JOURNAL OF VIROLOGY, Jan. 1988, p. 68–74
0022-538X/88/010068-07$02.00/0

Vol. 62, No. 1

Regulation of Adeno-Associated Virus Gene Expression in 293 Cells: Control of mRNA Abundance and Translation

JAMES P. TREMPE* AND BARRIE J. CARTER

Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Building 8, Room 304, Bethesda, Maryland 20892

Received 14 July 1987/Accepted 30 September 1987

We studied the effects of the adeno-associated virus (AAV) *rep* gene on the control of gene expression from the AAV p_{40} promoter in 293 cells in the absence of an adenovirus coinfection. AAV vectors containing the chloramphenicol acetyltransferase (*cat*) gene were used to measure the levels of *cat* expression and steady-state mRNA from p_{40}. When the *rep* gene was present in *cis* or in *trans*, *cat* expression from p_{40} was decreased 3- to 10-fold, but there was a 2- to 4-fold increase in the level of p_{40} mRNA. Conversely, *cat* expression increased and the p_{40} mRNA level decreased in the absence of the *rep* gene. Both wild-type and carboxyl-terminal truncated Rep proteins were capable of eliciting both effects. These data suggest two roles for the pleiotropic AAV *rep* gene: as a translational inhibitor and as a positive regulator of p_{40} mRNA levels. We also provide additional evidence for a *cis*-acting negative regulatory region which decreases RNA from the AAV p_5 promoter in a fashion independent of *rep*.

RNA transcription in the human parvovirus adeno-associated virus (AAV) is initiated from three distinct promoters at map positions 5, 19, and 40 (p_5, p_{19}, and p_{40}, respectively) and terminates at a single polyadenylation site (3, 11, 21, 22, 24). A long open reading frame (open reading frame 1) on the left side of the genome is accessible from p_5 and p_{19} transcripts, and genetic analysis has revealed that open reading frame 1 comprises the AAV replication (*rep*) gene (12, 28) and codes for six Rep proteins. A second open reading frame (open reading frame 2) on the right half of the genome is accessible from p_{40} transcripts and codes all or part of the three AAV capsid polypeptides VP3, VP2, and VP1 (1, 12, 14, 28). Complementation studies have demonstrated that the *rep* gene products exhibit a *trans*-acting function in AAV replicative-form DNA replication (12, 28).

Recent work in our laboratory using AAV as an expression vector showed that the *rep* gene also plays two other *trans* regulatory roles in gene expression from the p_{40} transcription unit (29, 30). When AAV vectors containing the gene for chloramphenicol acetyltransferase (CAT) under the control of p_{40} were transfected into 293 cells, the *rep* gene was found to be a negative regulator of *cat* expression (29). However, when the same vectors were transfected into HeLa cells, the *rep* gene was a positive effector of CAT enzyme levels (29). When adenovirus infection accompanied the DNA transfections, the *rep* gene increased the level of CAT activity in both cell types, regardless of transcription template replication (29). Positive regulation by *rep* was also shown with wild-type AAV in adenovirus-infected KB cells (17).

Using nuclease analyses of RNA from cells transfected with AAV-CAT vectors, we found that a *rep* gene product increased cytoplasmic p_{40} mRNA levels. The stimulation of p_{40} mRNA was observed whether the *rep* gene was present in *cis* or was supplied in *trans* by cotransfection with a wild-type pAV2 plasmid. In contrast to the increased message levels, *cat* expression was decreased when the *rep* gene product was present. Thus, the *rep* gene product appears to have two opposing roles in 293 cells: positively regulating mRNA levels and negatively regulating the level of the CAT enzyme. This appears to be a unique set of properties for any viral *trans*-acting proteins described thus far.

* Corresponding author.

MATERIALS AND METHODS

Cells. Human 293-31 cells (293 cells), an established line of adenovirus-transformed human embryonic kidney cells (9), were grown at 37°C as monolayer cultures in Eagle minimal essential medium supplemented with antibiotics and 10% fetal calf serum in 100-mm plastic dishes.

Growth and purification of plasmids. All the procedures for the construction, growth, and purification of recombinant plasmids have been described before (23, 28). Plasmids were maintained in *Escherichia coli* HB101 in ampicillin-containing medium, and all manipulations with recombinant DNA were performed in accordance with the guidelines of the National Institutes of Health.

Transfection of cells. Except when noted otherwise, 293 cells grown in monolayers in 100-mm dishes (5×10^6 cells per dish) were transfected with 10 μg of each plasmid DNA by the $CaPO_4$ procedure as described previously (10, 30). Control plasmid pBR322 or pAS1 (23), a derivative of pBR322, was used to adjust DNA $CaPO_4$ transfection mixtures so that all cultures received the same amount of DNA. The cell growth medium was replaced with fresh medium 4 h after transfection.

Fractionation of cells. At 40 h after transfection, cells were harvested, lysed, and fractionated into nuclear and cytoplasmic extracts by the Nonidet P-40 lysis procedure (23). A portion of the cytoplasmic extract was retained for the CAT assay (8), and the remainder was used for RNA purification (23). Polyadenylated RNA was separated from total cytoplasmic RNA by oligo(dT) cellulose chromatography.

Assay of CAT activity. CAT activity in transfected cell lysates was monitored by acetylation of [^{14}C]chloramphenicol, as measured by thin-layer chromatography of silica gel plates (8). Note that the cell extract used was the cytoplasmic fraction obtained from the Nonidet P-40 lysis procedure. This modified CAT assay (I. Miller, personal communication) was as efficient as the original procedure, which uses a whole-cell extract (8). Assay mixtures contained 25 μl of

cytoplasmic cell extract (equivalent to 2.5×10^4 cells) and were incubated at 37°C for 90 min. After chromatography, the thin-layer plates were analyzed by autoradiography, and radioactivity was then quantitated by liquid scintillation counting of the spots. Results are expressed as the percentage of [^{14}C]chloramphenicol acetylated.

Analysis of RNA. One-third of the cytoplasmic mRNA from a 10-cm plate was combined with the appropriate end-labeled [^{32}P]DNA probe in a 10-μl volume of 80% formamide containing 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA, and 400 mM NaCl. The mixture was heated for 15 min at 68°C and then hybridized at 48°C for 2.5 h. A 100-μl amount of S1 nuclease buffer (280 mM NaCl, 50 mM sodium acetate [pH 4.6], 4.5 mM $ZnSO_4$, 20 μg of denatured salmon sperm DNA per ml, and 27 U of S1 nuclease [P-L Biochemicals, Inc.]) was added, and the mixture was incubated for 40 min at 37°C. The mixture was then extracted once with phenol-chloroform and ethanol precipitated. The precipitate was suspended in 90% formamide–TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed in a 4% polyacrylamide–8 M urea gel, and the gel was fixed in 7% trichloroacetic acid, dried, and exposed to X-ray film.

The DNA probes used in the S1 nuclease analyses are shown in Fig. 1. pTS1 RNA from the cotransfections was analyzed with a 2,544-base-pair (bp) fragment extending from the *Sca*I site within the *cat* gene to a *Bgl*II site located within the polylinker sequence of the plasmid immediately to the left of the AAV terminus. pTS18 RNA from the cotransfections was analyzed with an analogous 1,658-bp fragment extending from the *cat* gene *Sca*I site to the *Bgl*II site. For the dose-response experiment (see Fig. 2), both pTS1 and pTS18 RNAs were analyzed with the same end-labeled probe that extended from the *Pvu*II site within the *cat* gene to the *Bam*HI site in plasmid pTS1. Since both plasmids synthesized the same p_{40} CAT constructs, the same hybridization probe could be used. pJDT269 RNA was analyzed with a 3,245-bp fragment extending from the first *Sca*I site in the capsid gene to the *Bgl*II site. Each of these fragments was dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals) and end labeled with T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP at the *Sca*I site.

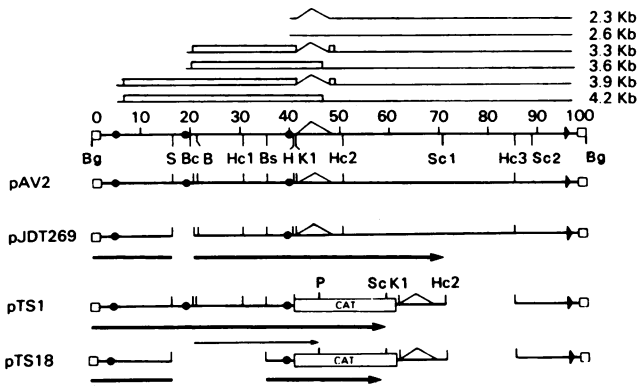

FIG. 1. Structure of the AAV genome and the AAV recombinant plasmids used in this work. The structures of the cytoplasmic RNA species are indicated in the top portion. Open reading frame 1 (open rectangles) codes for at least six Rep proteins (only four are indicated); p_{40} transcripts code for AAV capsid proteins. Kb, Kilobases. The structure of the AAV genome is shown below the RNA map on a scale of 100 map units (1 map unit is approximately 47 nucleotides). Open boxes at the extreme ends of the genome indicate the terminal repeat sequences containing the replication origins. Transcriptional signals are indicated as follows: promoters (solid circles), polyadenylation site (arrowhead), and intron (caret). Relevant restriction endonuclease sites are designated as follows: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II, Bs, *Bst*EII; H, *Hin*dIII; Hc, *Hin*cII; P, *Pvu*II; S, *Sal*I; and Sc, *Sca*I. Multiple sites are designated with an additional numeral. The four plasmid constructions used in this work are shown in the bottom portion, except that the pBR322 sequences are eliminated for clarity. Below the maps, the S1 nuclease hybridization probes are indicated as arrows: the light arrow indicates the p_{40} mRNA-specific probe used in Fig. 2, and the heavy arrows indicate the probes used in the cotransfections in Fig. 3 and 4. Symbols and abbreviations are the same as in the AAV genome structure.

RESULTS

Structure of AAV vectors. Maps of the AAV genome and AAV plasmids used in this study are shown in Fig. 1. pAV2 contains the entire wild-type AAV genome inserted into a pBR322 derivative via *Bgl*II linkers (20). The three transcriptional promoter elements at map units 5, 19, and 40 are indicated by closed circles. When pAV2 is transfected into tissue culture cells in the presence of a coinfecting adenovirus, AAV sequences are excised from pAV2 and replicated as in a wild-type (*rep*$^+$) virus infection (20). pJDT269 is a replication-defective (*rep*$^-$) deletion mutant of pAV2 that lacks 150 bp between the *Sst*I and *Bcl*I restriction endonuclease sites (28). pTS1 contains the coding sequence for CAT inserted at the *Hin*dIII site of pAV2 (30). pTS1 is a *cap* mutant because of the deletion of the Hc2/Hc3 fragment; however, although approximately 300 nucleotides have also been removed from the carboxyl terminus of open reading frame 1 pTS1 retains a Rep$^+$ phenotype (30). pTS18 is a *rep*$^-$ derivative of pTS1 that lacks sequences between the *Sst*I and *Bst*EII restriction endonuclease sites (29).

Dose response of CAT mRNA and expression. We previously reported that in 293 cells, the expression of CAT activity from chimeric p_{40} AAV-CAT plasmid constructs was negatively regulated by a *trans*-acting function of the AAV *rep* gene (29). In the present work we analyzed both steady-state mRNA levels and CAT activity to distinguish the regulation of mRNA levels from translational regulation.

Individual cultures of 293 cells were transfected with increasing amounts of the *rep*$^+$ vector pTS1 or the *rep*$^-$ vector pTS18, but the total amount of DNA transfected was adjusted to 15 μg with pBR322. At 40 h after transfection, the cultures were harvested, and portions of the cytoplasmic fraction were used for the assay of CAT activity and for the isolation of RNA. For each vector the level of CAT activity increased with increasing amounts of DNA transfected (Fig. 2), but in the *rep*$^+$ pTS1 transfections, a plateau was reached at 10 μg of DNA. In contrast, in the *rep*$^-$ pTS18 transfections, CAT activity continued to increase with the DNA dose. At the highest concentrations of transfected AAV-CAT DNAs, pTS18 produced 10-fold more CAT activity than did pTS1. The absolute enhancement of CAT activity from pTS18 over that from pTS1 increased with increasing amounts of DNA transfected.

The steady-state level of CAT mRNA was measured from these transfections in a quantitative S1 nuclease assay (Fig. 2). Total cytoplasmic RNA was hybridized to an excess of a 5′-end-labeled probe that extended from the *Pvu*II site in the *cat* gene to the *Bam*HI site at nucleotide 1049 in the AAV genome. This probe protected 175 nucleotides of p_{40} mRNA from both pTS1 and pTS18. With increasing amounts of either *rep*$^+$ pTS1 or *rep*$^-$ pTS18 there was a corresponding

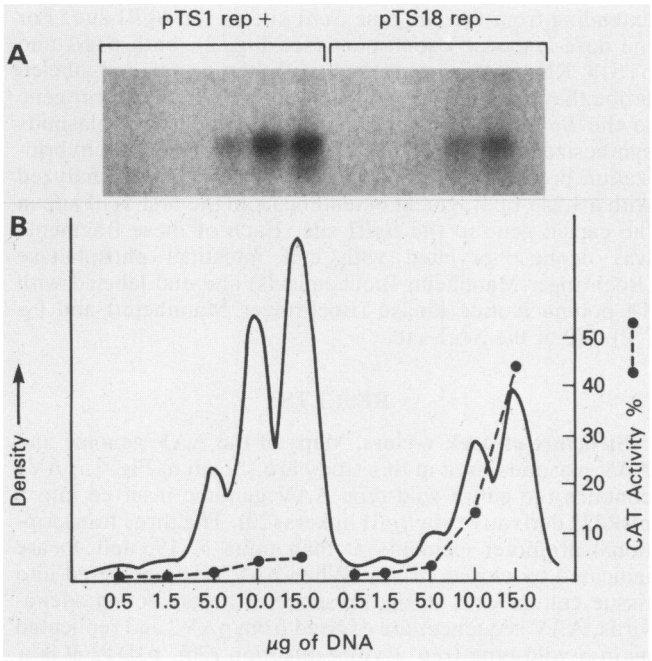

FIG. 2. Dose Response of AAV-CAT vectors. Increasing amounts of the rep^+ pTS1 and rep^- pTS18 plasmids (indicated at the bottom of panel B) were transfected into cultures of 293 cells. After 40 h, the cells were fractionated, and S1 nuclease RNA analysis and CAT activity assays were performed (A). Autoradiogram of the S1 nuclease assay with the p_{40} mRNA-specific probe shown in Fig. 1. (B) Densitometer tracing of the autoradiogram in panel A superimposed on the plot of CAT activity from the same transfections. Note that densitometry was performed by scanning across the gel. CAT activity is expressed as the percentage of acetylation of [^{14}C]chloramphenicol.

increase in the amount of the protected 175-bp fragment (Fig. 2A). A densitometer tracing of the autoradiogram (Fig. 2B) revealed that the rep^+ pTS1 transfections yielded a twofold-higher level of p_{40} transcripts than did the corresponding rep^- pTS18 transfections. Thus, a functional *rep* gene resulted in the activation of the p_{40} promoter or in the stabilization of CAT mRNAs while causing a concomitant decrease in CAT activity. The apparently opposing results between the level of the CAT mRNAs and their subsequent expression suggests an additional role for the pleiotropic *rep* gene, that of a translational inhibitor.

***trans* activation of p_{40} mRNA and *cis* regulation of p_5 mRNA.** The experiment in Fig. 2 did not determine whether the decreased level of p_{40} mRNA from pTS18 reflected *cis* effects of deletion of regulatory sequences upstream of p_{40} or *trans* effects of mutation of the *rep* gene. This question was analyzed in transfection complementation experiments. In the S1 nuclease analysis in these experiments, we used DNA probes (Fig. 1) which would detect RNA from the three promoters in pTS1 or from the two promoters in pTS18.

We first analyzed RNA transcribed from pTS1 or pTS18 transfections (Fig. 3). The pTS1 DNA probe revealed the S1 nuclease products of 2,357, 1,671 and 690 nucleotides expected for AAV-CAT RNAs from p_5, p_{19}, and p_{40}, respectively. Similarly, the pTS18 DNA probe revealed the truncated 1,357-nucleotide product expected from the p_5 RNA and the 690-nucleotide product expected from the p_{40} RNA. Interestingly, pTS18 yielded a much larger amount of p_5 RNA relative to p_{40} RNA than did pTS1. Since the DNA probes were different, the relative amounts of pTS1 and pTS18 transcripts could not be quantitatively compared. However, the results (Fig. 3) suggest that while pTS18 produced a somewhat decreased amount of p_{40} mRNA (consistent with Fig. 2), the amount of p_5 mRNA produced from pTS18 appeared to represent an actual increase in this mRNA. This result suggests that pTS18 had deleted from it a negative regulatory region which decreased the p_5 mRNA level.

We then performed experiments (Fig. 4) in which 293 cells were transfected with either rep^+ pTS1 or rep^- pTS18 complemented with rep^+ pAV2 or rep^- pJDT269. S1 nuclease analysis of the RNA (Fig. 4) was performed with the same DNA probes as those used in Fig. 3. When an intact *rep* gene was supplied in *trans* (from pAV2), there was a significant increase in the amount of p_{40} CAT mRNA from pTS18 as compared with the amount in the presence of the rep^- complementing plasmid pJDT269. Thus, the increase in the steady-state p_{40} message was due to *trans* activation by the AAV *rep* gene, and deletion of the upstream sequences had no significant role in p_{40} activation.

The complementing *rep* gene did not affect the amount of the p_5 message from pTS18. This result supports the idea that the elevated level of p_5 mRNA from pTS18 was indeed due to the removal of a *cis*-acting, negative regulatory sequence. *rep* gene-dependent *trans* activation of p_5 was reported in cells coinfected with AAV and adenovirus (17). However, we did not detect similar effects with pTS18, perhaps because of the high level of RNA from p_5 relative to that from p_{40}. When pTS1 was cotransfected with pAV2 or pJDT269 (Fig. 4), the presence of an additional *rep* gene from pAV2 had no significant effect on the amount of the p_{40} message but did result in some increases in the amounts of p_5 and p_{19} transcripts. Thus, the *rep* gene may indeed provide some *trans* activation of pTS1-driven p_5 and p_{19} mRNAs, perhaps by counteracting the normal *cis*-acting, negative regulation of p_5 mRNA in AAV.

As noted above (Fig. 2), the relative negative effect of *rep* on CAT activity varied with the amount of input plasmid

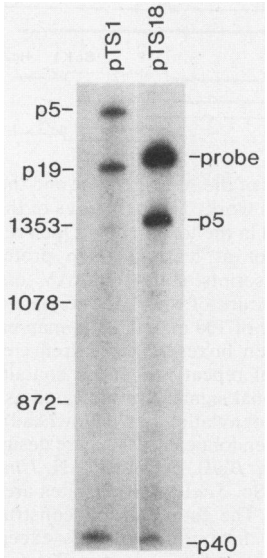

FIG. 3. S1 nuclease analysis of cytoplasmic RNA transcribed from pTS1 or pTS18 transfected into 293 cells. Cytoplasmic RNA was isolated at 40 h after transfection and analyzed with the probes shown in Fig. 1 (heavy arrows), which detected chimeric AAV-CAT RNAs transcribed from all the AAV promoters. The numbers at the left are DNA size markers.

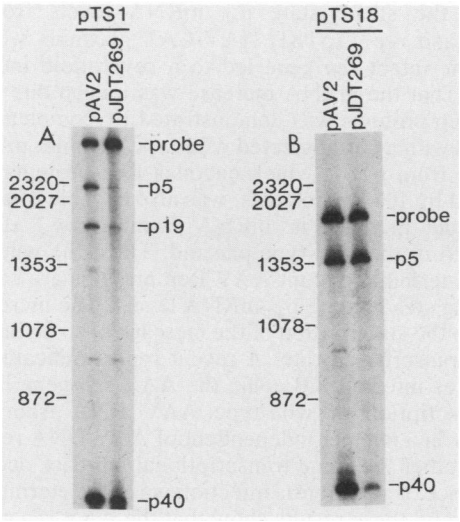

FIG. 4. S1 nuclease analysis of AAV-CAT RNA in the complementation assay for the effect of *rep*. Cultures of 293 cells were transfected with pTS1 or pTS18 complemented with pAV2 or pJDT269. Cytoplasmic mRNA was isolated and analyzed as described in the text. (A) Transcripts from the *rep*$^+$ pTS1 construct were detected with the pTS1-specific probe (Fig. 1 and 2) after cotransfection with wild-type pAV2 or *rep*$^-$ pJDT269. (B) Transcripts from the *rep*$^-$ pTS18 construct were detected with the pTS18-specific probe (Fig. 1 and 3) after cotransfection with pAV2 or pJDT269. Transcripts from the AAV promoters are shown. DNA size markers (numbers on the left) were end-labeled ϕX174 *Hae*III or λ *Hin*dIII DNA fragments.

DNA. However, the data in Table 1 show that at the level of input DNA used in the experiments in Fig. 3 and 4, there was indeed a negative *trans*-acting regulation of *cat* expression by the complementing *rep* gene. The data in Fig. 4 and Table 1 verify that the *rep* gene functions in *trans* to both increase mRNA levels and decrease gene expression from the AAV p_{40} promoter. Additional assays in which extracts from cells transfected with *rep*$^+$ and *rep*$^-$ plasmids were mixed showed that the negative regulation of *cat* expression was not due to the production of an inhibitory substance which interfered with the CAT assay itself (data not shown).

***rep* gene *trans* activation of wild-type p_{40} mRNA.** To verify that the *rep*-mediated increase in RNA was not due to the chimeric structure of the AAV-CAT messages, we also measured AAV-specific p_{40} mRNA levels from cotransfections of rep$^-$ pJDT269 with pTS1 or pTS18 (Fig. 5). The

TABLE 1. Expression of *cat* from AAV vectors in 293 cells

Vector(s)[a]	CAT activity[b]
pTS1	9.2
pTS1 + pJDT269 (*rep*$^-$)	8.5
pTS1 + pAV2 (*rep*$^+$)	12.6
pTS18	43.2
pTS18 + pJDT269 (*rep*$^-$)	35.7
pTS18 + pAV2 (*rep*$^+$)	13.7

[a] Cultures of 293 cells (5×10^6 cells) were transfected with 10 μg of pTS1 or pTS18 together with 10 μg of a complementary plasmid (pAS1, pJDT269, or pAV2).

[b] CAT activity was measured at 40 h after transfection as the percentage of acetylation of [^{14}C]chloramphenicol by 40 μl of extract (2.5×10^4 cells) at 1 h at 37°C.

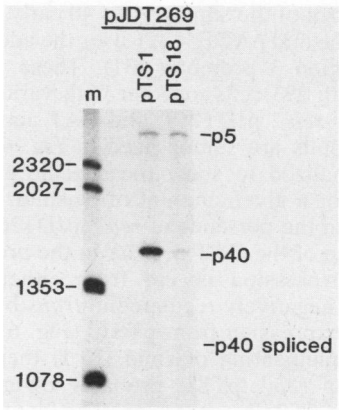

FIG. 5. pJDT269 cytoplasmic RNA transcripts detected after cotransfection of 293 cells with pJDT269 and either pTS1 or pTS18. S1 nuclease analysis used the end-labeled pJDT269 DNA probe shown in Fig. 1. The positions of S1 products from the p_5 and spliced or unspliced p_{40} RNAs are shown. M, Molecular weight markers.

hybridization probe derived from pJDT269 was end labeled at the *Sca*I site at nucleotide 3392 and extended to the *Bgl*II site at the left-hand end of the AAV sequence (Fig. 1). Unspliced pJDT269 transcripts from the p_5 (2,958 nucleotides) and p_{40} (1,538 nucleotides) promoters as well as a spliced transcript (1,164 nucleotides) were detected with this probe. It is noteworthy that in these transfections, very few of the AAV transcripts from pJDT269 were spliced, whereas in normal AAV infections in the presence of adenovirus, nearly all of the p_{40} mRNA was spliced. We have noted elsewhere (32) that adenovirus is required for efficient splicing of AAV RNA.

A comparison of the unspliced p_{40} transcripts from the cotransfections with pTS1 and pTS18 again revealed the *trans* activation of these RNAs by the pTS1 *rep* gene (Fig. 5). The increase was comparable to the pAV2-induced stimulation of the p_{40} transcripts from pTS18 (Fig. 4). However, the *trans*-acting *rep* gene from pTS1 is truncated at its 3′ terminus by about 300 nucleotides, owing to the insertion of the *cat* gene at the *Hin*dIII site during plasmid construction. The p_5 *rep*-encoded protein produced by pTS1 is approximately 10 kilodaltons smaller than the wild-type Rep78 protein (J. P. Trempe, E. Mendelson, and B. J. Carter, Virology, in press). Therefore, the carboxyl terminus of the Rep78 protein is not required to elicit increased RNA levels or decreased gene expression from the p_{40} promoter. Since pJDT269 produces wild-type p_{40} mRNAs, these results show that the *trans*-acting effect of *rep* on the increase in mRNA levels is not merely a function of the chimeric structure of the AAV-CAT constructs.

The deletion in pJDT269 spans 150 nucleotides between the *Sst*I and *Bcl*I sites flanking the p_{19} promoter. The relative level of p_5 transcripts was higher than that of p_{40} transcripts from pJDT269 in the absence of a functional *rep* gene from pTS18. Also, the pTS1 *rep* gene, which readily increased pJDT269 p_{40} mRNA levels, had no apparent effect on pJDT269 p_5 mRNA levels (Fig. 5). This result was not due to defective Rep proteins produced by pTS1, because similar results were found with wild-type Rep proteins produced by pAV2 in the cotransfection with *rep*$^-$ pTS18 (Fig. 4).

Effect of *rep* on *cat* expression from heterologous promoters. We also examined the effect of *rep* on the expression of *cat* from mRNAs transcribed from heterologous promoters. Plasmid pSVCAT contains the coding sequence for CAT

under the control of the simian virus 40 early gene promoter (8), whereas plasmid pADE3CAT uses the adenovirus type 5 early gene region 3 promoter (31). These plasmids were transfected with 293 cells together with various amounts of rep^+ pAV2 or rep^- pJDT269, and CAT activity was measured. The results are summarized in Fig. 6, in which the data are normalized to show the specific effect of *rep* in *trans*. Thus, for a given amount of plasmid, the amount of CAT activity in the presence of rep^- pJDT269 is expressed as a percentage of the CAT activity in the presence of rep^+ pAV2. The expression of *cat* from either heterologous promoter was negatively regulated in *trans* by the AAV *rep* gene, as was expression from pTS18 (Fig. 6). At very high ratios of complementing plasmid (10:1) there was even a small effect on rep^+ pTS1, probably owing to the large increase in Rep protein expression. The fact that *cat* expression from pSVCAT and pADE3CAT appeared to be inhibited by *rep* more readily than did that from pTS18 probably reflects the much lower amounts of RNA transcribed from these heterologous promoters than from p_{40}. Indeed, the lower levels precluded a reliable analysis of RNAs from pSVCAT and pADE3CAT. Nevertheless, the negative effect of *rep* on these plasmids was consistent with the translational control shown above for RNA from p_{40}.

DISCUSSION

The AAV Rep proteins are pleiotropic effectors of at least three different functions. The Rep proteins are required for AAV DNA replication in *trans* perhaps by acting to recognize the AAV DNA replication origins (12, 27). The additional functions of the Rep proteins are the positive and negative controls of AAV gene expression (17, 29). These effects appear to be dependent upon the host cell type and whether adenovirus helper functions are provided (29). The experiments described here provide evidence that in adenovirus-transformed 293 cells the *rep* gene exerts both positive and negative controls on p_{40} transcription units at different levels.

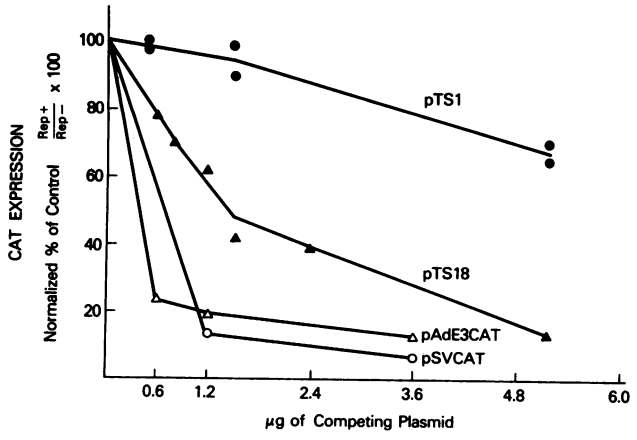

FIG. 6. Effect of AAV *rep* in transient assays on *cat* expression from RNA driven by heterologous promoters. Cultures of 293 cells (10^6 cells per 35-mm dish) were transfected with 0.5 μg of a CAT vector (pTS1, pTS18, pADE3CAT, or pSVCAT) and the indicated amounts of the complementing plasmid (pAV2 or pJDT269). For each CAT vector used the amount of DNA per culture was adjusted to 6 μg with control plasmid DNA. To normalize the data for each individual CAT vector, we calculated the amount of activity at a given concentration of complementing pAV2 (rep^+) plasmid as a percentage of the activity at the same concentration of complementing pJDT269 (rep^-) plasmid.

When the steady-state p_{40} mRNA levels from rep^- (pTS18) and rep^+ (pTS1) AAV-CAT plasmids were compared, the intact *rep* gene led to a severalfold increase in mRNA. That the mRNA increase was indeed due to *trans*-acting Rep proteins was demonstrated by complementation of pTS18 with a cotransfected wild-type plasmid, pAV2. The *rep* gene from pTS1, which encodes Rep proteins that are truncated by 100 amino acids, was also capable of eliciting a comparable increase in mRNA from a rep^- derivative (pJDT269) of the wild-type plasmid. Thus, the wild-type or carboxyl-terminal mutant AAV Rep proteins are capable of increasing steady-state p_{40} mRNA levels. The increase may be due to the stabilization of the message or to an increase in the p_{40} transcription rate. A recent report indicated that in adenovirus-infected KB cells, the AAV *rep* gene increased the transcription of wild-type AAV RNA from the p_{40} promoter in a manner independent of AAV DNA replication (17). Whether the same transcriptional increase occurred in the absence of adenovirus infection was not determined (17). Collectively, these results show that the AAV *rep* gene does *trans* activate the level of RNA from the AAV p_{40} promoter, which normally is required for AAV capsid protein synthesis. It remains to be determined whether this activation of RNA reflects an increased transcription rate, transport, or stability of RNA, although evidence in adenovirus-infected KB cells has suggested an effect on the transcription rate (17).

The negative regulation by *rep* of the expression of *cat* occurred in the same 293 cells in which *rep* positively activated mRNA levels, but the negative effect appeared to be of a larger magnitude. The negative effect appeared to reflect translational inhibition, since the chimeric AAV-CAT mRNA in the cytoplasm appeared to have a normal structure. However, we have not directly tested this RNA in an in vitro translation assay. Also, it appears that specific mRNA sequences may not be required, since the heterologous mRNAs had no obvious noncoding sequence homology with AAV-CAT mRNAs.

Since this work was completed, Labow et al. (16) reported that the AAV *rep* gene inhibited the transformation of mouse melanoma cells to geneticin resistance by the bacterial *neo* gene driven by several heterologous eucaryotic promoters, including the simian virus 40 early promoter, the herpesvirus thymidine kinase promoter, or the mouse metallothionein promoter. They also observed the inhibition of *cat* expression from pSVCAT in transient assays. Although the mechanisms of these effects were not analyzed, these results are consistent with our observation of translational inhibition. We have also observed *rep*-mediated negative regulation of *neo* driven by AAV p_{40} or the herpesvirus thymidine kinase promoter (E. Mendelson, M. Smith, and B. Carter, unpublished data) and of *cat* expression driven by the mouse metallothionein or mouse mammary tumor virus promoters (J. Trempe, unpublished data). The observations in mouse cells (16) also showed that the negative regulation was not simply due to the adenovirus E1A gene expressed in 293 cells. Also, we have observed strong negative regulation of *cat* expression from the AAV p_{40} promoter in mouse NIH 3T3 cells (data not shown).

The negative regulation in 293 cells presents an interesting paradox in that the Rep protein(s) provided in *trans* increased the cytoplasmic p_{40} transcripts but decreased the translation of RNA. Perhaps the binding of Rep proteins to RNA stabilizes the mRNA but inhibits translation. It is also unclear which Rep proteins mediate the various pleiotropic effects. We reported that the p_5 Rep proteins are mainly

nuclear in location, whereas the p_{19} Rep proteins are also found in the cytoplasm, as measured by immunoblotting (25). In contrast, immunofluorescence revealed Rep proteins mainly in the nucleus (Trempe et al., in press). The p_5 protein is required for DNA replication and positive regulation (12, 17, 28, 29). Also, in transient assays the p_5 protein is required for negative regulation (29). However, in assays with the dominant selection marker *neo*, the p_{19} protein alone may be able to mediate negative regulation (16; E. Mendelson, M. Smith, I. Miller, and B. Carter, submitted for publication).

Whatever the mechanism, the AAV *rep* gene may be unique thus far among eucaryotic *trans*-acting regulatory genes in mediating both positive transcriptional or post-transcriptional activation and negative translational regulation. Interestingly, the human immunodeficiency virus *tat* gene also mediates both transcriptional and translational regulation. However, in this case, translational regulation is a positive effect, and both effects of *tat* appear to be restricted to human immunodeficiency virus mRNA (5, 7, 27).

In the course of this work we also found evidence for a *cis*-acting region between the *Sst*I and *Bst*EII sites (map units 17 to 36) which appears to be a negative regulator of the level of RNA from the p_5 promoter. This negatively acting region was also observed by Labow et al. (17) with AAV mutants in adenovirus-infected KB cells. The complementation experiments indicate that the action of this region is independent of that of the *rep* gene products or of AAV DNA replication. The precise location of this region is not known, and it does not simply reflect deletion of the p_{19} promoter, as in pTS18. Interestingly, in pJDT269, which has a deletion between map units 17 and 20, there may be a somewhat lower elevation of p_5 mRNA (compare Fig. 4 and 5). Also, other experiments (data not shown) involving the deletion of map units 20 (*Bcl*I site) to 36 (*Bst*EII site), which leaves the p_{19} promoter intact, still reveal greatly increased p_5 mRNA.

It is now apparent that the AAV growth cycle is governed by a complex regulatory system expressed by AAV itself in addition to any regulation imposed by the helper virus. The precise role of these regulatory events must be interpreted with some caution, but the Rep proteins are at least formally analogous to "early" regulatory proteins of many other viruses, and the capsid proteins are analogous to "late" structural proteins. Whether a temporal relationship exists in the order of appearance of various Rep and capsid proteins remains to be established.

The positive effect of *rep* in increasing the level of p_{40} mRNA can be readily understood, since it would increase the amount of capsid protein and promote efficient particle assembly. The negative regulation of p_{40} mRNA expression by *rep* is more difficult to understand directly, but it might serve early in the growth cycle to prevent the excessive production of capsid protein. This may be important in early rounds of replication to prevent packaging of displaced single-stranded DNA and allow a more rapid increase in the pool of replicating molecules. Later in replication the negative effect may be overcome by the helper virus (29).

The negative regulatory features of the Rep proteins may also have other important roles in inhibiting or altering the expression of helper virus or cellular functions. AAV inhibits the replication of adenovirus DNA by at least 10-fold and the production of infectious adenovirus to an even larger extent (2, 19). Also, AAV inhibits tumorigenesis by adenovirus type 12 (6) and decreases the tumorigenicity of both adenovirus-transformed hamster cells (6, 26) and *ras*-transformed mouse cells (15).

The Rep proteins may also have important functions in the absence of helper virus. Although it is now known that AAV can replicate in a small fraction of some cells (1 to 3%) after metabolic insult of the cells, this replication is inefficient (33). Indeed, in the absence of helper virus, AAV integrates as a provirus into at least 10 to 20% of infected cells (4, 13, 18). Thus, in the absence of generally favorable conditions for replication, the integration of AAV appears to be preferred. The negative regulatory role of *rep* may be important in maintaining the proviral state. These considerations suggest that AAV may generally be a nondefective but "repressed" virus. The repression may be mediated by cellular factors as well as the *rep* gene. In this respect, the *cis*-acting negative region in AAV may also be important in down-regulating p_5, which otherwise appears to be a strong promoter. Elsewhere, we have shown that p_{19} is a relatively weak promoter but can be activated by the adenovirus E1A gene (30).

LITERATURE CITED

1. **Becerra, S. P., J. A. Rose, M. Hardy, B. M. Baroudy, and C. W. Anderson.** 1985. Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon. Proc. Natl. Acad. Sci. USA **82**:7919–7923.
2. **Carter, B. J., C. A. Laughlin, L. M. de la Maza, and M. Myers.** 1979. Adeno-associated virus auto-interference. Virology **92**: 449–462.
3. **Carter, B. J., C. A. Laughlin, and C. J. Marcus-Sekura.** 1983. Parvovirus transcription, p. 153–207. *In* K. Berns (ed.), The parvoviruses. Plenum Publishing Corp., New York.
4. **Cheung, A. M. K., M. D. Hoggan, W. W. Hauswirth, and K. I. Berns.** 1980. Integration of the adeno-associated virus genome into cellular DNA in latently infected Detroit 6 cells. J. Virol. **33**:739–748.
5. **Cullen, B. R.** 1986. *trans*-activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46**:973–982.
6. **de la Maza, L. M., and B. J. Carter.** 1981. Inhibition of adenovirus oncogenicity by adeno-associated virus DNA. JNCI **67**:1323–1326.
7. **Feinberg, M. B., R. F. Jarrett, A. Adovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46**:807–817.
8. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chlormamphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
9. **Graham, F. L., J. Smiley, W. C. Russell, and R. Naiva.** 1977. Characteristics of a human cell line transformed by DNA from adenovirus type 5. J. Gen. Virol. **36**:59–72.
10. **Graham, F. L., and A. J. van der Erb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
11. **Green, M. R., and R. G. Roeder.** 1980. Transcripts of the adenovirus-associated virus genome: mapping of the major RNAs. J. Virol. **36**:79–82.
12. **Hermonat, P., M. A. Labow, R. Wright, K. I. Berns, and N. Muzyczka.** 1984. Genetics of adeno-associated virus: isolation and preliminary characterization of mutants of adeno-associated virus type 2. J. Virol. **51**:329–339.
13. **Hoggan, M. D., G. F. Thomas, and F. B. Johnson.** 1973. Continuous carriage of adeno-associated virus genomes in cell culture in the absence of adenovirus, p. 243–249. *In* Proceedings of the Fourth Lepetit Colloquium, Cocoyac, Mexico. Elsevier/North-Holland Publishing Co., Amsterdam.
14. **Janik, J. E., M. M. Huston, and J. A. Rose.** 1984. Adeno-associated virus proteins: origin of the capsid components. J. Virol. **52**:591–597.
15. **Katz, E., and B. J. Carter.** 1986. Effect of adeno-associated virus on transformation of NIH 3T3 cells by *ras* gene and on

tumorigenicity of an NIH 3T3 transformed cell line. Cancer Res. **46**:3023–3026.
16. **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.
17. **Labow, M. A., P. L. Hermonat, and K. I. Berns.** 1986. Positive and negative auto-regulation of the adeno-associated virus type 2 genome. J. Virol. **60**:251–258.
18. **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.
19. **Laughlin, C. A., M. W. Myers, D. L. Risin, and B. J. Carter.** 1979. Defective-interfering particles of the human parvovirus adeno-associated virus. Virology **94**:162–174.
20. **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.
21. **Laughlin, C. A., H. Westphal, and B. J. Carter.** 1979. Spliced adeno-associated virus mRNA. Proc. Natl. Acad. Sci. USA **76**:5567–5571.
22. **Lusby, E. W., and K. I. Berns.** 1982. Mapping the 5′ termini of two adeno-associated virus RNAs in the left half of the genome. J. Virol. **41**:518–526.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Marcus, C. J., C. A. Laughlin, and B. J. Carter.** 1981. Adeno-associated virus RNA transcription in vivo. Eur. J. Biochem. **121**:147–154.
25. **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.
26. **Ostrove, J., D. H. Duckworth, and K. I. Berns.** 1981. Inhibition of adenovirus-transformed cell oncogenicity by adeno-associated virus. Virology **113**:521–523.
27. **Rosen, C. A., J. G. Sodroski, W. C. Gohg, A. I. Dayton, J. Lippke, and W. A. Haseltine.** 1986. Post-transcriptional regulation accounts for the *trans*-activation of the human T-lymphotropic virus type III. Nature (London) **319**:555–559.
28. **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.
29. **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.
30. **Tratschin, J.-D., M. H. P. West, T. Sandbank, and B. J. Carter.** 1984. A human parvovirus, adeno-associated virus, as a eucaryotic vector: transient expression and encapsidation of the procaryotic gene for chloramphenicol acetyltransferase. Mol. Cell. Biol. **4**:2072–2081.
31. **Weeks, D. L., and N. C. Jones.** 1983. E1A control of gene expression is mediated by sequences 5′ to the transcriptional starts of the early viral gene. Mol. Cell. Biol. **3**:1222–1234.
32. **West, M. H. P., J. P. Trempe, J. D. Tratschin, and B. J. Carter.** 1987. Gene expression in adeno-associated virus vectors: the effects of chimeric mRNA structure, helper virus and adenovirus VA_I RNA. Virology **160**:38–47.
33. **Yakobson, B., T. Koch, and E. Winocour.** 1987. Replication of adeno-associated virus in synchronized cells without the addition of a helper virus. J. Virol. **61**:972–987.