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The Adeno-Associated Virus (AAV) Rep Protein Acts as both a Repressor and an Activator To Regulate AAV Transcription during a Productive Infection

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Adeno-associated virus (AAV) uses three promoters, p5, p19, and p40, to regulate viral gene expression. The p5 and p19 promoters direct the synthesis of the viral regulatory proteins, Rep78 and -68 and Rep52 and -40, respectively. The p5 Rep proteins bind a linear 22-bp sequence, the Rep binding element (RBE), that is within both the terminal repeat (TR) and the p5 promoter. In the absence of helper virus, all four Rep proteins have been shown to reduce transcription from the viral p5 and p19 promoters. In this report, we focus on the roles of these proteins and the RBEs in controlling transcription during a productive infection, that is, in the presence of adenovirus. We find that in the presence of adenovirus, the p5 RBE represses p5 transcription while the RBE in the TR activates p5. However, both the TR RBE and the p5 RBE transactivate the p19 and p40 promoters. The fact that the p5 RBE-Rep complex can transactivate p19 and p40 while repressing p5 suggests that Rep78/68 is both a repressor and a transactivator. Rep repression of p5 is specific for the p5 RBE, as other p5 promoter elements do not support this activity. We also demonstrate that in the presence of adenovirus, the p19 Rep proteins, which do not bind to the RBE, can eliminate repression of the p5 promoter by Rep78 and Rep68. This may occur by the association of Rep52 with Rep78 or Rep68 to produce a Rep78/68-Rep52 protein complex which can be detected in vivo by immunoprecipitation. Finally, two Rep mutants that were deficient in RBE binding and transactivation but positive for p5 repression were identified. These mutants may define interaction domains involved in making contacts with other proteins that facilitate repression. These observations suggest a mechanism for controlling the p5 and p19 mRNA levels during a productive AAV infection.

Adeno-associated virus (AAV) type 2 is a helper-dependent DNA virus that requires the coinfection of either adenovirus (Ad) or herpesvirus to propagate (8, 35). The 4.7-kb genome of AAV contains two open reading frames (ORFs) that encode the viral nonstructural (Rep) and capsid (Cap) proteins necessary for virion biosynthesis (18, 43, 44). Three promoters, at map positions 5, 19, and 40, are used to regulate the expression of these two ORFs (14, 15, 29, 31). The p5 and p19 promoters direct the expression of the Rep ORF and generate four proteins with apparent molecular masses of 78, 68, 52, and 40 kDa (36, 43, 47). The p40 promoter directs the expression of the viral capsid proteins VP1, VP2, and VP3 (4, 5, 23).

Several reports have demonstrated that in the absence of helper virus, AAV transcripts are not detected (12, 27, 28). The absence of p5 activity may be attributed, in part, to the cellular YY1 protein. YY1 has been shown to interact at two sites in p5, −60 and +1, relative to the start of p5 transcription (Fig. 1A) (41). These YY1 interactions have been shown to effectively silence p5 activity (41). The YY1 element at +1 may also serve as a TATA-independent initiation element (40, 48); however, the significance of this observation in regulating wild-type AAV promoter activity is not understood. The presence of an Ad coinfection results in transactivation of p5 and p19 promoter activity (9, 28, 46). The Ad component that is critical for the activation of AAV transcription has been shown to be the early region 1a protein (E1a) (28). E1a transactivates the p5 promoter by interactions with the cellular proteins major late transcription factor (MLTF) and YY1 (9, 41), and the YY1 and E1a proteins have been shown to interact directly by protein contacts between these polypeptides (30).

Genetic analysis of AAV has shown that *rep* is essential for both the regulation of AAV genes and the replication of viral DNA (18, 44). The p5 Rep proteins bind to a specific 22-bp Rep binding element (RBE) that is present within both the terminal repeat (TR) and the p5 promoter (34). Binding to the TR leads to nicking at the terminal resolution site and repair of the terminal repeat (21, 22, 42).

The biochemical activities of Rep involved in regulating transcription are not as clear. In the absence of helper virus, all four Rep proteins have been observed to repress p5 and p19 transcription (19, 25). Kyostio et al. have shown that repression at p5 is mediated in part by Rep binding to the p5 RBE (26). In addition, they proposed that the consensus ATP binding region within Rep contributes to the ability of Rep to repress transcription. The Rep proteins have also been shown to repress several heterologous promoters (17, 19). Mutational analysis of one such promoter, the human papillomavirus type 18 upstream regulatory region, was unable to define a specific target of Rep repression and suggested that Rep may interact with several cellular transcription factors (19).

In the presence of helper Ad, *rep* has been shown to transactivate transcription from all three AAV promoters (9, 27, 28, 32). Proximal promoter regions that are required to mediate

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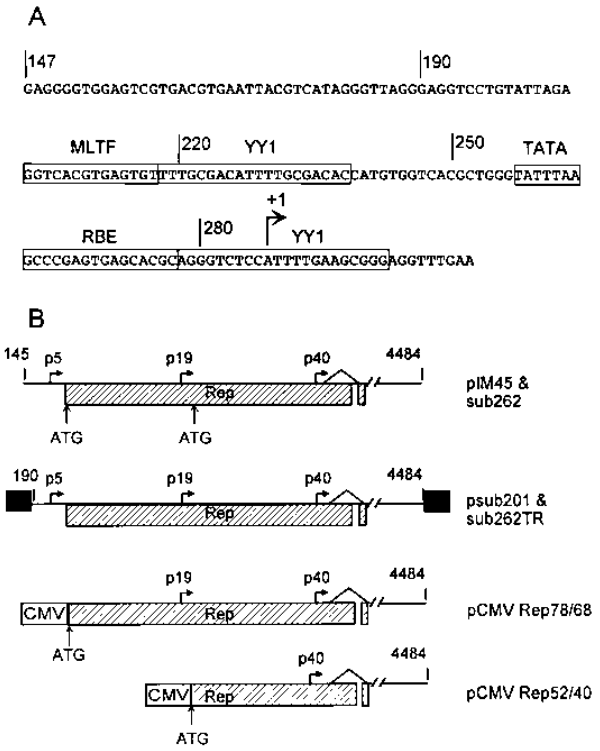

FIG. 1. (A) Sequence of the p5 promoter region (AAV nucleotides 147 to 308). Nucleotide positions are indicated by vertical lines and the corresponding nucleotide number. Boxed regions indicate sequence elements that bind the transcription factors YY1, MLTF, AAV Rep68 (RBE), and TATA binding protein, respectively. The start site of transcription is indicated by a bent arrow and labeled +1. (B) Plasmids used in this study. Vertical lines mark AAV nucleotide numbers for plasmids pIM45 and psub201. The *rep* coding region is indicated by a hatched box; the *cap* coding region is not shown. The p5 RBE mutants (*sub*262 and *sub*262TR) are in the background of pIM45 and psub201, respectively. Other p5 promoter mutants (*sub*YY1-60, *sub*MLTF, *sub*TATA, and YY1+1 mt1) are in the pIM45 background and are not shown (see Table 1). The p5, p19, and p40 promoter start sites are indicated by bent arrows. Filled boxes in the construct psub201 represent the AAV TR sequences. The positions of the 5′ and 3′ splice sites of AAV are marked by carets. The CMV promoter for constructs pCMVRep78/68 and pCMVRep52/40 is indicated as an open box.

Rep activation of p19 and p40 have been identified (32). As yet, the specific cellular and viral components that interact with these regions have not been identified. In addition, sequences outside the proximal promoter regions of p19 and p40 have been shown to be involved in Rep activation (32). Deletion of the p5 promoter region as well as a specific region in the p19 promoter results in a decrease in p19 and p40 transcripts. The requirement for these upstream sequences can be eliminated by the presence of a TR.

Given the evidence that the TR and the p5 promoter contain RBEs, as well as the requirement of Rep for p19 and p40 transcription, it seemed likely that Rep mediates the coordinate control of AAV transcription by interacting with the RBEs in the TR and the p5 promoter. In this report, we assess the function of the Rep proteins on p5 promoter activity in the presence of helper virus. Additionally, we examine the role of the p5 RBE and the TR in the regulation of the p5, p19, and p40 promoters. We observe that the Rep78 and -68 proteins and not the Rep52 and -40 proteins mediate repression of p5. The p19 Rep proteins appear to attenuate repression of p5, probably through direct contacts with the p5 Rep proteins. The p5 RBE or the TR facilitates transactivation of the p19 and p40 promoters. While the p5 RBE mediates repression of p5, the TR activates it. Finally, we examined a series of Rep mutants to identify interaction domains that play a role in repression of p5.

MATERIALS AND METHODS

Cell lines and transfections. HeLa cells and A549 cells (ATCC CCL 185) were maintained in Dulbecco's modified essential medium (DMEM) containing 10% bovine calf serum and grown in 10-cm-diameter culture dishes. DEAE-dextran was used to mediate DNA transfer into HeLa cells. Plasmid DNA was mixed with DEAE-dextran (molecular weight, 500,000; Pharmacia) at a final concentration of 0.1 mg/ml in serum-free 2× minimal essential medium (Gibco-BRL) and added to the cells for 30 min at 37°C and 5% CO_2. The transfection mixture was replaced with DMEM containing 2% calf serum and Ad type 2 (Ad2) at a multiplicity of infection (MOI) of 5.

Transfections for transcript analysis were performed in A549 cells, using cationic liposomes to deliver plasmid DNA. Liposomes were synthesized as described previously (13), and 12 μg of liposomes was added to 2.0 ml of serum-free DMEM followed by the addition of plasmid DNA. After the cells had been washed with phosphate-buffered saline, the transfection mixture was placed on the cells. Following a 3-h incubation at 37°C and 5% CO_2, the transfection mixture was replaced with 5 ml of DMEM containing 2% calf serum and Ad2 at an MOI of 5.

Plasmids. Plasmids pIM29 and pIM45 contain AAV nucleotides 145 to 4486 and 190 to 4486, respectively, in a pBSM13+ vector (Stratagene) (Fig. 1B) (32). The psub201 construct contains all wild-type (wt) AAV sequences except for nucleotides 145 to 190 (Fig. 1B) and has been described previously (39). The pCMVRep78/68 construct was made by inserting the *Bgl*II fragment of PIEplacZ (J. Glorioso, University of Pittsburgh), which contains the human cytomegalovirus immediate-early promoter (hCMV IEP), into the *Bam*HI site of pATGdXba (Fig. 1B). Plasmid pATGdXba was generated by deletion of nucleotides 145 to 320 from pIM45 while maintaining an *Xba*I restriction site in front of the initiation codon of the Rep78/68 ORF. pCMVRep52/40 was generated by insertion of the same *Bgl*II fragment containing the hCMV IEP into a pIM45-based plasmid that has had nucleotides 145 to 964 removed by a *Bgl*II-*Bcl*I restriction digest. Following ligation of these fragments, the Rep52/40 coding region was under the control of the hCMV IEP (Fig. 1B). The E1a-E1b-expressing plasmid, pXhoC, was generated by insertion of the left 15% of the Ad5 genome into the *Eco*RI and *Sal*I restriction sites of the pML2 cloning vector (P. Hearing, State University of New York at Stony Brook).

Plasmid RP5 was constructed to make antisense RNA homologous to the p5 promoter. An *Xba*I and *Bst*YI digestion fragment of pIM45 (AAV nucleotides 57 to 322) was inserted into the *Xba*I and *Bam*HI sites in the multiple cloning site of pBSM13+. The AAV fragment was oriented to use the T7 RNA polymerase promoter in the pBS vector. The probe when hybridized to p5 mRNA yields 162- and 164-bp protected fragments. Transcription from pCMVRep78/68 generates transcripts starting at AAV nucleotide 320 and when hybridized to the RP5 antisense RNA yields an RNase-resistant 129-bp fragment.

PLK215 was used to generate antisense RNA that detects the γ-actin transcripts (N. Reich, State University of New York at Stony Brook). A *Bam*HI-*Hin*dIII fragment from mouse γ-actin cDNA was inserted into pSP64 to utilize the SP6 RNA polymerase promoter. The probe when hybridized to human γ-actin transcripts yields a 135-bp protected fragment. Plasmids RP19 and RP40 were used to generate antisense messages directed at p19 and p40, respectively, and will be described elsewhere. The riboprobes from RP19 and RP40 generate protected fragments of 175 and 135 bp, respectively.

Substitution mutations were made by oligonucleotide mutagenesis as described previously (24) in the p5 promoter region of pIM45. These mutations are summarized in Table 1 and replaced the MLTF (*sub*MLTF), YY1-60 (*sub*YY1-60), and TATA (*sub*TATA) binding elements and the RBE (*sub*262). Additionally, a 2-bp mutation in the YY1+1 (YY1+1 mt1) binding element that has been reported to disrupt YY1 activity was made (Table 1) (41). Plasmid *sub*262TR was generated by replacing the *Xba*I fragment of psub201 with the *Xba*I fragment from the p5 RBE mutant plasmid *sub*262. The final *sub*262TR construct is similar in sequence to psub201 except for the p5 RBE mutation. Chloramphenicol acetyltransferase (CAT) reporter plasmids containing portions of the p5 promoter region (p5CAT constructs) were obtained from T. Shenk (Princeton University) and have been described elsewhere (9). Mutations in the *rep* coding region were made in the pIM29 background and have been described previously (33) (Table 2 and Fig. 1B).

CAT assays. Forty hours postinfection, cells were scraped into the medium and washed with phosphate-buffered saline. Cell were lysed in 0.5 ml of a solution containing 0.65% Nonidet P-40, 10 mM Tris-HCl (pH 8.0). 1 mM EDTA (pH 8.0), and 150 mM NaCl and heated for 5 min at 68°C. The reaction mixture contained, in a final volume of 0.1 ml, 2.7 mM chloramphenicol, 0.17 mM acetyl coenzyme A, 8 mM Tris-HCl (pH 7.8), 0.1 μCi of [^{14}C]acetyl coenzyme A, and 0.05 ml of lysate. The reaction mixture was incubated at 37°C for 1 h and extracted with an equal volume of ethyl acetate. The aqueous phase was removed, and the acetylated products were determined by scintillation counting.

EMSA. The probes for electrophoretic mobility shift assays (EMSAs) were generated by PCR using plasmid pIM45 or sub262 as the template for amplifi-

TABLE 1. Summary of p5 promoter mutants[a]

Mutant	Transcription element	Oligonucleotide	Substitution[b]
*sub*MLTF	MLTF	CCTGTATTAGATACACGCGTGTCGTTTTGCGACATTTTGC	*Mlu*I
*sub*YY1-60	YY1-60	CACGTGAGTGTTGAATTCTGGGATCCGACACCATGTGG	*Eco*RI/*Bam*HI
*sub*TATA	TATA	TCACGCTGGGCCGCGGGCCCGAGTGA	*Sac*II
*sub*262	RBE	TGGTATTTAAGAATTCTGGATCCACTAGGGTCTCCA	*Eco*RI/*Bam*HI
YY1+1 mt1	YY1+1	CGCAGGGTCTAAATTTTGAAGC	

[a] The p5 promoter mutations were constructed in the pIM45 plasmid background by oligonucleotide mutagenesis using the indicated oligonucleotides. Nucleotides that are underlined were substituted for the wt AAV sequence.
[b] Most of the substitutions inserted new restriction sites which are indicated.

cation. Primers and reaction conditions for p5 PCR were performed as described previously (34). Rep68 was expressed in a baculovirus expression system and purified to homogeneity as previously described (34). The Mono Q fraction was used with a protein concentration of 0.22 mg/ml, determined by the Bradford assay (Bio-Rad). Prior to incubation in the EMSA, Rep68 was treated with Tween 20 for 2 h on ice. Labeled p5 fragment (0.013 pmol) was incubated with Rep and electrophoresed on a 4% polyacrylamide gel as previously described (34). The RBE-specific competitor that was used was the A-25 substrate described previously (34).

Western blotting and immunoprecipitation experiments. A549 cells grown in 10-cm-diameter dishes were transfected with pIM45, pCMVRep78/68, or pCMVRep52/40 by using liposomes and infected with Ad2 at an MOI of 5. Twenty-four hours postinfection, the cells were harvested and nuclear extracts were made (1). To test expression of Rep78/68 and Rep52/40, one-fourth of the nuclear extract was run on a sodium dodecyl sulfate (SDS)–polyacrylamide gel followed by immunoblotting using anti-52/40, a monoclonal antibody which recognizes an epitope common to all four Rep proteins, or anti-78/68, a monoclonal antibody which recognizes only Rep78 and -68 (20). The antibody was detected by chemiluminescence (Amersham).

Anti-78/68 (20) was coupled to protein A-Sepharose (Sigma) as previously described (16) and used for immunoprecipitation experiments. Approximately 20 μg of coupled antibody (precoupling concentration) was added to one-half of the nuclear extract from the transfected cells containing Rep78/68, Rep52/40, or both. The reaction mixtures were incubated for 30 min on ice, with mixing every 10 min. The Sepharose was centrifuged for 20 s at 14,000 rpm, and the supernatant was removed. The Sepharose pellet was washed three times in a buffer containing 10% glycerol, 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol. Following the final wash, the Sepharose pellet was resuspended in 50 μl of 2× SDS-polyacrylamide gel electrophoresis loading buffer and boiled for 10 min. The samples were directly loaded onto an SDS–10% polyacrylamide gel alongside a set of protein molecular weight markers. Rep proteins were detected by immunoblotting using anti-52/40 (20).

RNA extraction. Forty-hours postinfection, the cells were harvested and 1/10 of each sample was used to isolate and quantitate input plasmid DNA (2). The Hirt DNA samples were digested with *Xba*I, electrophoresed on a 0.7% agarose gel in 1× Tris-borate-EDTA, and transferred to a nylon membrane (38). The blots were hybridized to an *Xba*I fragment of pIM45 containing AAV nucleotides 145 to 4484. The remaining cells were collected by centrifugation, and RNA was isolated by the acid guanidinium-phenol extraction protocol (11). Transfected plasmid DNA copurifies with RNA; therefore, samples were treated with RNase-free DNase I (Worthington Biochemicals) as described previously (2).

RNase protection analysis. To generate p5-antisense transcripts, plasmid RP5 was linearized by digestion with *Xba*I. The linearized template (100 ng) was mixed with 5 mM dithiothreitol, 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.025 mM UTP, 50 μCi of [^{32}P]UTP (800 Ci/mmol; Amersham), 2 U of RNasin (Promega), and 40 U of T7 RNA polymerase (Promega). This reaction mixture was incubated at 37°C for 60 min. The resulting antisense transcript had 162 and 164 bp of overlap with p5 transcripts. As a control for total RNA, an antisense probe targeted for γ-actin transcripts was used. Plasmid PLK215 was linearized with *Hin*fI, and the antisense transcript was generated by using SP6 RNA polymerase. Reaction conditions were identical to those for the p5-antisense transcript except that the reaction mixture was incubated at 40°C. The resulting antisense transcript provides a 135-bp protected fragment. The probes were gel purified on a 6% sequencing gel and eluted into a buffer containing 0.5 M ammonium acetate, 10 mM magnesium acetate, and 0.1% SDS for 1 h at 65°C. The probes were used immediately for hybridization.

Approximately 5 μg of total RNA (determined by A_{260}) was mixed with 2 × 10^5 cpm of each riboprobe. The RNA was collected by centrifugation at 12,000 × *g* for 10 min at 4°C. Hybridization at 55°C and RNase digestion were performed as described previously (37), and the reaction products were analyzed on a 6% sequencing gel. Following electrophoresis, the gels were dried and exposed to X-ray film. Bands corresponding to p5, p19, p40, and actin transcripts were quantitated with a Molecular Dynamics ImageQuant PhosphorImager. Quantitation of message levels was normalized for levels of both actin and input plasmid DNAs.

RESULTS

Rep proteins negatively regulate p5CAT activity in the presence and absence of an Ad coinfection. As mentioned earlier, several groups have reported that in the absence of Ad helper virus, the Rep proteins negatively regulate the p5 promoter (19, 25, 26).In contrast, it has also been reported that in the presence of Ad, Rep can both transactivate and repress p5 transcription (3, 27). To resolve Rep action at p5, expression of a CAT reporter under p5 control was used to assay promoter activity. A p5CAT expression plasmid, containing AAV nucleotides 143 to 310 (9), resulted in minimal CAT activity in the absence of Ad (Fig. 2A, −). Addition of a Rep-encoding plasmid (pIM45) to the p5CAT transfection reduced CAT activity twofold (Fig. 2A, pIM45). This result was consistent with several reports that demonstrate Rep repression of p5 in the absence of helper virus (19, 25, 26). When p5CAT-transfected cells were infected with Ad, a 12.5-fold increase in CAT activity was observed (Fig. 2A; compare p5CAT and Ad-2). If, however, the p5CAT transfection included the Rep-encoding plasmid (pIM45) and a coinfection with Ad, CAT activity was reduced to the levels seen with p5CAT alone (Fig. 2A; com-

TABLE 2. Effects of Rep mutations on p40 transactivation, TR binding, and p5 repression

Plasmid	Amino acid change	Transactivation[b]	TR binding[b]	Repression	Relative CAT activity[d]
None		−	−	−	1.0
pIM29	Wild type	+	+	+	0.06
*dl*720[a]	134–143	−	−	−	0.95
*dl*750	144–153	−	−	−	0.85
*dl*780	154–163	−	−	−	0.55
*dl*810	164–173	−	−	−	0.79
*dl*840	174–183	−	−	−	0.69
W242L	W242L	−	−	+	0.06
W242S	W242S	−	ND[c]	−	0.51
TN341IY	T341I, N343Y	−	+	+	0.01
P365A	P365A	+	+	+	0.08
P365T	P365T	+	+	+	0.03
E379K	E379K	−	+/−	+	0.01
P415H	P415H	−	−	+	0.04
*dl*1700	461–470, K460L	−	−	−	0.85
*dl*1730	471–480, F470L	−	−	−	1.19
*dl*1760	481–490	−	−	−	1.06
*dl*1790	491–500	+	+	+	0.20
*dl*1820	501–510	+	+	+	0.15
P505H	P505H	+	+	+	0.01

[a] Deletion mutation.
[b] Taken from reference 33. All of the mutants that were transactivation negative were also defective for DNA replication in vivo. Transactivation refers to the ability of the mutant to transactivate the p19 and p40 promoters in vivo.
[c] ND, not determined.
[d] p5CAT activity in the absence of Rep plasmid (none) was arbitrarily set at 1.0; other values are relative to this number.

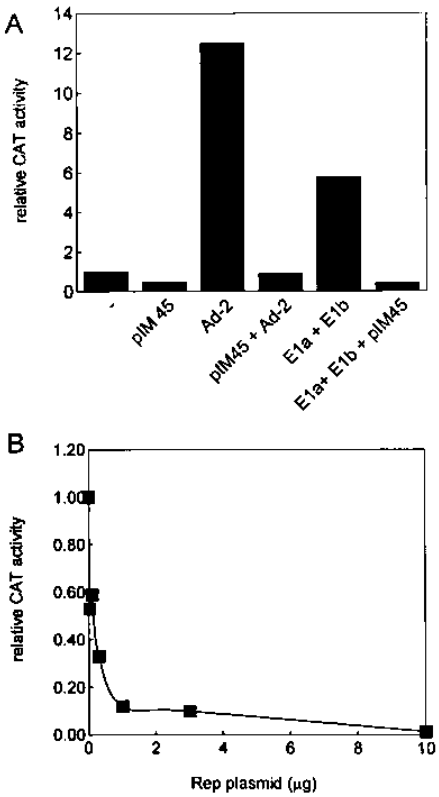

FIG. 2. (A) Rep represses p5CAT activity in the presence of Ad coinfection. Five micrograms of a p5CAT plasmid containing AAV nucleotides 143 to 310 was transfected into HeLa cells. CAT activity was measured from cells transfected with p5CAT alone (−) or in the presence of infection with Ad2 (Ad-2), cotransfection with 5 μg of E1a- and E1b-expressing plasmid (E1a + E1b), cotransfection with 5 μg of Rep expressing plasmid (pIM45), or combinations of the above. CAT activity is displayed relative to the activity of the p5CAT construct alone, which was arbitrarily set to 1. (B) Repression of p5CAT activity is a function of the concentration of Rep plasmid. Five micrograms of a CAT construct containing AAV nucleotides 216 to 310 was transfected alone or with increasing concentrations of a Rep expression plasmid (pIM45) and infected with Ad2. CAT activity in the absence of Rep was set at 1.0. Changes in CAT activity are relative to this value.

pare − and pIM45 + Ad-2). These results indicated that Rep acts as a repressor of p5CAT activity in the presence and absence of Ad infection. The results were consistent with those reported by Beaton et al. (3) and contradicted a study by Labow et al. (27), in which it was observed that Rep was essential for p5 transactivation in the presence of an Ad infection.

It also was established previously that the Ad E1a activator protein transactivates the p5 promoter through interactions with the cellular MLTF and YY1 proteins that directly bind the p5 promoter (Fig. 1A) (9, 41). It has been proposed that Rep negatively regulates p5 by inhibiting YY1 from binding to p5 (26). To provide evidence that the function of Rep repression was to counter E1a activity, we tested the ability of Rep to counteract the transactivation properties of the Ad E1 region by using p5CAT as the reporter. Plasmid pXhoC, which contains the left 15% of Ad5 and expresses the E1a and E1b genes under the control of their endogenous promoters, was used as an effector plasmid. CAT activity increased about sixfold when the E1-expressing plasmid was transfected with p5CAT (Fig. 2A, E1a + E1b), and this result confirmed earlier observations of E1 activation of p5 (9, 28). CAT activity returned to inactivated levels when Rep was added to the transfections (Fig. 2A, E1a+ E1b + pIM45).

To evaluate if repression of p5 activity was a function of the concentration of the Rep-expressing plasmid, Ad-infected HeLa cells were transfected with a constant amount of a p5CAT reporter plasmid, containing AAV nucleotides 216 to 310, and increasing amounts of pIM45. At the lowest concentration of pIM45 (0.03 μg), a twofold reduction in activity was seen (Fig. 2B). The activity of p5CAT continued to decrease upon addition of higher amounts of Rep plasmid, and addition of 10 μg of Rep plasmid resulted in complete repression of p5CAT activity.

Although the results of the CAT experiments confirmed previous reports that Rep could efficiently repress p5 promoter activity in both the presence and absence of helper virus, it has been suggested that Rep can affect CAT activity posttranscriptionally (45). To verify that Rep was repressing p5 transcription, the steady-state levels of p5 transcripts were monitored by using RNase protection analysis. Rep repression was measured by comparing p5 transcription from plasmids pIM45 (wt) and *dl*720 (a *rep* deletion; Fig. 1B and Table 2) in both the presence and absence of an Ad infection. Quantitation of the p5 message levels was normalized for both γ-actin mRNA and input plasmid DNA.

In the absence of helper virus, p5 mRNA could not be detected from either the wt plasmid or the *rep* mutant plasmid (Fig. 3A, lanes 5 and 6). This result was consistent with several reports that demonstrate the need for an Ad coinfection in order to stimulate AAV p5 transcription (28, 32, 41). Transcription from both the wt and mutant plasmids was apparent when an Ad infection accompanied the plasmid transfection (Fig. 3A, lanes 2 and 3). As expected, the p5-antisense transcript hybridized and protected two species of RNA corresponding to initiations at nucleotides 285 and 287 (31). Transcript accumulation from the *rep* mutant plasmid was three- to fourfold higher than that from the wt plasmid (Fig. 3A; compare lanes 2 and 3). These results were consistent with the results of the CAT experiments and demonstrated Rep repression of p5 in the presence of helper Ad infection. However, the level of mRNA repression was not as great as the level of CAT repression, suggesting that Rep may affect CAT expression

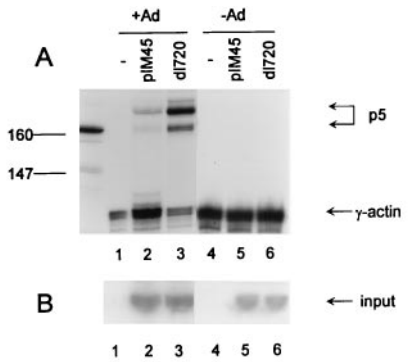

FIG. 3. Loss of Rep function causes an increase in the steady-state levels of p5 transcripts in the presence of Ad. (A) A549 cells were mock transfected (−) or transfected with 5 μg of a wt Rep (pIM45) or a mutant Rep (*dl*720) plasmid. One set of transfections was infected with Ad (+Ad); the other was not (−Ad). The RNA from these cells was isolated, and p5 and γ-actin transcripts were measured by RNase protection. 3′-labeled pBR322 DNA digested with *Msp*I was used as a marker (far left lane), and nucleotide lengths of marker bands are indicated to the left. Arrows indicate the positions of the p5 and γ-actin protected fragments. (B) Measurement of input plasmid DNA. One-tenth of the transfected cells was used to isolate low-molecular-weight DNA. The samples were digested with *Xba*I, electrophoresed on a 0.7% agarose gel, and transferred to a nylon membrane. The blots were hybridized to a ^{32}P-labeled DNA fragment containing AAV nucleotides 190 to 4487.

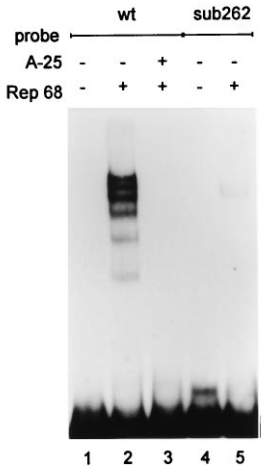

FIG. 4. Mutation of the p5 Rep binding site disrupts Rep68 interaction in an EMSA. A 170-bp 5′-end-labeled DNA fragment (AAV nucleotides 136 to 305) was generated by PCR, using either a wt or p5 RBE mutant (*sub*262) plasmid as the template for amplification. These fragments were incubated with (+) or without (−) purified Rep68. A 200-fold molar excess of an unlabeled oligonucleotide competitor containing the TR RBE (A-25 [7]) was added to the reaction in lane 3.

posttranscriptionally in addition to its effect on p5 mRNA levels.

The p5 RBE activates the p19 and p40 promoters in the presence of Ad. We previously reported that the p5 promoter region is required for Rep-mediated transactivation of the p19 and p40 promoters in the absence of a TR (32). The ability of the TR, an RBE, to replace this function suggested that a similar RBE in p5 was required for p19 and p40 activation. We and others subsequently demonstrated a specific interaction between the p5 promoter and purified Rep68, and the site of interaction was shown to be positioned between the TATA and YY1 initiators (26, 34). The presence of the p5 RBE suggested that this RBE may be responsible for directing p5-mediated activation of p19 and p40. To test this possibility, a substitution mutation encompassing the Rep binding site (AAV nucleotides 262 to 277) was introduced into plasmid pIM45. This mutation, *sub*262 (Fig. 1B and Table 1), replaces all of the p5 RBE with *Bam*HI and *Eco*RI restriction sites, without changing the distance between the TATA element and the YY1 element at position +1 relative to the transcript initiation site. To confirm that the substitution mutation disrupted the p5-Rep interaction, EMSAs were performed with p5 promoter probes derived from either pIM45 or *sub*262. Incubation of the wt p5 fragment with purified Rep68 resulted in the formation of a series of protein-DNA complexes (PDCs) as expected (Fig. 4, lane 2). These PDCs were efficiently competed when a 200-fold molar excess of an unlabeled TR A-stem RBE competitor was included in the reaction (Fig. 4, lane 3). Mutation of the p5 RBE site reduced Rep68 PDC formation to a level that was nearly undetectable (Fig. 4, lane 5).

The effect of the p5 RBE on p19 and p40 promoter activity was measured by RNase protection of p19 and p40 transcripts from pIM45 (wt) or *sub*262 (p5 RBE mutant). In the absence of a p5 RBE, the levels of both p19 and p40 activity were reduced fivefold compared to wt activity (Fig. 5B and C; compare lanes 1 and 2). This result demonstrated that the p5 RBE was the specific element within the p5 promoter that was necessary for Rep-mediated transactivation of the p19 and p40 promoters in the presence of Ad.

Theability of the TR to replace the function of the p5 RBE was tested by measuring the activity of the wt and *sub*262 p5 promoters in the presence of the TR. The presence of TRs in the absence of a p5 RBE (*sub*262TR) returned p19 and p40 activities to a level that was equal to that of a plasmid with both an intact p5 RBE and TRs (psub201) (Fig. 5B and C; compare lanes 3 and 4). Thus, the p5 RBE and the RBE within the TR were equivalent and redundant transcriptional elements required for Rep-mediated transactivation of the p19 and p40 promoters.

The p5 RBE represses p5 transcription in the presence of Ad, while the TR RBE transactivates p5. It has been reported by Kyostio et al. (26) that in the absence of helper virus, the p5 RBE in part mediates the ability of Rep to repress p5 transcription. Figure 6A illustrates that this is also true in the presence of Ad coinfection. Mutation of the p5 RBE resulted in a threefold increase in p5 transcription compared to an intact p5 promoter (Fig. 5A; compare lanes 1 and 2). This level of increase was similar to the increase in p5 transcription when the Rep coding region was mutated (Fig. 3A; compare lanes 2 and 3) and somewhat lower than the 8- to 10-fold effect seen by Kyostio et al. (25, 26) and Horer et al. (19) in the absence of

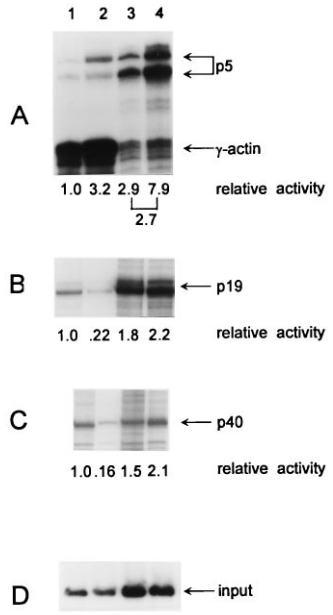

FIG. 5. The p5 RBE binding site is required for transactivation of the p19 and p40 promoters and repression of p5 transcription in the presence of Ad coinfection. (A) A549 cells were transfected with 5 μg of one of the following plasmids: pIM45 (lane 1), which contains only a wt p5 RBE; *sub*262 (lane 2), which contains neither a p5 RBE nor a TR; psub201 (lane 3), which contains both a wt TR and a wt p5 RBE; or *sub*262TR (lane 4), which contains a wt TR and a mutant p5 RBE. All cells were subsequently infected with Ad2 at an MOI of 5. Forty hours postinfection, RNA was isolated and the p5 and γ-actin transcripts were measured by RNase protection. Indicated are the 164- and 162-bp p5 and the 135-bp γ-actin protected fragments. p5 transcripts, γ-actin transcripts, and input DNA (D) were quantitated on a PhosphorImager, and the levels of p5 were normalized for both total RNA (γ-actin) and input plasmid DNA. The level of wt p5 (pIM45) RNA was arbitrarily set at 1, and all other transcript values relative to this value are shown below the lanes. (B) The same samples were used to measure the levels of p19 transcripts by RNase protection. The arrow marks the major 175-bp p19 RNase-resistant RNA. As in the case of the p5 transcripts, the levels of p19 transcript were normalized for both total RNA (γ-actin) and input plasmid DNA, and the value for each experiment relative to that for pIM45 is shown below each lane. (C) p40 transcripts were also measured by RNase protection using an antisense RNA directed to p40 transcripts. The 135-bp p40 protected fragment is indicated by the arrow. The levels of p40 were normalized for total RNA recovery and input DNA as described above. The relative abundance p40 RNA is shown below each lane. (D) Measurement of input plasmid DNA. Hirt extracts were digested with *Xba*I, run on a 0.7% agarose gel, blotted to a nylon membrane, and hybridized to a DNA fragment containing AAV nucleotides 190 to 4487.

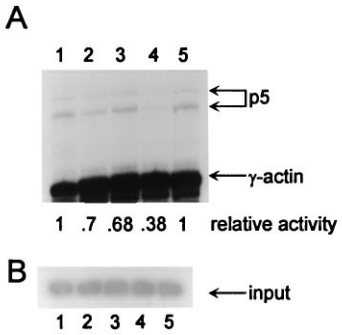

FIG. 6. Activities of p5 promoter elements in the presence of Rep and Ad. (A) Substitution mutations were made to replace the MLTF site (*sub*MLTF; lane 2), the YY1 site at position −60 (*sub*YY1-60; lane 3), and the TATA site (*sub*TATA; lane 4). Additionally, a 2-bp mutation in the YY1 site at position +1 (*sub*YY1+1; lane 5) was made. The effects of these mutations were compared to that of the wt pIM45 plasmid (lane 1). These plasmids were transfected into A549 cells, and the cells were then infected with Ad2 at an MOI of 5. p5 and γ-actin RNAs were measured by RNase protection experiments. Bands corresponding to the 164- and 162-bp p5 protected fragments and the 135-bp γ-actin fragment are indicated on the right. p5 transcripts, γ-actin transcripts, and input DNA (B) were quantitated with a PhosphorImager. The levels of p5 transcript were normalized for both total RNA (γ-actin) and input plasmid DNA. The level of wt p5 (pIM45) transcript was arbitrarily set at 1, and all transcript values relative to this value are shown below the lanes. (B) Measurement of input plasmid DNA. Low-molecular-weight Hirt DNA was isolated as for Fig. 5 and analyzed by Southern hybridization.

Ad infection. The presence of the TR, however, did not affect the approximately threefold increase in p5 activity when the p5 RBE was removed (Fig. 5A; compare lanes 3 and 4). Thus, regardless of whether the TR was present, the p5 RBE repressed p5 activity approximately threefold. In contrast, the presence of the RBE within the TR consistently increased p5 activity approximately threefold (Fig. 5A; compare lanes 1 and 3 and lanes 2 and 4). Furthermore, the two effects were additive; mutation of the p5 RBE and the addition of the TR increased p5 transcription nearly eightfold (Fig. 5A; compare lanes 1 and 4).

The p5 promoter also contains several other transcription factor binding sites that have been shown to be required for p5 promoter activity (9, 41, 48). To determine whether any of these elements contributed to Rep repression of p5, mutations that specifically disrupted the MLTF, YY1-60, TATA, and YY1+1 binding elements were made (Fig. 1A and Table 1). Previously, the effects of these promoter elements had been characterized in the absence of Rep (9, 41, 48). To determine if the function of each of these elements was altered in the presence of Rep, RNA was isolated from cells transfected with each of these constructs, assayed by RNase protection, and compared to the wt p5 promoter (pIM45) in the presence of Ad. None of the mutations resulted in an increase in p5 transcription (Fig. 6), thus demonstrating that the p5 RBE was the only promoter element that directs p5 repression in the presence of Ad. Three mutations, however, did result in a loss of p5 activity. Mutation of the MLTF, YY1-60, and TATA elements reduced p5 transcript accumulation two- to threefold (Fig. 6A, lanes 2 to 4). The loss of activity was consistent with earlier observations by Shenk and colleagues (9, 41, 48) in the absence of Rep protein. Disruption of the YY1+1 element, however, did not have any adverse effects at p5 (Fig. 6A, lanes 4 and 5). The p5 promoter has both the typical TATA element and a TATA-independent initiator sequence (YY1+1) (40), and these two elements are likely to be redundant. Therefore, YY1 binding at the initiator element may not be particularly significant as long as the TATA element has not been disrupted.

Rep52/40 can antagonize the p5-repressing activity of Rep78/68. Although Rep repressed p5 promoter activity, it was not clear from these experiments which Rep proteins were required for this activity. It has been observed previously that in the absence of helper virus, Rep78 and -68 can repress p5 transcription 5- to 10-fold, while and Rep52 and -40 repressed 2- to 5-fold (19, 25). To determine whether the same Rep proteins were responsible for repression of p5 in the presence of Ad, the constructs pCMVRep78/68 and pCMVRep52/40 were used as effector plasmids. These constructs place the Rep78/68 and Rep52/40 coding regions under the control of the CMV IEP (Fig. 1B). As expected, all four types of proteins, as well as a number of proteolytic breakdown products, were present in extracts from pIM45-transfected A549 cells (Fig. 7, lane 2). In contrast, pCMVRep78/68-transfected cells produced predominantly Rep78 and -68 (presumably because induction of p19 mRNA synthesis was reduced due to the absence of the p5 RBE). pCMVRep52/40-transfected cells synthesized only Rep52 and -40 (Fig. 7, lanes 3 and 4). Thus, the use of these two effector plasmids allowed us to separately examine the effect of Rep78 and -68 or Rep52 and -40 expression on p5 mRNA synthesis in the presence of Ad.

Two plasmids, the wt plasmid pIM45 and the *rep* deletion plasmid *dl*720, were used as reporter plasmids (Fig. 1B and Table 2). Both contain wt p5 promoter regions, but *dl*720 contains a deletion within the Rep coding region which renders this mutant defective for DNA replication and transactivation (32, 33) as well as repression of p5CAT (see below and Table 2). As expected, mutation of the Rep coding region caused a four- to fivefold increase in p5 activity compared to the wt plasmid pIM45 (Fig. 8A [compare lanes 1 and 2] and C). Cotransfection of pCMVRep78/68 and *dl*720 effectively restored p5 repression to approximately the wt level (Fig. 8A [lanes 1 and 5] and C). However, addition of pCMV78/68 to pIM45 (which effectively increased the level of Rep78 and -68) did not cause any additional repression of p5 (Fig. 8A [compare lanes 1 and 5] and C).

In contrast to what has been observed in the absence of Ad infection (19, 25), cotransfection of pCMVRep52/40 with *dl*720 in the presence of Ad (that is, with the synthesis of Rep52 and -40 alone) did not repress the p5 promoter (Fig. 8A [compare lanes 2 and 8] and C). Furthermore, when pCMVRep52/40 was added to pIM45 (thus effectively increasing the intracellular ratio of Rep52/40 to Rep78/68), the activity of the p5 promoter was eightfold higher than that seen with pIM45

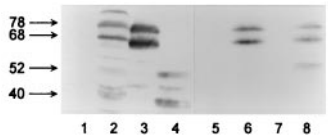

FIG. 7. Rep78/68 and Rep52 can form a complex in vivo. Nuclear extracts derived from A549 cells that were mock transfected (lane 1) or transfected with pIM45 (lane 2), pCMVRep78/68 (lane 3), or pCMVRep52/40 (lane 4) were run on an SDS–10% acrylamide gel and transferred to nitrocellulose. The Rep proteins were detected by Western blotting using anti-52/40, a monoclonal antibody that detects all four Rep proteins (20). Indicated to the left of the gel are the positions of Rep78, -68, -52, and -40. Additional bands are proteolytic breakdown products of the Rep proteins (not shown). Extracts from lanes 1 and 3 to 5 were then used in an immunoprecipitation experiment using protein A-Sepharose coupled to anti-78/68, a monoclonal antibody that recognizes only Rep78 and Rep68 (20). The anti-78/68 coupled antibody was then added to extracts from mock-transfected (lane 5), pCMVRep78/68-transfected (lane 6), or pCMVRep52/40-transfected (lane 7) cells or to extracts transfected with both pCMVRep52/40 and pCMVRep78/68 (lane 8). The antibody beads were collected by centrifugation, boiled in 2× SDS gel loading buffer for 10 min, and run on an SDS–10% polyacrylamide gel. Rep proteins were detected by immunoblotting using the anti-52/40 antibody.

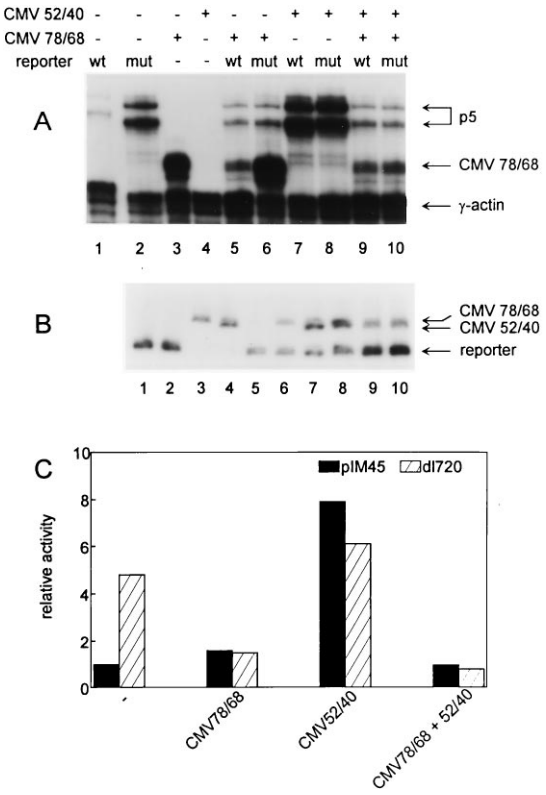

FIG. 8. Effects of Rep78/68 and Rep52/40 on p5 promoter expression. Five micrograms of wt (pIM45) or Rep mutant (mut; *dl*720) plasmid was transfected into A549 cells with (+) or without (−) 5 μg of pCMVRep78/68 (CMV 78/68) or 5 μg of pCMVRep52/40 (CMV 52/40). (A) RNase protection assays were used to measure p5 and γ-actin transcripts. The positions of the p5, CMV 78/68, and γ-actin transcripts are marked by arrows on the right. (B) Measurement of input plasmid DNA. Hirt DNA was digested with *Xba*I and analyzed by Southern hybridization as for Fig. 5. The positions of the reporter plasmids (pIM45 and *dl*720) and effector plasmids (pCMVRep78/68 and pCMVRep52/40) are indicated. (C) Graphical illustration of the relative effects of Rep78/68 and Rep52/40 on p5 promoter activity. The levels of p5 transcripts were measured and normalized for both total RNA (γ-actin) and input plasmid DNA. The level of activity from plasmid pIM45 (lane 1) was arbitrarily set at 1; changes in p5 activity are relative to this value.

alone (Fig. 8A [compare lanes 1 and 7] and Fig. 8C). This result meant that in the presence of Ad, Rep52/40 not only were incapable of repressing the p5 promoter by themselves but also were capable of eliminating repression by Rep78/68. Indeed, p5 activity was significantly higher (almost twofold) in the pIM45 and pCMVRep52/40 transfection than it was in the absence of any Rep protein (derepressed conditions) (Fig. 8A [compare lanes 2 and 7] and Fig. 9C), suggesting that under the appropriate conditions, Rep was capable of transactivating the p5 promoter. Finally, cotransfection of equal amounts of pCMVRep78/68 and pCMVRep52/40 returned repression of the p5 promoter back to normal levels, suggesting that the ratio of Rep52/40 to Rep78/68 determined whether the p5 promoter was repressed in the presence of Ad.

To confirm the observations that Rep52/40 could relieve repression of the p5 promoter in the presence of Ad, we titrated plasmid pCMVRep52/40 in the presence of a constant amount of the wt pIM45 plasmid (Fig. 9). As the amount of pCMVRep52/40 was increased, the level of p5 transcription increased to approximately sevenfold more than that seen in the absence of pCMVRep52/40 (Fig. 9A and C, lanes 1 to 5). Addition of pCMVRep78/68 returned p5 transcription back to normal levels (Fig. 9A and C, lanes 5 to 7). Thus, it appeared that the stoichiometry of Rep78/68 and Rep52/40 was important for proper regulation of p5.

Rep52associates with Rep78/68. One reason for the increase in p5 transcription in the presence of increased Rep52/40 may be that the larger and smaller Rep proteins can associate into heteromeric protein complexes, thereby changing the properties of Rep78/68. To see if we could detect a stable protein complex that contains both p5 and p19 Rep proteins, we prepared cellular extracts from cells that were transfected with pCMVRep78/68, pCMVRep52/40, or both and precipitated them with the anti-78/68 monoclonal antibody (20), which recognizes only the larger Rep78 and -68 proteins (Fig. 7). As expected the antibody was capable of precipitating Rep78 and -68 but not Rep52 and -40 (Fig. 7, lanes 6 and 7). When an extract that contained all four Rep proteins was treated with the antibody, a complex that contained Rep52 in addition to Rep78 and -68 was precipitated. No Rep40 was detected in these complexes. We concluded that Rep52 could form a stable complex with either Rep78 or Rep68 or with both.

Mutations in Rep that affect p5 repression activity. In our previous studies, a series of mutations were made throughout the Rep coding region in an attempt to identify domains responsible for DNA replication and transactivation functions. With few exceptions, the mutants that were incapable of transactivating the p19 and p40 promoters were also defective for

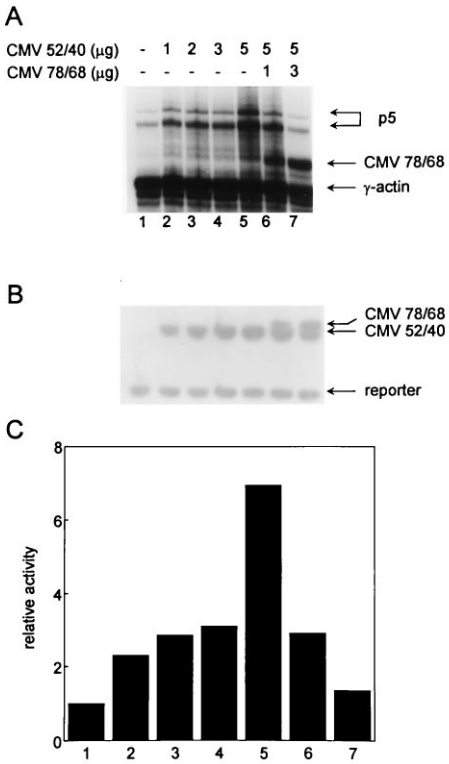

FIG. 9. The effect of Rep52/40 and Rep78/68 on p5 promoter activity is a function of their relative concentration. A549 cells were infected with Ad at an MOI of 5 and transfected with 5 μg of pIM45 and the indicated amounts of pCMVRep52/40 and pCMVRep78/68. (A) p5 and γ-actin RNAs isolated from these cells were measured by RNase protection. Indicated are the protected fragments corresponding to p5, γ-actin, and pCMVRep78/68 (CMV 78/68). (B) Measurement of input plasmid DNA. Hirt DNA was isolated and measured by Southern hybridization as for Fig. 5. Indicated on the right are the positions of plasmids pIM45, pCMVRep78/68, and pCMVRep52/40. Graphical illustration of p5 activity. The levels of p5 transcripts were measured and normalized for both total RNA (γ-actin) and input plasmid DNA. The level of activity from the plasmid pIM45 (lane 1) was set at 1; changes in p5 activity are relative to this value.

AAV DNA replication and for binding the RBE (33) (Table 2). To determine whether the regions that were previously found to be essential for transactivation and DNA binding were also required for p5 repression, the p5CAT reporter plasmid was used to measure the repression activity of each mutant Rep.

The results of these experiments are summarized in Table 2 and compared to the abilities of these proteins to transactivate the p40 promoter and bind the TR. In general, mutations that were defective for transactivation, TR binding, and DNA replication (not shown [33]) were also defective for repression of the p5 promoter. There were two exceptions, however. Mutant P415H was defective for transactivation of p40 transcription and for binding to a RBE but retained its ability to repress the p5 promoter. In addition, one of two substitutions at amino acid 242 (W242L but not W242S) was capable of repressing the p5 promoter but had lost its ability to replicate and bind to the RBE. Analyses using these two mutants suggest that the ability to repress the p5 promoter does not absolutely require binding to the RBE. Furthermore, the positions of these mutants may define interaction domains of Rep with other proteins that may be important for p5 repression.

DISCUSSION

The control of AAV transcription must be sufficiently flexible to accommodate both the latent and productive phases of viral infection. In the absence of Ad, expression of the *rep* gene has been shown to be cytostatic (49), and thus survival of the virus probably requires a mechanism for reducing transcription from the two *rep* promoters, p5 and p19, as well as the capsid promoter, p40. Indeed, with the exception of 293 cells, which constitutively express the Ad E1 genes, expression of the AAV genes is usually undetectable during latent infection or in the absence of Ad coinfection (27, 28) in human cells. In the presence of Ad, when AAV undergoes a productive infection, a mechanism must exist for efficiently inducing the three promoters. The primary proteins used for control of AAV transcription are the products of the viral *rep* gene (3, 6, 18, 32). In this report, we have focused on the role of the AAV Rep proteins and the *cis* active elements in the p5 promoter in controlling AAV transcription during a productive infection.

The p5 RBE activates p19 and p40 transcription and is required for repression of p5 in the presence of Ad. In previous work, we demonstrated that in the presence of Ad, a sequence within the p5 promoter was required for coordinate Rep-mediated induction of the p19 and p40 promoters (32). In the absence of Rep, or in the absence of both the p5 promoter and the TR, p19 and p40 transcription was reduced. Rep interaction with both the p5 promoter and the TR was through a specific 22-bp Rep binding sequence (34). In this report, we have demonstrated that binding of Rep to the p5 RBE is required for transactivation of the p19 and p40 promoters, in the absence of TRs (Fig. 10, cis, +Ad). In contrast, deletion of the p5 RBE relieved Rep-mediated repression of the p5 promoter. Thus, in the presence of Ad, Rep appears to be a transcription factor that can be both a repressor (of p5) and a transactivator (of p19 and p40).

The p5 RBE and the RBE within the TR were redundant with respect to p19 and p40 transactivation; the presence of either element ensured that the p19 and p40 promoters were transactivated by Rep (Fig. 10, cis, +Ad). This finding was consistent with our previous observations (32) and those of Beaton et al. (3), who suggested that the TR may function as an enhancer for AAV transcription. As yet, however, we have not convincingly shown that the RBE is a true enhancer which is independent of position and orientation. In contrast, the TR

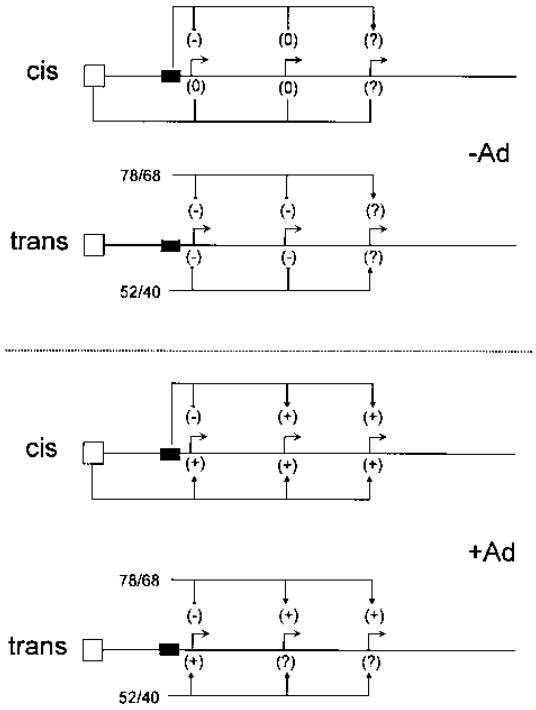

FIG. 10. Summary of observations from several reports, including experiments in this study, with respect to the regulation of transcription of the AAV promoters (3, 19, 25–27, 32, 45). The *cis* effects of the two strongest RBEs, the TR (open box) and the p5 RBE (filled box), as well as the *trans* effects of the p5 (Rep78/68) and p19 (Rep52/40) Rep proteins, in both the absence (−Ad) and presence (+Ad) of helper Ad are diagrammed. The start sites of the AAV p5, p19, and p40 promoters are indicated from left to right, respectively, and are denoted as bent arrows. The net effects of these elements at each of the AAV promoters are indicated by their effects on transcription: negative (−), positive (+), no effect (0), and unknown (?).

could not substitute for the p5 RBE to achieve repression of the p5 promoter (Fig. 10, cis, +Ad). Instead, the TR consistently increased p5 transcription as it did with p19 and p40 transcription. Furthermore, none of the other transcription elements within the p5 promoter, the two YY1 sites, the MLTF site, and the TATA box, were involved in Rep-mediated repression of p5 in the presence of Ad. Thus, regardless of whether the TR was present, the p5 RBE repressed the p5 promoter, and regardless of whether the p5 RBE was present, the TR consistently transactivated the p5 promoter (Fig. 10, +Ad, cis).

In contrast to our results in the presence of Ad coinfection, Kyostio et al. (25) have demonstrated that in the absence of Ad coinfection, the TR has no detectable effect on p5 or p19 transcription (Fig. 10, cis, −Ad). The only effect that was seen was that the p5 RBE acted as a negative regulatory element for p5; no p5 RBE effect (positive or negative) was seen at the p19 promoter. Thus, Rep binding to the RBEs within the TR and p5 promoter produces different results in the presence of Ad infection than in the absence of Ad infection.

Roles of Rep proteins in repression and transactivation. Several laboratories have demonstrated that in the absence of Ad, the Rep proteins repress the p5 and p19 promoters (19, 25). Both the p5-encoded Rep proteins, Rep78 and -68, and the p19-encoded Rep proteins, Rep52 and -40, are capable of repressing, albeit at different levels (Fig. 10, trans, −Ad). There have been conflicting reports concerning the role of Rep inregulating the p5 and p19 promoters in the presence of helper virus. Using CAT reporter plasmids, Beaton et al. (3)

have found that Rep negatively regulates both the p5 and p19 promoters. In contrast, Labow et al. (27) have reported that AAV transcripts are significantly reduced in the absence of functional Rep protein, suggesting that Rep is a transactivator in the presence of Ad for all three AAV promoters. Our previous results (33) as well as those presented here (Fig. 5) indicate that in the presence of Ad, Rep is a transactivator for the p19 and p40 promoters (consistent with results of Labow et al. [27]). However, we also have shown here that even in the presence of Ad, Rep continues to repress the p5 promoter (Fig. 10, trans, +Ad). This was shown both by p5-driven CAT assays and by determination of the steady-state levels of p5 mRNA (Fig. 2 and 3).

Our experiments suggest that in the presence of Ad, Rep represses p5 in part by counteracting the transactivation effects of the Ad E1a region (Fig. 1A). The mechanism of this interaction is not clear. Shenk and colleagues (9, 41, 48) have identified four sequence elements that are necessary for basal p5 transcription, MLTF, TATA, and two YY1 sites. The YY1 and MLTF elements are required for E1a-mediated activation of p5. Presumably, Rep repression of p5 is mediated by Rep interaction with one of these factors or, as suggested by Kyostio et al. (26), by preventing the binding of one of these factors.

We also examined the separate contributions of Rep78/68 and Rep52/40 to p5 repression in the presence of Ad. The results showed that Rep78/68, but not Rep52/40, facilitates p5 repression (Fig. 8 and 9; Fig. 10, trans, +Ad). These results were different from the observations made for p5 repression in the absence of Ad (19, 25). As mentioned earlier, in the absence of Ad, Rep52/40 was found to repress p5. Furthermore, we observed that Rep52/40, when present at high concentrations, resulted in a net increase in p5 transcription (Fig. 8 and 10), thus implying that Rep52/40 can reverse the negative effects of the Rep78/68 proteins at the p5 promoter. This observation might explain some of the contradictions noted earlier with respect to the role of the Rep proteins in p5 transcription. Although we have not detected transactivation of p5 by Rep in the presence of Ad (only repression), Labow et al. (27) saw no p5 transcripts in the absence of Rep, suggesting that Rep transactivated the p5 promoter. Furthermore, although we and others have been unable to detect p5 transcription in the absence of Ad in KB (27, 28), HeLa (12, 27), and A549 (Fig. 3) cells, other laboratories using 293 (25, 26) and HeLa (19) cells could detect p5 transcripts in the absence of Ad. Our observations suggest that if the ratio of Rep52/40 to Rep78/68 is high, repression of the p5 promoter is eliminated. Under these circumstances, Rep could still transactivate the p5 promoter through the TR, giving the appearance that Rep can transactivate the p5 promoter as well as the p19 and p40 promoters. This outcome would depend both on the basal level of transcription of the p5 promoter in a particular cell line and on the ratio of p5 to p19 transcripts.

Rep52 forms a complex with Rep78 or Rep68 in vivo. The fact that Rep52/40 can repress p5 and p19 in the absence of Ad (19, 25) (Fig. 10) and the fact that Rep52/40 can counteract repression by Rep78/68 in the presence of Ad (Fig. 8 to 10) both suggest that Rep52 and -40 exert their effects through protein-protein contacts with either cellular or viral transcription factors. The current biochemical and genetic data (10, 18, 22, 26, 33) (Table 2) indicate that neither Rep52 nor Rep40 is capable of binding to the RBE. Thus, although repression of p5 may occur in part by direct binding of Rep78/68 to the p5 promoter (reference 26 and this report), repression of the p5 and p19 promoters by Rep52/40 in the absence of helper must occur by protein-protein interactions rather than by interaction with the p5 or p19 RBEs. Similarly, the ability of Rep52/40 to attenuate Rep78/68 repression of p5 in the presence of Ad suggests that Rep interacts with a p5 transcription factor. These effects could be mediated in part by the formation of a complex between the larger Rep proteins and Rep52/40. In this report, we have demonstrated that a stable complex of Rep52 and either Rep78 or Rep68 can form in vivo. It was not clear from our study whether there was a preference for Rep78 or Rep68 in the complex, but clearly there was a preference for Rep52 (Fig. 7). We also note that although the Rep78/68-Rep52 complex is likely to be involved in the attenuation of Rep repression at the p5 promoter, it would not account for the fact that in the absence of Ad, Rep52 alone can repress the p5 and p19 promoters (19, 25). These observations suggest that the p19 Rep proteins and, most likely, the p5 Rep proteins interact with cellular transcription factors as well. This possibility is supported by the recent work of several groups who have demonstrated that Rep can repress heterologous promoters that do not contain an RBE (17, 19).

If the Rep proteins form complexes with themselves and perhaps cellular proteins, then it should be possible to identify the interaction domains within the Rep amino acid sequence. Interaction domains that are specifically involved in repression would have the property that mutations in these domains might be defective for RBE binding but retain their ability to repress the p5 promoter. For this purpose, we screened 18 deletions and amino acid substitutions within the *rep* coding region. We found two mutants that were completely defective for transactivation, DNA replication, and RBE binding but retained their ability to repress the p5 promoter. These two positions, amino acids 242 and 415, were both within the region of Rep that is common to all four Rep proteins. Amino acid 242 was interesting in that, depending on which amino acid substitution was made, repression activity was either eliminated or preserved. Additional experiments will be necessary to determine whether these Rep positions are involved in Rep-Rep interactions.

Multiple layers of Rep control. As mentioned earlier, given the life cycle of AAV, it would not be surprising to find that there are multiple layers of Rep control that behave differently in the presence and absence of helper virus. In the absence of Ad, autoregulation has been demonstrated for both p5 and p19 Rep proteins. Our studies have focused on the role of Rep in the presence of Ad and have revealed several new layers of control while confirming much of what was previously known (Fig. 10). First, when bound to the p5 RBE in the presence of Ad, Rep can simultaneously act as a repressor and a transactivator. Second, the position of the RBE (p5 or TR) affects whether Rep acts as a repressor or an activator of p5 transcription; only the p5 RBE-Rep complex appears to be capable of p5 repression. Finally, the p19 Rep proteins can modulate Rep78/68 activity in the presence of Ad, and this is most likely accomplished by Rep-Rep interactions. These observations suggest that during a productive AAV infection, a feedback loop exists to control the level of Rep78 and -68. Because the TR and p5 RBEs are redundant for p19 and p40 activation, the primary role of the p5 RBE appears to be to repress p5 transcription. This reduces the steady-state level of p5 transcripts relative to p19 and p40 transcripts. If the ratio of p19 to p5 transcripts increases, Rep52/40 attenuates repression at p5 to raise the level of Rep78/68. The net effect is a control loop which maintains a constant ratio of the p5 and p19 transcripts (Fig. 10, +Ad).

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