medium containing M9 salts (50), 2 mM MgSO₄, 0.1 mM CaCl₂, 1% (wt/vol) glucose, and 50 µg/ml ampicillin to an A₆₀₀ of 0.8 to 1.0 and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown for 3 h after induction and harvested by centrifugation. Purification procedures were performed at 4°C. Cells (25 g in a typical preparation) were suspended in 5 volumes of 25 mM Tris-HCl (pH 7.5 at 25°C), 1 mM EDTA, 1 mM dithiothreitol (DTT), 20% (vol/vol) glycerol, 250 mM NaCl, 0.1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.01 mg/ml lysozyme and incubated 30 min. The extract was centrifuged at 27,000 × g (average) for 30 min, and the supernatant was retained. Polyethylene glycol 8000 (0.25 volume of a 50% [wt/vol] solution) was slowly added to 10% (wt/vol) with stirring to the supernatant and stirred for 30 min after the polyethylene glycol was added. The suspension was centrifuged at 17,000 × g for 40 min, and the precipitate was resuspended with a Dounce homogenizer in 50 ml of buffer A (25 mM Tris-HCl [pH 7.5 at 25°C], 1 mM EDTA, 1 mM DTT, 20% [vol/vol] glycerol, 50 mM NaCl, and 1 mM PMSF). The solution was applied to a Q-Sepharose column (2.5 × 10.5 cm) equilibrated with the same buffer, and the column was washed with 4 bed volumes of the same buffer. The column was eluted with a 20-column-volume, linear gradient from 50 mM to 1 M NaCl in buffer A. Fractions were assayed for ATPase activity and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). MBPRep78-containing fractions were combined and concentrated with an Amicon ultrafiltration cell using a PM10 membrane. The concentrated material was applied to a Sephacryl S-300 gel filtration column (1.5 × 141 cm) previously equilibrated in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 20% (vol/vol) glycerol, and 200 mM NaCl and eluted at 10 ml/h. Fractions were examined by ATPase assays and SDS-PAGE, pooled, diluted to 50 mM NaCl, and applied to a column of S-Sepharose (1.5 × 15 cm) equilibrated in 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 20% (vol/vol) glycerol, and 50 mM NaCl. The column was eluted at 30 ml per hour with a 10-column-volume gradient of NaCl from 50 to 400 mM. Fractions were assayed for ATPase and examined by SDS-PAGE, and active fractions were stored at -80°C.

Purification of human TBP. Histidine-tagged human TBP was purified by the method of Maldonado et al. (36) with some modifications. *E. coli* BL21(DE3) cells were transformed with a His-tagged pET-TBP plasmid (36) (kindly provided by Kam Yeung, Medical University of Ohio). LB broth cultures containing 50 µg/ml of ampicillin were grown to an optical density at 595 nm of 0.7. TBP expression was induced by adding IPTG to a final concentration of 1 mM, and the cultures were incubated for a further 90 min. One liter of cells was harvested by centrifugation and resuspended in 50 ml of buffer B (20 mM Tris-HCl, pH 7.9, 10% [vol/vol] glycerol, 10 mM mercaptoethanol) containing 1 M KCl and 0.05% (vol/vol) NP-40. Lysozyme was added to a final concentration of 1 mg/ml. The suspension was incubated for 20 min on ice and then sonicated for three 45-s bursts using a Fisher sonic dismembrator. The lysate was centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was transferred to a fresh tube containing Ni²⁺-nitrilotriacetic acid resin previously equilibrated with buffer B containing 1

M KCl, 0.05% NP-40, and 1 mM imidazole. The slurry was transferred to a column, and the resin was washed with buffer B containing 1 M KCl, 0.05% NP-40, and 10 mM imidazole. The resin was then eluted using a step gradient of buffer B containing 1 M KCl and 0.05% NP-40 and 60 mM, 200 mM, and 500 mM imidazole. The 200 mM imidazole fraction was dialyzed against buffer A (20 mM HEPES-KOH, pH 7.9, 10% (vol/vol) glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) containing 25 mM KCl. The sample was then loaded onto a 1-ml Mono-S column and separated by a linear gradient of 25 mM to 1 M KCl in buffer A at a rate of 30 ml/h. TBP was eluted at approximately 530 mM KCl. Fractions containing TBP were either used directly or dialyzed against buffer A containing 25 mM KCl. Approximately 25 to 50 µg of TBP was obtained per liter of culture.

HeLa nuclear extracts. HeLa-S cells from 10 liters of culture were obtained from Biostest International Inc.-National Cell Culture Center as cell pellets on ice. Nuclear extracts were prepared by the method of Dignam et al. (16). Approximately 10 ml of extract at a protein concentration of 5 to 7 mg/ml was typically obtained from 5 × 10⁹ cells.

In vitro transcription reactions. Transcription reactions were performed using HeLa nuclear extracts and 150 ng of XbaI-linearized pTIGL (53). The pTIGL plasmid contains the AdMLP from nucleotides (nt) 6004 to 6053 of adenovirus type 5 (Ad5). The sequence contains the MAZ site that is located 5' to the TATA box and extends 9 nt downstream of the transcription initiation site. It lacks the upstream promoter element (UPE). The promoter directs the synthesis of a 388-nt G-less cassette. Reaction mixtures of 25 µl containing 60 to 80 µg of HeLa nuclear extract, 8.8 mM HEPES (pH 7.9), 44 mM KCl, 0.22 mM DTT, 0.09 mM EDTA, 8.8% glycerol, 6 mM MgCl₂, 0.4 mM each of ATP, CTP, and GTP, and 10 µCi of [γ -³²P]UTP (3,000 Ci/mmol) were incubated at 30°C for 60 min. RNase T₁ (50 units) was added and incubated for 10 min to remove background transcripts that did not originate from the AdMLP G-less cassette. Reaction products were phenol-chloroform extracted, ethanol precipitated, and analyzed on a 6% polyacrylamide-7 M urea gel in 0.5× Tris-borate-EDTA (TBE) (3). To examine transcription repression, individual Rep proteins were added to standard transcription reaction mixtures and compared to transcription reaction mixtures containing an equal volume of protein dialysis buffer. The amount of transcription was assessed by scanning gels using a Molecular Dynamics Storm 840 phosphorimager and ImageQuant software.

EMSAs. Rep binding to AdMLP DNA was analyzed by electrophoretic mobility shift assays. A 54-base-pair oligonucleotide containing the AdMLP from nt 6004 to 6058 in the Ad5 genome was end labeled with T4 polynucleotide kinase and [γ -³²P]ATP. Labeled DNA and protein in a 20-µl reaction mixture were incubated for 30 min at 25°C in binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM MgCl₂, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol) with 250 ng poly(dI-dC) and 0.5 µg bovine serum albumin per reaction mixture. When TBP was included in the assays, poly(dG-dC) (62.5 ng/ml) was used in lieu of poly(dI-dC). Samples were adjusted to 50 mM Tris-HCl (pH 7.5), 0.04% bromophenol blue, and 8% glycerol by the addition of a 5× concentrated stock solution, and 8 to 12 µl was applied to a 4% polyacrylamide gel in 0.5× TBE. Gels were run at 4°C at 300 V. Gels were dried, and radioactive bands were detected by phosphorimaging as described above. For competition studies, labeled DNA and unlabeled competitor were combined and the Rep protein was added. The oligonucleotides used are as follows: the 57-bp fragment (5'-GAATTCCTGAA GGGGGCTATAAAGGGGGTGGGGGCGCTTCGTCTCTCACTC TCTT 3'); Oligo-1, 18 bp (5'-TTCCTGAAGGGGGGC 3'); Oligo-2, 21 bp (5'-TATAAAGGGGGTGGGGGCGC 3'); Oligo-3, 18 bp (5'-GTTCTGCTCTCAC TCTCTT 3'). The first three nucleotides of the 57-bp fragment and Oligo-1 are not part of the Ad5 sequence. For TBP-AdMLP EMSAs, poly(dG-dC) was used as a competitor because TBP-DNA interactions are negligible when poly(dI-dC) is used (19).

DNase I protection assays. DNase I protection assays were performed as described previously (10). The AdMLP fragment was obtained by PCR amplification of a 336-bp DNA between nt 5893 and 6229 of the Ad5 genome using the following primers: 5'-GTCGTTGTCCACTAGG-3' and 5'-GATTGTCTTTCT GACCAG-3'. One of the primers was labeled with [γ -³²P]ATP and polynucleotide kinase prior to amplification.

Rep68-TBP cross-linking and immunoprecipitation. To measure TBP and Rep68 interactions, formaldehyde cross-linking experiments were performed. Twenty-microliter EMSA reaction mixtures were set up using the TBP conditions described above except 25 mM HEPES buffer (pH 7.5) was used instead of Tris-HCl. Rep68 in different concentrations was added to the mixtures and incubated for 10 min at room temperature. Unlabeled AdMLP (2.5 pM) was added, and the incubation was continued for 30 min. Formaldehyde in 1× binding buffer was added to a final concentration of 1% and incubated for 5 min. Ten microliters of 1 M glycine was added to inactivate the cross-linker. Three

hundred microliters of radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Na₂HPO₄, pH 7.3, 2 mM EDTA) containing preimmune rabbit serum was added to each reaction mixture. After the mixtures were incubated for 60 min at room temperature with periodic mixing, they were added to 60- μ l slurries of immunoprecipitin (Gibco-BRL) previously washed in RIPA buffer. The mixtures were incubated at room temperature for 60 min. The antibody-immunoprecipitin complexes were pelleted by centrifugation, and the supernatants were transferred to fresh tubes containing anti-Rep antisera and incubated overnight at 4°C. The mixtures were then added to 60- μ l slurries of immunoprecipitin that had been washed with RIPA buffer and incubated for 60 min at room temperature. The antigen-antibody-immunoprecipitin complexes were pelleted and washed three times with 500 μ l of RIPA buffer. The final pellets were resuspended in 1 $\times$ SDS-PAGE sample buffer (3) and boiled for 20 min to reverse the formaldehyde cross-links. The samples were pelleted, and supernatants were separated on 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose filter paper and analyzed by immunoblotting (3) using anti-TBP (sc421; Santa Cruz) and anti-Rep (52).

RESULTS

Rep-mediated inhibition of AdMLP-directed transcription.

We have demonstrated that AAV Rep68 inhibits AdMLP transcription during in vitro assays (40). Rep68 inhibited AdMLP-directed gene transcription more effectively than did Rep68 with a mutation in its purine nucleotide binding site. All of the Rep proteins have DNA-stimulated ATPase activity and function as DNA helicases (14, 48, 59). To determine which domains in Rep68 are responsible for the inhibition of transcription, in vitro transcription assays were performed using a G-less transcription cassette under the control of the AdMLP (53). The reaction mixtures were supplemented with Rep68 and Rep40 and their PNB mutant versions or with RepNT (Fig. 1A). RepNT is a 225-amino-acid, His-tagged, truncated Rep78/68 (truncated carboxyl terminus). This N-terminal domain mediates Rep78/68 binding to the AAV terminal repeat DNA elements (27, 42). Rep68 strongly suppressed transcription as described previously (Fig. 1A) (40). RepNT and Rep40, individually or in combination, inhibited AdMLP transcription only slightly. Thus, the DNA binding domain and the helicase domains (contained in Rep40) of Rep68 must be contiguous to achieve full transcription inhibition. Transcription inhibition could result from nonspecific interactions between Rep and the template DNA. To test this possibility, Rep68 was incubated with increasing concentrations of poly(dI-dC) or poly(dG-dC) and added to the in vitro transcription reaction mixture. In these reaction mixtures, Rep68 inhibited transcription from the AdMLP transcription template regardless of the amount of either competitor DNA (data not shown). To determine whether Rep68 acts preferentially on the template DNA or on some component of the HeLa nuclear extract, Rep68 was preincubated with the template DNA or the HeLa nuclear extract. Preincubation of Rep68 with the HeLa extract for 10 min, prior to addition of the other components of the transcription reaction mixture, resulted in the strongest inhibition (Fig. 1B, compare lanes 3 and 4). However, preincubation of Rep68 with the template did not result in greater inhibition of transcription (lane 2). To determine whether Rep68 interacts with a component of the HeLa extract, Rep68 was preincubated with increasing amounts of HeLa extract followed by template. This experiment showed that transcription increased with increasing amounts of HeLa extract (Fig. 1C). These experiments indicate that Rep68 does not inhibit transcription by random interactions with the transcription template and

FIG. 1. Rep-mediated inhibition of AdMLP transcription in vitro. A. In vitro transcription assays were performed with HeLa nuclear extracts, the linearized AdMLP and equimolar (200 nM) amounts of RepNT, Rep40, and Rep68. Lane 1, no added Rep proteins; lane 2, RepNT; lane 3, Rep40; lane 4, RepNT and Rep40 added together; lane 5, Rep68 alone. B. Effect of the order of addition on Rep68-mediated repression. Lane 1, no Rep68 added; lane 2, Rep68 preincubated with template 10 min before addition of HeLa extract; lane 3, Rep68 preincubated with HeLa extract 10 min before template addition; lane 4, Rep68 added 2 min after the start of transcription. C. Effect of increasing amounts of HeLa extract. Lane 1, control transcription with 64 μ g of extract and no Rep protein; lanes 2 to 4, Rep68 added at 0.4 μ M and increasing amounts of HeLa extract (64, 96, and 128 μ g), respectively. The RNA product of 388 nt is shown in all panels.

that Rep68 may interact with a specific region of the template and/or a component of the HeLa nuclear extract, resulting in greater inhibition of transcription.

Titration experiments to measure the effects of Rep proteins on AdMLP transcription demonstrated that wild-type Rep68 was a stronger inhibitor of transcription than Rep68PNB was (Fig. 2). Our previous studies indicated that Rep68PNB, at much lower concentrations (2 nM) than those used in Fig. 2, had no effect on in vitro transcription from the AdMLP (40). Clearly, higher concentrations of Rep68PNB inhibit transcription. Wild-type Rep52 inhibited transcription less effectively than Rep68 did but comparably to Rep68PNB. Rep40, Rep40PNB, and Rep52PNB were not inhibitory. The inability of Rep40 to inhibit transcription suggests that the carboxyl terminus of Rep52 contributes to the effect. However, the lack of an effect from Rep52PNB indicates that a functional helicase domain is involved in Rep52-mediated

FIG. 2. Effects of different Rep proteins on transcription from the AdMLP. A. Purified wild-type and PNB mutant Rep proteins were added to the standard transcription reaction mixture. Transcription reaction products were separated by denaturing gel electrophoresis, and the amount of radioactivity in the 388-nt RNA product was determined by using a phosphorimager. The results are reported as the level of transcription of the Rep-containing reaction mixtures relative to control reaction mixtures (normalized signal [Norm. Signal]). The results are the means $\pm$ standard deviations (error bars) of three determinations. B. Purified MBPRep78 ($\bullet$) and MBPRep68 ($\circ$) were added to standard transcription reaction mixtures as described above. The results are the averages of two determinations at each concentration.

inhibition. To determine whether there are functional differences between Rep78 and Rep68 in suppression of the AdMLP transcription, we performed similar experiments using MBP-Rep fusion proteins. These fusion proteins have been used extensively in the study of the enzymatic properties of the Rep proteins (12, 48, 55). While they have often been considered to have wild-type levels of enzyme activity, the MBP moiety at the N terminus (or the His tag at the C terminus) may have unknown effects on protein function. MBPRep78 and MBPRep68 suppressed AdMLP transcription with similar 50% inhibitory concentrations and to comparable levels (>90%). The addition of excess ATP or GTP to the transcription reaction mixtures had no effect on Rep inhibition, suggesting that nucleotide hydrolysis and depletion does not explain the Rep effects (data not shown). The results of these experiments suggest that the N terminus and a functional

helicase domain are required for full inhibition and that either Rep78 or Rep68 is a strong suppressor of AdMLP transcription *in vitro*. These results also indicate that the C terminus of Rep78 plays a minimal, if any, role in these *in vitro* assays.

Rep78/68 bind to the AdMLP. The results described above demonstrate that nonspecific DNA in the form of poly(dI-dC) or poly(dG-dC) did not alleviate Rep68-mediated inhibition of transcription. To determine whether Rep proteins interact with the AdMLP in a specific manner, electrophoretic mobility shift assays were performed with purified Rep proteins, poly(dI-dC), and a radiolabeled 57-bp DNA (nt 6004 to 6058) from the Ad5 genome. The sequence in the AdMLP to which this oligonucleotide corresponds and other competitor oligonucleotides we employed in the studies described here are summarized in Fig. 9. This 57-bp oligonucleotide extends 15 bp upstream of the TATA box to the transcription initiation site of the major late mRNAs. Rep68, MBPRep78, and RepNT shifted the AdMLP promoter fragment, whereas Rep52 and Rep40 did not (Fig. 3A). These data indicate that the N terminus of Rep78/68 is required for interaction with AdMLP. Thus, the same region of the protein that interacts with the AAV ITR mediates Rep interaction with AdMLP DNA. This observation of Rep interaction with a DNA target that lacks sequence similarity to known AAV Rep78/68 binding sites has also been observed in the human immunodeficiency virus HIV long terminal repeat and the human papillomavirus (HPV) p₉₇ promoters (4, 51).

To identify the Rep68 binding site in the AdMLP element, competition assays were performed using a molar excess of unlabeled oligonucleotides corresponding to the first 15 bp (Oligo-1), the next 21 bp (Oligo-2), and the next 18 bp (Oligo-3) of the AdMLP fragment (see Fig. 9). EMSAs were performed using Rep68 and increasing concentrations of competitive oligonucleotides (Fig. 3B). Unlabeled, full-length oligonucleotide competed effectively for Rep68 binding. Only Oligo-2 (nt 6019 to 6039 in the Ad5 genome) containing the AdMLP TATA box and the MAZ/Sp1 site (see Fig. 9) competed effectively for binding with the full-length AdMLP fragment. Oligo-2 did not compete for Rep68 binding as effectively as the full-length AdMLP fragment did, suggesting that sequences flanking Oligo-2 influence binding.

We examined the binding of Rep68 to the AdMLP quantitatively using electrophoretic mobility shift assays as shown in Fig. 4. The binding isotherm is complex and could not fit a simple hyperbola, assuming a 1:1 complex. Fits using the Hill equation were also unsatisfactory and show a systematic pattern in the residual values. The curves in Fig. 4 are fits to a third-order polynomial and have no theoretical significance. The behavior of the binding isotherm likely reflects a complex, cooperative assembly process. Electrophoretic mobility shift assays also have the limitation that they are not performed under conditions of thermodynamic equilibrium. When Rep68 was incubated with 500 ng of HeLa extract, the binding isotherm was shifted systematically to the left. There was a slightly lower mobility in the Rep-shifted bands in the HeLa-supplemented lanes. However, we were not able to determine whether slightly altered mobility was due to formation of a new Rep-DNA species or merely increased Rep binding.

The greatest effect on binding was observed at low concentrations of Rep68. Thus, HeLa extract stimulates Rep68 inter-

FIG. 3. Rep proteins bind to the AdMLP. A. Rep proteins were incubated with end-labeled AdMLP and separated by native gel electrophoresis. Lane 1, AdMLP without Rep; lane 2, RepNT at 900 nM; lanes 3 and 4, Rep40 at 460 and 920 nM, respectively; lanes 5 and 6, Rep52 at 440 and 880 nM, respectively; lanes 7 and 8, Rep68 at 400 and 800 nM, respectively; lanes 9 and 10, MBPRep78 at 450 and 900 nM, respectively. B. EMSAs were performed with the addition of 200- to 400-fold excess unlabeled oligonucleotide competitor DNAs. Lane 1, AdMLP alone. Lanes 2 to 10 all contain Rep68. Lane 2, no competitor; lanes 3 and 4, unlabeled full-length AdMLP added as a competitor; lanes 5 and 6, addition of Oligo-1; lanes 7 and 8, addition of Oligo-2 competitor; lanes 9 and 10, addition of Oligo-3 competitor.

action with the AdMLP, indicating stronger binding during the in vitro transcription reactions.

DNase I mapping of the Rep-AdMLP complex. The Rep binding site in the AdMLP was further defined using DNase I protection assays. Purified Rep68 was incubated with a labeled oligonucleotide corresponding to nt 5882 to 6219 of the Ad5 genome and treated with limiting amounts of DNase I. The digestion products were analyzed by denaturing gel electrophoresis. A DNase I protection assay of Rep binding to the Ad *E2a* promoter was included as a positive control (10). Rep68 protected a 38-bp region of the *E2a* gene between nt 27057 and nt 27085 as we observed previously (Fig. 5A). In the AdMLP

FIG. 4. Binding of Rep68 to the AdMLP. Rep68-AdMLP EMSAs were performed in triplicate, and the radioactivity was determined by phosphorimager analyses. Binding assays were performed with (filled circles) or without (half-filled circles) 500 ng of HeLa nuclear extract protein in the binding reaction mixture.

fragment, Rep68 protected approximately 36 bp that corresponds to nt 6011 to 6047 in the Ad5 genome (Fig. 5B). The protected region extends from the TATA box to the transcription initiation site and is shown diagrammatically in Fig. 9. The central part of the DNase I-protected region corresponded to Oligo-2 used in the competition experiments. The results of DNase I protection experiments show that the Rep68-protected region extends beyond the Oligo-2 sequence. EMSA and DNase I assays identify the same Rep binding sites. A second Rep68 footprint was detected upstream of the TATA box at nt 5966 to 5983 (Fig. 5C). The second protected region is located upstream adjacent to the upstream promoter element that is bound by the USF family of transcription factors. USF (47) has also been called MLTF (9) and UEF (38). The UPE motif is absent from the transcription template used in the in vitro transcription assays. However, Rep binding in this region may play a role in AAV and adenovirus interactions during coinfection.

Rep68 alters TBP-AdMLP interactions. The location of the Rep binding site overlapping the TATA box suggests that Rep may affect TBP binding to its cognate binding site. To assess TBP-AdMLP interactions, we performed gel mobility shift assays with purified human TBP, Rep68, Rep52, and Rep40 proteins, and poly(dG-dC). TBP interacts with the Ad TATA element more readily when poly(dG-dC) is used in lieu of poly(dI-dC) (19). Rep68 shifted the AdMLP fragment (Fig. 6A, lane 2), but neither Rep52 (Fig. 6B) nor Rep40 (Fig. 6C) bound to the DNA fragment. TBP interacted with the AdMLP as expected (Fig. 6A to C, lanes 3). When increasing concentrations of the Rep proteins were incubated with TBP, there was a loss of the TBP-AdMLP complex with Rep68 (Fig. 6A). There was a slight decrease in TBP-AdMLP complex at the

FIG. 5. Rep68 interaction with AdMLP protects it from DNase I digestion. DNase I protection assays were performed with an end-labeled E2a promoter fragment (A) or the AdMLP (B and C). The concentrations of Rep68 used in each reaction mixture are shown below each lane. The Ad5 nucleotide numbers are indicated to the left of each gel. The DNase I-protected regions are indicated by brackets to the right of the gel. Footprint I is indicated in panel B. Footprint II is indicated in panel C.

highest concentration of Rep52 (Fig. 6B). However, Rep40 did not affect TBP binding (Fig. 6C). The Rep68 EMSA revealed that the primary shifted band does not migrate in the gel as far as the band generated when the Rep protein is incubated with the labeled DNA fragment alone (Fig. 6A, compare lane 2 to

lane 6). This result suggests either that TBP increases Rep interaction with the AdMLP or that Rep and TBP physically interact in complex with the DNA. To determine whether Rep and TBP interact, chemical cross-linking and immunoprecipitations were performed using

FIG. 6. Rep and TBP interactions with the AdMLP. TBP and various concentrations of Rep68 (A), Rep52 (B), and Rep40 (C) were incubated with end-labeled AdMLP and separated by native gel electrophoresis. TBP (10 nM) was incubated with the labeled probe alone (lanes 3) or with increasing concentrations of the Rep proteins (lanes 4 to 6, 0.25, 0.5, and 1 μM, respectively). Lane 1, AdMLP alone; lane 2, Rep (0.8 μM) and AdMLP. Protein-DNA complexes are labeled TBP, Rep, or Rep/TBP to the left of the gel.

FIG. 7. Rep68 and TBP interactions as determined by formaldehyde cross-linking. Rep68, TBP, and AdMLP DNA were incubated using the conditions described for the EMSA experiments shown in Fig. 6. Mixtures were incubated for 30 min and then cross-linked with formaldehyde. The cross-linked complexes were immunoprecipitated with Rep antisera. The cross-links were reversed, and proteins were analyzed by SDS-PAGE and immunoblotting for TBP and Rep. The concentrations of Rep68 are indicated below the lanes. Lanes 13 and 14 contain purified TBP and Rep68, respectively.

the conditions of the mobility shift assays shown in Fig. 6. TBP, unlabeled AdMLP DNA, and increasing concentrations of Rep68 were incubated and then cross-linked with formaldehyde. The complexes were then immunoprecipitated with anti-Rep antibody (52). Cross-linking was reversed, and the complexes were analyzed by SDS-PAGE and immunoblotting with anti-TBP or anti-Rep. Figure 7 shows that when Rep68 is increased, more TBP is immunoprecipitated by the Rep antibody, indicating a greater amount of interaction. The interaction occurs regardless of the presence of AdMLP DNA (compare lanes 4 to 6 to lanes 9 to 11). This result confirms that Rep68 and TBP interact and that the complex in the EMSA shown in Fig. 6A may contain both proteins on the same DNA molecule.

DNase I mapping of the Rep-TBP-AdMLP complex. To map the combined Rep68 and TBP interactions, we performed DNase I protection assays. These assays were performed with poly(dG-dC) competitor or no competitor DNA. We did not perform these assays with poly(dI-dC) because of the negligible interactions between TBP and the TATA element with poly(dI-dC). TBP binding alone to the AdMLP protected 24 bp extending from approximately nt 6011 to nt 6035. TBP binding is also distinguished by nuclease-hypersensitive sites. This region contains the TATA element and was protected by TBP under both conditions (Fig. 8A and B, lanes 2). Rep68

FIG. 8. DNase I mapping of Rep68 and TBP interactions with the AdMLP. TBP and Rep68 were incubated with an end-labeled 336-bp AdMLP fragment from nt 5893 to 6229 in the Ad5 genome. The proteins were incubated at the indicated concentrations and treated with limiting DNase I. The products were separated by denaturing gel electrophoresis. A. Reactions were performed in the presence of 62.5 ng/ml of poly(dG-dC) competitor DNA. B. No competitor DNA was included. The numbers to the left of the panels are the nucleotide positions using the Ad5 numbering system. The locations of the Rep68- and TBP-interacting sites are indicated to the right of the gels. The positions of TBP- and Rep68-induced DNase I-hypersensitive sites are indicated by asterisks and arrowheads, respectively, to the left of the gels.

binding alone to the AdMLP protected an approximate region between nt 6035 to nt 6050 that extends into the transcription initiation region (INR) region of the promoter (Fig. 8A and B, lane 3). Nuclease-hypersensitive sites were also observed with Rep68. Nuclease hypersensitivity may be indicative of DNA bending. A second protected site of Rep68 interaction was detected between nt 6050 and 6086. TBP and the highest concentration of Rep68 demonstrated primarily Rep68-only DNase I sensitivity with poly(dG-dC) (Fig. 8A, lane 4). In contrast, the highest Rep68 concentrations in the absence of competitor DNA resulted in protection of both the TATA element and the downstream Rep binding site (Fig. 8B, lane 4). This indicates that both proteins bind to the same DNA fragment. This interaction is also similar to the Rep68-protected region shown in Fig. 5. As the Rep68 concentration is decreased, the DNase I sensitivity pattern becomes more like that of TBP alone, as demonstrated by the reappearance of the TBP nuclease-hypersensitive sites. DNase I protection experiments support our conclusion from EMSA and chemical cross-linking experiments that purified TBP and Rep68 protect distinct regions of the AdMLP and that these proteins can bind simultaneously to the AdMLP in the absence of a competitor DNA.

DISCUSSION

The roles of the AAV Rep78/68 proteins in viral replication are well documented (39). However, much less is known about how these pleiotropic effector proteins regulate viral gene expression. We know that Rep78/68 function as transcriptional activators of the AAV p_{40} promoter and repressors of the p_5 promoter. These effects depend on Rep interaction with RBS elements in the ITR and in the vicinity of the p_5 and p_{19} promoters (33, 37). During AAV and Ad coinfections, Ad early (30) and late (J. Timpe, K. Verrill, and J. Trempe, submitted for publication) gene expression is inhibited by AAV replication and presumably by the Rep proteins. The Rep proteins associate with the Ad E2a and AdMLP transcription promoters as determined by chromatin immunoprecipitation assays *in vivo* and EMSA analyses *in vitro* (10). The AAV Rep proteins also exert strong inhibitory effects on numerous cellular and viral transcription promoters in transient plasmid transfection assays (1, 23, 24, 28, 30, 31, 34). Most of the known Rep protein-interacting partners are transcription factors or are involved in transcription regulation. The mechanisms whereby these proteins interact with Rep78/68 to regulate AAV, Ad, and cellular gene expression are poorly understood.

To investigate how Rep proteins regulate gene expression, we performed a series of *in vitro* transcription assays using the AdMLP and purified Rep proteins. Initial studies showed that a His-tagged Rep68 protein suppressed gene expression from the AdMLP in *in vitro* assays (40). To define the functional domains of Rep that are required for this inhibition, *in vitro* transcription reactions were performed with purified Rep68, Rep52, and Rep40 and their purine nucleotide binding site mutants. Our results indicate that full-length Rep68 is required for suppression of transcription. The lack of inhibition by Rep40 suggests that the N terminus of Rep68 is necessary for inhibition of transcription. The Rep68 N terminus is the ITR interaction domain. Therefore, Rep68 binding to the transcrip-

tion template may be required for inhibition. We also found that MBPRep78 and MBPRep68 suppressed transcription at comparable levels. This result suggests that the Rep78 carboxyl terminus plays no role in blocking transcription from the AdMLP *in vitro*. The only known role for the C termini of Rep78 and Rep52 is their interaction with the protein kinase X homolog PrKX (13, 18). The C terminus contains a region homologous to a protein kinase inhibitor that mediates inhibition of PKA/PKX activity, resulting in less transcriptionally active CREB protein available for binding to ATF sites (17). There are several ATF sites in the Ad early promoters, and this mode of inhibition likely contributes to Rep-mediated inhibition of Ad replication. Since there are no ATF sites in the AdMLP, transcription from this promoter would probably not be affected by diminished PrKX-mediated phosphorylation of CREB.

The purine nucleotide binding site plays a role in transcription inhibition of the p_5 promoter (33). Alteration of Lys340 to a His in the Walker A motif of the Rep helicase domain diminishes Rep68's ability to inhibit AdMLP transcription and eliminates Rep52 inhibitory capabilities. Rep68PNB retains the ability to bind AdMLP, and this interaction is indistinguishable from that of wild-type protein in EMSA experiments (results not shown). Thus, Rep68 may act catalytically on the transcription template to diminish transcription. The role of the purine nucleotide binding site or helicase domain is less clear. Depletion of the purine nucleotide pool in the *in vitro* transcription reactions by Rep-mediated hydrolysis is likely not the reason for inhibition. Other experiments contained 10-fold-greater concentrations of ATP or GTP and still demonstrated Rep-mediated inhibition (results not shown). How wild-type Rep52 exerts its effects remains to be defined. Although Rep52 does not bind to AdMLP DNA in EMSAs, it exerts moderate transcription inhibition. The Lys340His mutation in Rep52 abolished the inhibitory effect. Rep52, through its carboxyl terminus, may interact with components of the initiation or elongation complex, thus allowing Rep52 helicase activity to disrupt transcription.

The requirement for the Rep78/68 N terminus suggests that direct interaction with the DNA template is necessary for suppression. Rep protein binding to transcription regulatory elements has been described for AAV p_5 and p_{19} , Ad E2a, HPV p_{97} , human immunodeficiency virus long terminal repeat, and the cellular E2F promoters (1, 4, 5, 24, 33, 51, 58). In the AAV and Ad promoter sequences, there are sequence similarities with the Rep binding site from the AAV ITR elements. Rep binding to the HPV p_{97} and cellular E2F promoters has not been fully characterized with respect to the locations of binding sites. There is no obvious similarity between the ITR and HPV p_{97} . However, there is limited sequence similarity between the ITR and the cellular E2F promoter. Our observation of Rep binding to DNA structures lacking similarity to the ITR sequence suggests that perhaps secondary structures are involved in Rep recognition. At least 18 Rep binding sites have been identified within or in close proximity to human genes (56). How these binding sites affect gene expression has not been thoroughly investigated.

Our observations that the Rep proteins bind to the AdMLP and E2a promoter (10) and their concomitant inhibition of transcription suggests that promoter binding is important in

FIG. 9. AdMLP and locations of Rep and TBP binding. The locations of the major transcription factor binding elements of the AdMLP are labeled above the DNA sequence. The transcription initiation site is indicated by the underlined residue in the INR region. The locations of the competitive oligonucleotides used in Fig. 3 are indicated above the transcription factor binding sites. The locations of the DNase I-protected footprints are shown below the sequence. The RepI and RepII sites were determined in the absence of TBP but in the presence of poly(dI-dC) (Fig. 5). The RepIII and TBP sites were determined with poly(dG-dC) or without competitor DNA (Fig. 8). The nucleotide numbers are from the Ad5 numbering scheme.

Rep-mediated inhibition of transcription. However, there are no obvious Rep-specific binding sites (GAGC)² in the AdMLP. The inability of competitor DNA oligonucleotides to overcome Rep68-mediated inhibition of transcription suggests that non-specific association of Rep with the transcription template is not the mechanism of inhibition. However, a specific interaction was observed in EMSA analyses. RepNT interacted with the full-length AdMLP, but Rep40 did not, suggesting that the ITR binding domain was required. Localization of the Rep binding site using competitive, unlabeled oligonucleotides revealed that the Rep binding site spanned the TATA element. Fine structure mapping using DNase I assays revealed slightly different results, depending on which competitor DNA was included in the reaction mixture. In the presence of poly(dI-dC), Rep68 protected two regions in the AdMLP: a 17-bp element upstream of the UPE and approximately 36 bp that extended from slightly upstream of the TATA element to just upstream of the INR element (RepII and RepI sites, respectively, in Fig. 9). In the presence of poly(dG-dC), Rep68 protected a shorter region that extended from just downstream of the TATA element into the INR (RepIII in Fig. 9). Others have reported differences in protein-DNA interactions when different, nonspecific DNA competitors were used (35). The common region bound by Rep68 with either competitor contains no similarity to known Rep protein binding sites but a GC-rich region that contains 69% GC base pairs (Fig. 9). EMSAs of the GC-rich regions around the TATA box are bound by the common cellular transcription factors MAZ and Sp1 (43). There is also evidence that this region promotes binding of the TBP/transcription factor IIB (TFIIB) complex (57). The lack of a canonical Rep binding site in this region is puzzling and suggests that Rep may recognize structural features beyond simple sequence. An interesting sequence arrangement in the middle of the competitive Oligo-2 is G₅TG₅. This arrangement of GC-rich sequence suggests that this region may be bent. Octamers in which seven of eight residues are G or C are predicted to be the most bent of all possible octamers (21). The DNase I-protected region has a predicted curvature of six degrees per helical turn of the DNA when analyzed by the bend.it software package (<http://hydra.icgeb.trieste.it/~kristian/dna/index.html>). Rep68 binds to the T-shaped ITR at the A-stem as well as to the GC-rich B and C loops of the structure (46). We are currently investigating the topology

of the Rep-AdMLP binding site to determine whether Rep interacts with bent, GC-rich DNA.

The close proximity of the TATA element and the Rep binding site in the AdMLP prompted an investigation of whether the Rep proteins alter TBP interaction with its cognate binding site. The Rep52 and Rep40 proteins did not appreciably alter TBP binding to the AdMLP. However, Rep68 bound to the promoter with TBP. The EMSA results also suggested that both Rep and TBP proteins may interact on the same promoter DNA. Protein-DNA cross-linking experiments demonstrated that Rep68 and TBP physically interact in solution and that the interaction is not dependent upon DNA interaction. DNase I protection assays suggest that Rep68 and TBP could coexist on the AdMLP. These observations indicate that direct DNA binding of the larger Rep proteins and/or TBP interactions are required for Rep-mediated suppression of AdMLP transcription. These observations are similar to those of others who have reported Rep and TBP interactions. A direct physical interaction between Rep and TBP has been demonstrated in *in vitro* assays (26). A direct physical interaction was not observed on the HPV p₉₇ promoter where it was demonstrated that Rep78 disrupts binding of TBP to the promoter (51). Recently, the Rep binding site downstream of the TATA element in the AAV p₅ promoter was shown to be required for Rep-dependent amplification of this region (20). In these studies, the authors showed that both proteins could bind simultaneously to the promoter and that the TBP binding stimulated amplification. The RBS in p₅ is involved in Rep-mediated autoregulation of gene expression (33). Repression of the p₅ promoter requires Rep binding to the RBS and a functional purine nucleotide binding site in the protein. This is similar to our observations that Rep binding at a position downstream of the TATA element and functional ATPase activity are required to achieve full suppression of AdMLP transcription *in vitro*.

The proximity of the Rep binding site and the TATA element suggests that Rep alters the assembly or conformation of the RNA polymerase II preinitiation complex. Crystal structures of TBP, TFIIB, and 16-bp synthetic promoter reveal that TBP-induced bending allows TFIIB to interact with the major groove of the DNA upstream and downstream of the TATA element (41). Rep interaction with the DNA around the TATA element or its physical interaction with TBP may pre-

clude TFIIB assembly into the preinitiation complex. Our studies have provided the first evidence that Rep proteins alter the conformation of the preinitiation complex and provide new evidence defining Rep protein roles in transcription regulation. Further studies are under way to dissect the assembly of transcription complexes in the presence of the AAV Rep68 protein.

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