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Negative Regulation of the Adeno-Associated Virus (AAV) P_5 Promoter Involves both the P_5 Rep Binding Site and the Consensus ATP-Binding Motif of the AAV Rep68 Protein

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Transcript levels from the P_5 promoter of adeno-associated virus type 2 (AAV) are negatively regulated by the AAV Rep78 and Rep68 proteins in the absence of helper virus. We have identified a Rep-responsive negative *cis* element of the P_5 promoter between the P_5 TATA box and transcription start site by using 5′ and 3′ deletions of the P_5 promoter fused to the chloramphenicol acetyltransferase gene. This element contains four imperfect GAGC repeats similar to the Rep recognition sequences (RRSs) in the AAV inverted terminal repeats and in the AAV preferred integration locus in chromosome 19. Band shift analyses showed that human 293 cell nuclear extracts containing Rep68 or Rep68/K340H, a putative nucleoside triphosphate (NTP)-binding-site mutant of Rep68, formed Rep-specific complexes with this P_5 RRS DNA. Within the P_5 RRS, mutation of a cytosine at position 273 in the AAV sequence to guanine abolished Rep68 binding to the DNA. A mutation in the P_5 RRS within a full-length AAV genome, which abolished Rep binding, resulted in a 40 to 50% reduction in the ability of wild-type Rep68 to inhibit the accumulation of P_5 transcripts in vivo. In contrast, the Rep68/K340H mutant was unable to down-regulate this mutated promoter. These results indicate that there are at least two mechanisms involved in the negative regulation of P_5 transcript levels by Rep68; one involves Rep68 binding to the P_5 RRS, and another requires the region of Rep68 containing the consensus NTP-binding motif. Furthermore, our studies of AAV genomes containing mutated RRS- and/or YY1-binding elements suggest that transcription factor YY1 binding to the transcription start site of P_5 interferes with Rep68 repression of the P_5 promoter.

Adeno-associated virus type 2 (AAV) is a nonpathogenic human parvovirus that requires coinfection with adenovirus or herpesvirus as a helper virus for efficient replication (5). In the absence of the helper virus, the AAV DNA integrates into the host genome and is maintained as a latent provirus. The linear single-stranded viral genome of 4.7 kb contains two open reading frames (ORFs) flanked by inverted terminal repeats (ITRs) at either end (6, 50). The first ORF encodes the nonstructural (NS) Rep proteins; the larger Rep proteins, Rep78 and Rep68, are made from transcripts initiating from the P_5 promoter, while the smaller Rep proteins, Rep52 and Rep40, are expressed from the downstream P_{19} promoter (6, 37, 51, 55). The second ORF of AAV encodes the viral capsid proteins (VP-1, VP-2, and VP-3) expressed from the P_{40} promoter (50, 53).

The AAV Rep proteins have multiple functions and are involved in both the replication and gene regulation of the virus. Several of the known properties of the Rep proteins, such as the binding to the ITR DNA, the ATP-dependent site-specific endonuclease, and the DNA helicase activities, are important for its replication function (21–23, 49). The Rep proteins also contain a consensus nucleoside triphosphate (NTP)-binding motif which is involved in the endonuclease, helicase, and ATPase activities of Rep78 and Rep68 (10, 12, 34, 42, 57). This motif has also been shown to be necessary for the cell cytotoxicity by the analogous NS proteins of the autonomous parvoviruses B19 and minute virus of mice (MVM) (28, 38). The Rep functions important for gene regulation have been less well characterized and may vary depending on the presence or absence of the helper virus. In the absence of a helper virus, the larger Rep proteins, Rep78 and Rep68, efficiently inhibit the production of P_5 and P_{19} transcripts, while Rep52 and Rep40 have a lesser effect on these transcript levels (25). A mutant Rep78 with an alteration of a lysine to a histidine in the putative NTP-binding site (K340H) retains the ability to decrease P_5 mRNA levels but lacks the ability to down-regulate P_{19} mRNA levels (25). The presence of helper virus results in the activation of AAV promoters by Rep protein(s) (2, 26), suggesting interaction with or modification of Rep proteins by host or helper virus proteins. Mutation of two amino acids (T341I and N342Y) in the putative NTP-binding site of Rep abolished the ability of the AAV Rep proteins to activate the AAV P_{19} and P_{40} promoters in the presence of helper virus (34). Furthermore, the ability to activate promoters by an analogous NS protein of MVM was also dependent on the conserved lysine in the NTP-binding site, since its change to serine abolished MVM P_{38} activation (24).

The complexity of the AAV life cycle is reflected in the regulation of the viral promoters by proteins produced by the helper virus and the host cell, as well as by AAV. Consequently, several known and putative *cis* elements for both viral and host *trans* activators and repressors have been localized in the AAV P_5 promoter. These include *cis* elements such as the binding sites for CREB/ATF (17, 29) and major late transcription factor (MLTF; USF) (8) in the region upstream of the P_5 TATA box. The P_5 promoter also contains two binding sites for the host cell transcription factor YY1 (46, 47). One is located upstream from the TATA box at −60 (in relation to the tran-

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scription start site), where YY1 binding results in repression of P_5 expression in the absence of helper virus (47). In the presence of E1a protein produced by the coinfecting adenovirus, this site mediates transcription activation of the P_5 promoter by a direct E1a-YY1 interaction (47). Binding of YY1 to the second binding site located at the transcription start site (+1) enhances and directs the accurate positioning of P_5 transcript initiation (46). Recently, a Rep68-binding site has been located just upstream of the +1 YY1-binding site, although its function in AAV gene regulation was not characterized (35).

In this study, the objective was to analyze the mechanism of the repression of the P_5 promoter by Rep68. The Rep-responsive negative *cis* element within the P_5 promoter was localized between the P_5 TATA box and transcription initiation site and matches the recently reported Rep-binding sequence (35). We also report a mutational analysis of this binding site and its functional significance in the context of the full-length AAV genome during conditions nonpermissive for AAV replication.

MATERIALS AND METHODS

Cells and viruses. Adenovirus-transformed human embryonic kidney 293-31 cells (293 cells) (18) were maintained as monolayer cultures and grown in Eagle's minimal essential medium (Quality Biological, Inc., Gaithersburg, Md.) supplemented with 10% fetal calf serum (Inovar, Gaithersburg, Md.), 2 mM L-glutamine, and 100 U each of penicillin, neomycin, and streptomycin (Life Technologies, Gaithersburg, Md.) per ml.

Plasmids. The AAV plasmids used in this study were constructed by using standard techniques (44). Plasmid pHIVrep*am*Δ (encoding the protein designated Rep*am*) is similar to pHIVrep*am* described previously (1) except that the right-hand ITR has been deleted by *Sna*BI-*Sph*I digestion. The overhangs of the larger fragment were then filled in by T4 DNA polymerase, and the blunt ends were religated. The right-hand ITR was also deleted from plasmid pSK9, which contains the AAV Rep68-coding region expressed from the long terminal repeat (LTR) promoter of human immunodeficiency virus type 1 (HIV-1) (42). pSK9/NTP is derived from pSK9 and contains the same mutation in the region encoding the NTP-binding site as pHIVrepNTP (42).

The plasmids containing the 3′ deletion series of P_5-chloramphenicol acetyltransferase gene (*cat*) fusions (Fig. 1A) were derived from plasmid pAV2, which contains the entire AAV genome (27). pAV2 was cleaved with *Pvu*II and *Sph*I and religated in order to delete the *Nru*I site in the pBR322-derived region. A 2.4-kb fragment downstream from the P_{40} promoter was removed by cleavage with *Hin*dIII and *Kpn*I and replaced with the *Hin*dIII-*Kpn*I fragment of plasmid pTS1 (52), which contained the *cat* gene. The resulting plasmid was cleaved with *Nru*I at AAV nucleotide 658 and then digested with BAL 31 exonuclease. Aliquots taken at various times during the BAL 31 treatment were cut with *Hin*dIII, and the termini were filled in with the Klenow fragment of DNA polymerase and blunt-end ligated. Of the plasmids generated, two in which a *Hin*dIII site had been reconstituted, plasmids pYT45 (17) and pYT36, were identified. The *Sna*BI-*Nde*I fragments of pYT45 and pYT36, which included the right-hand ITR, were deleted to produce pRO45 (17) and pRO36, respectively. pRO45 and pRO36 contain AAV nucleotides 1 to 263 and 1 to 505, respectively, ligated to the *cat* gene. pRO1472 was made by inserting an annealed synthetic oligonucleotide pair (AAV nucleotides 266 to 321) flanked by *Hin*dIII-compatible ends into *Hin*dIII-cleaved pRO45 (17). Two tandem repeats of AAV nucleotides 266 to 287 with *Hin*dIII-compatible ends were inserted into the *Hin*dIII site of pRO45 to make pRO1511. An insertion of a single copy of the same oligonucleotide pair, but in the reverse orientation, resulted in pRO1517. Because of the presence of the *Hin*dIII site, pRO1472 and pRO1511 have nucleotides 264 and 265 changed from CC to TT. An oligonucleotide pair containing AAV nucleotides 287 to 321 with *Hin*dIII-compatible ends was inserted into the *Hin*dIII-cleaved pRO45 in the correct orientation to make pRO1535 or in the reverse orientation to make pRO1538.

pNTC244 and pNTC3 (see Fig. 5A) contain a wild-type AAV genome and an AAV genome with an amber mutation in the *rep* gene, respectively, cloned into pTZ19U (Bio-Rad, Melville, N.Y.), and they have been described before (9). Mutations between the TATA box and the transcription initiation site in pNTC3 and pNTC244 were created by PCR. A 5′ primer (5′ GGCCCGAGATATC GATCAGGGTCTCCATTTTG 3′) containing the native *Ava*I site and a novel *Eco*RV site (mRRS-3 [RRS = Rep recognition sequence]) and a 3′ primer located at AAV positions 1062 to 1085, downstream from a *Bam*HI site, were used to synthesize a 0.8-kb fragment. This fragment was cleaved with *Ava*I and *Bam*HI, gel purified, and then cloned into pNTC3 that had been partially cleaved with *Ava*I and *Bam*HI. A double mutation containing both the *Eco*RV site (mRRS-3) and an altered +1 YY1 site that abolishes YY1 binding (47) was constructed similarly except that the 5′ primer (5′ GGCCCGAGATATCGAT CAGGGTCTAAATTTTGAAGC 3′) additionally contained mutations at nucle-

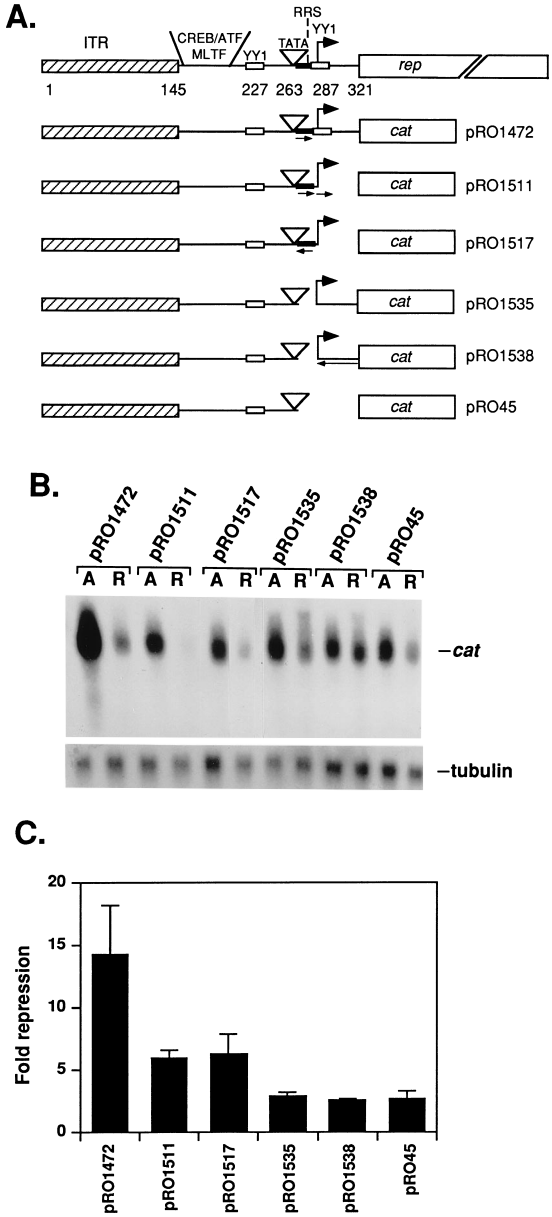

FIG. 1. Analysis of 3′ deletion constructs of P_5-*cat* fusions. (A) Structures of the 3′ deletion constructs of P_5-*cat* fusions. Numbers indicate positions in the AAV nucleotide sequence. ITR (hatched box), binding sites for CREB, ATF, MLTF, YY1 (at −60 and +1), and Rep68 (RRS), TATA box, transcription start site (bent arrow), and the beginning of the *rep* ORF (position 321) are shown. The short arrows indicate the number of copies and the orientation of the region between the TATA box and transcription start site. The long arrow indicates the reversal of the region between the transcription start site and the beginning of the *rep* ORF. (B) Northern analysis of RNA from cells transfected with the P_5-*cat* constructs. The P_5-*cat* constructs (10 μg) indicated at the top were cotransfected with plasmids (5 μg) producing either Rep*am* (lanes A) or Rep68 (lanes R) into human 293 cells. The cytoplasmic RNA was isolated after 48 h and analyzed by Northern blotting using a ^{32}P-labeled *cat* fragment. Hybridization for tubulin gene transcripts was performed to indicate the intactness of the RNA. The RNA blot shown represents one of the three independent experiments performed. (C) Quantitation of P_5 repression by Rep68, using 3′ deletion constructs. Three independent transfection experiments were performed and analyzed by Northern blotting. The *cat* transcripts were cut and counted, and the values were normalized for transfection efficiency by values obtained from DNA slot blots (P_5-*cat* constructs). The level of repression is expressed as fold repression by comparing the unrepressed (in the presence of Rep*am*) and repressed (in the presence of Rep68) levels. Error bars indicate standard deviations between experiments.

otides 285 and 286 (cytosines to adenines) of the AAV nucleotide sequence. A derivative of the pNTC3 construct with the same altered YY1-binding site but a wild-type P_5 RRS was also created by using a 5′ primer (5′ GGCCCGAGT GAGCACGCAGGGTCTAAATTTTG 3′) with this mutation.

For electrophoretic mobility shift analyses, several double-stranded oligonucleotides containing flanking *Bam*HI overhangs (5′ GATC 3′) were cloned into *Bam*HI-digested pBlueScriptII SK$^+$ (pBluescript; Stratagene, La Jolla, Calif.). These include the P_5 RRS (AAV nucleotides 259 to 285), various mutant forms of the P_5 RRS (with AAV cytosines 265, 273, and/or 277 changed to guanines) (see Fig. 4), P_{19} (AAV nucleotides 847 to 874), the HIV-1 LTR (40) (+30 to +54 relative to the mRNA start site), and a series of oligonucleotide pairs (AAV nucleotides 259 to 298) containing wild-type or mutant forms of the P_5 RRS and the +1 YY1-binding site (see Fig. 5A). The YY1-binding-site mutations were designed such that they abolish YY1 binding to the DNA (47). For competition assays, double-stranded oligonucleotides flanked by *Bam*HI overhangs and containing only the wild-type (5′ AGGGTCTCCATTTTGAAGCGG 3′) or mutated (5′ AGGGTCTAAATTTTGAAGCGG 3′) +1 YY1-binding site were cloned into pBluescript.

The presence of the mutations or cloned double-stranded oligonucleotides in all constructs was confirmed by DNA sequencing (45) using a Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). All plasmids used for transfections were grown in *Escherichia coli* DH5 (Life Technologies, Inc.), prepared by alkaline lysis and purified on cesium chloride gradients (44).

DNA transfections. Transfections for RNA analyses were performed by the calcium phosphate coprecipitation method (19). For transfections, 6 to 10 μg of the template plasmids and 3 to 5 μg of *rep* plasmids were used as indicated in the figure legends. All transfections were adjusted with vector DNA (pBR322) to contain a total of 15 μg of DNA. Human 293 cells were plated on 100-mm-diameter dishes at a density of 3×10^6 cells per plate 24 h prior to transfection. All cells were harvested 48 h after transfection and used for RNA isolation.

RNA isolation and Northern (RNA) analysis. Cytoplasmic RNA was isolated and analyzed as described previously (25). The 0.7-kb *Hin*dIII-*Sca*I fragment of pRO5 and tubulin DNA (Oncor, Inc., Gaithersburg, Md.) were used for the detection of *cat* and tubulin gene transcripts, respectively. For the detection of *rep* transcripts, a 1.6-kb *Hin*dII fragment of pNTC3 was used. The fragments were labeled by the random-priming method (16), using [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; DuPont NEN, Boston, Mass.) and a random-primer labeling kit (Boehringer Mannheim, Indianapolis, Ind.). The transcript levels were quantitated by cutting and counting the radioactive signals in a liquid scintillation counter. These values were normalized by plasmid uptake into the nuclei, as measured by DNA slot blot analysis (25).

Primer extensions. Ten micrograms of cytoplasmic RNA was mixed with 40 pmol of ^{32}P-labeled primer (5′ GCCATTGGGATATATCAACGGTGGT 3′) complementary to the *cat* gene (10^5 cpm) in primer extension buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dNTP, 0.5 mM spermidine). The primer was annealed by heating the tubes at 70°C for 10 min followed by slow cooling to room temperature. The volume was doubled to 20 μl by adding primer extension buffer, sodium pyrophosphate to a final concentration of 2.8 mM, and 2 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.). The reaction mixtures were incubated at 42°C for 1 h, after which 20 μl of loading dye was added. Samples were heated at 90°C for 10 min and loaded on an 8% polyacrylamide sequencing gel. A ^{32}P-end-labeled primer or [^{35}S]dATP (DuPont NEN) was used for the labeling of sequencing reactions that served as DNA size markers.

Gel mobility shift assays. Annealed oligonucleotide pairs or oligonucleotide pairs cloned into pBluescript were used in band shift analyses. The plasmids containing the double-stranded oligonucleotides were cut with *Xba*I and *Hin*dIII to release a 70- to 90-bp fragment containing the oligonucleotide pair flanked with DNA sequences of the multiple cloning site of pBluescript. Both the annealed oligonucleotide pairs and the restriction fragments were labeled with Klenow polymerase and [α-^{32}P]dCTP (3,000 Ci/mmol; DuPont NEN). The labeled fragments were then purified on a 6% polyacrylamide gel. Nuclear extracts (600 mM NaCl) of 293 cells transfected with pHIVrep*am*, pSK9, or pSK9/NTP were prepared as described previously (42). The protein concentrations were equalized with extraction buffer, and the samples were diluted 10-fold in buffer A (25 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20% [vol/vol] glycerol) to reduce the NaCl concentration. Labeled DNA (0.02 or 0.002 pmol) was incubated with 0.44 μg of nuclear extract protein for 20 min at 4°C in a 20-μl volume containing 5.5 μg of acetylated bovine serum albumin (BSA; New England Biolabs, Beverly, Mass.), 50 mM NaCl, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2% (vol/vol) glycerol, 0.01% (vol/vol) Nonidet P-40, and 1 μg of poly(dI-dC) (Boehringer Mannheim). A rabbit antibody against the S18K oligopeptide (Rep78 amino acids 516 to 533) was added in a volume of 1 μl where indicated (37). In some experiments, various amounts of ATP were added to the mixture. The samples were electrophoresed in a 4% polyacrylamide gel as described previously (21) except that the gel was run at 4°C.

RESULTS

Localization of a negative Rep-responsive *cis* element in the AAV P_5 promoter. The AAV Rep proteins have been previously shown to inhibit transcript accumulation from the AAV P_5 promoter in 293 cells in the absence of the helper virus (25). To localize a negative Rep-responsive element in the AAV P_5 promoter, several 3′ and 5′ deletions of the P_5 promoter and upstream regions were constructed and fused to the *cat* gene (Fig. 1A and data not shown). The constructs were tested in human embryonic kidney cells (293 cells) which have been immortalized with the adenovirus E1a and E1b genes (18). Expression of these adenovirus proteins enhances the levels of AAV P_5 and P_{19} transcripts but is not sufficient for AAV replication in the absence of the helper virus (8, 27). The effect of Rep proteins on *cat* transcript levels from the P_5-*cat* fusions was measured by Northern analysis after cotransfection of the fusion plasmids with plasmids producing either wild-type Rep68 (pSK9) or a truncated Rep*am* protein (pHIVrep*am*) as a negative control. The *cat* transcript levels were normalized for transfection efficiency by measuring the template (P_5-*cat* fusions) uptake in DNA slot blots. CAT activities were not measured because the Rep proteins may negatively regulate translation of the *cat* mRNA (54). Similarly, expression of a control reporter gene was not used because of the effects of Rep proteins on heterologous promoters.

We first analyzed the effects of Rep68 on the 3′ deletion constructs of the P_5 promoter fused to the *cat* gene (Fig. 1B and C). Plasmids pRO1472, pRO1511, and pRO1517 all contained the previously identified Rep68-binding site between the P_5 TATA box and the transcription start site (35) and were all clearly down-regulated approximately 6- to 14-fold, depending mainly on the level of unrepressed transcription. Furthermore, the orientation of the region between the P_5 TATA box and the transcription start site did not appear to be critical, since in construct pRO1517, this region was reversed. Further deletions from the 3′ direction (pRO1535, pRO1538, and pRO45), which deleted the Rep68-binding site, however, abolished efficient down-regulation (to threefold or less) by Rep68 without changing the unrepressed level of expression compared with pRO1511 and pRO1517 (Fig. 1B). These results suggested that the region between the TATA box and transcription start site containing the Rep68-binding site was required for efficient Rep68 repression. However, the remaining repression activity by Rep68 suggested that other factors besides this *cis* region may also be involved.

We also analyzed the effects of Rep68 on the 5′ deletion constructs, all of which contained the known Rep68-binding site between the TATA box and transcription start site (35). The Northern analysis indicated that all of the 5′ deletion constructs (deletions up to AAV base 217) tested were down-regulated to some degree by the Rep68 protein (data not shown). However, the differences in fold repression were strongly influenced by the differences in the unrepressed levels of expression, and thus comparison of repression efficiencies of the various constructs was difficult. The results showed that the AAV ITR and several upstream *cis* elements such as binding sites for CREB, ATF, or MLTF (USF) were not required for the negative effect by Rep68. Furthermore, a construct containing a deletion to just upstream of the −60 YY1-binding site (AAV nucleotide 217) and a mutation in the −60 YY1-binding site (47) had reduced mRNA levels but was still repressed by Rep.

Since several of the 3′ deletion constructs contained an altered DNA sequence around the transcription start site, due to the deletions and reversals, the initiation sites of *cat* tran-

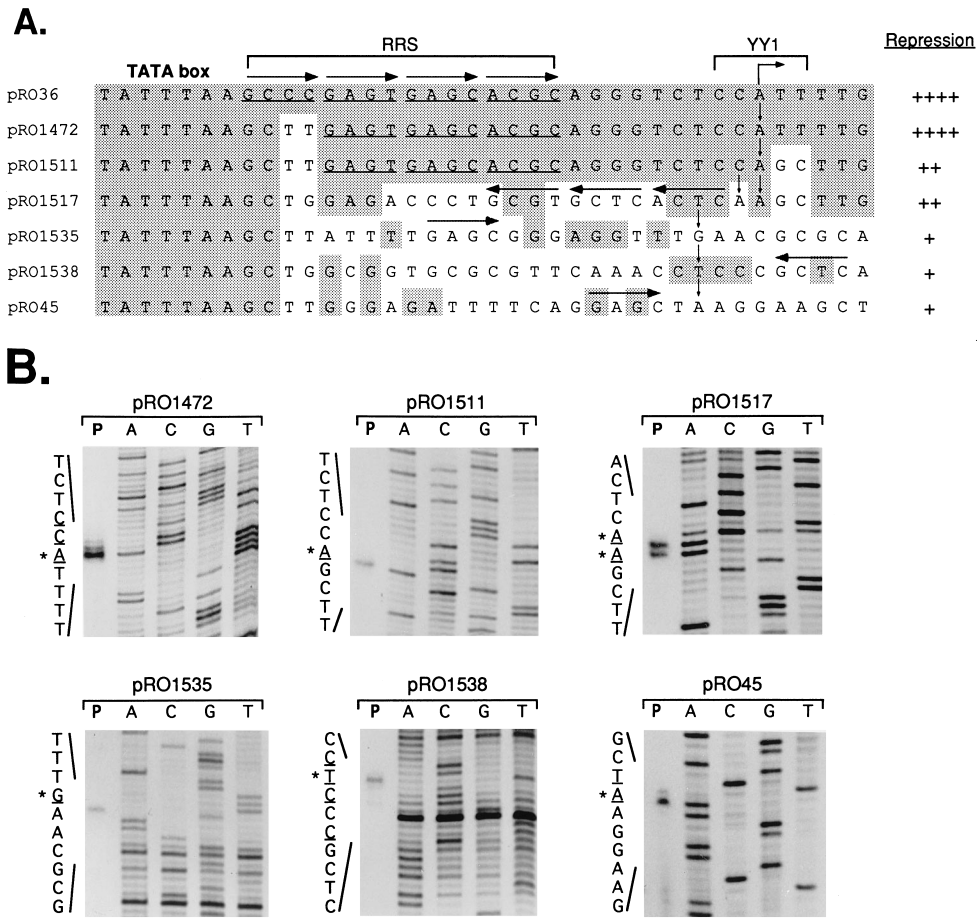

FIG. 2. Mapping of transcription start sites in 3′ deletion constructs of P_5-*cat* fusions and their locations with respect to the P_5 TATA box. (A) Summary of primer extension results with P_5-*cat* constructs. The DNA sequence in the P_5 promoter between the TATA box and transcription start site are shown (AAV positions 255 to 292). Some constructs contain additional changes due to deletions and reversals of sequences (see Fig. 1A). Regions identical to the wild-type AAV sequence are shaded. The wild-type AAV start site at 287 is shown as a bent arrow; the vertical arrows indicate the major transcript start sites determined by three primer extension assays. The horizontal arrows show the positions of the native or serendipitous GAGC boxes. The YY1-binding site in the transcription initiation site is also indicated. On the right side are shown the relative levels of repression by Rep68 on each construct. (B) Primer extension analysis of P_5-*cat* constructs. All nucleotides detected as start sites for transcripts are underlined; the major start sites are indicated by asterisks. Cytoplasmic RNA from transfections containing the P_5-*cat* constructs (10 μg) indicated and pHIVrep*am* (5 μg) was annealed to a primer complementary to the *cat* gene. The primer extension products (lane P) were run on an 8% polyacrylamide sequencing gel, and the start site was localized by using standard sequencing reactions (lanes A, C, G, and T) as DNA markers.

scripts from these constructs were analyzed by primer extension using a primer complementary to the *cat* gene (Fig. 2). Previous results have located the start site of the P_5 mRNA at nucleotide 287 of the AAV sequence (32). Our analyses showed that in constructs (pRO36, pRO1472, and pRO1511) containing the native AAV transcription initiation site, which is normally within a known YY1-binding site (46), single major transcripts were initiated accurately at the correct AAV start site 26 bp downstream from the end of the P_5 TATA box at 261. The alteration in the YY1-binding site in pRO1517 resulted in the appearance of two equally strong start sites located one nucleotide from each other. The effect of mutations at the +1 YY1-binding site resulting in multiple start sites has been reported before (46). Interestingly, the lack of the normal initiator sequence in the other constructs (pRO1535, pRO1538, and pRO45) did not appear to decrease the levels of the *cat* mRNA relative to pRO1511 (Fig. 1B) or result in multiple strong start sites (Fig. 2B). With pRO1535 and pRO1538, transcription was initiated consistently 23 bp from the TATA box, using either purines or pyrimidines as starting nucleotides. Similarly, with the pRO45 construct, which lacks all of the native AAV sequence downstream from the P_5 TATA box, the transcript initiated 23 bp downstream of the TATA box (13 bp upstream of the ATG of the *cat* gene). These results indicated that the P_5 repression by Rep68 required the Rep68-binding region between the TATA box and the mRNA initiation site (AAV nucleotides 265 to 287). The alterations in the YY1-binding site at the transcription start site also correlated with the reduced repression by Rep68, suggesting that Rep-mediated repression might be affected by host transcription factor YY1 binding to the transcription start site (Fig. 2A). Therefore, we next analyzed the properties and the significance of Rep68 binding to the P_5 promoter as well as the role of YY1 binding to the P_5 transcript start site.

Wild-type and NTP-mutant Rep68 proteins bind to the region between the P_5 TATA box and the transcription initiation site. The region between the P_5 TATA box and transcription initiation site that was important for the P_5 negative regulation by Rep68 contained four imperfect GAGC repeats. These repeat sequences are similar to those of the GAGC repeats observed previously in the RRSs identified in the AAV ITRs and within the preferred AAV integration locus on human

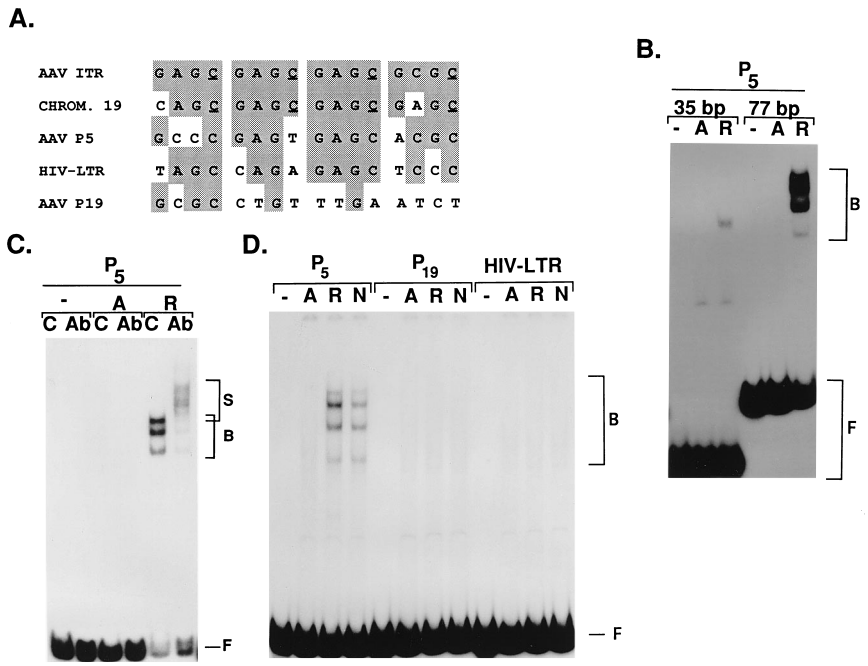

FIG. 3. Rep68 forms specific protein-DNA complexes with the P_5 RRS. (A) Sequence comparison of the P_5 negative *cis* element with the RRSs located in the AAV ITRs and the AAV integration site in human chromosome (CHROM.) 19. Sequence identity with the AAV ITR sequence is indicated by shading. Underlined cytosines in the human chromosome 19 sequence and in the AAV ITR are complementary to guanines which have been previously shown to be important for Rep binding (42, 56). For comparison, also shown are the region between the TATA box and the transcription start site from the AAV P_{19} promoter and the Rep-responsive negative element from the HIV-1 LTR. (B) Effect of length of the P_5 RRS-containing DNA on the Rep-specific binding. An oligonucleotide pair containing the P_5 RRS was ^{32}P end labeled by fill-in and resulted in a 35-bp fragment used for the band shift assay (35 bp). The oligonucleotide pair was also cloned into the multiple cloning site of pBluescript. The P_5 RRS together with a part of the multiple cloning site of pBluescript was then excised as a 77-bp fragment, labeled with ^{32}P, and used for the band shift assay (77 bp). The DNA was incubated with no extract (−) or nuclear extracts prepared from 293 cells transfected with a plasmid producing Rep*am* (A) or Rep68 (R). The samples were electrophoresed in a 4% polyacrylamide gel run at 4°C. The Rep-specific shifted (B) and unshifted (F) fragments are indicated. (C) The Rep68-specific P_5 DNA-protein complexes are supershifted with an anti-Rep antibody. The incubation mixtures contained either BSA as a control (C) or anti-Rep/S18K antibody (Ab) together with the nuclear extracts and the 77-bp P_5 RRS DNA. The gel mobility shift analysis was performed as described above. The supershifted fragments are indicated by S. (D) Rep68- and Rep68/K340H-containing extracts result in a P_5-specific mobility shift. For comparison, the DNA from the HIV-1 LTR or from the region between the TATA box and the transcription start site of P_{19} are not shifted by the Rep68-containing extracts. ^{32}P-labeled fragments containing the cloned oligonucleotides were incubated with no extract (−) or nuclear extracts prepared from 293 cells transfected with a plasmid producing Rep*am* (A), Rep68 (R), or Rep68/K340H (N).

chromosome 19, which have been shown to be bound by Rep proteins (Fig. 3A) (12, 56). Furthermore, McCarty et al. (35) have recently demonstrated that the P_5 RRS can also be bound by purified Rep68 produced in Sf9 insect cells. Since our experiments were performed in human 293 cells, we analyzed the abilities of Rep proteins in 293 cell nuclear extracts to bind to this P_5 sequence. The band shift reaction mixtures contained equal amounts of nuclear proteins, and results of Western blot (immunoblot) analyses indicated that the concentrations of Rep68 and Rep68/K340H were within twofold of each other within their respective extracts (not shown).

The gel mobility shift analyses with Rep68-containing extract and an annealed oligonucleotide pair containing the P_5 RRS showed a Rep68-specific shifted band which was not seen with Rep*am*-containing extract (Fig. 3B). This 35-bp double-stranded oligonucleotide produced a single Rep68-specific shifted band and thus did not produce the characteristic multiple shifted band pattern observed previously for Rep68 binding to the RRS sequences within the ITR or chromosome 19 (42, 56). However, when the annealed oligonucleotide pair was cloned into pBluescript and isolated within a larger fragment (77 bp), the formation of the Rep68-specific DNA-protein complexes was enhanced and the typical multiple band pattern was detected (Fig. 3B). The effect of the length of the fragment in stabilizing Rep binding has also been observed with the linear ITR DNA by using a purified maltose-binding protein–Rep68 fusion protein (13). Consequently, all of the oligonucleotide pairs analyzed in band shift analyses were cloned into pBluescript and tested as larger fragments. The retarded complexes contained Rep protein(s), as the shifted bands were supershifted by an anti-Rep antibody (Fig. 3C). The specificity of the anti-Rep antibody has been shown before (42). Furthermore, the Rep68-dependent shift was specific for the P_5 RRS (region between the TATA box and the transcription initiation site), since a region from the P_{19} promoter between the TATA box and the mRNA start site or a Rep-responsive region from the HIV-1 LTR (40) produced no Rep68-specific shifted bands (Fig. 3D). Nuclear extracts containing Rep68/K340H produced Rep-specific shifted bands with P_5 DNA similar in intensity and pattern to the bands produced by wild-type Rep68. This result indicated that the NTP mutant of Rep68 was comparable to wild-type Rep68 in its DNA-binding properties analyzed in vitro.

Previous methylation interference assays of Rep binding to the AAV integration site in human chromosome 19 or to the AAV ITR indicated the importance of four guanines complementary to the underlined cytosines shown in Fig. 3A (42, 56). One of these residues is not present in the P_5 RRS, while the remaining three are all conserved in the RRS of P_5, the AAV ITR, and chromosome 19. To analyze the significance of these three cytosines (and/or their complementary guanines) in the P_5 RRS, we tested several altered P_5 RRS sequences flanked

		Relative Rep68-specific binding (%)
AAV P_5	G C C C G A G T G A G C A C G C	100
oSK93/94	- - - G - - - - - - - - - - - -	30
oSK95/96	- - - G - - - - - - - G - - - -	< 10
oSK97/98	- - - G - - - - - - - - - - - G	< 10*
oSK101/102	- - - - - - - - - - - G - - - G	< 10
oSK103/104	- - - - - - - - - - - G - - - -	< 10
oSK105/106	- - - - - - - - - - - - - - - G	27
P_5/mRRS-2	- - - G - - - - - - - G - - - G	< 10
P_5/mRRS-3	- - - - - - - A T - T - G A T -	< 10

FIG. 4. Mutational analysis of the P_5 RRS. Several 31-base oligonucleotides containing the indicated changes in the P_5 RRS were synthesized (only the core 16-mers containing the imperfect GAGC repeats are shown). After being annealed with their complementary oligonucleotides (oSK series), they were cloned into pBluescript and used to isolate a 77-bp fragment. For band shift analyses, the labeled fragments were incubated with 293 nuclear extracts containing either Rep*am* or Rep68, and the samples were electrophoresed in a 4% polyacrylamide gel run at 4°C. The radioactive bands were cut and counted in a scintillation counter. The background with the Rep*am* extract was subtracted, and the amount of Rep68 binding was calculated as a percentage of Rep68 binding to the wild-type P_5 RRS. The sequences with below 10% binding did not show any detectable Rep68-specific band shifts even after long exposures (4 days), except for construct oSK97/98 (*), which after a long exposure had very faint shifted bands.

by a portion of the pBluescript multiple cloning site sequence for Rep68 binding, using 293 nuclear extracts (Fig. 4). The band shift analyses were performed with equal picomole amounts of DNA fragments. A DNA fragment (mRRS-2) that had all three cytosines changed did not show any detectable Rep-specific binding (Fig. 4), indicating the importance of these cytosines and/or their complementary guanines in the P_5 RRS for Rep68 binding. We then changed these cytosines either as a single mutation or as double mutations. The results indicated that a mutation of the cytosine (AAV nucleotide 273) in the third GAGC repeat abolished the Rep-specific shift both as a single mutation (oSK103/104) and as part of a double mutation (oSK95/96 and oSK101/102). Mutation of the other two cytosines alone had a smaller effect (oSK93/94 and oSK105/106).

We also tested Rep68 binding to another mutant P_5 RRS (mRRS-3). This sequence had changes in the second, third, and fourth GAGC boxes which created an *Eco*RV site but had all three conserved cytosines intact (Fig. 4). Interestingly, the mutations in mRRS-3 also completely abolished Rep68-specific binding in vitro. These results indicate that although the RRS is somewhat degenerate, multiple sequence elements are required for Rep68 binding. It is also possible that in vivo, the Rep68-binding properties are affected by other components such as DNA conformation and the presence of other proteins on the P_5 promoter.

Mutations both in the P_5 Rep-binding region within the context of the entire AAV genome and in the consensus NTP-binding motif of Rep68 eliminate the negative regulation by Rep68. The correlation between Rep68 binding to the P_5 RRS and negative regulation of P_5 transcript levels was tested by mutating the P_5 RRS in a plasmid (pNTC3) containing the entire AAV genome (Fig. 5A). This construct produces unrepressed levels of P_5 and P_{19} transcripts as a result of an amber mutation in the *rep* gene (9, 25). The P_5 mRRS-3 mutation was used to abolish Rep68-specific binding, since the creation of a novel *Eco*RV site facilitated the screening of the constructs.

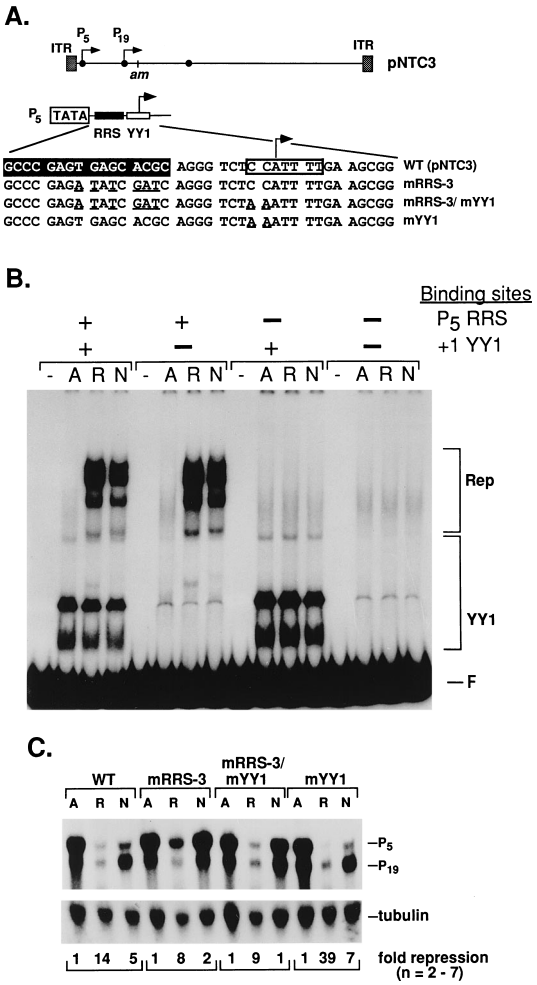

FIG. 5. Effects of P_5 RRS mutations in the context of a full-length AAV genome on the negative regulation of P_5 by wild-type Rep68 and Rep68/K340H. (A) Construction of mutated P_5 promoter sequences into the pNTC3 background. Mutations (underlined) that abolished Rep68 binding (mRRS-3) and/or YY1 binding (mYY1) were created by PCR using 5′ primers containing the sequences shown and were introduced into pNTC3 as described in Materials and Methods. (B) Band shift analyses with DNA containing the P_5 RRS- and YY1-binding sites. Oligonucleotides containing either wild-type or mutated P_5 RRS and +1 YY1-binding sites (AAV nucleotides 259 to 298) with *Bam*HI overhangs were synthesized, annealed, and cloned into pBluescript. A 90-bp fragment was isolated, labeled, and then incubated with no extract (−) or extract containing Rep*am* (A), Rep68 (R), or Rep68/K340H (N) in band shift analyses. The samples were electrophoresed in a 4% polyacrylamide gel run at 4°C. The Rep-specific and YY1-specific shifted bands are indicated on the right. F, free DNA. (C) Northern analysis of RNA from 293 cells transfected with different AAV genomes. The cells were transfected with 6 μg of pNTC3 (wild type [WT]) or pNTC3 derivatives containing a mutated P_5 promoter sequence (mRRS-3, mRRS-3/mYY1, or mYY1) plus 3 μg of plasmids encoding Rep*am* (A), Rep68 (R), or Rep68/K340H (N). The RNA blots were hybridized with a ^{32}P-labeled AAV fragment. The tubulin gene transcripts were analyzed to show the intactness of the RNA. To quantitate the mRNA levels, several independent transfection experiments were performed (n = 7 for pNTC3 and pNTC3/mRRS-3, n = 3 for pNTC3/mRRS-3/mYY1, and n = 2 for pNTC3/mYY1). The values at the bottom indicate levels of repression expressed as fold repression by comparing the unrepressed (in the presence of Rep*am*) and repressed (in the presence of Rep68 or Rep68/K340H) P_5 transcript levels. These values were calculated by cutting and counting the P_5 transcripts and normalizing the values for transfection efficiency by counts obtained from DNA slot blots (pNTC3 and its derivatives; data not shown).

Plasmids containing mutations in the +1 YY1-binding site (47) alone or in combination with the P_5 mRRS-3 mutation were also made to test the possible effect of the YY1-binding site at +1 on Rep68-mediated repression. This possibility was sug-

gested by the partially overlapping regions of Rep68- and YY1-binding sites as determined by DNase I protection assays (35, 47). Furthermore, our findings with the assays of 3′-deleted P_5-*cat* constructs showed that the constructs not efficiently repressed by Rep68 also lacked the +1 YY1-binding site (Fig. 1 and 2).

The effects of mutations in the Rep68- and YY1-binding sites were first analyzed by gel mobility shift assays (Fig. 5B). The cloned oligonucleotide pairs with combinations of wild-type or mutant Rep- and YY1-binding sites (Fig. 5A) were analyzed for Rep68- and YY1-specific retarded complexes, using 293 nuclear extracts containing Rep68, Rep68/K340H, or Rep*am*. A double mutant (mRRS-3/mYY1) abolished all detectable Rep68- and YY1-related DNA-protein complexes as expected. Three protein-DNA complexes which were dependent on the presence of a wild-type YY1-binding site were identified. The amount of each of these complexes was not affected by the presence of wild-type or NTP-mutant Rep68 in the nuclear extract. The YY1 specificity of these three complexes was tested by a competition experiment with various amounts of unlabeled DNA containing either wild-type or mutant YY1-binding sites. The wild-type YY1-binding site competed with the formation of all three DNA-protein complexes, while the mutant binding site did not (data not shown). Labeled DNA fragments containing the wild-type P_5 RRS showed the typical Rep68-specific, multiple shift pattern both in the presence and in the absence of a wild-type YY1-binding site (Fig. 5B). When both the wild-type Rep68- and YY1-binding sites were present on the same DNA fragment, only the Rep68- and YY1-specific bands were detected, and no novel bands suggestive of simultaneous binding to the same DNA molecules were observed. Thus, YY1 and Rep binding on the same DNA may be mutually exclusive. However, since not all of the labeled DNA was shifted, it is possible that by random chance Rep68 and YY1 did not interact with the same DNA molecules. We tried band shift experiments with lesser amounts of template DNA, but this abolished both YY1- and Rep68-specific binding (data not shown).

The significance of the Rep68-binding site in the full-length AAV genome (pNTC3 and derivatives) for Rep68-mediated repression was tested in vivo in 293 cells (Fig. 5C). Rep68 decreased P_5 transcript levels from the wild-type P_5 promoter approximately 14-fold as shown before (25). The NTP mutant also reduced the P_5 mRNA levels but was less efficient than the wild-type Rep68. The mutation in the Rep68-binding site (pNTC3/mRRS-3) reduced the ability of Rep68 to decrease transcript levels by about 40 to 50% compared with its ability to repress the wild-type P_5 promoter. In contrast, the Rep68/K340H mutant was virtually unable to inhibit the P_5 transcript levels from the altered P_5 promoter. Thus, a wild-type NTP-binding motif of Rep68 was necessary for P_5 repression in the absence of stable Rep68 binding to the P_5 RRS. When the mRRS-3 mutation was cloned into a wild-type AAV genome (Rep^+; pNTC244), similar results were obtained; the ability of additional Rep68 to inhibit P_5 mRNA levels was decreased, and there was no inhibition by the Rep68/K340H mutant in *trans* (data not shown). Although not the sole determinant, these results indicate the importance of Rep68 binding to the P_5 RRS in repression. This binding site was particularly important for the ability of the NTP mutant to repress. The band shift analyses indicated that neither Rep68 nor Rep68/K340H in the 293 nuclear extracts bound the mRRS-3 sequences in vitro (Fig. 5B and data not shown). However, we do not know whether this mutation abolishes Rep68 binding in vivo, where DNA structure and other DNA-binding proteins may modify Rep68-binding properties.

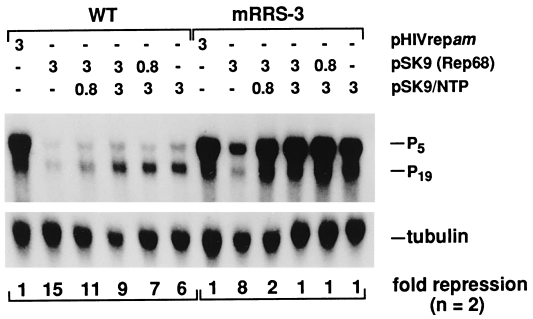

FIG. 6. The NTP mutant of Rep68 interferes with the ability of the wild-type Rep68 to negatively regulate the P_5 mRNA levels. The Northern blot shows the analysis of the RNA from 293 cells transfected with 6 μg of pNTC3 or pNTC3/mRRS-3 plus different amounts of plasmids encoding Rep*am* (3 μg), Rep68 (0.8 or 3 μg), and Rep68/K340H (0.8 or 3 μg) as indicated at the top. Amounts of total transfected DNA were equalized by the addition of vector plasmid pBR322. The Northern blot and the quantitations (two independent transfections) were performed as described for Fig. 5C. The samples were compared with the control, pNTC3 (wild-type [WT] P_5 promoter) plus pHIVrep*am*, which represents no repression (= 1).

The effect of YY1 binding at +1 on Rep68-mediated repression was studied next (Fig. 5C). The mutations in the +1 YY1-binding sites in the constructs (pNTC3/mRRS-3/mYY1 and pNTC3/mYY1) reduced P_5 mRNA levels approximately to 50 to 70% of that from pNTC3 in the presence of Rep*am*. The pNTC3/mYY1 construct, which contained the wild-type P_5 RRS, was very efficiently down-regulated by the wild-type Rep68. There was an approximately two- to threefold increase in the level of P_5 repression by Rep68 in the absence of YY1 binding, suggesting that the YY1 binding interfered with Rep68 repression. The results with the double mutant (pNTC3/mRRS-3/mYY1) containing both the mutated Rep68- and YY1-binding sites show the importance of normalizing the data. The Northern analyses indicated that Rep68, but not the NTP mutant, was able to repress comparably to the wild-type P_5 promoter. However, normalizing the P_5 transcript levels with the template amount showed that Rep68 repression of the double mutant was comparable to its repression of the pNTC3/mRRS-3 construct. This finding implied that the YY1-binding-site mutation had no effect in the presence of mutated Rep68-binding site.

Rep68/K340H interferes with the ability of wild-type Rep68 to negatively regulate P_5 mRNA levels. To further analyze the properties of Rep68/K340H in P_5 negative regulation, we performed a mixing experiment with wild-type Rep68 and the NTP mutant in vivo (Fig. 6). The Rep68- and Rep68/K340H-producing plasmids were cotransfected in different ratios (4:1, 1:1, and 1:4) with template plasmid pNTC3 or pNTC3/mRRS-3. On the wild-type P_5 promoter (pNTC3), mixing Rep68/K340H with Rep68 reduced the ability of wild-type Rep68 to decrease P_5 mRNA levels (from 15- to 9-fold). In the case of the mutant P_5 promoter (pNTC3/mRRS-3), the NTP mutant was clearly dominant over the wild-type Rep68 at all tested ratios, resulting in a complete lack of down-regulation of P_5 transcript levels. Additionally, Rep68/K340H was unable to down-regulate the P_{19} transcript levels on either the wild-type or mRRS-3 template and was dominant over wild-type Rep68 at the P_{19} promoter. These results suggested that the Rep proteins regulated P_5 and P_{19} mRNA levels as multimeric complexes and that the presence of the NTP mutant in the Rep complex rendered the complex functionally defective.

ATP increases Rep68 binding to the P_5 RRS. The absence of negative regulation by Rep68/K340H on the mutated P_5 RRS

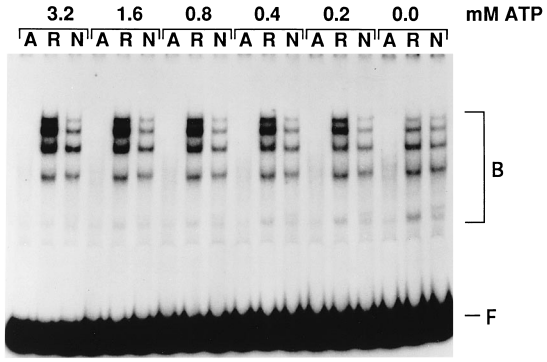

FIG. 7. Effect of ATP on Rep68-dependent P_5 RRS binding. Band shift analysis was performed with the cloned P_5 RRS (77-bp) fragment, using 293 nuclear extract containing Rep*am* (A), Rep68 (R), or Rep68/K340H (N). Different concentrations of ATP were included in the incubations as indicated at the top. The gel mobility shift analysis was performed as described in Materials and Methods. The positions of bound (B) and free (F) DNA are indicated at the right.

(mRRS-3) indicated the importance of the NTP-binding motif of Rep68 in negative regulation. To investigate whether the presence of ATP modified the binding characteristics of Rep68, gel shift analyses with the P_5 RRS were performed with various concentrations of ATP. The results in Fig. 7, as well as results of other experiments in which the radioactive bands were cut out and counted (data not shown), showed that the addition of ATP increased the formation of Rep68-specific DNA-protein complexes about two- to fourfold and peaked between 1.6 and 3.2 mM ATP. The presence of ATP preferentially enhanced the formation of the more slowly migrating complexes. These results suggested that in the presence of ATP, some properties of Rep68 were changed. In contrast, the presence of increasing concentrations of ATP did not reproducibly or significantly enhance (never greater than 30%) retarded complexes formed by the Rep68/K340H-containing nuclear extract, indicating that the effect of ATP was mediated by the putative NTP-binding motif of Rep68. We also performed a mixing experiment with Rep68 and Rep68/K340H in the presence of 3.2 mM ATP. These results showed an additive formation of protein-DNA complexes by the wild-type and mutant Rep68, and we did not see any indication of a dominant-negative effect on Rep68 binding by Rep68/K340H in the presence of ATP (data not shown).

We also performed gel shift analysis in the presence of 3.2 mM ATP, using the HIV-1 LTR RRS-like motif and the mRRS-3 sequence (data not shown). No Rep-specific complexes were observed in the presence of ATP with these sequences, as with earlier experiments in the absence of ATP (Fig. 3D).

DISCUSSION

The Rep78 and Rep68 proteins of AAV have pleiotropic effects on gene expression from both AAV and heterologous promoters. In this study, we have investigated how Rep proteins mediate their negative effect on expression of the AAV P_5 promoter. The results indicate that there are at least two mechanisms that contribute to the reduction of P_5 transcript levels. One mechanism involves the binding of Rep proteins to the AAV P_5 promoter, specifically between the TATA box and the transcription initiation site. The other mechanism may be independent of stable Rep binding to DNA and requires the consensus NTP-binding motif of the Rep proteins.

Our deletion analyses indicated that the P_5 promoter region between the TATA box and the transcription initiation site is important for the negative regulation by Rep68. This region is similar to the RRSs identified previously in the AAV ITR and in the AAV integration site in human chromosome 19, which contain three perfect GAGC repeats and are bound by Rep proteins (12, 13, 22, 23, 42, 56). Although the P_5 RRS contains only one perfect GAGC box, it is bound by Rep68, as shown by the gel shift analyses. McCarty et al. (35) have also demonstrated Rep68 binding to P_5 by gel shifts with purified Rep68 from recombinant baculovirus-infected Sf9 cells. Their DNase I protection assays localized Rep68 binding to an approximately 26-bp region, which coincides with the location indicated in our studies. Similarly located negative regulatory elements have been detected in several herpesvirus promoters, such as the binding sites for the cytomegalovirus IE2 protein and the herpes simplex virus ICP4 protein (11, 30, 39). This location suggests a mechanism of repression in which Rep binding overlaps or interferes with the assembly of the transcription initiation complex. The negative regulation by Rep68 may be affected by the binding of the ubiquitous eukaryotic transcription factor YY1 to the P_5 transcription start site. The lack of a YY1-binding site at +1 in the P_5 promoter resulted in enhanced Rep68-mediated repression in the presence of the Rep68-binding site. The down-regulation of P_5 transcript levels by Rep68 in the absence of a YY1-binding site indicates that YY1 binding at the transcription start site is not necessary for regulation by Rep68, and thus Rep proteins probably affect additional components in the process of transcription complex assembly. It is tempting to speculate that Rep68 bound to the P_5 RRS simultaneously interacts with some of the basal transcription factors, thereby possibly interfering with the recruitment of RNA polymerase II or other factors. Several viral transcription factors have been previously reported to interfere with transcription initiation by interacting with TATA-binding protein and/or TFIIB (7, 48, 58).

The data presented here demonstrate an interesting ability of the wild-type Rep68 to inhibit P_5 transcript levels, even in the presence of a mutated P_5 RRS, which did not allow Rep68 binding in vitro. Whether the remaining Rep68-mediated repression was due to weak Rep68 binding to the mutated P_5 RRS which was undetectable in our gel shift assays, was due to Rep68 binding to somewhere else in the AAV genome, or was entirely independent of Rep68 binding to DNA is unclear. This property was dependent on the wild-type consensus NTP-binding motif, since Rep68/K340H was unable to repress the mutated P_5 promoter, in contrast to wild-type Rep68. Rep-mediated negative regulation of promoters which show weak or no detectable Rep binding to the DNA has been observed previously (25, 35, 40). The AAV P_{19} promoter region is only weakly bound by Rep68 (35), and we have previously shown that in contrast to wild-type Rep68, the NTP mutants of Rep68 or Rep78 are unable to repress P_{19} transcript levels (Fig. 5C and 6) (25). Mixing with the NTP mutant also interferes with the ability of the wild-type Rep68 to inhibit P_{19} mRNA levels (Fig. 6). Similarly, expression of CAT activity from the HIV-1 LTR is negatively regulated by the wild-type Rep proteins in the absence of detectable Rep binding to the Rep-responsive, negative element (40). The wild-type Rep proteins can also inhibit HIV-1 replication, whereas the Rep proteins with the K340H mutation cannot (43). The putative NTP-binding motif of Rep68 may be crucial for protein-protein interactions that normally stabilize Rep68 binding to the P_5 promoter region. An altered protein structure of the NTP mutant or its putative inability to bind NTP could contribute to a lack of protein-protein interaction. ATP has been reported to enhance DNA

binding of several sequence-specific DNA-binding proteins, many of which, such as the yeast origin-binding complex, simian virus large T antigen, the NS1 protein of MVM, polyomavirus large T antigen, bovine papillomavirus E1, and herpes simplex virus UL9, are involved in the recognition of origins of replication (3, 4, 14, 15, 20, 31, 33). Similar to the increased DNA binding of the MVM NS1 protein to the MVM origin of replication in the presence of ATP, our results with Rep68 showed that the addition of ATP could increase the formation of protein-DNA complexes mediated by the wild-type protein but not by an NTP-binding-site mutant (14). It is possible that Rep undergoes a change in conformation upon binding and possible hydrolysis of ATP. The possibility that ATP causes a rearrangement of Rep multimers is supported by the increased appearance of more slowly migrating DNA-protein complexes in the presence of ATP, similar to the observations with the polyomavirus large T antigen (31). Lastly, some of the enzymatic activities of Rep proteins which are dependent on the putative NTP-binding regions such as ATPase or DNA-RNA helicase activities may also be involved in AAV gene regulation (57).

Multiple Rep68 molecules may be involved in negative regulation of the P_5 promoter. This possibility was suggested by the mixing experiments in vivo that demonstrated the negative dominance of Rep68/K340H over the wild-type Rep68 protein on the mutant P_5 promoter. The negative dominance of Rep proteins with the K340H mutation has previously been detected with the DNA helicase, DNA-RNA helicase, and terminal resolution site endonuclease activities of Rep proteins in vitro (41, 42, 57) as well as AAV replication in vivo (10). The action of Rep proteins as multimeric complexes is also suggested by the multiple shifted band patterns observed in the gel shift assays with the P_5 RRS. The multiple shifts are similar to those previously detected for Rep binding to the AAV ITR and to the preferred AAV integration site in chromosome 19 both with purified Rep proteins and with Rep-containing nuclear extracts (12, 13, 36, 42, 56).

Knowledge of the role of individual bases in RRS binding or the number of perfect GAGC repeats required for Rep binding is important in evaluating the Rep effects on AAV and heterologous gene expression. A sequence comparison shows that the third GAGC box is the most conserved of the four boxes among the RRSs (Fig. 3A). While this third GAGC box was also reported to be important in Rep binding to the AAV ITR, its alteration did not obliterate Rep68 binding if the first two GAGC boxes were left intact (36). The P_5 RRS contains only one perfect GAGC box, which is surrounded by imperfect boxes. We analyzed the sequence requirements for Rep68-specific binding to the P_5 RRS by changing nucleotides that have been previously shown to be important for Rep binding to the AAV integration site in chromosome 19 and the AAV ITR by methylation interference assays (42, 56). Our studies indicated that the alteration of a complementary cytosine in the third box of the P_5 RRS had the strongest effect on Rep68 binding. The importance of the flanking sequence is apparent in the comparison between the P_5 RRS and the HIV-1 LTR RRS-like region (40). Both of these elements contain only a single perfect GAGC box and are surrounded by approximately equal numbers (8 of 12 and 7 of 12) of residues in common with the AAV ITR (Fig. 3A), but the HIV-1 LTR sequence showed no detectable Rep binding (Fig. 3D) (40). Thus, a single GAGC box alone is not sufficient for stable Rep binding. While we did not test the region downstream from the P_5 TATA box in pRO1535, pRO1538, or pRO45 for Rep68 binding, a sequence analysis of these constructs also revealed a single, serendipitous GAGC box located in the vicinity of the transcription initiation site (Fig. 2A). Similarly, the single GAGC boxes in these constructs were not enough for efficient repression by Rep68 (Fig. 1). Further studies will be necessary to evaluate what flanking sequences are required for Rep68 binding.

In summary, our studies indicate mechanisms of negative regulation by Rep proteins that require either DNA binding in the vicinity of the transcription initiation site or the wild-type consensus NTP-binding motif of Rep68. The mechanism by which Rep68 inhibits P_5 transcription in the absence of the specific DNA-binding site may be similar to the mechanism by which it affects some heterologous promoters and may involve Rep interactions with basal transcription factors. Furthermore, in the advent of the development of AAV vectors for human gene therapy, it is necessary to evaluate the effects of Rep proteins on cellular gene expression.

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