JOURNAL OF VIROLOGY, May 1994, p. 2947–2957
0022-538X/94/$04.00+0

Vol. 68, No. 5

Analysis of Adeno-Associated Virus (AAV) Wild-Type and Mutant Rep Proteins for Their Abilities To Negatively Regulate AAV p_5 and p_{19} mRNA Levels

SIRKKA R. M. KYÖSTIÖ,* ROLAND A. OWENS, MATTHEW D. WEITZMAN,† BETH ANN ANTONI,‡ NOR CHEJANOVSKY,§ AND BARRIE J. CARTER‖

Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892

Received 29 November 1993/Accepted 27 January 1994

The *rep* gene of adeno-associated virus type 2 (AAV) encodes four overlapping Rep proteins that are involved in gene regulation and replication of the virus. We studied here the regulation of mRNA transcribed from the AAV p_5 and p_{19} promoters, using transient expression in human 293 cells followed by Northern (RNA) blot analysis of the mRNA. The p_5 transcript encodes the larger Rep proteins, Rep78 and Rep68, while the p_{19} transcript encodes the smaller proteins, Rep52 and Rep40. A plasmid (pNTC3) containing the entire AAV genome with an amber mutation in the *rep* gene accumulated higher levels of p_5 and p_{19} mRNA than a plasmid containing the wild-type AAV genome. Addition of increasing amounts of the wild-type *rep* gene in *trans* from a heterologous promoter inhibited p_5 and p_{19} mRNA accumulation from pNTC3, indicating that the levels of both transcripts were decreased by the Rep proteins. Cotransfections with plasmids producing individual wild-type Rep proteins in *trans* showed that p_5 and p_{19} mRNA accumulation was inhibited 5- to 10-fold by Rep78 and Rep68 and 2- to 3-fold by Rep52 and Rep40. Analysis of carboxyl-terminal truncation mutants of Rep78 showed that the ability of Rep78 to decrease p_5 and p_{19} mRNA levels was lost when 159 or more amino acids were deleted. Rep78 and Rep68 mutants deleted for the methionine at residue 225 showed decreased abilities to down-regulate both p_5 and p_{19} transcript levels, while mutants containing a substitution of glycine for the methionine resembled the wild-type Rep78. A Rep78 protein with a mutation in the putative nucleoside triphosphate binding site inhibited expression from p_5 but not from p_{19}, suggesting that the regulation of p_5 transcript levels by Rep78 and Rep68 differs from that of p_{19}. A deletion analysis of AAV *cis* sequences revealed that an intact terminal repeat was not required for negative regulation of p_5 and p_{19} transcript levels and that the regulation of p_{19} mRNA levels by Rep78 did not require the presence of the p_5 promoter.

Adeno-associated virus type 2 (AAV) is a nonpathogenic human parvovirus. It has a linear, single-stranded DNA genome (4,680 bases) with 145-base inverted terminal repeats (ITRs) at both ends (Fig. 1) (5, 42). The genome has two open reading frames (ORFs) encoding the nonstructural and structural viral proteins (42). The structural capsid proteins are encoded by the right half of the genome and are expressed from the p_{40} promoter (47). The left half of the genome contains the ORF for the *rep* gene, which encodes four nonstructural Rep proteins (29, 44, 48). Rep78 and Rep68 are expressed from the p_5 promoter and are the translation products of unspliced and spliced mRNAs, respectively. Rep52 and Rep40 are synthesized from unspliced and spliced mRNAs, respectively, transcribed from the p_{19} promoter (5). Also, alternate splicing of p_5 and p_{19} mRNAs may generate Rep68′ and Rep40′ which differ from Rep68 and Rep40 by the insertion of 9 amino acids near the carboxyl terminus (35, 47).

* Corresponding author. Mailing address: Laboratory of Molecular and Cellular Biology, Bldg. 8, Rm. 309, NIDDK, National Institutes of Health, Bethesda, MD 20892. Phone: (301) 496-3359. Fax: (301) 402-0053. Electronic mail address: moorer@helix.nih.gov.

† Present address: Institute for Human Gene Therapy, Wistar Institute, Philadelphia, PA 19104.

‡ Present address: New Jersey Center for Advanced Biotechnology and Medicine, Piscataway, NJ 08854.

§ Present address: Entomology Department, Institute for Plant Protection, Bet Dagan, 50-250, Israel.

‖ Present address: Targeted Genetics Corporation, Seattle, WA 98101.

The roles of Rep proteins in the AAV life cycle have been studied primarily during AAV replication, which usually requires the presence of a helper virus, such as adenovirus or herpesvirus (4). The larger Rep proteins, Rep78 and Rep68, specifically bind to the AAV origins of replication, the ITRs, and possess helicase and endonuclease activities needed in the early steps of AAV replication (2, 17–19, 34, 41). The smaller Rep proteins, Rep52 and Rep40, are essential for the accumulation of single-stranded progeny genomes used for packaging (8).

Besides their role in replication, the Rep proteins are also involved in the regulation of AAV genes. In the presence of helper virus, the Rep proteins positively regulate AAV gene expression (24, 30, 45). The Rep proteins activate the AAV p_{40} promoter in adenovirus-infected human HeLa, KB, or 293 cells (24, 30, 45) as well as increase the p_{19} mRNA levels in helper virus-infected HeLa cells (30). A Rep-dependent increase of p_5 transcript levels in helper virus-infected KB cells from a mutant AAV genome lacking a region within the *rep* gene has also been reported (24). However, both the p_5 and p_{19} transcript levels from the wild-type AAV genome detected in helper virus-infected cells are always low compared with that of p_{40} transcripts (24). The individual roles of different Rep proteins in the positive regulation have not been clarified.

Little is known about the roles of Rep proteins in the absence of helper virus. Under these nonpermissive conditions, AAV efficiently integrates into host chromosomal DNA and persists as a latent infection, with little morphological change in the host cell (5, 22, 27). Subsequent infection with

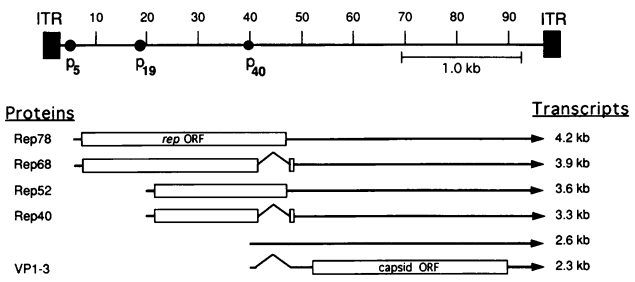

FIG. 1. Organization of the AAV genome, transcripts, and proteins. The 4,680-nucleotide-long AAV genome is shown at the top as a thin line with the ITRs indicated by solid boxes at the ends. The three AAV promoters are indicated as solid circles. The structures (lines with arrows) and sizes of the AAV mRNAs and ORFs (open boxes) for Rep and capsid proteins are indicated. The introns are shown as carets. For simplicity, the alternative splicing for the spliced mRNAs is omitted (47).

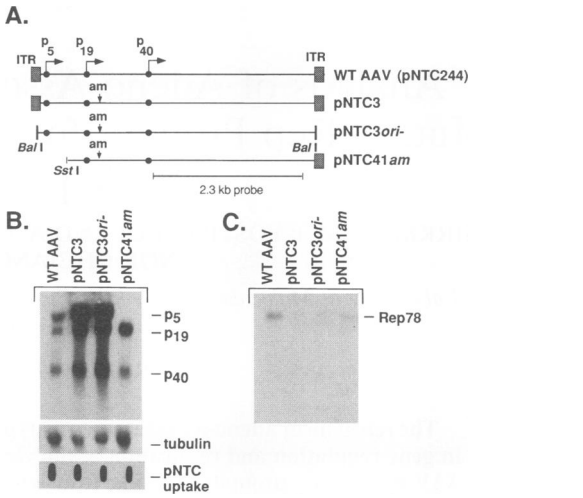

FIG. 2. AAV genomes containing an amber codon in the *rep* gene accumulate p_5 and p_{19} transcripts. (A) Structures of recombinant AAV plasmids. The locations of mutations (vertical arrows) and restriction enzyme sites (vertical bars) for *Sst*I and *Bal*I in the plasmids are shown. The vector DNA is not shown here for clarity. Other symbols: solid circles, AAV transcription promoters; am, amber termination codon; bent arrows, transcription start sites. (B) Transcription products from wild-type and mutant AAV genomes in the absence of adenovirus. The 293 cells were transfected with the plasmids designated at the top and harvested after 48 h. The RNA was analyzed by Northern analysis and probed with a 2.3-kb, ^{32}P-labeled *Pst*I fragment of the AAV genome (shown in A) or with ^{32}P-labeled tubulin DNA. Transcripts from the AAV p_5, p_{19}, and p_{40} promoters are indicated. Plasmid (pNTC) uptake was measured by slot blots, with a 1.6-kb, ^{32}P-labeled *Hin*cII fragment of pNTC3 as a probe. (C) Production of Rep proteins from the transfected plasmids. Nuclear proteins were isolated, and the Rep proteins were detected by immunoblotting with an anti-Rep antibody (anti-Rep78.93). The position of the Rep78 protein is indicated on the right.

helper virus, however, can result in rescue and replication of the AAV genome in the nucleus to produce infectious progeny virus. During latency, very little expression of AAV genes has been detected (27, 49) despite the fact that some characterized AAV integration sites are located in transcriptionally active CpG-rich regions (21, 43). This low level of AAV gene expression might be explained by the ability of AAV to regulate negatively its own gene expression in the absence of helper virus. Experiments using transient transfection assays showed that the level of chloramphenicol acetyltransferase (CAT) activity from the p_{40} promoter was repressed by Rep protein(s) both in *trans* and in *cis* in uninfected human 293 cells (45, 46). Similarly, cotransfection of HeLa cells with a plasmid containing a *rep* gene inhibited the CAT activity from p_5 and p_{19} promoter-*cat* fusions (3).

In this study, we have characterized further the negative regulation of AAV gene expression by the Rep proteins in nonpermissive conditions. Our objectives were (i) to identify the individual Rep proteins responsible for the negative regulation, (ii) to localize putative domains within Rep proteins required for this regulation, and (iii) to analyze possible differences between p_5 and p_{19} regulation. Because of the putative translational effects of Rep proteins on reporter gene expression (46), we chose to analyze the negative regulation at the RNA level in uninfected human 293 cells. In contrast to HeLa or KB cells, the AAV transcripts are detectable in 293 cells in the absence of helper virus because the adenovirus E1a and E1b proteins constitutively expressed in these cells result in higher levels of AAV p_5 and p_{19} transcripts (6, 15, 46). These adenovirus proteins, however, are not sufficient for replication and productive infection of AAV (26). Our results indicate that uninfected 293 cells transfected with a plasmid, pNTC3, containing an amber mutation in the *rep* gene, accumulate p_5 and p_{19} transcripts as a result of the lack of functional Rep protein(s). The ability of individual Rep proteins to inhibit this accumulation of p_5 and p_{19} transcripts from pNTC3 was tested by cotransfection of plasmids that expressed individual Rep proteins from a heterologous promoter. The results showed that of the wild-type Rep proteins only Rep78 and Rep68 efficiently inhibited p_5 and p_{19} mRNA accumulation. Furthermore, one Rep78 mutant (Rep78/K340H) had different effects on the p_5 and p_{19} transcripts levels, suggesting that the mechanisms of the inhibition of the transcript levels from these two promoters are distinct.

MATERIALS AND METHODS

Cells and viruses. Adenovirus-transformed human embryonic kidney 293-31 cells (293 cells) (15) were maintained as monolayer cultures and grown in Eagle's minimal essential medium (Quality Biological, Inc., Gaithersburg, Md.) supplemented with 10% fetal calf serum (GIBCO/BRL, Gaithersburg, Md.), 2 mM L-glutamine, and penicillin, neomycin, and streptomycin (GIBCO/BRL) (100 U/ml each).

Plasmids. The AAV plasmids used in this study were constructed by using standard techniques (28). The construction of the pNTC series of plasmids (Fig. 2A) has been described previously (7, 8). pNTC244 carries the entire wild-type AAV genome inserted into phagemid pTZ19U (Bio-Rad, Richmond, Calif.). pNTC3 has an amber mutation immediately downstream from the AAV p_{19} promoter at AAV nucleotide 1033 (7). pNTC41*am* was derived from pNTC3 by deleting an *Sst*I fragment which removed the p_5 promoter (8). pNTC3*ori*− (pSK20) was constructed by cloning a *Bal*I fragment (AAV nucleotides 121 to 4553) of pNTC3 into *Sma*I-digested pTZ19U, which results in an AAV genome lacking the terminal 120 bp of the left ITR and 128 bp from the right ITR.

A series of plasmids containing the AAV *rep* gene expressed from the long terminal repeat (LTR) promoter of the human immunodeficiency virus type I (HIV-1) (the HIV-LTR promoter) (1, 14, 35) were used (see Fig. 3A and 4). In all of the plasmids, the left-hand AAV ITR and the p_5 promoter (AAV

nucleotides 1 to 263) were replaced by the HIV-LTR promoter (1, 35). pHIVrep contains the wild-type *rep* gene. pHIVrep*am* and pHIVrepNTP are identical to pHIVrep, except that pHIVrep*am* contains the same amber mutation as pNTC3 and produces a truncated Rep protein (residues 1 to 237 of Rep78) (1, 35), while pHIVrepNTP (34) contains mutations at AAV nucleotides 1338 and 1340 which change lysine 340 of Rep78 to histidine in the putative nucleoside triphosphate (NTP) binding site (designated the NTP mutant) (9). pHIVRepK*am*, pHIVRepH*am*, pHIVRepBs*am*, and pHIVRepBa*am* have a linker containing amber termination codons in all reading frames inserted into the *Kpn*I (replacing codon 531 of Rep78), *Hin*dIII (codon 523), *Bst*EII (codon 463), and *Bam*HI (codon 244) sites of pHIVrep, respectively (35). pSK1 produces wild-type Rep78 and was derived from pHIVrep by deleting the *Sna*BI-*Sph*I fragment which contained the right-hand ITR. pSK9, pSK10, and pSK14 contain the ORFs for Rep68 (major splice site), Rep68′ (minor splice site), and Rep40′ (minor splice site), respectively, expressed from the HIV-LTR and are all derivatives of pSK1. The construction of these plasmids is described elsewhere (35). pSK12 and pSK13 produce Rep52 and Rep40, respectively, expressed from the HIV-LTR. These two plasmids contain DNA fragments synthesized by PCR. The 5′ primer contained an additional *Hin*dIII site created by a point mutation 15 bases upstream of the p_{19} initiation ATG codon. The 3′ primer includes the natural AAV *Hin*dIII site located downstream from p_{19}. This PCR product containing AAV sequences 973 to 1885 was digested with *Hin*dIII and cloned into *Hin*dIII-cleaved pBennCAT (14), creating pSK11. The *cat* gene of pSK11 was removed by digestion with *Sal*I and replaced with the corresponding *Sal*I fragment from pSK1 or pSK9 to create pSK12 or pSK13, respectively. pSK3 and pSK4 were derived from pSK1 and express mutant Rep78 proteins. pSK3 contains a deletion of the p_{19} initiator methionine codon (AAV nucleotides 993 to 995), while pSK4 has a mutation which replaces the same codon with a glycine codon (GGG) (35). These plasmids were also used, respectively, to construct pSK5 and pSK7. pSK3 and pSK4 were digested with *Bam*HI and *Xho*I to delete a 1.2-kb fragment which was replaced with a corresponding fragment from pSK9 to create pSK5 or pSK7, respectively. pSK5 encodes a Rep68 protein containing a deletion of the p_{19} initiator methionine codon, while in pSK7 this codon is replaced with a glycine codon. The presence of the mutations in all constructs was confirmed by DNA sequencing (39) with a Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). All plasmids were grown in *Escherichia coli* HB101 or DH5 and were purified on cesium chloride gradients (28).

DNA transfections. Transfections for RNA or protein analyses were performed in parallel by the calcium phosphate coprecipitation method (16). For cotransfections, 6 μg of pNTC3 and 6 μg of the complementing *rep* plasmids were used, unless otherwise indicated. All transfections had 12 or 20 μg of DNA adjusted with vector DNA (pTZ19U or pBR322). 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 for protein or RNA.

Protein extraction and Western (protein) analysis. Nuclear proteins were isolated as previously described (35). For analysis of total cell proteins, the cells were scraped and washed with ice-cold phosphate buffered saline (PBS), pH 7.4, (Quality Biological, Inc.) and then lysed in 0.5 ml of cold lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM disodium EDTA, 150 mM NaCl, 1% sodium deoxycholate, 0.25 trypsin inhibitory units of aprotinin [Sigma Chemical Co., St. Louis, Mo.] per ml, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium dodecyl sulphate [SDS]). The cellular DNA was sheared by sonication with a model W-375 Sonicator (HeatSystems-Ultrasonics, Inc., Plainview, N.Y.) at setting 5 for 15 s. Total protein was measured by using the bicinchoninic acid (BCA) assay reagent (Pierce, Rockford, Ill.). Samples containing equal amounts of protein were boiled for 5 min in sample buffer (50% glycerol, 100 mM sodium phosphate [pH 7.0], 5% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue) and electrophoresed in a 12.5% polyacrylamide gel containing 0.1% SDS (25). A Multiphor II Nova Blot apparatus (Pharmacia-LKB, Piscataway, N.J.) was used for the electrophoretic transfer of proteins from the gel to a polyvinylidene difluoride membrane (GIBCO/BRL). Immunoblotting (Western blotting) was performed by using the PhotoBlot chemiluminescent system according to the manufacturer's instructions (GIBCO/BRL). The membrane was incubated with rabbit anti-Rep antibodies (1:800 dilution) raised against either S18K oligopeptide (Rep78 amino acids 516 to 533 [29]) or Rep78.93 (*E. coli*-synthesized truncated Rep78 [48]). The membrane was then incubated first with biotin-conjugated goat anti-rabbit immunoglobulin G antibody (1:14,000 dilution) and then with streptavidin-conjugated alkaline phosphatase (1:3,000 dilution) and finally was treated with the chemiluminescent substrate. The treated membranes were then exposed to Kodak (Rochester, N.Y.) X-Omat XAR-5 X-ray film.

Isolation of RNA and Northern (RNA) analysis. All solutions used for RNA isolation and electrophoresis were treated with diethylpyrocarbonate prior to use (28). Transfected cells were washed twice on ice with PBS and then scraped into PBS. Cytoplasmic RNA from the cells was isolated by using an RNA isolation kit (5 Prime→3 Prime, West Chester, Pa.) according to manufacturer's instructions. Equal amounts of RNA, based on the measurement of optical density at 260 nm, were electrophoresed in a 1.3% agarose-formaldehyde gel in 1× MOPS (morpholinepropanesulfonic acid) buffer (28) and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) by capillary action in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (28) overnight.

For detection of transcripts, various AAV fragments of pNTC3 and tubulin DNA (Oncor, Inc., Gaithersburg, Md.) were labeled by the random priming method of Feinberg and Vogelstein (11) using [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; NEN-DuPont, Boston, Mass.) and a random-primer labeling kit from Boehringer Mannheim (Indianapolis, Ind.). The pNTC3 fragments used as probes are indicated in Results and in the figure legends.

Nitrocellulose filters were prehybridized for at least 1 h at 65°C and then hybridized with the appropriate probe in hybridization solution (5× Denhardt's solution [28], 0.5% SDS, 6× SSC, 0.05% sodium pyrophosphate, and 100 μg of denatured calf thymus DNA per ml) at 65°C for 12 to 24 h. The filters were washed first in 2.0× SSC–0.1% SDS at room temperature for 15 min and then with 0.1× SSC–0.1% SDS at 65°C for 1 h. Filters were autoradiographed either at room temperature or at −70°C on Kodak X-Omat XAR-5 film. The transcript levels were quantitated by cutting and counting the radioactive signals in a liquid scintillation counter.

Slot blot analysis for plasmid uptake. Nuclei from the cytoplasmic RNA isolation procedures were dissolved in 250 μl of 0.2 M NaOH and boiled for 15 min in order to hydrolyze RNA and denature plasmid DNA. The samples were chilled and neutralized with an equal volume of 2.5 M ammonium acetate. Serial dilutions of samples were performed in 6× SSC to verify linearity and loaded on a slot blot apparatus (Hybri-

Slot Manifold; Bethesda Research Laboratories, Inc.). For analysis of template plasmid uptake (pNTC3, pNTC3*ori*−, or pNTC41*am*), the membranes were hybridized with a 1.6-kb, ^{32}P-labeled *Hin*cII fragment of pNTC3 (not present in the HIV-LTR constructs) under conditions described for Northern analysis. Various concentrations of pNTC3 were used as positive controls, while similar concentrations of pHIVrep*am* were used as negative controls. Radioactive signals were counted in a scintillation counter, and values were used to normalize transcript levels obtained from RNA analyses.

RESULTS

AAV genomes containing amber mutations in the *rep* gene have higher levels of p_5 and p_{19} transcripts than the wild-type genome. The pNTC series of plasmids used in this study is shown in Fig. 2A. Plasmid pNTC244 contains the cloned wild-type AAV genome. Two of the mutant constructs, pNTC3 and pNTC3*ori*−, contain all of the AAV promoters, but as a result of an amber stop codon, produce a truncated Rep protein (amino acids 1 to 237) from the p_5 promoter (7). pNTC41*am* contains the same amber mutation but also lacks both the ITR and p_5 promoter region (AAV nucleotides 1 to 814) (8). The constructs were transfected into uninfected human 293 cells, and the cells were harvested for analyses of cytoplasmic RNA or nuclear Rep proteins. The AAV transcripts were identified by hybridization with a 2.3-kb *Pst*I fragment (AAV nucleotides 1958 to 4258) which detects transcripts from the p_5, p_{19}, and p_{40} promoters (Fig. 2A). Transcript levels from three independent transfections were quantitated and normalized by plasmid uptake into the nucleus measured by DNA slot blots. Hybridizations for tubulin transcripts were performed to analyze the quality of the RNA. Tubulin was not used to normalize AAV transcript levels, since it is not clear whether Rep proteins affect tubulin transcription.

The AAV p_5, p_{19}, and p_{40} promoters of the wild-type plasmid (pNTC244) accumulated low levels of the corresponding unspliced 4.2-, 3.6-, and 2.6-kb mRNAs (Fig. 2B). The spliced transcripts (3.9, 3.3, and 2.3 kb) were not detected because of inefficient splicing of AAV transcripts in the absence of adenovirus (1, 46). The mutated genomes, pNTC3 and pNTC3*ori*−, expressed levels of p_5 transcripts elevated approximately five- to sixfold above those of the wild type. Since no difference was observed between pNTC3 and its derivative, pNTC3*ori*−, the intact ITR was not required for the elevated levels. The p_{19} transcript levels from these constructs and from pNTC41*am* lacking the p_5 promoter region were increased two- to threefold compared with the wild-type levels. Since all the plasmids were equally transfected into 293 cells, as analyzed for plasmid uptake by DNA slot blots of nuclear extracts, the differences in transcript levels were not due to different amounts of templates. Furthermore, because the transfected AAV genomes do not replicate in the absence of helper virus, the template levels did not change during the course of the experiment.

The wild-type genome (pNTC244) has been shown to produce all four Rep proteins in the presence of adenovirus (8, 9). To determine whether the Rep proteins are detectable in the absence of the helper virus, we performed protein analysis of nuclear extracts in parallel with the RNA blotting (Fig. 2C). The levels of Rep proteins synthesized in the absence of helper virus were low, and only Rep78 was clearly detectable from the wild-type construct. The products from the spliced p_5 (Rep68) and p_{19} (Rep40) transcripts were not visible, reflecting the inefficient splicing of AAV transcripts in the absence of helper virus (1, 46). The smaller Rep proteins, Rep52 and Rep40, and the amber truncation products, were also difficult to detect as a result of their low levels in the nuclear extract compared with those in the cytoplasmic fraction (1, 29). They may also be underestimated because they probably lack some epitopes recognized by the antibody used (anti-Rep78.93 [32, 48]).

Since none of the mutated genomes produced the wild-type Rep proteins, these results suggested that the increased p_5 and p_{19} transcript levels from these genomes may be due to the lack of wild-type Rep protein(s). Thus, the wild-type Rep proteins may negatively regulate the transcription from the p_5 and p_{19} promoters or alter the stability of the mRNA.

Rep proteins inhibit p_5 and p_{19} mRNA accumulation in *trans*. To test whether the accumulation of p_5 and p_{19} transcripts from pNTC3 was due to the absence of wild-type Rep proteins, the wild-type *rep* gene was supplied in *trans* from pHIVrep (Fig. 3A). This construct produces high levels of Rep proteins from a heterologous promoter, HIV-LTR (1, 34, 35). Plasmid pHIVrep*am* was used as a negative control since it produces the same truncated Rep protein from the HIV-LTR as pNTC3. Both pHIVrep and pHIVrep*am* produce shorter transcripts from the HIV-LTR and p_{19} than pNTC3 because of the deletion of a *Hin*cII fragment (AAV nucleotides 2397 to 3987) in the capsid region. Increasing amounts of pHIVrep or pHIVrep*am* were cotransfected with pNTC3 into 293 cells, and the transcript levels were analyzed by RNA blotting with an *Sst*I-*Hin*dIII fragment of pNTC3 (AAV nucleotides 813 to 1883; Fig. 3B) which detects p_5 and p_{19} transcripts from pNTC3 and the HIV-LTR and p_{19} transcripts from the pHIV plasmids equally well. The amounts of p_5 and p_{19} transcripts from pNTC3 were quantitated, and the values were normalized to the amount of template (pNTC3) uptake into the nucleus, as measured by DNA slot blot analysis.

The wild-type Rep protein supplied in *trans* inhibited the accumulation of p_5 and p_{19} mRNAs from the template (pNTC3). An approximate fivefold decrease in the level of p_5 expression was observed when 1.5 μg of pHIVrep was cotransfected with 6 μg of pNTC3; a similar amount of pHIVrep*am* had a minimal effect (Fig. 3B and C). The accumulation of p_{19} transcripts from pNTC3 was inhibited about two- to threefold. Similarly, the p_{19} levels from the complementing pHIVrep were inhibited compared with that from pHIVrep*am*. The decreased p_5 and p_{19} mRNA levels caused by cotransfection with pHIVrep were similar to those detected from a wild-type AAV genome (Fig. 2B). Thus, the fold inhibition for the p_5 and p_{19} expression from pNTC3 by the Rep proteins produced from pHIVrep correlated well with the fold increase of p_5 and p_{19} levels from pNTC3 compared with that from the wild-type genome, pNTC244 (Fig. 2B). The slight decrease in p_5 and p_{19} mRNA levels from pNTC3 with increasing amounts of cotransfected pHIVrep*am* may reflect competition for transcription factors binding to both AAV promoters and the HIV-LTR. Similar moderate dose-dependent inhibition by pHIVrep*am* or by pHIVLTR, which contains no AAV sequences, has been observed before on HIV CAT expression (1). Alternatively, the amber truncation mutant may have a weak ability to inhibit p_5 and p_{19} mRNA accumulation, as suggested by the dose-dependent inhibition curve (Fig. 3C).

Levels of *rep* transcripts from pHIVrep lower than that of pHIVrep*am* could be due to the weak ability of Rep proteins to repress expression from the HIV-LTR (1). There is a report suggesting that Rep proteins negatively regulate CAT activity from the HIV-LTR (38), although our results do not indicate a strong or consistent negative effect at the RNA level. In summary, supplying the functional *rep* gene in *trans* restored the down-regulation of p_5 and p_{19} transcript levels from

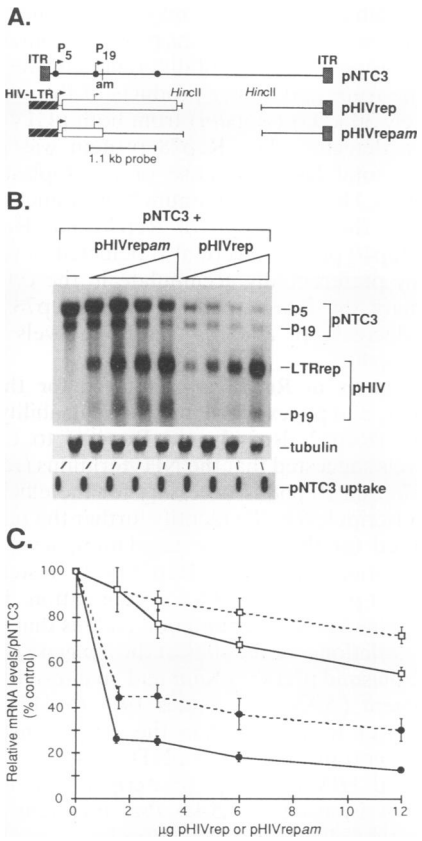

FIG. 3. Inhibition of p_5 and p_{19} transcript accumulation by Rep protein(s). (A) Constructs used for transfection. *Hin*cII sites used to delete AAV capsid genes are indicated. ORFs encoding Rep and Rep*am* (truncated Rep) are shown as open boxes. Other symbols: solid circles, AAV promoters; diagonally striped box, HIV-LTR promoter; am, amber termination codon; bent arrows, transcription start sites. (B) Northern analysis. Plasmid pNTC3 (6 μg) was cotransfected into 293 cells with increasing amounts of either pHIVrep or pHIVrep*am* (1.5, 3.0, 6.0, and 12.0 μg) and equalized with pBR322 DNA. Cytoplasmic RNA was isolated after 48 h and analyzed by Northern blot hybridized with a 1.1-kb ^{32}P-labeled *Sst*I-*Hin*dIII fragment of AAV DNA (shown in A) or with a ^{32}P-labeled tubulin DNA probe. The positions of the transcripts produced from pNTC3 and pHIVrep or pHIVrep*am* are shown on the right. –, control lane. Template pNTC3 uptake into the nucleus was measured by slot blot analysis, with a 1.6-kb, ^{32}P-labeled *Hin*cII fragment of pNTC3 as a probe. (C) Quantitation of transcript levels. The radioactive p_5 and p_{19} transcripts from pNTC3 and plasmid uptake signals were cut and counted in a scintillation counter. Two independent experiments were averaged, with the range shown by the error bars. Values at each point are expressed as the transcript/plasmid uptake ratio as a percentage of transcript levels from the control (pNTC3 plus pBR322). These values were plotted against micrograms of cotransfected plasmid (pHIVrep or pHIVrep*am*). □, pHIVrep*am*; ●, pHIVrep; ——, p5 mRNA; – – –, p19 mRNA.

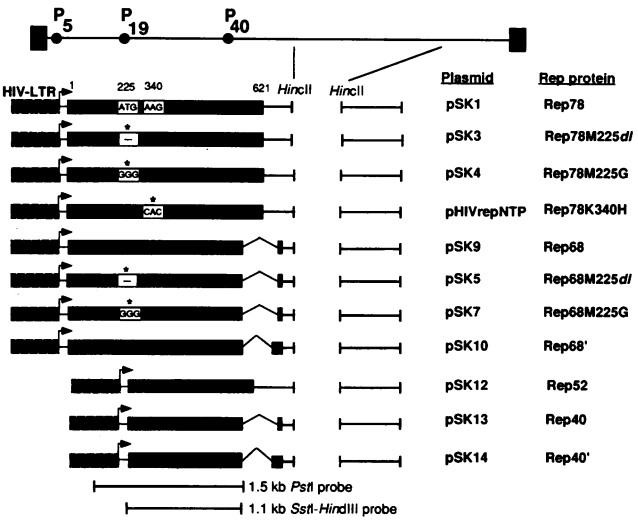

FIG. 4. Series of pHIV plasmids constructed to express individual Rep proteins from the HIV-LTR. The plasmid names and Rep proteins produced from each construct are shown on the right. The thin line at the top represents AAV DNA containing the native AAV promoters (solid circles). The HIV-LTR (diagonally striped box) was used to replace the p_5 or p_{19} promoter. Other symbols: ATG, codon for the methionine located at amino acid 225 (in Rep78 and Rep68) or 1 (in Rep52 and Rep40); – – –, deletion of ATG codon; GGG, substitution of a glycine codon for the methionine codon; AAG, a lysine codon replaced with a histidine codon (CAC) (at amino acid 340 in Rep78) in the putative NTP binding site; large caret, location of the intron deleted by using the major splice site; small caret, location of the intron deleted by using the minor splice site (intron and second exon not drawn to scale); asterisks, mutated sequences or deletions. The numbers above the constructs refer to the amino acid positions in Rep78. The AAV sequences used as probes for the subsequent RNA blots are indicated on the bottom.

pNTC3 to the same levels observed from the wild-type AAV genome (pNTC244).

Wild-type Rep78 and Rep68 proteins have the greatest ability to negatively regulate p_5 and p_{19} transcript levels. As described above, plasmid pHIVrep in *trans* inhibited p_5 and p_{19} mRNA accumulation from the template plasmid, pNTC3. We have previously shown that pHIVrep produces mainly Rep78 protein (1, 34, 35). To determine whether the other Rep proteins can affect p_5 and p_{19} transcript levels from the template plasmid, pNTC3, we used plasmid constructs that each produced only one Rep protein expressed from the HIV-LTR (Fig. 4). Template pNTC3 was cotransfected with plasmids expressing Rep78 (pSK1), Rep68 (pSK9), Rep68′ (pSK10), Rep52 (pSK12), Rep40 (pSK13), or Rep40′ (pSK14) as a source of complementing Rep protein, and the levels of p_5 and p_{19} mRNA from pNTC3 were analyzed by RNA blotting. Transcript levels were also quantitated and normalized by pNTC3 uptake.

On the basis of two independent transfections, Rep78 decreased the accumulation of p_5 transcripts 5-fold, while Rep68 and Rep68′ inhibited p_5 mRNA levels 10-fold (Fig. 5A and C). The smaller Rep proteins, Rep52, Rep40, and Rep40′, each decreased p_5 expression two- to threefold. The p_{19} transcript levels were also efficiently decreased by Rep68 (fivefold), while Rep78, Rep52, Rep40, and Rep40′ were equally capable of lowering p_{19} expression (two- to threefold).

All the *rep* transcripts from the HIV-LTR were equivalent and indicated little visible differential effect by the various Rep proteins. The p_{19} transcript levels from the constructs producing Rep*am* and Rep78 mirrored the p_{19} transcript levels from pNTC3, while the p_{19} levels from the Rep68 constructs were slightly higher. It is possible that efficient inhibition of p_{19} mRNA accumulation by Rep68 requires an upstream p_5 promoter or is affected by the presence of HIV-LTR.

The production of each Rep protein from the HIV-LTR was further tested by immunoblotting of proteins from the trans-

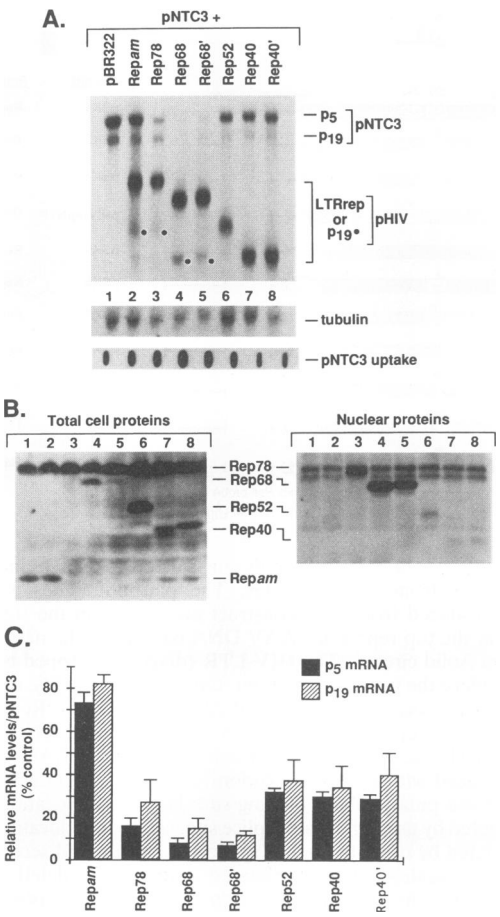

FIG. 5. Regulation of p_5 and p_{19} expression by wild-type Rep proteins. Template plasmid pNTC3 and test plasmids (pHIV series or pBR322) were cotransfected into 293 cells, and the cells were harvested after 48 h. The test plasmids cotransfected and the Rep proteins produced were as follows: pBR322, pHIVrep*am* (Rep*am*), pSK1 (Rep78), pSK9 (Rep68), pSK10 (Rep68′), pSK12 (Rep52), pSK13 (Rep40), and pSK14 (pSK40′). (A) Northern analysis of the *rep* transcripts produced from the transfected plasmids. The transcripts were analyzed by hybridizing with a 1.1-kb ^{32}P-labeled *Sst*I-*Hin*dIII fragment of pNTC3 (shown in Fig. 4) or tubulin DNA. The mRNA detected from each plasmid is indicated on the right. LTRrep is the transcript expressed from the HIV-LTR. The p_{19} transcripts from pHIV constructs are indicated by dots. Template (pNTC3) uptake was measured as described in the legend to Fig. 3. (B) Production of the Rep proteins expressed from the HIV-LTR and pNTC3 analyzed for total cell (left) and nuclear (right) proteins. Protein extracts from transfected cells were analyzed by immunoblotting with an anti-Rep antibody (anti-Rep78.93). The positions of the Rep and the amber truncation proteins produced are shown between the panels. A cross-reacting, predominantly cytoplasmic cellular protein comigrates with Rep78. Lanes correspond to those in A. (C) Quantitation of transcript levels. The radioactive p_5 and p_{19} transcripts and plasmid uptake signals were cut and counted in a liquid scintillation counter. The results of two independent experiments were averaged, with the range shown by the bars. Values are expressed as the p_5 and p_{19} transcripts/plasmid uptake ratio as a percentage of the control (pNTC3 plus pBR322).

fected 293 cells by using both total cell proteins and nuclear extracts (Fig. 5B). The immunoblots using anti-Rep78.93 antibody (48) showed that each Rep protein was expressed from the appropriate HIV-LTR plasmid (Fig. 5B). The amounts of Rep proteins detected, however, may not be directly comparable, since the various Rep proteins probably contain different numbers of epitopes as a result of differences in length. In total cell lysate, the truncated amber products of the expected size (approximately 30 kDa) (Rep*am*) from both pHIVrep*am* and pNTC3 were detected. The Rep78 protein was difficult to detect in the total lysate because of a cytoplasmic, cross-reacting protein. However, an immunoblot of nuclear proteins clearly showed the production of Rep78 and Rep68. Both Rep52 and Rep40 proteins were also detected in the nucleus, although they preferentially accumulate in the cytoplasm (1, 29). In summary, the data here indicate that Rep78 and Rep68 are able to decrease p_5 and p_{19} transcript levels more than Rep52 and Rep40.

Multiple regions of Rep78 are required for the negative regulation of p_5 and p_{19} expression. The weak ability of the p_{19} Rep proteins (Rep52, Rep40, and Rep40′) to decrease p_5 transcript levels suggested that the NH_2 terminus (residues 1 to 224) of Rep78 and Rep68 is necessary for the efficient inhibition of p_5 transcript levels. To identify further the region within Rep78 required for the negative regulation, several COOH-terminal truncation mutants of Rep78 were tested for their abilities to affect p_5 and p_{19} mRNA accumulation. The mutant plasmids contained amber termination codons that were introduced into restriction enzyme sites of the *rep* gene (Fig. 6A). In addition, the plasmid pHIVrepK*am* had an internal deletion of a *Kpn*I fragment (AAV nucleotides 1911 to 4151) and thus produced shorter transcripts than the other constructs. The plasmids were cotransfected with pNTC3, and the production of p_5, p_{19}, and HIV-LTR *rep* transcripts was observed by Northern analysis, using a 1.5-kb *Pst*I fragment of pNTC3 (AAV nucleotides 496 to 1958) as a probe.

The truncated Rep proteins, RepH*am* and RepK*am*, were still able to decrease p_5 and p_{19} transcript levels (Fig. 6B). These mutants lacked 99 and 91 amino acids, respectively, from the COOH terminus of Rep78 and thus did not contain the region encoded by the intron spliced from the transcript for Rep68. Furthermore, like Rep68, the RepH*am* and RepK*am* proteins inhibited p_5 and p_{19} mRNA accumulation more strongly than the wild-type Rep78. This may indicate that approximately 100 carboxyl-terminal residues are not required for the negative regulation and, in fact, may make the repressor function of Rep78 weaker. However, since quantitations of Rep78 levels were difficult to perform because of the comigrating, cross-reacting protein, we cannot exclude differences in protein levels as an explanation for different levels of inhibition. The RepBa*am* and RepBs*am* truncation mutants that contained the amino-terminal 243 and 462 amino acids of Rep78, respectively, had no effect on p_5 or p_{19} transcript levels. The inability of RepBa*am* to inhibit was not surprising, since this protein is close to the length of Rep*am* (237 residues), which was used as a negative control.

The p_{19} transcript levels from the complementing pHIV plasmids, except from the RepH*am* and RepK*am* constructs, mimicked the p_{19} levels from the pNTC3 within the same transfection. This was previously seen with Rep68 constructs (Fig. 5A) and may reflect the context of the p_{19} promoter.

The immunoblot analysis of total cell proteins showed that all mutant constructs produced Rep proteins of the expected size and that the amounts were approximately equivalent (Fig. 6C). All of these Rep proteins have also been detected previously in nuclear extracts, although the RepBs*am* protein is preferentially found in the cytoplasm (32, 35). The strong signal across the top of the immunoblot represents a cross-reacting cellular protein which comigrates with Rep78.

Different regions of Rep78 and Rep68 are responsible for p_5

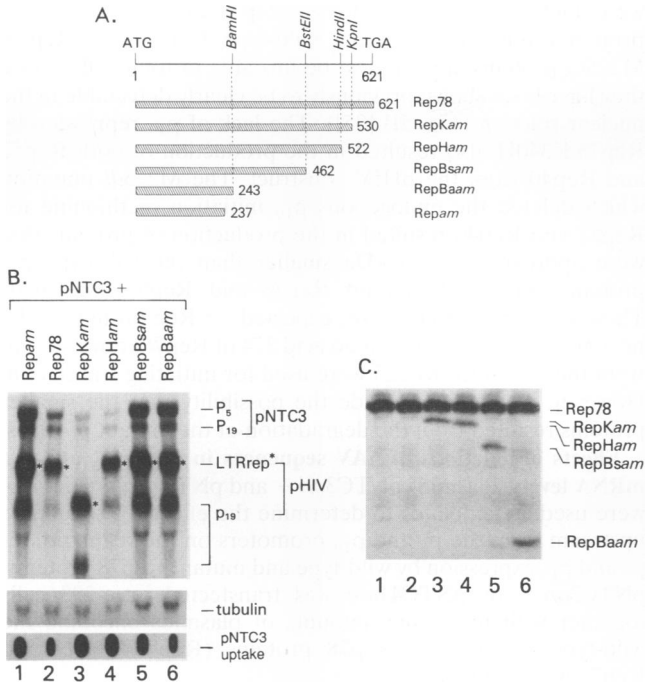

FIG. 6. Production of the truncated Rep proteins and their abilities to inhibit p_5 and p_{19} expression in *trans*. Plasmids encoding truncated Rep78 proteins were cotransfected with pNTC3 into 293 cells, and the cells were harvested after 48 h for cytoplasmic RNA or total proteins. The plasmids used for transfections (and Rep proteins produced) were pHIVrep*am* (Rep*am*), pSK1 (Rep78), pHIVrepK*am* (RepK*am*), pHIVrepH*am* (RepH*am*), pHIVrepBs*am* (RepBs*am*), and pHIVrepBa*am* (RepBa*am*). (A) Map of the truncated Rep78 proteins. The locations of the restriction enzyme sites where termination codons were introduced are indicated at the top. The length (number of amino acids) and the designation of each protein are shown on the right. (B) Northern analysis of transcription levels. The RNA blot was hybridized with a ^{32}P-labeled, 1.5-kb *Pst*I fragment of pNTC3 (shown in Fig. 4) or with a tubulin DNA probe. The mRNAs synthesized from each plasmid are indicated on the right. *, transcripts from the HIV-LTR (LTRrep). Template (pNTC3) uptake was measured as described in the legend to Fig. 3. (C) Production of each truncated protein, as analyzed by immunoblot with whole cell extracts and an anti-Rep antibody (anti-Rep78.93). The location of each protein is shown on the right. A cross-reacting cellular protein comigrates with Rep78. Lanes correspond to those in B.

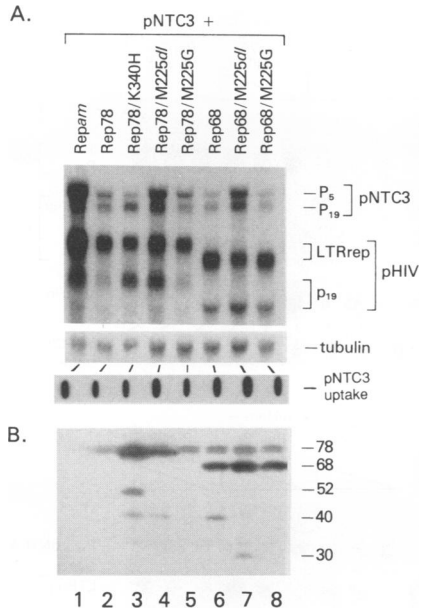

FIG. 7. Regulation of p_5 and p_{19} expression by wild-type and mutant Rep proteins. 293 cells were cotransfected with pNTC3 and one of the following pHIV plasmids (producing the Rep protein indicated in parentheses): pHIVrep*am* (Rep*am*), pSK1 (Rep78), pHIVrepNTP (Rep78/K340H), pSK3 (Rep78/M225*dl*), pSK4 (Rep78/M225G), pSK9 (Rep68), pSK5 (Rep68/M225*dl*), and pSK7 (Rep68/M225G). (A) Effect of mutant Rep78 or Rep68 proteins on p_5 and p_{19} transcript levels. *rep* mRNA levels were analyzed by Northern blotting, with the ^{32}P-labeled 1.5-kb *Pst*I fragment of pNTC3 (shown in Fig. 4) as a probe. ^{32}P-labeled tubulin DNA was used as a control. Template (pNTC3) uptake was measured as described in the legend to Fig. 3. (B) Production of the Rep proteins analyzed by immunoblotting with anti-Rep antibody (S18K). The positions and apparent molecular weights (in thousands) of the Rep proteins are indicated on the right. The signal at the top of panel B represents a cross-reacting cellular protein.

and p_{19} regulation. To define further the regions within Rep78 important for the negative regulation of p_5 and p_{19} expression, we tested the abilities of several additional mutant Rep78 and Rep68 proteins to affect p_5 and p_{19} transcript levels from pNTC3 in *trans*. The mutant proteins expressed from the HIV-LTR (Fig. 4) contained an alteration in the putative NTP binding site (K340H), a deletion of the methionine at residue 225 (M225*dl*) or a substitution of this methionine with glycine (M225G) (Fig. 4). Since the methionine at 225 is encoded by the initiation codon for the p_{19} Rep proteins, the corresponding plasmids are not able to produce Rep52 or Rep40 proteins. The plasmids producing the mutant proteins were cotransfected with pNTC3, and the cytoplasmic RNA was measured by Northern analysis, using a 1.5-kb *Pst*I fragment of pNTC3 as a probe (Fig. 4).

As expected, the positive controls, Rep78 and Rep68, inhibited p_5 and p_{19} transcript accumulation from pNTC3 (Fig. 7A). The Rep78/K340H protein also decreased the level of p_5 transcripts to a level comparable with that of Rep68, but it did not efficiently lower the level of p_{19} transcripts from pNTC3. This difference was also observed for the p_{19} transcripts initiated from the construct producing the mutant Rep protein. The fact that expression from p_5, but not p_{19}, was clearly inhibited by the Rep78/K340H mutant indicates that different regions of Rep78 may be required for regulation of p_5 versus p_{19}.

The two Rep78 constructs containing mutations in the methionine codon (M-225) revealed different effects on *rep* mRNA accumulation (Fig. 7A). The Rep78/M225*dl* mutant had reduced ability to inhibit p_5 and p_{19} mRNA accumulation compared with the wild-type Rep78. Although it is not clear in Fig. 7, Rep78/M225*dl* was not totally incapable of inhibiting the RNA accumulation. A titration experiment with pNTC3 or pNTC3*ori* − and increasing amounts of this mutant indicated a weak dose-dependent inhibition of both p_5 and p_{19} mRNA levels (data not shown for pNTC3; Fig. 8A for pNTC3*ori* −). In contrast, Rep78/M225G lowered both p_5 and p_{19} mRNA levels comparably to wild-type Rep78. Similar results were observed when the same mutations (M225*dl* and M225G) were introduced into Rep68 (Fig. 7A).

The transcript levels from the HIV-LTR did not appear to be affected by the Rep proteins produced from these complementing plasmids, since the amounts of the chimeric *rep* transcripts were even. The p_{19} transcript levels from these

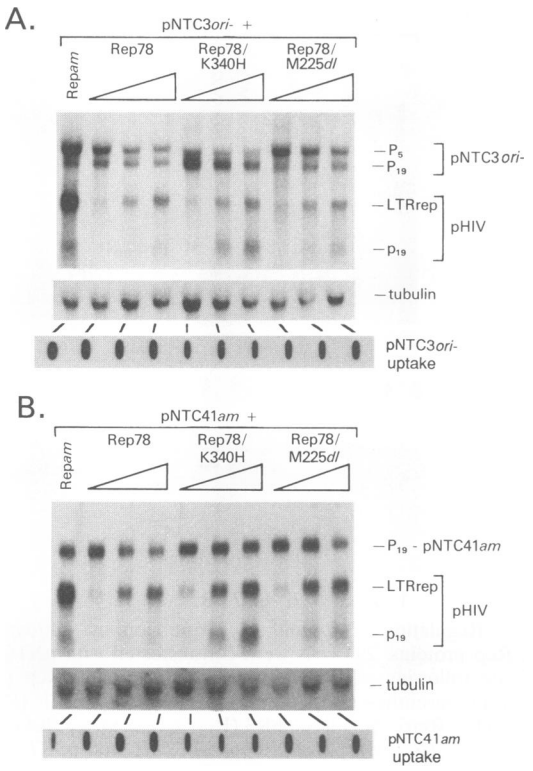

FIG. 8. The effect of deleting upstream AAV DNA sequences on the regulation of AAV p_5 and p_{19} expression by the wild-type and mutant Rep78 proteins. The template plasmids containing amber mutations (Fig. 2A) were pNTC3*ori*− (lacking approximately 120 nucleotides from each ITR) or pNTC41*am* (lacking nucleotides 1 to 814 of the AAV sequence). Six micrograms of plasmid pNTC3*ori*− (A) or pNTC41*am* (B) was cotransfected with plasmids producing Rep*am* (6.0 μg of pHIVrep*am*) or increasing amounts (0.6, 3.0, and 6.0 μg) of wild-type Rep78 (pSK1), Rep78/K340H (pHIVrepNTP), or Rep78/M225*dl* (pSK3). The cytoplasmic RNA was isolated after 48 h and analyzed by RNA blotting. The RNA blots were hybridized with the ^{32}P-labeled, 1.5-kb *Pst*I fragment of pNTC3 (shown in Fig. 4) or with a tubulin DNA probe. The transcripts produced from the various promoters (p_5, p_{19}, and LTRrep) and plasmids (pNTC3*ori*−, pNTC41*am*, and pHIV) are indicated on the right. Template plasmid (pNTC3*ori*− and pNTC41*am*) uptake was measured as described in the legend to Fig. 3.

constructs behaved differently, depending on whether p_{19} mRNA was produced from various Rep78 or Rep68 constructs. In the case of Rep78 constructs, the p_{19} levels from the cotransfected, complementing plasmids mimicked the p_{19} levels from pNTC3, as was described previously for Fig. 5A and 6B. In contrast, the p_{19} transcript levels from different Rep68 constructs were consistently slightly higher, as was also visible in previous analyses (Fig. 5 and 6).

The M225*dl* and M225G mutations in Rep78 and Rep68 prevent the expression of Rep52 and Rep40 proteins from the respective pHIV-LTR plasmids (8, 35). Our finding that Rep78 and Rep68 containing the M225G mutations still clearly inhibited p_5 expression supports our earlier conclusion that Rep52 and Rep40 have little effect on the regulation of p_5 expression.

Western analysis of total cell proteins showed that mutant proteins of the expected size were produced (Fig. 7B). While the levels of the different Rep68 proteins produced were equivalent, the levels of Rep78/K340H and Rep78/M225*dl* were higher than the wild-type Rep78 and Rep78/M225G protein levels. Although the wild-type Rep78 and Rep78/M225G proteins appeared to be unstable in total cell lysates, they have been shown previously to be clearly detectable in the nuclear fraction (Fig. 5B [35]). The lack of p_{19} repression by Rep78/K340H also resulted in the production of both Rep52 and Rep40 from the pHIV construct. The M225*dl* mutation which deleted the endogenous p_{19} initiation methionine for Rep52 and Rep40 resulted in the production of proteins that were approximately 10 kDa smaller than the wild-type p_{19} proteins from both mutant Rep78 and Rep68 constructs. These correspond to the sizes expected for Rep proteins if the next methionine codon (amino acid 274 of Rep78) downstream from the codon for M-225 were used for initiating translation. However, we cannot exclude the possibility that the smaller proteins resulted from the degradation of the mutant proteins.

Effects of deletions of AAV sequences in *cis* on p_5 and p_{19} mRNA levels. Plasmids pNTC3*ori*− and pNTC41*am* (Fig. 2A) were used as templates to determine the effects of sequences upstream from the p_5 and p_{19} promoters on the regulation of p_5 and p_{19} expression by wild-type and mutant Rep78 proteins. pNTC3*ori*− or pNTC41*am* was transfected into 293 cells together with increasing amounts of plasmids encoding the wild-type and mutant Rep78 proteins (Rep78/K340H and Rep78/M225*dl*) (Fig. 8).

The lack of ITR sequences (AAV nucleotides 1 to 120) in pNTC3*ori*− did not alter the regulation of p_5 or p_{19} mRNA levels by any of the Rep78 proteins tested (Fig. 8A). Both the wild-type and mutant Rep78 proteins in *trans* regulated the p_5 and p_{19} transcript levels from the pNTC3*ori*− template similarly to the pNTC3 template (Fig. 7A). The inability of the NTP mutant (Rep78/K340H) to down-regulate p_{19} mRNA levels is clear, although a slight decrease of p_{19} transcript levels was seen at the highest level of the mutant protein. Also visible in Fig. 8 is the decreased ability of Rep78/M225*dl* to inhibit both p_5 and p_{19} mRNA accumulation. The p_{19} expression from the complementing pHIV plasmids (encoding Rep78/K340H and Rep78/M225*dl*) consistently mimicked the p_{19} expression from the cotransfected pNTC3. In summary, an intact ITR is not required for the inhibition of p_5 or p_{19} transcript levels by Rep78.

The results with the pNTC41*am* construct which lacks the ITR and p_5 promoter region (AAV nucleotides 1 to 814) revealed three features (Fig. 8B). First, because no p_5 transcripts are produced from pNTC41*am*, the dose-dependent negative regulation of p_{19} transcripts by Rep78 was more evident than that in Fig. 8A. Second, the levels of p_{19} mRNA from pNTC41*am* in the presence of Rep78/K340H remained unchanged and there was no dose-dependent inhibition as seen with pNTC3*ori*−. Third, similarly to p_5 transcripts (Fig. 8A), the ability of Rep78/M225*dl* to down-regulate p_{19} transcript levels was decreased. As was described above, the weaker negative regulation of p_{19} expression by the mutant Rep proteins was also seen from the p_{19} promoter in the cotransfected HIV-LTR plasmids.

The abilities of the other wild-type Rep proteins, Rep68, Rep52, and Rep40 to inhibit p_{19} mRNA accumulation from pNTC41*am* were also tested (Fig. 9). Rep68 caused a stronger inhibition than Rep78, similar to results in Fig. 5. The smaller Rep proteins affected pNTC41*am* p_{19} expression less than Rep68 but were approximately as efficient as Rep78, as was already described for Fig. 5. This indicates that negative regulation of p_{19} expression by Rep proteins is possible in the absence of the upstream p_5 promoter.

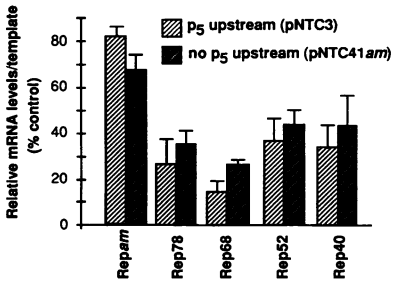

FIG. 9. Quantitation of p_{19} transcript levels from templates pNTC3 and pNTC41*am* which either contain or lack the upstream p_5 promoter region. The values for pNTC3 are those obtained for the quantitation of transcript levels indicated in Fig. 5C. Values for p_{19} transcript levels from pNTC41*am* were measured from two independent transfection experiments using 6 μg of pNTC41*am* and 6 μg of constructs producing Rep*am* (pHIVrep*am*), Rep78 (pSK1), Rep68 (pSK9), Rep52 (pSK12), or Rep40 (pSK13). The p_{19} transcripts were quantitated by cutting and counting in a liquid scintillation counter and normalized by template DNA uptake measured by slot blot. Values are expressed as the p_{19} transcripts/template uptake ratio as a percentage of the control (pNTC3 or pNTC41*am* plus pBR322).

DISCUSSION

We have shown here that AAV Rep proteins, Rep78 and Rep68, translated from the p_5 transcripts, efficiently down-regulate the mRNA levels from both the AAV p_5 and p_{19} promoters. This conclusion is based on the following results. First, the mutant AAV genomes (pNTC3, pNTC3*ori*−, and pNTC41*am*) containing amber mutations in the *rep* gene accumulated higher levels of p_5 and p_{19} transcripts in the cytoplasm of human 293 cells than the wild-type genome (pNTC244). Second, the complementation of the AAV genomes containing amber mutations in the *rep* gene (pNTC3, pNTC3*ori*−, and pNTC41*am*) with Rep78 or Rep68 in *trans* restored the down-regulation of p_5 and p_{19} mRNA to wild-type levels. This confirms that the increase in p_5 and p_{19} transcript levels from the amber constructs was due to the absence of the wild-type Rep proteins. The reduced p_5 or p_{19} levels with some of the Rep proteins were not due to limiting amounts of template, since the template (pNTC3, pNTC3*ori*−, or pNTC41*am*) uptake into the nucleus was consistent, as measured by slot blots. The production of a correct-size Rep protein was also confirmed by immunoblots.

The mechanism of the negative regulation observed in our studies remains to be determined. Thus, whether the changes in the steady-state levels of mRNA resulted from a change at the level of transcript synthesis or message stability is not clear. The negative regulation of p_5 and p_{19} expression by Rep proteins has been detected previously in HeLa cells by Beaton et al. (3). In their studies, cotransfection of a wild-type AAV genome producing Rep protein(s) decreased CAT activity from p_5 (AAV nucleotides 144 to 310) or p_{19} (814 to 964) promoters fused to the *cat* gene, implying that the regulation occurred at transcriptional level. Our preliminary data (not shown) also suggest that the inhibition of p_5 levels in 293 cells is mediated at the level of transcription initiation, since regions downstream of the transcript initiation site are not necessary for the negative regulation. Furthermore, it has recently been shown by gel shift and DNaseI protection analysis that purified Rep68 binds between the p_5 TATA box and the transcription initiation site (36), indicating that Rep proteins can interact with AAV DNA in regions which are important for the control of transcription. In our studies, the specificity of negative regulation by Rep proteins for AAV promoters was supported by the fact that the levels of *rep* transcripts produced from a heterologous promoter (HIV-LTR) were subject to little or no change by the Rep proteins. All the wild-type Rep proteins produced in this study have previously been shown to be present in the nucleus, as would be expected for proteins involved in transcription regulation (1, 29, 35). Although Rep B*sam* was underrepresented in the nuclear fraction, all the mutant Rep proteins could also be detected in the nucleus because of either the presence of a putative nuclear localization sequence or the small size of the protein (35, 51). Lastly, the negative regulation of p_5 and p_{19} transcript levels is not restricted to 293 cells, since the phenomenon has been previously detected in uninfected HeLa cells (3). Thus, the negative regulation by the Rep proteins does not appear to require the presence of adenovirus E1a and E1b proteins expressed in 293 cells (15).

Our results indicate that control of p_5 versus p_{19} expression by Rep proteins can be separated and thus may occur by different mechanisms. First, as also shown by Beaton et al. (3) in HeLa cells, the negative regulation of p_{19} in 293 cells did not require the presence of the p_5 promoter, since the deletion of the AAV nucleotides 1 to 814 (which includes the p_5 promoter) did not abolish the ability of Rep78 or Rep68 to decrease p_{19} expression. Second, the Rep78/K340H protein in *trans* still efficiently down-regulated p_5 transcript levels but was greatly reduced in its ability to decrease the transcript levels from the p_{19} promoter (Fig. 7A and 8A). These results indicate that p_5 and p_{19} transcript levels can be regulated independently. However, simultaneous control of both p_5 and p_{19} by the Rep proteins in vivo may enhance the negative effect. In the presence of the upstream p_5 promoter, we observed a weak dose-dependent inhibition of p_{19} expression by the NTP mutant (Rep78/K340H) while in the pNTC41*am* construct lacking the p_5 promoter, there was no visible inhibition by the increasing amounts of the NTP mutant. Furthermore, there was a slight decrease in the abilities of the wild-type Rep proteins, especially Rep68, to regulate p_{19} mRNA in the absence of the upstream p_5 region (Fig. 9). This may also explain why the p_{19} mRNA levels from Rep68 HIV-LTR constructs lacking the p_5 promoter region were consistently higher than those from the p_{19} promoter in the cotransfected pNTC3 template containing the upstream p_5 promoter.

The inability of the NTP-mutant Rep protein (K340H) to negatively regulate the p_{19} promoter indicates another functional importance of the putative NTP binding site. A mutant AAV genome containing this K340H mutation is also dominant-negative for replication (9) and the Rep78/K340H protein is dominant-negative for in vitro AAV terminal resolution (33). Furthermore, the K340H mutation abolishes the ability of Rep78 to inhibit HIV-1 replication (1, 38). Previous reports of studies using a different Rep-NTP mutant (T341I, N342Y) have indicated that the activation of AAV p_{19} and p_{40} promoters in the presence of adenovirus is also dependent on the wild-type NTP binding site (31). However, it still remains to be seen whether the putative NTP binding site actually binds NTP.

The Rep68 and Rep78 proteins require multiple functional domains for efficient inhibition of p_5 and p_{19} transcript levels. On the basis of our results with the carboxyl-terminal deletion mutants and the reduced ability of Rep52 and Rep40 to decrease p_5 and p_{19} expression, we conclude that a large region (amino acids 1 to 522) of the protein is required for maximal negative regulation. Similar requirements for multiple regions of the Rep protein have been demonstrated for other Rep functions (30, 31, 35, 51). Rep68 and some of the COOH-

terminal truncation mutants of Rep78 had increased abilities to down-regulate p_5 mRNA accumulation. It is not clear whether some of the features of the COOH-terminal region of Rep78 missing in Rep68, such as two putative zinc fingers and 10 of a total of 12 cysteines, or greater production or stability of the Rep68 is responsible for this differential effect. The site-specific mutants also showed some interesting features. The ability of the Rep78/K340H mutant to decrease p_5 mRNA levels indicated that the putative NTP binding site is not required for this function. The Rep mutant containing a deletion at the codon for amino acid 225 (M225*dl*) showed decreased efficiency to lower both p_5 and p_{19} mRNA accumulation. The fact that a deletion rather than a substitution of the methionine residue affected the mutant Rep protein's ability to regulate p_5 expression suggests that the methionine itself is not important but rather its deletion may have altered the overall protein conformation. The AAV genome containing this mutation (M225*dl*) is also unable to replicate (8), and the mutant Rep78 protein produced from this genome binds weakly to the AAV terminal hairpin (35).

The physiological significance of the negative regulation of p_5 and p_{19} is not clear. There are several stages of the AAV life cycle during which inhibition of *rep* gene expression might be advantageous, and it is possible that repression occurs through more than one mechanism. In the absence of helper virus, AAV establishes a latent infection by integrating into host chromosomal DNA, probably as a result of conditions unable to support AAV replication (5, 27, 50). Only very low levels of AAV gene expression have been detected in latently infected human cells by Northern analysis (27, 49). This silent stage may be due to binding of cellular repressors, such as YY1, which are known to repress p_5 expression in the absence of helper virus (40). However, integrated heterologous genes can be efficiently expressed from the AAV p_5 promoter in the absence of *rep* genes (12, 13). Furthermore, several integration sites for AAV have been localized to CpG-rich regions of chromosomal DNA which are suggestive of transcriptionally active sites (21, 43). This suggests an additional safety mechanism in which low levels of AAV Rep proteins actively reduce transcription from their own promoters during latency. This may aid in maintaining the latent stage and would decrease the synthesis of structural and nonstructural AAV proteins and thus minimize the activation of host immune defenses. Likewise, suppression of Rep protein levels would reduce the known inhibitory effects of Rep proteins on heterologous genes and on cellular proliferation and thus ensure the replication of the provirus with the cellular DNA (20, 23).

The Rep proteins appear to have multiple properties in gene regulation during the AAV life cycle. In the presence of helper virus, where replication of AAV occurs, AAV p_5, p_{19}, and p_{40} promoters are activated by the Rep proteins (24, 30). However, analysis of the AAV transcript levels during replication has shown very low levels of p_{19} and p_5 transcripts (5 to 10%) compared with that of p_{40} (24). It is possible that the AAV promoters are differentially regulated at various times after infection. Under these conditions, the putative *trans*-activating and repressing properties of Rep proteins on p_5 and p_{19} expression could be important for production of appropriate levels of Rep proteins in order to control AAV gene expression precisely. Several regulatory proteins from herpesviruses (e.g., human cytomegalovirus IE2 protein) have the ability both to activate and to repress their own expression at various times during the virus life cycle and, consequently, strongly autoregulate their own expression to maintain proper levels of the regulatory proteins (10, 37). The mechanism for the seemingly opposite functions for the herpesvirus proteins appears to depend on the presence of other viral and host proteins (10). It would not be surprising if AAV Rep proteins could also respond to changing environmental conditions such as the presence of helper virus, various hosts, or phases of the host cell cycle.

In summary, we have shown that AAV Rep68 and Rep78 proteins are able to inhibit efficiently p_5 and p_{19} transcript levels under nonpermissive conditions. This ability may be important to down-regulate AAV transcription from the integrated AAV provirus stage, when the flanking genomic environment might otherwise enhance AAV gene expression. We are currently studying the mechanism of p_5 promoter repression by Rep proteins and hope that these studies will further clarify the regulation of AAV gene expression and its physiological significance in the AAV life cycle.

ACKNOWLEDGMENTS

We thank Nancy Nossal, Deborah Hinton, and Robert Moore for critically reviewing the manuscript.

REFERENCES

1. **Antoni, B. A., A. B. Rabson, I. L. Miller, J. P. Trempe, N. Chejanovsky, and B. J. Carter.** 1991. Adeno-associated virus Rep protein inhibits human immunodeficiency virus type 1 production in human cells. J. Virol. **65**:396–404.
2. **Ashktorab, H., and A. Srivastava.** 1989. Identification of nuclear proteins that specifically interact with adeno-associated virus type 2 inverted terminal repeat hairpin DNA. J. Virol. **63**:3034–3039.
3. **Beaton, A., P. Palumbo, and K. I. Berns.** 1989. Expression from the adeno-associated virus p_5 and p_{19} promoters is negatively regulated in *trans* by the *rep* protein. J. Virol. **63**:4450–4454.
4. **Carter, B. J.** 1990. Adeno-associated virus helper functions, p. 255–282. *In* P. Tijssen (ed.), Handbook of parvoviruses, vol I. CRC Press, Inc., Boca Raton, Fla.
5. **Carter, B. J., J. P. Trempe, and E. Mendelson.** 1990. Adeno-associated virus gene expression and regulation, p. 227–254. *In* P. Tijssen (ed.), Handbook of parvoviruses, vol I. CRC Press, Inc., Boca Raton, Fla.
6. **Chang, L.-S., Y. Shi, and T. Shenk.** 1989. Adeno-associated virus P5 promoter contains an adenovirus E1A inducible element and a binding site for the major late transcription factor. J. Virol. **63**:3479–3488.
7. **Chejanovsky, N., and B. J. Carter.** 1989. Replication of a human parvovirus nonsense mutant in mammalian cells containing an inducible amber suppressor. Virology **171**:239–247.
8. **Chejanovsky, N., and B. J. Carter.** 1989. Mutagenesis of an AUG codon in the adeno-associated virus *rep* gene: effects on viral DNA replication. Virology **173**:120–128.
9. **Chejanovsky, N., and B. J. Carter.** 1990. Mutation of a consensus purine nucleotide binding site in the adeno-associated virus *rep* gene generates a dominant negative phenotype for DNA replication. J. Virol. **64**:1764–1770.
10. **Cherrington, J. M., E. L. Khoury, and E. S. Mocarski.** 1991. Human cytomegalovirus *ie2* negatively regulates α gene expression via short target sequence near the transcription start site. J. Virol. **65**:887–896.
11. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
12. **Flotte, T. R., S. Afione, R. Solow, M. L. Drumm, D. Markakis, W. B. Guggino, P. L. Zeitlin, and B. J. Carter.** 1993. Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. J. Biol. Chem. **268**:3781–3790.
13. **Flotte, T. R., R. Solow, R. A. Owens, S. Afione, P. L. Zeitlin, and B. J. Carter.** 1992. Gene expression from adeno-associated virus vectors in airway epithelial cells. Am. J. Respir. Cell Mol. Biol. **7**:349–356.
14. **Gendelman, H. E., W. Phelps, L. Feigenbaum, J. M. Ostrove, A. Adachi, P. M. Howley, G. Khoury, H. S. Ginsberg, and M. A.**

Martin. 1986. Trans-activation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc. Natl. Acad. Sci. USA **83**:9759–9763.
15. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from adenovirus type 5. J. Gen. Virol. **36**:59–72.
16. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
17. **Im, D.-S., and N. Muzyczka.** 1989. Factors that bind to adeno-associated virus terminal repeats. J. Virol. **63**:3095–3104.
18. **Im, D.-S., and N. Muzyczka.** 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell **61**:447–457.
19. **Im, D.-S., and N. Muzyczka.** 1992. Partial purification of adeno-associated virus Rep78, Rep52, and Rep40 and their biochemical characterization. J. Virol. **66**:1119–1128.
20. **Khleif, S. N., T. Myers, B. J. Carter, and J. P. Trempe.** 1991. Inhibition of cellular transformation by the adeno-associated virus *rep* gene. Virology **181**:738–741.
21. **Kotin, R. M., R. M. Linden, and K. I. Berns.** 1992. Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. EMBO J. **11**:5071–5078.
22. **Kotin, R. M., M. Siniscalco, R. J. Samulski, X. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns.** 1990. Site-specific integration by adeno-associated virus. Proc. Natl. Acad. Sci. USA **87**:2211–2215.
23. **Labow, M. A., L. H. Graf, Jr., and K. I. Berns.** 1987. Adeno-associated virus gene expression inhibits cellular transformation by heterologous genes. Mol. Cell. Biol. **7**:1320–1325.
24. **Labow, M. A., P. L. Hermonat, and K. I. Berns.** 1986. Positive and negative autoregulation of the adeno-associated virus type 2 genome. J. Virol. **60**:251–258.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Laughlin, C. A., J.-D. Tratschin, H. Coon, and B. J. Carter.** 1983. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. Gene **23**:65–73.
27. **Laughlin, C. A., C. B. Cardellichio, and H. C. Coon.** 1986. Latent infection of KB cells with adeno-associated virus type 2. J. Virol. **60**:515–524.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Mendelson, E., J. P. Trempe, and B. J. Carter.** 1986. Identification of the *trans*-acting Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. J. Virol. **60**:823–832.
30. **McCarty, D. M., M. Christensen, and N. Muzyczka.** 1991. Sequences required for coordinate induction of adeno-associated virus p19 and p40 promoters by Rep protein. J. Virol. **65**:2936–2945.
31. **McCarty, D. M., T.-H. Ni, and N. Muzyczka.** 1992. Analysis of mutations in adeno-associated virus Rep protein in vivo and in vitro. J. Virol. **66**:4050–4057.
32. **Owens, R. A.** Unpublished data.
33. **Owens, R. A., and B. J. Carter.** 1992. In vitro resolution of adeno-associated virus DNA hairpin termini by wild-type Rep protein is inhibited by a dominant-negative mutant of Rep. J. Virol. **66**:1236–1240.
34. **Owens, R. A., J. P. Trempe, N. Chejanovsky, and B. J. Carter.** 1991. Adeno-associated virus Rep proteins produced in insect and mammalian expression systems: wild-type and dominant-negative mutant proteins bind to the viral replication origin. Virology **184**:14–22.
35. **Owens, R. A., M. D. Weitzman, S. R. M. Kyöstiö, and B. J. Carter.** 1993. Identification of a DNA-binding domain in the amino-terminus of adeno-associated virus Rep proteins. J. Virol. **67**:997–1005.
36. **Pereira, D., and N. Muzyczka.** 1993. Personal communication.
37. **Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward.** 1988. *trans*-Activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J. Virol. **62**:1167–1179.
38. **Rittner, K., R. Heilbronn, J. A. Kleinschmidt, and G. Sczakiel.** 1992. Adeno-associated virus type 2-mediated inhibition of human immunodeficiency virus type 1 (HIV-1) replication: involvement of $p78^{rep}/p68^{rep}$ and the HIV-1 long terminal repeat. J. Gen. Virol. **73**:2977–2981.
39. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
40. **Shi, Y., E. Seto, L.-S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. Cell **67**:377–388.
41. **Snyder, R. O., D.-S. Im, and N. Muzyczka.** 1990. Evidence for covalent attachment of the adeno-associated virus (AAV) Rep protein to the ends of the AAV genome. J. Virol. **64**:6204–6213.
42. **Srivastava, A., E. W. Lusby, and K. I. Berns.** 1983. Nucleotide sequence and organization of the adeno-associated virus 2 genome. J. Virol. **45**:555–564.
43. **Tazi, J., and A. Bird.** 1990. Alternative chromatin structure at CpG islands. Cell **60**:909–920.
44. **Tratschin, J. D., I. L. Miller, and B. J. Carter.** 1984. Genetic analysis of adeno-associated virus: properties of deletion mutants constructed in vitro and evidence for an adeno-associated virus replication function. J. Virol. **51**:611–619.
45. **Tratschin, J. D., J. Tal, and B. J. Carter.** 1986. Negative and positive regulation in *trans* of gene expression from adeno-associated virus vectors in mammalian cells by a viral rep gene product. Mol. Cell. Biol. **6**:2884–2894.
46. **Trempe, J. P., and B. J. Carter.** 1988. Regulation of adeno-associated virus gene expression in 293 cells: control of mRNA abundance and translation. J. Virol. **62**:68–74.
47. **Trempe, J. P., and B. J. Carter.** 1988. Alternate mRNA splicing is required for synthesis of adeno-associated virus VP1 capsid protein. J. Virol. **62**:3356–3363.
48. **Trempe, J. P., E. Mendelson, and B. J. Carter.** 1987. Characterization of adeno-associated virus rep proteins in human cells by antibodies raised against rep expressed in *Escherichia coli*. Virology **161**:18–28.
49. **Walz, C., and J. R. Schlehofer.** 1992. Modification of some biological properties of HeLa cells containing adeno-associated virus DNA integrated into chromosome 17. J. Virol. **66**:2990–3002.
50. **Yakobson, B., T. Koch, and E. Winocour.** 1987. Replication of adeno-associated virus in synchronized cells without the addition of helper virus. J. Virol. **61**:972–981.
51. **Yang, Q., A. Kadam, and J. P. Trempe.** 1992. Mutational analysis of the adeno-associated virus *rep* gene. J. Virol. **66**:6058–6069.